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Abstract – A method aiming at investigating possible bioactive conformations of acyl homoserine lactone (AHL) quorum sensing (QS) modulators is established. The method relies on the exhaustive conformational analysis of AHLs by varying torsion angles around the amide group then on the selection of the closest conformation to those known from co-crystallized XRD data of AHL-receptor complexes. These latter are then docked as rigid ligand within the receptor binding site, leading to conformations, interactions with binding site residues which are highly consistent as compared with the data arising from XRD studies.

The method is first validated using AHLs for which XRD data of their complexes with their cognate receptor are available, then extended to examples for which the binding mode is still unknown.

Three compounds were used to validate the method: hexanoyl homoserine lactone (HHL) as an example of autoinducer, 3-oxo-butanoyl homoserine lactone (OBHL), as a representative model of 3-oxo-AHLs, and 4-(4-chlorophenoxy)butanoyl homoserine lactone (CPOBHL) as an example of a QS inhibitor. The conformational analysis of these three compounds to their cognate protein (TraR, SdiA, LasR and CviR) provides the data which enable the next rigid docking step. Further rigid docking of the closest conformations compared to the known bioactive ones within the binding sites allows to recover the expected binding mode with high precision (atomic RMSD < 2Å). This "conformational analysis/torsion angle filter/rigid ligand docking" method was then used for investigating three non-natural AHL-type QS inhibitors without known co-crystallized XRD structures, namely was 2-hexenoyl homoserine lactone (HenHL), 3-oxo-4-phenylbutanoyl homoserine lactone (OPBHL) and 3-(4-bromophenyl)propanoyl homoserine lactone (BPPHL). Results provide insights into their possible binding mode by identifying specific interactions with

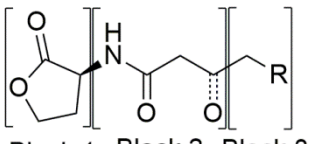
some key residues within the receptor binding site, allowing discussion of their biological activity.

Key words: Conformational analysis, Acyl homoserine lactone, Quorum sensing, Docking

1. Introduction

Acyl homoserine lactones (AHLs) are used by Gram negative bacteria to communicate in order to synchronize their behavior according to their environment. AHLs, referred to as autoinducers, are biosynthesized by proteins of the LuxI family, and known to bind transcriptional regulator of the LuxR family.[1, 2] This system called quorum sensing is extensively studied, especially to interfere with bacterial communication.[3-5] The structure of AHLs differs according to bacteria species, with different alkyl chain lengths, and presence or not of a 3-oxo functional group. For example, 3-oxo-hexanoyl homoserine lactone (OHHL) is found in *Vibrio fischeri* (recognized by luxR), 3-oxo-octanoyl homoserine lactone (OOHL) in *Agrobacterium fabrum* previously referred to as *Agrobacterium tumefaciens* (recognized by TraR), 3-oxo-dodecanoyl homoserine lactone (ODHL) in *Pseudomonas aeruginosa* (recognized by LasR) and hexanoyl homoserine lactone (HHL) in *Chromobacterium violaceum* (recognized by CviR).

The molecular structure of AHL can be divided in three blocks: block 1, the lactone moiety; block 2, the 3-oxo amide functional group; and block 3, the alkyl chain.[6]



In terms of conformation of homoserine lactones,[7-10] conformational analysis studies have shown that they can adopt different conformations depending on the occurrence or not of a $n \rightarrow \pi^*$ interaction between *p*-type lone pair (*n*) of the *N*-acyl oxygen of the amide with the π^* orbital of the lactone carbonyl functional group. Notably the study by Raines and co-workers using DFT calculations showed particularly the importance of this $n \rightarrow \pi^*$ interaction for free AHLs whereas for bound conformations this interaction do not exist.[8] The importance of the solvents effects was further highlighted by the study by Kelleher and co-workers who showed, also using DFT calculations, that $n \rightarrow \pi^*$ interactions become stronger with the polarity of the solvent.[9, 10]

All functional groups found in AHLs and their close analogues are known to influence their molecular recognition by their cognate receptors, through hydrogen bonds and hydrophobic interactions. As example, the XRD data for the complex between OOHL and TraR (pdb code 1L3L)[11] shows that the lactone is involved in a hydrogen bond with Trp57, the amide with Asp70 (NH) and Tyr53 (C=O), and the 3-oxo functional group with a water molecule (Fig. 1). Similar binding modes are obtained from X-ray structures of SdiA in complex with 3-oxo-hexanoyl homoserine lactone (OHHL) and 3-oxo-octanoyl homoserine lactone (OOHL) (pdb code 4Y15 and 4Y17),[12] and of LasR with 3-oxo-dodecanoyl homoserine lactone (ODHL) (pdb code 3IX3). For the complex of CviR and the native ligand hexanoyl homoserine lactone (HHL) lactone (pdb code 3QP1),[13] the same overall binding mode was observed, except the hydrogen bond with the water molecule due to the absence of the 3-oxo functional group. For the inhibitor 4-(4-chlorophenoxy)butanoyl homoserine lactone (CPOBHL) in complex with CviR (pdb code 3QP5), a similar hydrogen bond network is observed (the lactone and Trp84 and the amide with Asp97 (NH) and Tyr80 (C=O)) as well as aromatic interactions between the 4-chlorophenyl substituent and Tyr88 (Fig. 1).[13]

In this work, using XRD data from six co-crystallized AHLs-proteins complexes, representative of the structural diversity of AHLs and of the receptors, we developed a "conformational analysis/torsion angle filter/rigid ligand docking" method which was then applied to AHL-type QS inhibitors for which no XRD data support any binding mode. Technically, three compounds were used for the first validation phase, namely hexanoyl homoserine lactone (HHL) as an example of 3-CH_2 AHL autoinducer, 3-oxo-butanoyl homoserine lactone (OBHL) a representative model example of natural 3-oxo AHLs, and the known QS inhibitor 4-(4-chlorophenoxy)butanoyl homoserine lactone (CPOBHL) as example of AHL analogue modified on the side chain. The method was then applied in a second phase using three AHL-type QS modulators with unreported XRD data reflecting the same structural diversity, namely TraR antagonist 2-hexenoyl homoserine lactone (HenHL), as example of close analog of AHL,[14, 15], 3-oxo-4-phenylbutanoyl homoserine lactone (OPBHL), as an example of 3-oxo-AHL analog, and the interesting broad spectrum antagonist[16, 17] 3-(4-bromophenyl)propanoyl homoserine lactone (BPPHL) as example of aromatic AHL analog.

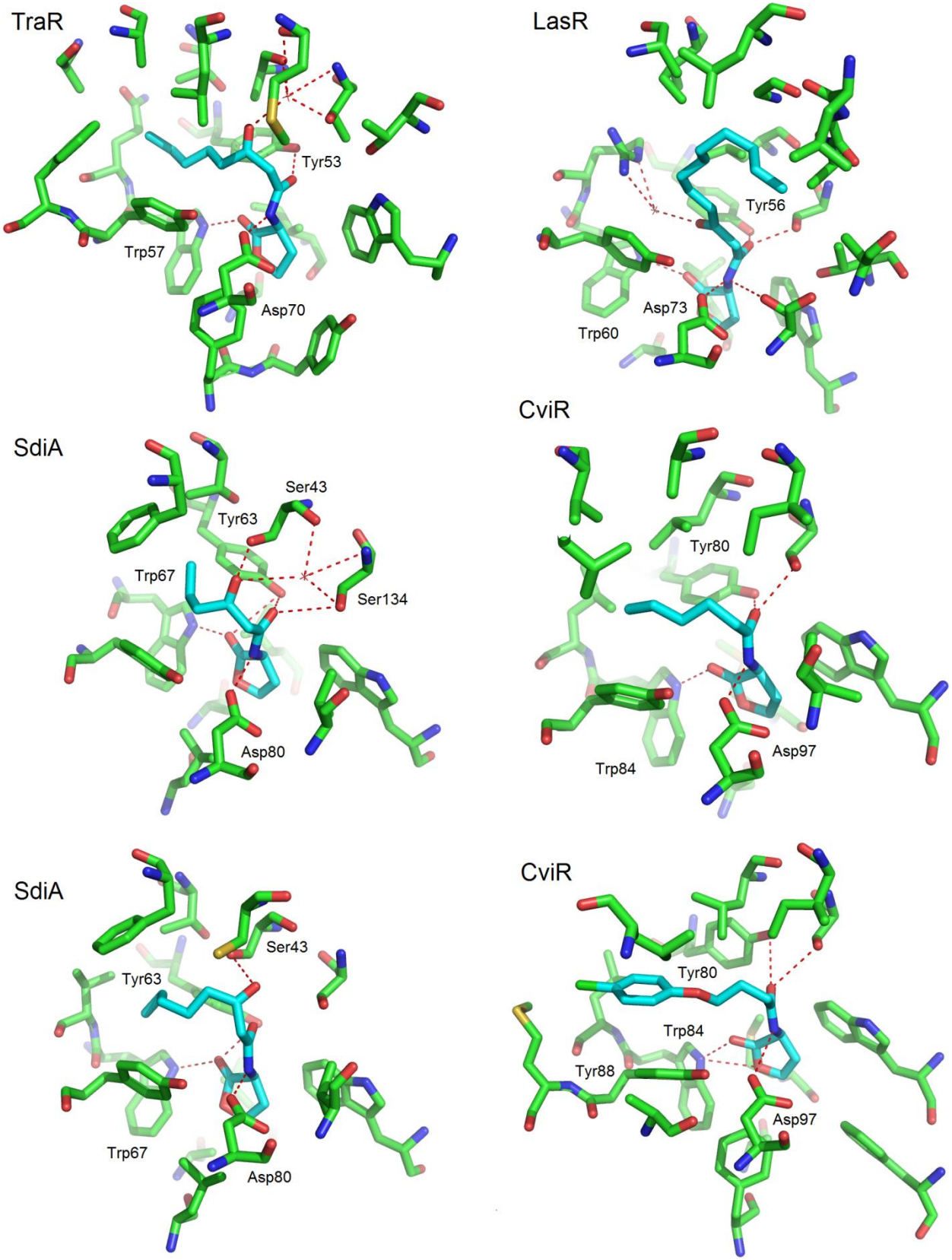

TraR
Tyr53
Trp57
Asp70
LasR
Tyr56
Asp73
Trp60
SdiA
Ser43
Tyr63
Trp67
Ser134
Asp80
CviR
Tyr80
Trp84
Asp97
SdiA
Ser43
Tyr63
Trp67
Asp80
CviR
Tyr80
Trp84
Tyr88
Asp97

Figure 1: Bound (bioactive) conformations (cyan) of acylhomoserine lactones: 3-oxo-hexanoyl homoserine lactone (SdiA pdb 4Y15), hexanoyl homoserine lactone (CviR pdb 3QP1), 3-oxo-octanoyl homoserine lactone (TraR pdb 1L3L and SdiA pdb 4Y17), 3-oxo-dodecanoyl homoserine lactone (LasR pdb 3IX3) and 4-(4-chlorophenoxy)butanoyl homoserine lactone (CviR pdb 3QP5).

2. Computational methods

For conformational analyses,[7] the three compounds, hexanoyl homoserine lactone (HHL), 3-oxo-butanoyl homoserine lactone (OBHL) and 4-(4-chlorophenoxy)butanoyl homoserine lactone (CPOBHL) were constructed using Vega ZZ[18, 19], and charges were assigned with the SP4 force field and Gasteiger.[20] The obtained 3D models were then subjected to a systematic conformational search[21-24] with the implemented AMMP program[25] (SP4 force field) by varying five torsion angles using a 60° increment (7776 conformations) for HHL, three torsion angles using a 30° increment (1728 conformations) for OBHL and six torsion angles using a 60° increment (46656 conformations) for CPOBHL (in all cases, the amide bond is in *s-trans* conformation). Increments of 30° or 60° in torsion angles were chosen for keeping the method practical in terms of calculation time and number of generated data. Indeed, for compounds that require variations of 3 or 4 torsion angles, an increment of 30° lead to 1728 or 20736 conformations respectively, which is easily analyzed, whereas for 5 or 6 torsion angles, 30° would lead to 248832 or 2985984 conformations, respectively, less appropriate for next steps of the method. In those cases, an increment of 60° was preferred , leading to 7776 or 46656 conformations, respectively, though leading to meaningful docking results as measured by low

RMSD value < 2 Å.[26] For OBHL which is a 3-oxo amide, the method was performed on its 3-oxo tautomeric forms, the most predominant form,[9] rather than on the enol tautomeric one. The generated conformations were then analyzed to define the most stable ones for the three compounds, and the closest conformations as compared with the known bound conformations to TraR, SdiA, LasR or CviR (pdb codes 1L3L, 4Y15, 4Y17, 3IX3, 3QP1 and 3QP5) were selected. These latter were then docked as rigid ligands within their cognate receptor binding site using Arguslab[27] with the following parameters: Docking box: X = Y = Z = 15 Å, ligand option: rigid; calculation type: Dock; Docking engine: GADock (Genetic Algorithm);[28] Genetic algorithm dock settings: default advanced parameters. The "conformational analysis/torsion angle filter/rigid ligand docking" method was then applied to three examples for which no X-ray data is available, HenHL, OPBHL and BPPHL. For HenHL and BPPHL, the method was executed using four torsion angles with 30° increments which led to 20736 conformations. For OPBHL, five torsion angles and 60° increments were used, leading to 7776 conformations.

3. Results and discussion

Docking experiments with stochastic genetic algorithm frequently give different binding modes rendering difficult their validation.[29, 30] As a consequence, conformational and docking studies were first applied to AHLs which have been co-crystallized with transcriptional factor of the LuxR family namely TraR, SdiA, LasR or CviR in order to validate the method i.e. to retrieve the co-crystallized conformation. The method, validated with co-crystallized AHLs was then applied to AHLs with unknown binding mode from XRD data (Figure 2).

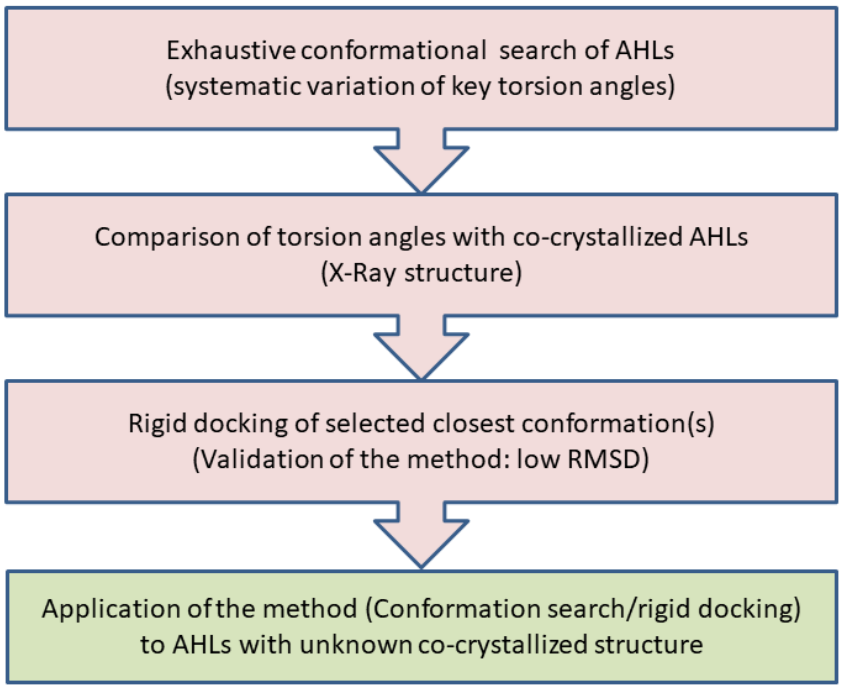
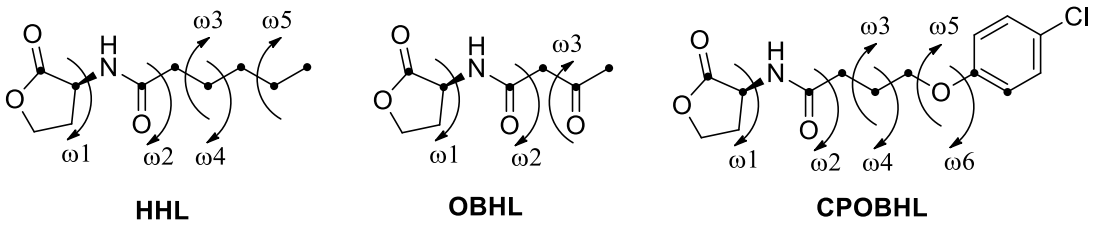


Figure 2: Description of the "conformational analysis/torsion angle filter/rigid ligand docking" method.

Conformational analyses of HHL, OBHL and CPOBHL were thus achieved by varying key torsion angles defined by the atoms marked in bold. For OBHL, an increment of 30° for the three torsion angles was chosen to generate 1728 conformations. For HHL and CPOBHL, an increment of 60° was selected to limit the number of conformations to 7776 and 46656 respectively.



3.1. Conformational analysis and docking studies of AHLs with known XRD data

3.1.1. Hexanoyl homoserine lactone (HHL)

Among the 7776 conformations generated for HHL, the most stable conformation was obtained with a calculated energy value of 21 kcal.mol^{-1}. To retrieve the bound conformation of HHL in complex with CviR, ω1 to ω5 were measured and the conformation with closest torsion angle values was selected. The conformation 6106 was recovered for its similarity in the key angles as compared to the known bound conformer (Table 1). These two conformations (the lowest energy conformation and the conformation 6106) are very close in energy (only 1 kcal/mol different) though dissimilar in terms of torsion angles values. No $n \rightarrow \pi^*$ interaction, known to be observed preferentially for non-bound AHLs [8, 10] was observed here logically, as only "bound-like" conformations are selected during the protocol.

Table 1. HHL: values in degree of the torsion angles for the most stable, the bound conformation and the closest conformation (energy in kcal/mol)

	ω1	ω2	ω3	ω4	ω5	energy
Lowest (4162)	180	60	60	180	180	21
Bound (CviR)	240	240	70	150	150	--
6106	240	240	60	180	180	22

A rigid docking was then applied to the conformation 6106 within the binding site of CviR (Figure 3). The conformation 6106 fits very well with a RMSD value of 0.84 Å[26] showing the robustness of the method using conformational search and subsequent rigid ligand docking.

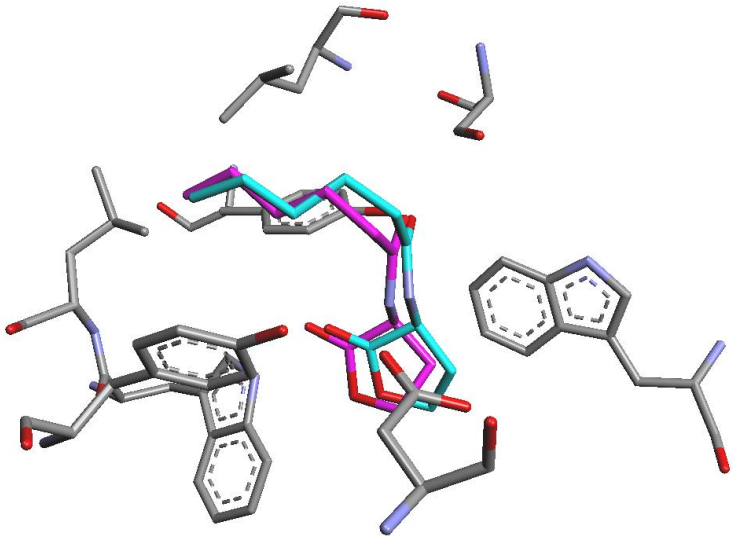


Figure 3: Rigid ligand docking result of HHL within the binding site of CviR (co-crystallized conformation is colored in cyan and conformation 6106 in magenta).

3.1.2. 3-Oxo-butanoyl homoserine lactone (OBHL)

3-Oxo-butanoyl homoserine lactone (**OBHL)** was chosen as a representative example of 3-oxo homoserine lactones, possessing the key 3-oxo functional group known to be involved in additional H-bonds with a water molecule. In the family of 3-oxo-AHLs, the butyl analog (C4) was chosen because it is long enough to mimic longer alkyl chains, which only differ at the side-chain extremity with successive anti or gauche relationships for each additional C-C bond (as shown for HHL), though not modifying the main lactone-amide-3-oxo overall conformation.[7] Therefore, using OBHL in our study is the way to access the key ω1 to ω3 angles during the conformational analysis, while keeping practical both the calculation time and data analysis. This choice is validated by the consistency of the docking results (see below) with known XRD data for short or longer 3-oxo-AHLs.

Among the 1728 conformations generated, the most stable one was obtained with an energy value of 10 kcal.mol^{-1}. To retrieve the bound conformation of OBHL in complex with TraR, SdiA and LasR, ω1 to ω3 were measured, and the conformations with closest torsion angle values compared to those of the bound compounds were selected. Thus, conformations 1263 for TraR, 1251 for the C6-type SdiA, 1371 for C-8-type SdiA and 1267 for LasR in complex with 3-oxo-dodecanoyl homoserine lactone were selected (Table 2).

Table 2. OBHL: values in degree of the torsion angles for the most stable and the bound conformations and the closest conformations (energy in kcal/mol)

	ω1	ω2	ω3	energy	RMSD
Lowest (1037)	210	60	120	10	--
Bound (TraR)	250	260	54	--	--
1263	240	270	60	22	0.76
Bound (SdiA 4Y15)	245	245	90	--	--
1251	240	240	90	12	1.14
Bound (SdiA 4Y17))	260	190	90	--	--
1372	270	180	90	24	1.31
Bound (LasR)	240	260	190	--	--
1267	240	270	180	25	0.99

A rigid docking was then applied to the four selected conformations within the binding site of the three corresponding proteins (Figure 4). The conformations of the bound OBHL overlap very well with those of the native autoinducers, with RMSD values ranging from 0.76 to 1.31 Å when comparing the common part of the molecules showing the robustness of the method and confirming that the C-4 derivative is a meaningful model for longer chains (Table 2).

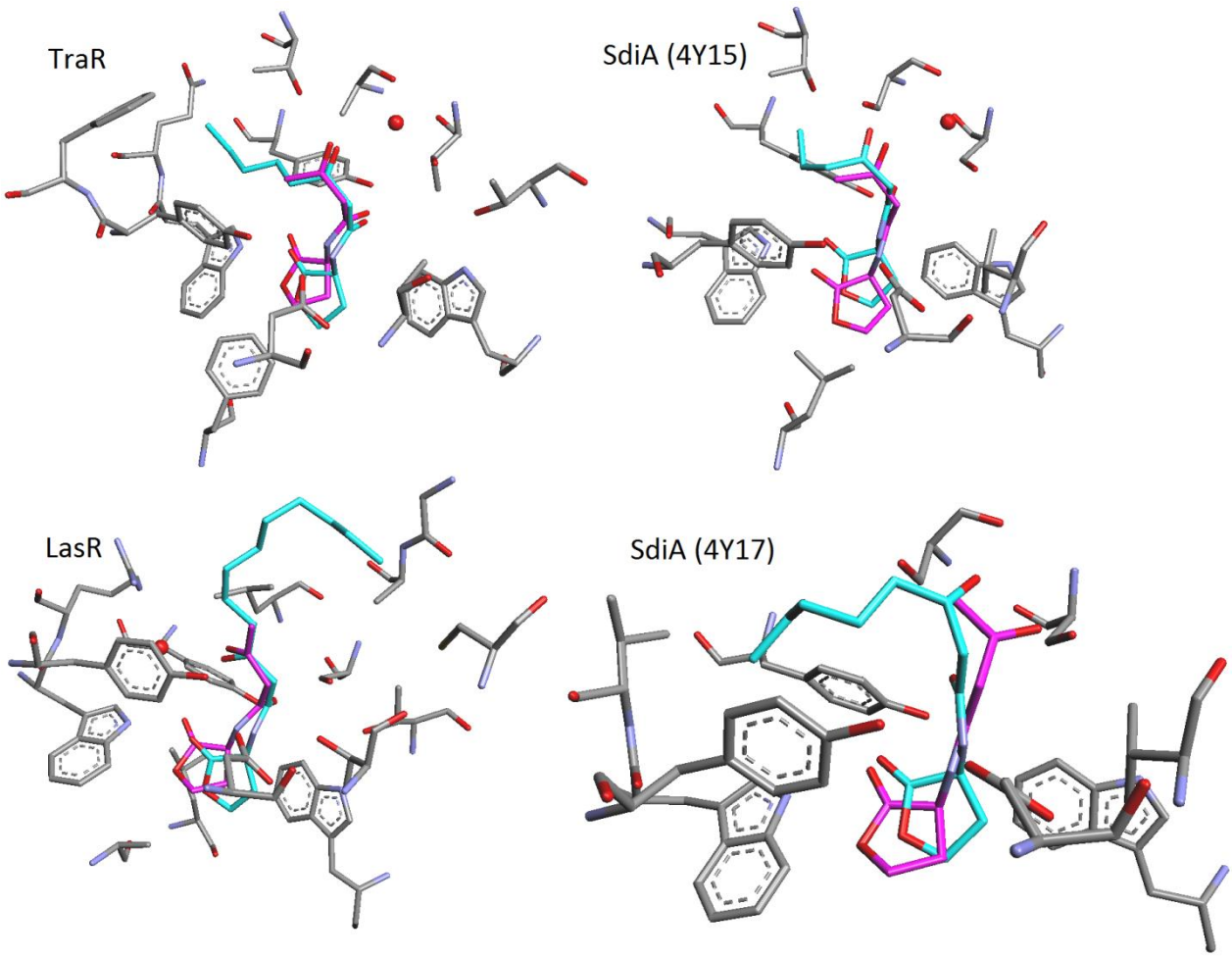


Figure 4: Rigid ligand docking results of the selected conformations for OBHL within the binding site of TraR, SdiA and LasR. (The co-crystallized conformations with OOHL, OHHL, ODHL are colored in cyan)

3.1.3. 4-(4-Chlorophenoxy)butanoyl homoserine lactone (CPOBHL)

Finally, we performed the study of the QS inhibitor CPOBHL which has been co-crystallized with CviR. The six torsion angles were subjected to a systematic conformational search by varying with a 60° increment generating 46656 conformations. To retrieve the bioactive conformation, the 6 torsion angles were estimated for the known bound conformation (table 3). In

this case, three conformations of CPOBHL showing similar structural closeness to the known bound conformation were selected, *i.e.* conformations 36633, 36591 and 36597 (table 3).

Table 3. CPOBHL: values in degree of the torsion angles for the most stable, the bound conformation and the closest conformations (energy in kcal/mol)

	ω1	ω2	ω3	ω4	ω5	ω6	energy	RMSD
Lowest (27203)	180	120	300	300	180	240	30	--
Bound (CviR)	250	240	60	146	150	136	--	--
36633	240	240	60	180	180	120	35	1.78
36591	240	240	60	120	120	120	39	1.84
36597	240	240	60	120	180	120	39	1.16

The selected conformations were then docked as rigid ligands within the binding site of CviR (Figure 5). All conformations fit well (RMSD < 2 Å) with the best docking result obtained for the conformation 36597 (yellow conformation).

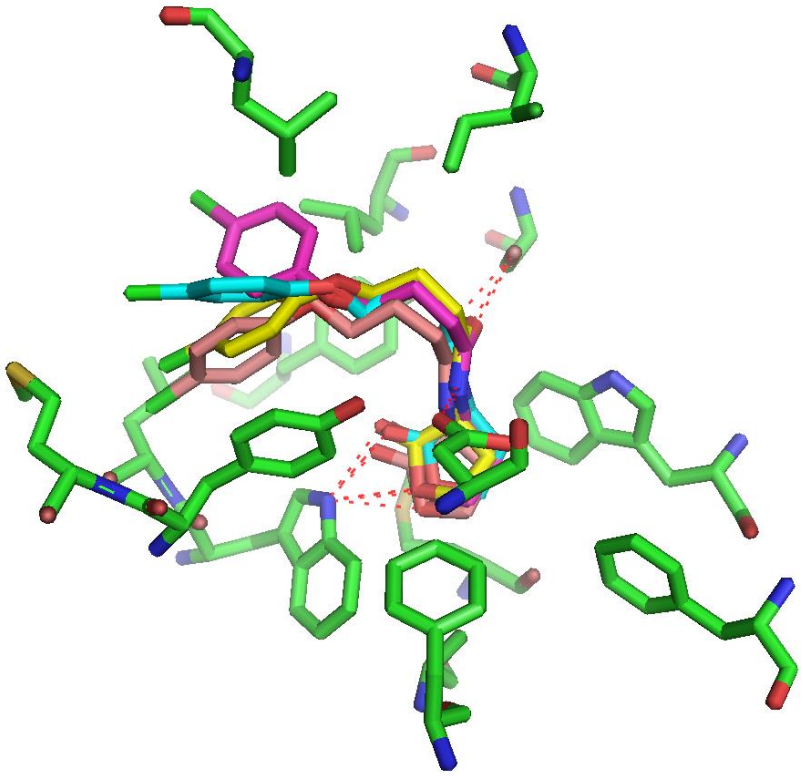
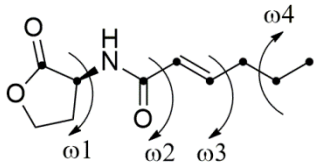


Figure 5: Rigid ligand docking results of selected conformations of OBHL within the binding site of CviR (magenta 36591, brown 36633, yellow 36597 and cyan co-crystallized conformation).

3.2. Application to AHLs without known XRD data

3.2.1. 2-Hexenoyl homoserine lactone (HenHL)



2-hexenoyl homoserine lactone (HenHL)

Having the protocol validated by the above results, we applied it for investigating the possible binding mode of 2-hexenoyl homoserine lactone (HenHL), a TraR antagonist [14, 15] for which no binding mode has been shown. After conformational analysis, the closest torsion angles values as compared to the natural ligand were selected for rigid ligand docking experiments. The retained conformation 14779 exhibits a rigid planar system in s-trans conformation for the conjugated double bond with the carbonyl of the amide functional group (Table 4).

Table 4. HenHL: values in degree of the torsion angles for the most stable and the closest conformation compared to the natural ligand (energy in kcal/mol)

	ω1	ω2	ω3	ω4	energy
Lowest (11347)	180	180	270	180	20
Closest (14779)	240	180	210	180	24

Next, the rigid ligand docking experiment showed that the rigidity of the alkenyl chain due to the planarity of the enamide moiety induces a different orientation of the acyl chain in the

hydrophobic zone of the binding site compared to the TraR autoinducer OOHL, while the hydrogen bonds network remains the same. The particular orientation of the acyl chain leads to new hydrophobic interactions between the enamide functional group and Tyr61, not seen in the case of the OOHL binding mode, which may explain the antagonistic activity of this compound (Figure 6).

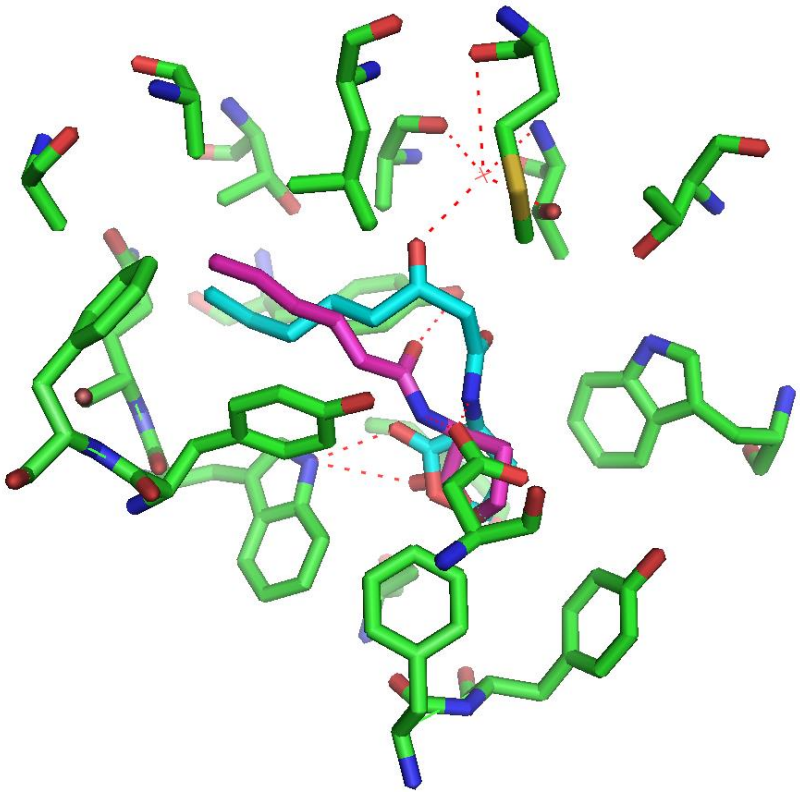
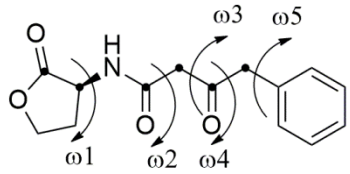


Figure 6: Rigid ligand docking result for 2-hexenoyl homoserine lactone (HenHL), (magenta) within the binding site of TraR. The co-crystallized natural ligand is colored in cyan.

3.2.2. 3-Oxo-4-phenylbutanoyl homoserine lactone (OPBHL)



3-oxo-4-phenylbutanoyl homoserine lactone (OPBHL)

Among 3-oxo-AHL analogues, the method was then applied to 3-oxo-4-phenylbutanoyl homoserine lactone (OPBHL), a very potent TraR antagonist with a 3-oxo functional group.[16] The conformational analysis allowed to select the closest conformation as compared to the TraR natural ligand (Table 5).

Table 5. OPBHL: values in degree of the torsion angles for the most stable and the closest conformations compared to the natural ligand (energy in kcal/mol)

	ω1	ω2	ω3	ω4	ω5	energy
Natural ligand	250	260	54	220	180	--
Lowest (4256)	180	60	240	60	60	15
Closest (6112)	240	240	60	240	180	25

The retained conformation 6112 was then submitted to rigid ligand docking within the binding site of TraR. The rigid ligand docking result shows that this compound fits very well within the TraR binding site. The overall orientation of the molecule is similar compared to the native ligand (in cyan) with a comparable hydrogen bond network, but new attractive interactions with Tyr53 and Tyr61 of the phenyl group are clearly observed (Figure 7). These attractive interactions appear as sensible causes for explaining the potent antagonistic activity of this compound.

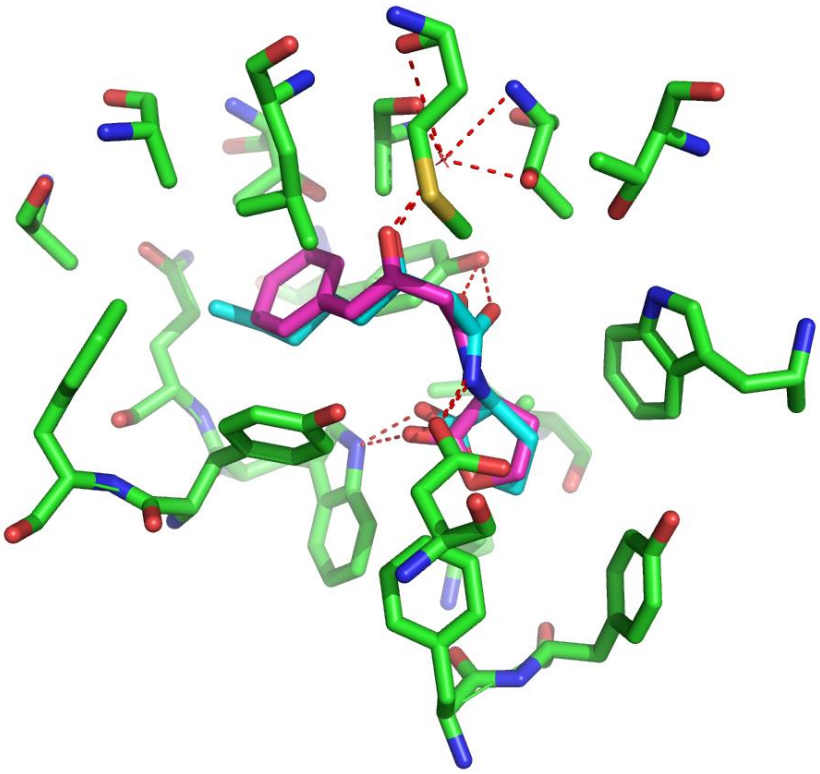
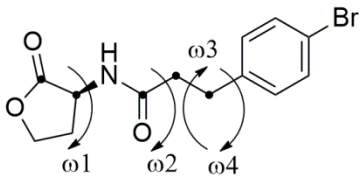


Figure 7: Rigid ligand docking result of the selected conformation for 3-oxo-4-phenylbutanoyl homoserine lactone (OPBHL) within the binding site of TraR (magenta 6112 and cyan co-crystallized conformation of 3-oxo-octanoyl homoserine lactone).

3.2.3. 3-(4-Bromophenyl)propanoyl homoserine lactone (BPPHL)



3-(4-bromophenyl)propanoyl homoserine lactone (BPPHL)

Finally, the interesting broad spectrum antagonist 3-(4-bromophenyl)propanoyl homoserine lactone (BPPHL) [16, 17] was investigated through the same protocol as above. Conformational

analysis was performed and two close conformations as compared to the natural ligand were selected, namely conformations 15152 and 15153 (Table 6).

Table 6. BPPHL: values in degree of the torsion angles for the most stable and the closest conformations compared to the natural ligand (energy in kcal/mol)

	ω1	ω2	ω3	ω4	energy
Natural ligand	250	260	54	220	--
Lowest (13474)	210	270	180	270	30
Closest (15152)	240	270	60	210	31
Closest (15153)	240	270	60	240	43

The retained conformations 15152 and 15153 were then submitted to rigid ligand docking within the binding site of TraR as reference protein. Results reveal a similar overall orientation as compared to the natural ligand but with significant differences in several interactions. While the hydrogen bonds involving the NH and the carbonyl of the amide functional group with Asp70 and Tyr53 are conserved, the carbonyl of the lactone is deprived of the hydrogen bond with Trp57 and the intra-cyclic oxygen of the lactone is involved in a hydrogen bond with this residue. On the chain side, the 3-(4-bromophenyl)propanoyl chain induces attractive interactions with Tyr53 and Tyr61. These attractive interactions and this new hydrogen network can be considered as possible causes for explaining the antagonistic activity of this compound (Figure 8).

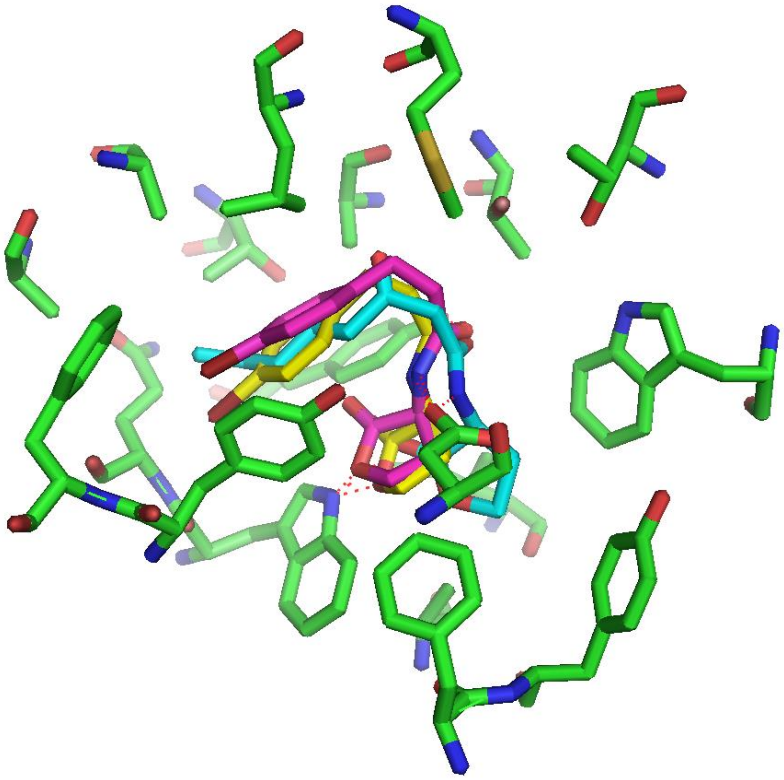


Figure 8: Rigid ligand docking results of selected conformations for 3-(4-bromophenyl)propanoyl homoserine lactone (BPPHL) within the binding site of TraR (yellow 15152, magenta 15153 and cyan co-crystallized conformation of 3-oxo-octanoyl homoserine lactone).

4. Conclusion

A method based on conformation analyses, torsion angle filter and subsequent rigid ligand docking experiments has been applied to AHLs and analogues to investigate bioactive conformations. This method allows the data for the conformations recognized by their cognate transcriptional regulator to be retrieved, ready to be used as starting files for the subsequent rigid ligand docking experiments. This latter step led to low RMSD values indicating the robustness of

the method. The proposed protocol shows excellent consistency between calculation and X-ray structural data.

The method allowed to investigate the putative binding mode of three AHL-type QS inhibitors analogues for which no X-ray data is available. For 2-hexenoyl homoserine lactone and 3-oxo-4-phenylbutanoyl homoserine lactone, new interactions in the hydrophobic zone are observed, and and for broad spectrum QS inhibitor 3-(4-bromophenyl)propanoyl homoserine lactone, attractive interactions for the side chain and a different hydrogen network on the lactone-amide moiety have been found. By providing insights in the modes of action of reported QS modulators, the present method can therefore be considered as a complementary tool in studies directed to the design of new AHL analogues as QS modulators.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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