Anti-Virulence Strategy Targeting Sortase A

A Structural Investigation of the Sortase A Enzyme, and the Identification, Synthesis, and Evaluation of Sortase A Inhibitors

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To my father

Abstract

The emergence of multi-resistant bacteria and their continuous spread is one of the greatest challenges when treating bacterial infections. Increased understanding of bacterial pathogenesis has revealed new strategies for treating bacteria-mediated diseases. Targeting virulence factors or virulence-mediated mechanisms is one strategy which is believed to cause less selective pressure and thereby resistance development since it would not affect bacterial growth or survival. The bacterial enzyme sortase A (SrtA) anchors the majority of virulence associated proteins to the bacterial cell wall and is a promising target for development of anti-virulence drugs. This thesis describes the investigation of SrtA conformations, derived from MD simulations, and their performance in virtual screening (VS) using a diverse set of active inhibitors and their decoys. From the performance results, SrtA structures can be selected for further docking studies and VS. Further, novel SrtA inhibitors were discovered using high throughput and fragment based screening (HTS and FBS) as starting points for hit selection. Hits were synthetically modified and evaluated using several different biochemical and biophysical assays. The HTS resulted in the discovery of substituted thiadiazoles with inhibitory activities in the low micromolar range. They probably act by binding covalently to the active site cysteine of SrtA. The fragment screening resulted in the discovery of substituted pyrazoles and isoxazoles as promising starting points for further development into more potent SrtA inhibitors. A hybrid compound combining the knowledge from the HTS and FBS was developed. The hybrid is a potent non-covalent inhibitor as opposed to the HTS compounds. The flavone morin and its effects on SrtA were also investigated, showing that morin might act as both an inhibitor and an activator. Morin seems to bind to the SrtA dimer interface inducing a conformational change in the protein allowing various fragments to bind more efficiently to the active site. This sheds further light on the importance of investigating the inhibitory mechanism of already existing SrtA inhibitors as to get a better understanding of their mode of action, which will be crucial for the development of more potent SrtA inhibitors.

Keywords: Sortase A, structural investigation, molecular dynamics, virtual screening, SrtA inhibitors, fragment based lead generation, FBLG, high-throughput screening, HTS, allosteric modulation.

List of Publications

This thesis is based on the following publications and manuscripts, which are referred to in the text by the Roman numerals.

I **Exploration of multiple sortase A protein conformations in virtual screening**
Chunxia Gao, Ivana Uzelac, Johan Gottfries, Leif A. Eriksson
Scientific reports **2016**, 6:20413 (DOI: 10.1038/srep20413)

II **Discovery and development of substituted thiadiazoles as inhibitors of *Staphylococcus aureus* sortase A**
Patrick M. Wehrli,* Ivana Uzelac,* Tomas Jacso, Thomas Olsson, Johan Gottfries
Bioorganic and Medicinal Chemistry **2019**, 27:115043
(DOI: 10.1016/j.bmc.2019.115043)

III **Identification, synthesis, and evaluation of substituted pyrazoles and isoxazoles as *Staphylococcus aureus* sortase A inhibitors**
Ivana Uzelac, Tomas Jacso, Thomas Olsson, Patrick M. Wehrli, Johan Gottfries
Submitted Manuscript

IV **Is morin both an activator and an inhibitor of sortase A?**
Ivana Uzelac, Chunxia Gao, Tomas Jacso, Thomas Olsson, Leif A. Eriksson, Johan Gottfries
Manuscript

* Equally contributing authors.

The Author's Contribution to Papers I–IV

I Contributed to designing the study and evaluation and selection of actives. Performed some of the MD simulations and dockings. Provided minor contribution to the writing of the manuscript.

II Contributed to the formulation of the research problem. Performed the biophysical and biochemical assays of synthetically developed hits. Performed also extensive data analysis and the investigation of oxidation of sortase A and the mode of action of the inhibitors. Significant contribution to writing of the manuscript.

III Formulated the research problem, performed the synthesis and the majority of the assays, analyzed the data, and wrote the manuscript.

IV Formulated the research problem, performed the majority of the experimental work, analyzed the data and interpreted the results, and wrote the manuscript.

List of Abbreviations

Ala (A)	Alanine
Abz	2-Aminobenzyl
Arg (R)	Arginine
CPMG	Carr-Purcell-Meiboom-Gill
CWA	Cell wall anchored
Cys (C)	Cysteine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dnp	2,4-Dinitrophenyl
Et	Ethyl
Glu (E)	Glutamic acid
FBLD	Fragment based lead discovery
FBS	Fragment based screening
FRET	Fluorescence resonance energy transfer
Gly (G)	Glycine
h	Hour(s)
His (H)	Histidine
HMQC	Heteronuclear multiple-quantum correlation
HRMS	High resolution mass spectrometry
HSQC	Heteronuclear single-quantum correlation
HTS	High-throughput screening
IC_{50}	Half maximal inhibitory concentration
Ile (I)	Isoleucine
Lys (K)	Lysine
LCMS	Liquid chromatography–mass spectrometry
Leu (L)	Leucine
MD	Molecular dynamics
MoA	Mode of action
MRSA	Methicillin-resistant *Staphylococcus aureus*

n.a.	Not applicable
n.d.	Not determined
NMR	Nuclear magnetic resonance
PAINS	Pan-assay interference compounds
PDB	Protein Data Bank
Pro (P)	Proline
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
SAR	Structure-activity relationship
SD	Standard deviation
Sol	Solubility
SPR	Surface plasmon resonance
SrtA	Sortase A protein
THF	Tetrahydrofuran
Thr (T)	Threonine
Trp (W)	Tryptophan
VS	Virtual screening
X	Variable amino acid

Table of Content

1.1 THE GLOBAL PROBLEM OF ANTIBIOTIC RESISTANCE

The emergence and continuous spread of bacteria showing resistance to multiple antibiotics, such as methicillin-resistant *Staphylococcus aureus* (MRSA), is a major public health problem.[1] Bacterial resistance to antibiotics was first discovered not long after the beginning of the antibiotic era. However, the number of multidrug resistant bacteria has grown dramatically over the past decades, which calls for new strategies for treating bacterial infections.[2-3]

Traditional approaches for treatment of infectious diseases rely on the inhibition of vital bacterial functions such as cell wall synthesis, DNA replication, and protein synthesis.[4] These approaches exert substantial stress on the target bacterium favoring the selection of resistant subpopulations. The evolution of bacterial resistance is however a natural process, and most likely resistance to many, if not all, natural product based antibiotics already existed before their discovery by man, and would have existed even in absence of human mismanagement.[3, 5] Still, the unnecessary prescription and overuse of antibiotics, as well as the use of antibiotics for non-curative reasons, contribute to the fast emergence and to the global spread of bacterial resistance.[6]

In 2015, the World Health Organization (WHO) initiated a global action plan with the objective to improve awareness and understanding of antimicrobial resistance, strengthen the surveillance and research, reduce incidence through prevention measures, optimize the use of antibiotics, and to ensure sustainable investment which takes into account the needs of all countries.[7]

Diminished pharmaceutical investment also adds to the problem of lacking therapies and discovery of novel drugs to treat the increasing number of antibiotic-resistant bacterial infections. Antibiotic management policies and regulatory hurdles limit the return of investment. This, along with the inevitable emergence of new resistance strains and short treatment time compared to other chronic diseases make the discovery and development of antibiotics far less profitable and less appealing.[6, 8]

Numerous international and national initiatives aimed at encouraging the research and development (R&D) of antimicrobials have been implemented.[9] An extensive review by Renwick *et al.*[10] presents a framework on assessment of incentive strategies for discovery and development of novel antibiotics. Although current programs are important initial steps in R&D of novel antibiotics they lack in coordination across all incentives and tend

to prioritize funding projects in early-stage discovery rather than late-stage clinical development.

This is a challenging task and the ideal solution would address both the public health priorities, i.e. the growing need for a sustainable solution, and at the same time tackle the shortage of new compounds on the market and operate within implementation constraints.[10] In addition, truly novel antimicrobials with novel mechanisms of action effective against the most resistant pathogens need to be discovered and developed.

1.2 BACTERIAL RESISTANCE

Bacteria have evolved strategies to withstand environmental challenges such as antibiotic attacks.[11] These strategies include development of mechanisms that permit the bacteria to thrive in the presence of increasing concentrations of an antibiotic. The bacteria acquire and spread resistance by mutations in genes, which are often associated with the mechanisms of action of the antibiotic, or by horizontal gene transfer (HGT), where the bacterium obtains foreign DNA material.[3, 12] Some mechanisms of resistance include reduction of the intracellular concentration of the antibiotic by increased efflux or reduced permeability.[13-14] Other mechanisms involve modification of the antibiotic target by genetic mutation or post-translational modifications which prevent efficient antibiotic binding.[15-16] Direct modification of the antibiotic itself can also occur by addition or modification of a functional group that prevents the antibiotic to bind to its target.[17] *Staphylococci* use mainly two mechanisms for resistance towards β-lactam antibiotics, i.e. expression of an enzyme which modifies the antibiotic by hydrolyzing the β-lactam ring, and the acquisition of a gene encoding a modified penicillin-binding protein that is resistant to β-lactames.[18]

1.3 BACTERIAL VIRULENCE AND ANTI-VIRULENCE STRATEGIES

1.3.1 Virulence

Research involving strategies based on the inhibition of bacterial virulence has gained increased attention.[19-20] The word virulence originates from the Latin word *virulentus* which means "full of poison". Virulence is traditionally described as the capacity of a microbe to cause disease. This is a microbe-centered view distinguishing pathogens from non-pathogens by their expression of virulence factors.[21] Virulence can also be seen as a dynamic phenomenon that not only includes the microbial characteristic but host-related factors as well.[22] In this thesis, the term "bacterial virulence" is used in the former sense.

1.3.2 Anti-virulence strategies

As traditional antibacterial agents target bacterial viability, which strongly favors resistant subpopulations, other strategies for treating infectious diseases are necessary. Targeting virulence has emerged as a promising strategy for treating bacterial infections while evading the problem of resistance. Anti-virulence strategies target virulence-associated mechanisms without inhibiting bacterial growth or killing the bacteria. This would reduce the selection pressure.[19] This strategy has the advantage of having fewer undesirable effects than traditional strategies, maintaining a normal and healthy host microbiota. Anti-virulence drugs may allow the immune system to clear the disease and could be used in combination therapies with other antimicrobials. They further offer new pharmacological targets and the possibility of generating drugs with novel mechanisms of action.

There are currently numerous strategies for inhibiting bacterial virulence under investigation, including inhibition of adhesion and biofilm formation, interfering with gene regulation and bacterial signaling, and inhibition of toxins and specialized secretion systems.[19, 23] Resistance to anti-virulence drugs is however slowly emerging,[24] therefore the robustness of different anti-virulence strategies and their evolution of resistance needs to be evaluated.

1.3.3 Targeting adhesion and biofilm formation

Bacterial adhesion to host cells is a critical step for effective colonization of a host and promotion of disease.[19] Bacterial attachment to host cells also promote biofilm formation protecting the bacteria from the immune system.[25]

Adhesion is often the first step in the infection process, mediated by cell-wall anchored (CWA) proteins[26] which interact with specific receptors on the host cells and initiate the attachment. There are three main types of adhesion-receptor interactions; i) lectin-carbohydrate; ii) protein-protein; and iii) hydrophobin-protein interactions. Lectin-carbohydrate recognition is most common.[27]

Anti-adhesion strategies aim to inhibit the interactions between bacteria and host and to reduce the establishment of infection, e.g. by direct inhibition of adhesins.[28] Another strategy is to inhibit the cell-to-cell signaling by targeting the mechanism of quorum sensing (QS), which is not only involved in adhesion but also in the expression of virulence genes and biofilm formation.[29]

Anti-adhesion strategies involve inhibition of the assembly of CWA proteins on the bacterial cell surface and can be categorized into distinct structural and functional groups, microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)

being the largest group.[26] Gram-positive bacteria use sortase enzymes for the display of CWAs by facilitating the attachment of such proteins to the bacterial cell wall. Sortase A is one of four sortase classes and the most essential for bacterial virulence and has become a potential anti-virulence target for preventing adhesion and biofilm formation.[30]

1.4 SORTASE A

1.4.1 Biological function

Sortase A (SrtA) is a membrane bound cysteine transpeptidase found in most Gram-positive bacteria.[31] It catalyzes the covalent anchoring of surface proteins to the bacterial cell wall, including virulence factors such as MSCRAMMs.[26, 32] SrtA anchors specific precursor surface proteins consisting of a hydrophobic region, a positively charged tail, and a Leu-Pro-X-Thr-Gly (LPXTG) motif.[33] The catalytic site of SrtA contains the highly conserved triad, His120, Cys184, and Arg197 (*S. aureus* SrtA numbering). The mechanism for the surface anchoring of proteins by SrtA is illustrated in Figure 1. SrtA recognizes the LPXTG motif in a surface protein and cleaves it between threonine (T) and glycine (G) resulting in formation of an acyl-enzyme intermediate between the active site cysteine of SrtA and the threonine at the *C*-terminal end of the surface protein. The *N*-terminal primary amine of the cell wall precursor lipid II performs a nucleophilic attack at the thioester bond between SrtA and its cleaved substrate, thereby forming an amide bond between the *C*-terminal threonine and lipid II. The product is then incorporated into the cell wall via transglycosylation and transpeptidation.[33-34] The structure of SrtA will be described in more detail in section 3.2.1.

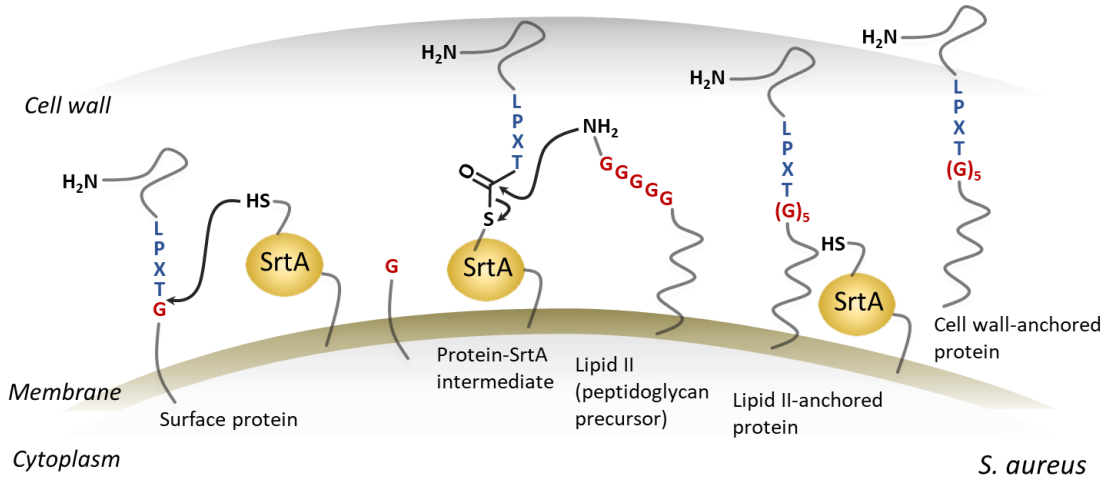


Figure 1. The role of SrtA in anchoring surface proteins to the cell wall envelope of Gram-positive bacteria.

1.4.2 SrtA mechanism

Understanding the exact molecular mechanism of SrtA catalysis has been difficult with conflicting mechanistic models published.[35-38] Frankel *et al.*[39] re-evaluated the overall kinetic mechanism of *S. aureus* SrtA and parts of its reaction mechanism. They observed that SrtA acylation is the rate-limiting step during the transpeptidation reaction. For the detailed mechanism, they propose a reverse protonation of His120 and Cys184 (Figure 2A). The nucleophilic Cys184 thiolate attacks the carbonyl carbon between Thr and Gly in the substrate, forming a tetrahedral intermediate. His120 then protonates the Gly-leaving group resulting in the acyl-enzyme intermediate (Figure 2B).

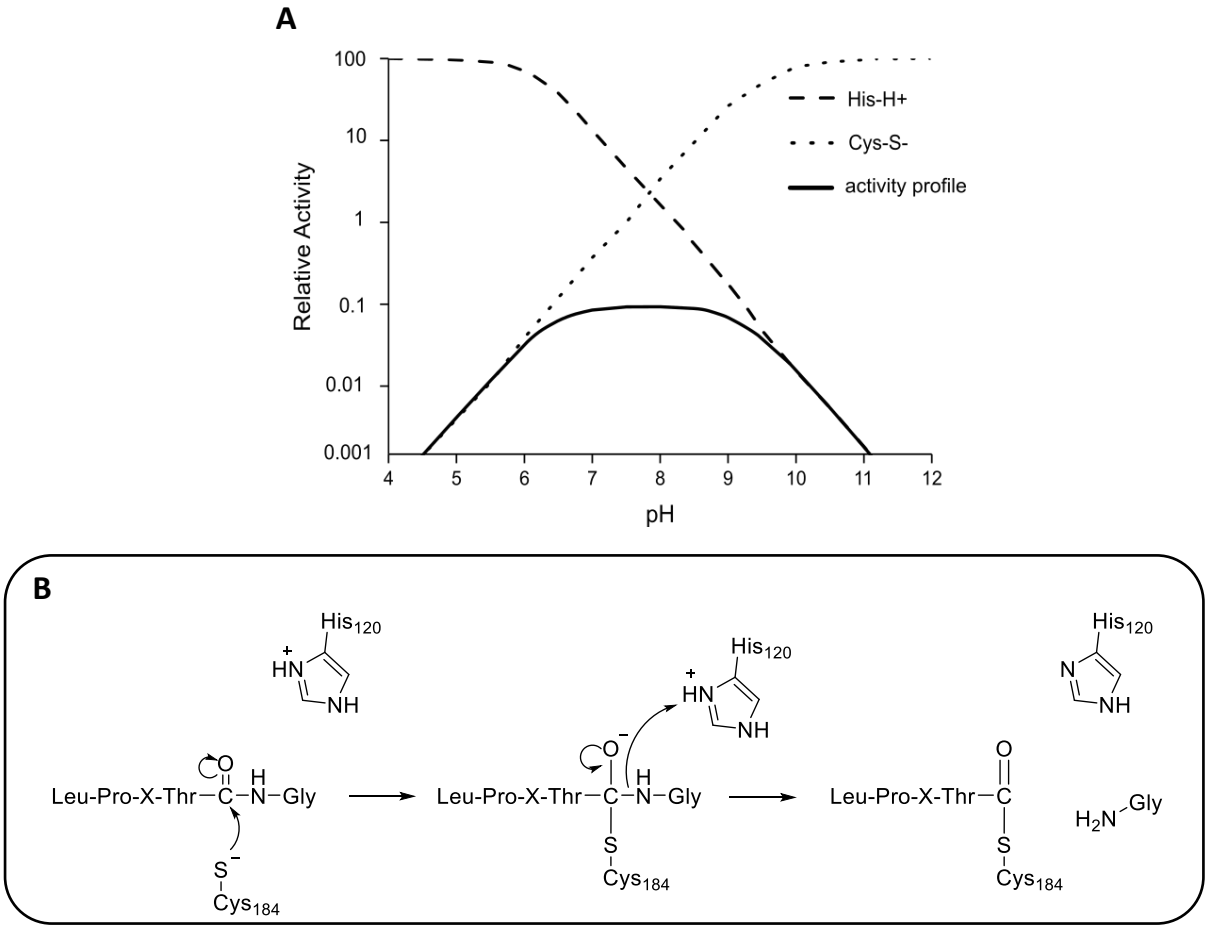


Figure 2. Reverse protonation model for SrtA-catalyzed transpeptidation.[39] A) Simulated SrtA activity invoking the reverse protonation of Cys184 and His120. The relative activity is plotted as a function of pH showing the bell-shaped activity profile of SrtA which fits the overlapped region of reverse protonation of Cys184 and His120. B) Reaction mechanism for SrtA acylation.

1.4.3 SrtA as a drug target

Class A sortases have attracted significant interest as potential drug targets as they are essential for virulence in a number of clinically important pathogens such as MRSA. SrtA plays a housekeeping role in a wide range of Gram-positive bacterial species by anchoring

the majority of surface proteins to the cell wall. Other sortase classes have more specialized roles, anchoring far fewer proteins and are also involved in other processes such as iron uptake.[40]

Deletion of the SrtA gene in *S. aureus* results in a significant decrease in bacterial virulence through loss of binding activity to host proteins such as IgG, fibronectin, and fibrinogen.[41-42] This has also been reported for several other Gram-positive bacteria, such as *Listeria monocytogenesis*[43] and *Streptococcus pneumoniae*.[44] In addition, gene deletion further results in bacteria being more susceptible to macrophage killing.[45] The presence of SrtA in many different pathogens may also allow for development of broad-spectrum drugs. To date, no eukaryotic SrtA homologue has been identified lowering the risk of undesired side effects when targeting SrtA.[46] Several SrtA inhibitors have already been published, confirming SrtA as a druggable target.[47]

1.4.4 Discovery of srtA inhibitors

Identification of "true hits", i.e. compounds with the desired activity confirmed through orthogonal testing, is essential in the early drug discovery. Many compound libraries include reactive, promiscuous, and assay interfering compounds such as Pan-Assay Interference Compounds (PAINS)[48] that makes the recognition of true hits from false hits and false positives challenging. These compounds need to be excluded or considered with caution in further investigation.[49] When a true hit is identified it can be used in further lead optimization in order to obtain pharmaceutically useful drugs.

Discovery strategies used for identifying SrtA inhibitors include screening of natural products or small compound libraries,[47, 50] high throughput screening campaigns (HTS) of small molecules,[51] and computational methods such as virtual screening (VS).[46, 52] The type of inhibitors discovered range from natural product, analogues of natural products, small molecules, and peptides.[53] The following sections will focus on natural products and small molecule inhibitors of SrtA.

1.4.5 Natural products and derivatives thereof as srtA inhibitors

Screening of natural products has led to the discovery of several SrtA inhibitors with IC_{50} values in the micromolar range (Figure 3). One of the first discovered inhibitors was β-sitosterol-3-*O*-glucopyranoside (**1**),[54] extracted from *Fritillaria verticillat*. However, it showed to be bactericidal against *S. aureus*, which is the case for most of the natural SrtA inhibitors. This indicates that they may also act on other targets than SrtA. Other discovered natural products that also show bactericidal effects are berberine chloride (**2**),[55] topsentin (**3**),[56] isoaaptamine (**4**),[57] curcumin (**5**),[58] rosmarinic acid (**6**),[59] and chlorogenic acid (**7**).[60]

Flavonoids including morin (**8**),[61] myricetin,[62] isovitexin,[63] acacetin,[64] and quercetin[65-66] constitute another class of natural products that inhibit SrtA and biofilm formation of *S. aureus* without affecting bacterial growth, thus maintaining the bacterial viability.

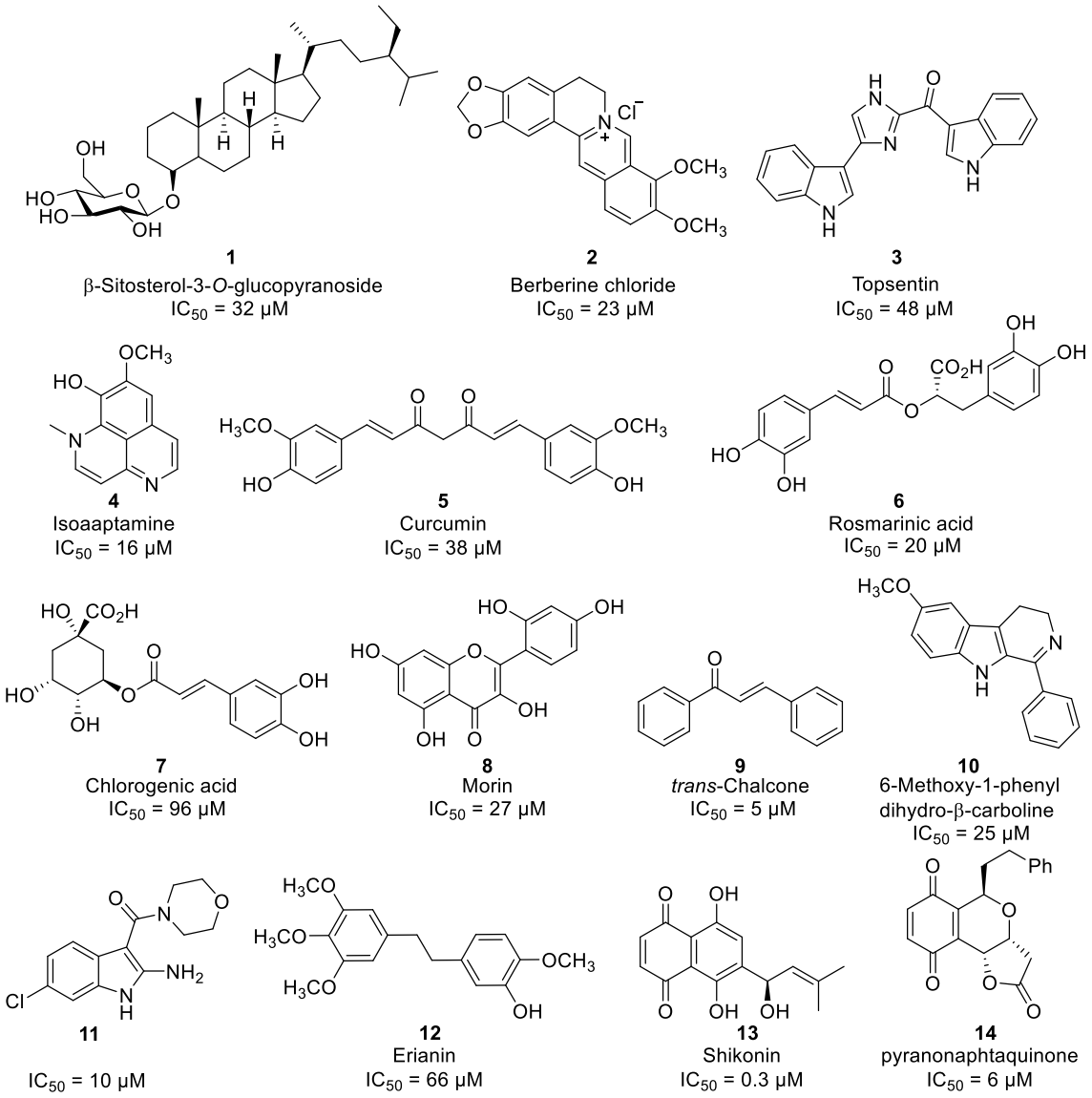


Figure 3. Natural products (**1–14**) as SrtA inhibitors.

Further, *trans*-chalcone (**9**)[67] was shown to inhibit SrtA and biofilm formation in *Streptococcus mutans* by binding irreversibly to the SrtA cysteine. Compound **10** is an improved analogue of indole-containing natural products showing inhibitory activity against SrtA with no inhibition of bacterial growth.[68] The use of indole as scaffold in compound collections screened for inhibitory activity of SrtA, lead to the discovery of **11**.[69] This study further showed that the free amine and the morpholine oxygen are essential for activity. When removed a total loss of activity is observed, while removal of chlorine showed a two-fold loss in activity. Erianin (**12**), a natural product extract recently discovered, although similar

to some of the other natural product-based inhibitors it does not affect bacterial growth.[70] The naphtoquinones shikonin (**13**) and alkannin were discovered when screening a library containing 2000 approved drugs of or candidates in clinical trials. Both compounds showed to inhibit SrtA with IC_{50}-values in sub-micromolar range, however they also inhibit bacterial growth.[71] The study also presents pyranonaphtaquinone (**14**)[71] as a potent inhibitor with minimal effect on bacterial growth from screening of a natural product-based library.

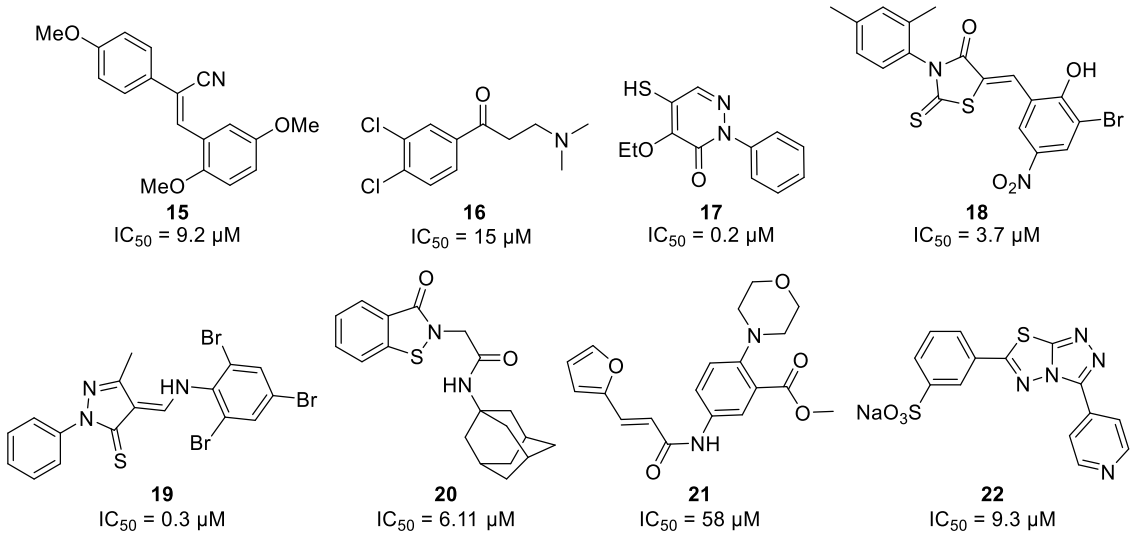


Figure 4. Small molecule SrtA inhibitors (**15–22**).

1.4.6 Small molecule sortase A inhibitors

A number of SrtA inhibitors have been discovered using HTS and *in silico* screening of small molecules libraries (Figure 4). In one study,[50] the diarylacrylonitrile **15** was the most potent inhibitor optimized from a hit discovered by HTS. It inhibits SrtA reversibly, however at high concentrations it also inhibits bacterial growth which would suggest off-target or toxic effects. Aryl(β-amino)ethyl ketone (AAEK) **16** showed to inhibit SrtA irreversibly, forming a covalent bond to the SrtA cysteine.[72] Suree *et al.*[51] discovered three classes of small molecule SrtA inhibitors, pyridazinones (**17**), rhodanines (**18**), and pyrazolethiones (**19**), from optimization of HTS hits. These compounds inhibit SrtA reversibly with IC_{50} values in the sub-micromolar range. The most active inhibitor was **17**, which is also the most potent inhibitor reported to date (IC_{50} = 0.2 μM). Another study using HTS for generation of hits discovered irreversible benzisothiazolinone-based inhibitors such as **20**, where the 3-oxobenzo[*d*]isothiazol-2(3*H*)-yl moiety is responsible for the covalent binding to the active site cysteine. They are also able to react with other Cys-containing enzymes and show cytotoxicity, which does not make them good lead candidates.[73] VS of a small compound library further led to the discovery of the optimized

hit **21**.[74] Structure activity relationship (SAR) studies showed that the double bond was crucial for its activity. A new class of bicyclic triazolo-thiazole derivatives was discovered by *in silico* screening of a small compound library by combining scaffold hopping and molecular docking, using topsentin (**3**, Figure 3) as the starting point. Subsequent optimization of one of the hits resulted in the identification of **22**, acting as a reversible inhibitor with no influence on bacterial growth.[56]

Research in anti-virulence inhibitors using SrtA as the target has resulted in the discovery and development of potent inhibitors with IC_{50} values in the low micromolar range. Despite the efforts, many of the inhibitors developed exhibit selectivity issues and toxic effects with unknown mechanism of action (MoA) and need further optimization to be therapeutically useful. In addition, further investigations of ligand-SrtA binding modes are required.

2 AIMS OF THE THESIS

The overall aim of the work presented in the thesis was to investigate structures of the sortase A enzyme for virtual screening. Another aim was to discover new sortase A inhibitors using a variety of screening methods and to design, synthesize, and biologically evaluate these inhibitors and their modes of action based on these results.

The specific objectives of the thesis were:

- Exploring the flexibility of sortase A using molecular dynamics simulations and assessing the performance of different conformations in virtual screening (Paper I).

- Discovery and development of sortase A inhibitors by high-throughput screening and fragment based screening (Papers II and III).

- Investigate the mode-of-action of morin (Paper IV)

3 CONFORMATIONAL INVESTIGATION AND VIRTUAL SCREENING OF SORTASE A INHIBITORS (PAPER I)

Three-dimensional protein structures are the basis of structure-based drug design. At present, the PDB[75] holds more than 140,000 protein structures. Proteins are flexible entities and dynamics plays a key role for their function. Conformational changes are often observed between PDB structures of the same protein upon substrate or ligand binding.[76] The experimental data of structures determined by X-ray diffraction from crystals, or using NMR spectroscopy or cryo-electron microscopy (CryoEM), are averages.[77] Therefore, theoretical techniques are often used to obtain a representation of the dynamic properties, of e.g. a protein or protein-ligand binding, by conformational sampling. The conformational ensemble can be used as an alternative to the single structures from PDB.[78]

3.1 COMPUTER-AIDED DRUG DESIGN

3.1.1 Molecular dynamics simulations

Classical molecular dynamics (MD)[79] simulation is a computational method used to simulate the motions of atoms and molecules. Trajectories of particles, starting from a defined conformation, are determined by solving Newton's law of motion. The total force acting on the system is described by a force field and determines the evolution of the system after a small time step. In 1959 the first MD simulation was accomplished by Alder and Wainwright using a hard-sphere model,[80] a method which is widely applied in modelling of biomolecules but also in other areas.[81]

A general workflow of the MD procedure is shown in Figure 5, starting by defining the force field and molecular topology. The interatomic forces over a small time step are then computed followed by solving Newton's equation of motion, to obtain new velocities and positions. New geometries can then be determined by repeating the steps until the energy or geometry stabilizes.

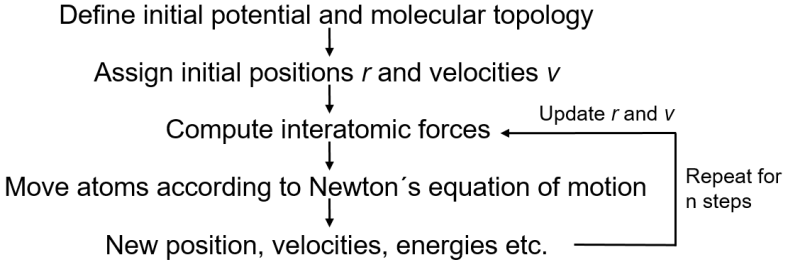


Figure 5. Molecular dynamics workflow.

3.1.2 Protein-ligand docking

Molecular docking is widely used to predict the most favorable structure of the intermolecular complex formed between two molecules, in this case the ligand and its target protein.[82] First efforts of molecular docking involved docking of rigid bodies, which is the most basic approach to sample the conformational space, a "key and lock" approach.[83] It has evolved since then, incorporating flexibility of the ligand and even protein flexibility. Docking using flexible proteins still remains less common due to the complexity of the systems.[84] The flexible-ligand approach is most commonly used and involves docking of different ligand conformations and orientations (poses) within a given target protein. The ligands are often docked in a predefined pocket of the protein. Different search algorithms generate the different poses and their binding affinity is calculated and ranked using scoring functions. Scoring functions can be divided into three classes.[84] Force-field based scoring,[85] which sums the ligand binding energy of intermolecular and intramolecular energies using force fields; empirical scoring,[86] sums several parameterized functions to reproduce experimental data such as binding energies and conformations, and depends highly on the training set; and knowledge-based scoring functions,[87] use data from already known protein-ligand complexes to estimate atomic interactions.

Virtual screening (VS) is the docking of large numbers of compounds in a protein target and the ranking of actives early in the docked compound library (the "early recognition problem"), and can be evaluated using different methods.[88-89] The enrichment factor (EF),[90] is the measure of how many more actives are found within a defined fraction of the VS, relative to a random distribution. While EF addresses the early recognition problem by focusing on the true positive fraction it misses the ranking or the goodness of the VS. Receiver operating characteristics (ROC),[91] area under the ROC curve (AUC),[91] is the probability that an active compound will be ranked earlier than an inactive one. It summarizes the quality of the VS but is not sensitive to early recognition. Robust initial enhancement (RIE)[92] uses a continuously decreasing exponential weight as a function of rank, which addresses the limitations of EF but lacs the advantages of ROC. The Boltzmann-enhanced discrimination of ROC (BEDROCK),[89] addresses the early recognition in a ranking method.

3.2 VIRTUAL SCREENING OF SORTASE A INHIBITORS

3.2.1 SrtA structure

There are several SrtA enzyme structures available in the PDB, determined by either NMR spectroscopy or X-ray crystallography. The SrtA structures consist of an eight-stranded β-

barrel fold including conserved active site residues and the catalytic His120, Cys184, and Arg197. In addition, SrtA consists of a hydrophobic *N*-terminus that is presumably embedded in the membrane and a *C*-terminal catalytic region. SrtA mutants, with removed transmembrane parts, show activity and are usually used in both experimental and computational studies.[93] The structures available in PDB exist in both apo (unbound) and holo (bound) forms and although they are homologues, they are structurally different. The two substrate bound structures 2KID[94] (NMR structure covalently bound to LPAT*) and 1T2W[95] (crystal structure, C184A mutation, bound to LPETG) differ significantly in how the substrate is oriented and in the positioning of the β6/β7 and β7/β8 loops (Figure 6). In the crystal structure, the LPETG substrate is bound to a shallow solvent-exposed part of the pocket located away from the active site. The catalytically important residues are also improperly oriented and positioned for catalysis, probably due to the C184A mutation preventing covalent binding. In the NMR structure, the substrate is positioned deeper in the pocket as a result of the opening of the β7/β8 loop, in addition Cys184 and His120 are properly aligned for partaking in thioester formation. It was first argued that the crystal structure represents only non-specific binding of the substrate and that the NMR structure is more representative for binding. This has been questioned by Suliman *et al.*[96] who suggest that SrtA undergoes a range of structural rearrangements upon ligand binding. They hypothesize that the crystal structure represents the initial substrate binding prior to catalysis, which after substantial conformational change moves the substrate deeper into the active site pocket for catalysis to occur.

3.2.2 Virtual screening of SrtA inhibitors

VS has been applied for identification of novel hits as potential SrtA inhibitors and has resulted in the identification of several compound classes.[52, 74, 97-99] Different *in silico* approaches have been used where Chan *et al.*[52] performed one of the very first VS of potential SrtA inhibitors. An initial docking of a small molecule library was made in the 2KID structure followed by re-docking the best scored ligands in MD cluster centroids resulting in 15 hits for further experimental evaluation. Another group has used flexible docking to place ligands into the active site resulting in the identification of **21** and other analogues.[74] Triazolo-thiazole derivatives such as **22** were discovered by scaffold hopping using a template ligand in combination with molecular docking in the 2KID structure using both experience-based and force field-based scoring function (Figure 4).[98] VS using a pharmacophore model of already known inhibitors as filter was another interesting approach.[99] Here the 2KID structure was also used for the docking, generating indole

derivatives as the top ranked ligands followed by MD simulations to further investigate the stability of the best ligand-protein complex.

Although the *in silico* methods have generated several possible SrtA inhibitors only a few would be suitable as starting points for further optimization because of selectivity issues and toxicity. This could possibly be improved by using more appropriate libraries but also by evaluating existing structures for the VS.

3.3 EXPLORATION OF SORTASE A CONFORMATIONS IN VIRTUAL SCREENING (PAPER I)

In this study, several SrtA structures have been studied for improving VS performance. Protein conformations and their performance in molecular docking were explored because of large differences in the reported SrtA structures and their binding properties. MD simulations were used to sample the geometries of both apo and holo SrtA NMR and crystal structures used in the docking.

3.3.1 Molecular dynamics simulations

Molecular dynamics simulations over 200 ns were performed using four SrtA structures, apo and holo NMR structures (PDB ID: 1IJA and 2KID) and apo and holo X-ray structures (PDB ID: 1T2P and 1T2W). Cα root mean square deviations (RMSD), root mean square fluctuations (RMSF), and the radius of gyration were used to assess the structural variations from each simulation. The RMSD for the substrate bound SrtA X-ray structure was stable at ~1.5 Å after 5 ns whereas the other three structures stabilized after longer simulation times at higher RMSD. For both X-ray and NMR apo structures the loop regions showed to be rather dynamic from RMSF, particularly loop β6/β7 (Figure 6). This is not the case for the two substrate-containing structures where this loop is less dynamic due to immobilization of the loop by substrate binding. From the radius of gyration three of the structures showed to be quite stable. It was only the holo SrtA NMR structure that displayed more dynamic movement probably due to the more extended β7/β8.

The active site flexibility was also investigated, selecting only residues in the active site for the RMSD calculation. Large flexibility was observed for both NMR structures and the apo X-ray structure while the holo SrtA X-ray structure was stable at ~2.5 Å. The holo SrtA NMR structure showed to have a more closed conformation due to the orientation of loop β7/β8, whereas the holo SrtA X-ray structure showed a more open form because of the positioning of the substrate. As expected, both apo structures showed fluctuations in the active site. From the MD simulation of each of the four SrtA structures, twenty snapshots

with evenly spaced intervals of 10 ns were retrieved and used as the docking targets in the VS in order to sample the flexibility of their active sites.

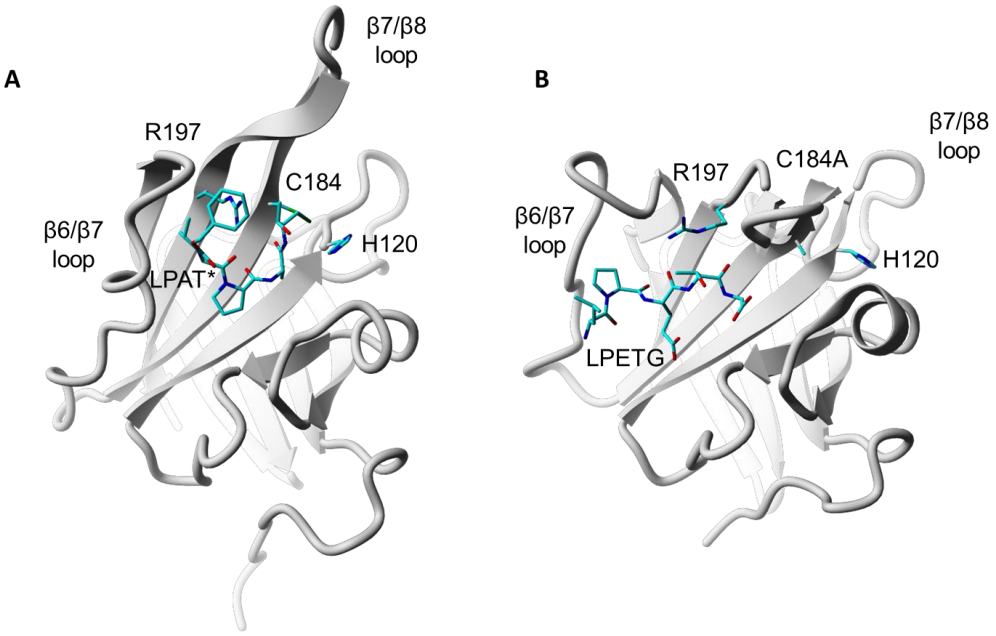


Figure 6. A) Holo SrtA NMR structure (PDB ID: 2KID) with LPAT* covalently bound to the active site Cys184. B) Holo SrtA X-ray structure (PDB ID: 1T2W) with LPETG bound to the active site. The catalytic amino acids Arg197, Cys184, and His120 and the β6/β7 and β7/β8 loops are annotated.

3.3.2 Virtual screening and evaluation of docking performance

Ten active compounds were selected based on structural diversity amongst published SrtA inhibitors (Figure 7). For each active compound, 50 decoys were selected from the ZINC database that are physically similar but topologically dissimilar to the actives to evaluate the VS.[100] All compounds were docked using Glide[101] with extra precision (XP) scoring functions. The virtual screening was evaluated using EF, ROC, BEDROC, AUC, and RIE, described in section 3.1.2 above (Tables 1–4, Paper I). EF and BEDROC (α = 160.9) showed that the two NMR structures (2KID and 1IJA), which adopt a more "closed" conformation during the MD simulation, performed better than the crystal structures, which adopt a more "open" conformation. The substrate bound NMR structure performed slightly better than the one without substrate and gave two structures with EF = 20. The ranking performance for each conformation evaluated using AUC showed that, overall, both substrate bound structures performed better than the apo structures. The apo NMR structure however had the highest AUC (0.8) after 70 ns. RIE and BEDROC gave similar overall results for all except the apo crystal structure which performed worse.

From the evaluation, a few of the snapshots generated from the MD simulations having the highest combined scores may be used in VS of SrtA inhibitors. The study may also be

extended in several ways. A different selection strategy of snapshots might give different outcomes, selecting the most stable conformations instead of snapshots every 10 ns. Another important aspect is that the actives were selected based on diversity and this might not be suitable for SrtA, which has a very flexible binding site. In a study by Lou *et al.*[97] 11 actives were selected and 210 decoys generated, docked in *S. mutans*, and scored by multiple scoring functions to evaluate the VS. They managed to obtain a high AUC (0.877) and this might be because all selected actives came from the same structure class, the flavonoids. Dividing the actives into structure classes and performing parallel docking in order to obtain a starting structure may be a better strategy but it may also cause bias towards that particular structure class and other types of structures may therefore be missed. The actives need to be selected more carefully, including information on binding mode or confirmation that they bind at the desired site, in this case the catalytic site. The large and flexible binding pocket of SrtA has thus shown to be a challenging target for VS.

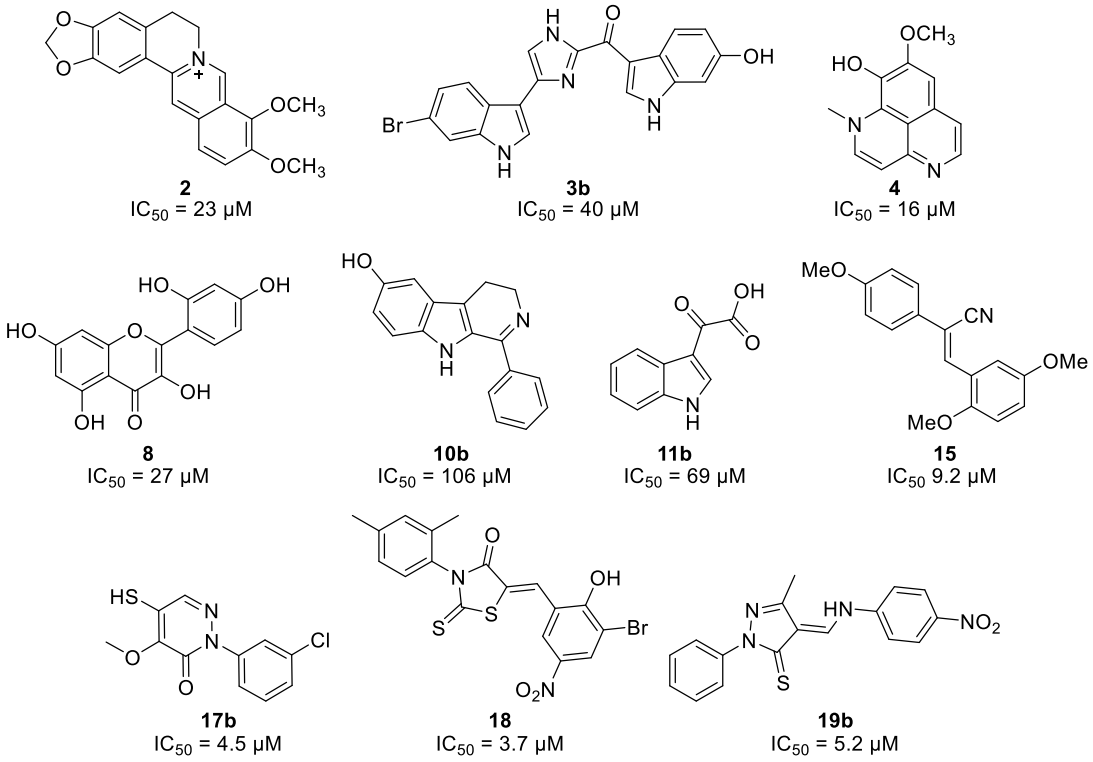


Figure 7. Selected known SrtA inhibitors used in the VS.

3.3.3 Summary Paper I

Investigation of SrtA conformations and their VS performance has been explored. MD snapshots were used as structures in the VS to sample the flexibility of SrtA. The active site is surrounded by several loops which makes it dynamic and flexible and a challenging target for VS. From analyzing the VS performance snapshots can be selected for further

docking studies. The study may also be extended by investigating how different ways of selecting MD snapshots, actives, and libraries for docking may influence the VS performance. Since SrtA has an active site surrounded by loops, other docking approaches may be more suitable for modeling the effect the ligand has on the protein structure, such as induced fit docking.

4 DISCOVERY OF NOVEL SORTASE A INHIBITORS (PAPERS II AND III)

Even though several potent SrtA inhibitors have been discovered, many of them lack selectivity, show toxic effects, and need to be further optimized to be useful as potential anti-virulence drugs. Herein, a high throughput screening (HTS) and a fragment based screening (FBS) was performed with the aim to find new starting points for optimizing hits into SrtA inhibitors. Hits were synthetically modified and evaluated using different biochemical and biophysical assays.

4.1 COVALENT VS. NON-COVALENT INHIBITORS

Covalent inhibitors are often associated with toxicity. While many drugs act by covalently modifying their target, such as acetylsalicylic acid[102] and penicillins, or for which metabolites are covalent inhibitors, such as omeprazole.[103] A common focus of modern drug discovery has been to maximize the strength of noncovalent molecular interactions rather than developing covalent inhibitors. In 2005 Robertson[104] wrote 'drug discovery programs never set out to make irreversible inhibitors'. Despite this 35% of the enzyme targets in that same study are irreversibly inhibited.

Non-covalent interactions, such as hydrogen bonds, ionic interactions, and hydrophobic interactions, are weaker than covalent bonds. Covalent inhibition includes both reversible and irreversible inhibition. Reversible inhibition can be divided into two classes; one typically involves initial non-covalent binding followed by covalent bond formation, which is reversible. For the other class, the inhibitor is recognized as a substrate, which is then cleaved by the enzyme. Irreversible inhibition includes for example residue-specific reagents, which are selective towards particular nucleophiles rather than particular binding sites.

Some of the potential advantages of the sustained duration of action by covalent inhibitors can be a higher biochemical efficiency, usage of lower doses, and the ability to overcome competing endogenous ligands. Such inhibitors may also be used for targeting shallow "undruggable" binding sites.[105] Potential disadvantages associated with covalent inhibitors are the difficulty to assess their selectivity and reactivity, possible modification of off-targets, and that they are not suitable for mechanisms requiring short residence time or if the target protein has a rapid turnover.

4.2 FRAGMENT BASED LEAD DISCOVERY (FBLD) VS. HIGH THROUGHPUT SCREENING

In early drug discovery, the identification of hits with the desired activity or binding to the target is essential for further use in hit to lead optimization giving more pharmaceutically relevant compounds.

In this work, hits acting on SrtA were identified using both HTS and FBLD. FBLD has emerged as an alternative to, and is often used in parallel with, HTS for identification of new chemical leads.[106] Traditional HTS is the process by which large numbers of compounds can be tested for activity in an automated fashion. FBLD involves screening of low molecular weight molecules that typically follow the "rules of three" (Ro3),[107] i.e. i) molecular weight ≤ 300 Da; ii) clogP ≤3; iii) number of hydrogen bond acceptors (HBA) and donors (HBD) ≤ 3; and iv) number of rotatable bonds ≤ 3. Thus they are equivalent to Lipinski's rule of five, but for fragments.

Fragment libraries further have the advantage of being smaller (10^3-10^4 compounds) than traditional HTS libraries (>10^5 compounds) because of fewer possible fragment sized molecules than lead- or drug-sized ones (Table 1). The chemical space can therefore be explored more efficiently even though the library is significantly smaller.[108] Although HTS hits usually bind with higher affinity, optimization can be challenging because of their complexity, especially for more intricate targets because of the difficulty of finding the key interactions in a multifunctional compound. Hit rates for detecting binding of a small molecule fragment have shown to be higher than for detecting hits of full-sized ligands.[109] This has directed more attention into identification and optimization of low molecular weight compounds when screening. Fragments can be optimized into larger molecules in a stepwise and controlled manner by subsequent linking,[110] growing,[111] or merging[112] of fragments.

Detecting small molecules with low affinity requires highly sensitive biophysical assays, e.g. NMR[113] spectroscopy, surface plasmon resonance (SPR),[114] thermal shift (T_s) assays, and X-ray crystallography.[115] Problems with biochemical and cell based assays are often that they are not suitable for detecting weak binders. In addition, high concentrations regularly lead to false positives in biochemical assays. The high concentrations necessary for FBLD require that the fragments are relatively soluble, which can be an advantage later on in the optimization phase. Advantages and limitations of FBLD and HTS strategies are listed in Table 1.

Table 1. Advantages and limitations of FBLD and HTS.

FBLD	HTS
Smaller libraries ~10^3 compounds (<300 Da)	Larger libraries >10^5 compounds (>300 Da)
Higher coverage of chemical space	Lower coverage of chemical space
Requires well characterized targets	Broad range of targets
Low-affinity hits (K_d ~ μM to mM)	High-affinity hits (IC_{50} in nM to μM range)
Step-by-step optimization increasing molecular size	More complex optimization
Biophysical screening methods (low/medium-throughput, require larger amounts of compound and protein)	Biochemical assays (high-throughput, require less protein)

4.2.1 Compound libraries

There are a number of commercially available libraries which range in size and focus. Generally, libraries should not contain functional groups that may contribute to additional reactivity, toxicity, or false positives[116-117] such as reactive covalent modifiers (e.g. Michael acceptors and epoxides), chelators, or aggregators. Such compounds can be found as hits in an assay without specific binding affinities and are referred to as PAINS.[48] The removal of these compounds is especially important when a fragment library is screened, to prevent interaction between fragments since they are often screened in mixtures. The selection of assays is also of particular importance as different assays identify different hits. Orthogonal secondary assays are therefore often used to confirm hits.

4.3 BIOPHYSICAL AND BIOCHEMICAL METHODS

Screening of small molecules often requires other techniques than screening of fragments because of the large difference in affinity.

In this study, ligand-detected NMR spectroscopy was used as the primary assay for the fragment screen, which was followed up by protein-detected NMR experiments. Two functional assays (FRET based and HPLC based assays) were used to confirm biological activity. The HTS was performed using a FRET based assay as the initial assay and ligand- and protein-detected NMR experiments to confirm binding.

4.3.1 NMR spectroscopy techniques for screening of fragments

Protein-detected NMR spectroscopy pioneered the field in the 1990s, applicable both to detect weakly binding fragments and to guide optimization.[118] Ever since NMR spectroscopy was first used in screening of fragments, several approaches have been applied to facilitate the process of FBS. The most common methods used in primary screening of fragments in 2017 were one-dimensional ligand-detected NMR methods.[119] The methods are fast with higher throughput than for protein-detected experiments and are not limited by the size of the biomolecule. Further, expensive isotopically labelled biomolecules are not needed, the methods do not require high concentrations of the target, and they allow measuring of mixtures of fragments in one sample. Ligand-detected NMR methods exploit the differences in the physical properties of small molecules in solution and when bound to the target. Although it measures the signal from the fragment when it is in solution, it also gives information about the fragment in its bound state. Since the fragment, when bound to the target, adopts the properties of the target rather than a small molecule, and is in fast equilibrium between free and bound state.

There are several one-dimensional ligand-detected NMR methods available such as saturation transfer difference (STD),[120] WaterLOGSY,[121] and Carr-Purcell-Meiboom-Gill (CPMG)[122-123] relaxation dispersion. In this study CPMG relaxation dispersion experiments were used to determine binding of fragments to SrtA. CPMG relaxation dispersion exploits the different T2 relaxation properties of a ligand in its free and bound state. Larger molecules, such as proteins, have slower tumbling rates than smaller molecules in solution, which leads to faster relaxation of transverse magnetization (shorter T2). When a ligand binds to its target it will adopt the relaxation properties of the target. In CPMG relaxation dispersion experiments the spectrum is acquired after a delay time which acts as a T2 filter, resulting in a decrease in intensity of signals from bound ligands (Figure 8).[124] The reversibility of binding can be determined with competitive binding experiments using a known binder to compete for the binding site, which results in subsequent recovery of the signal.

Protein-observed NMR methods[118] are more time consuming than ligand-observed methods because they require two-dimensional or higher dimensionality experiments. Isotopically labelled target and higher concentrations of the target are needed making these experiments more costly. Protein-observed NMR methods however offer structural information not available from any other NMR-based method.

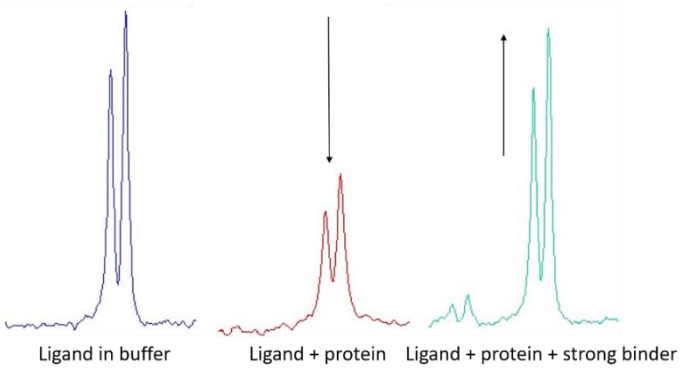


Figure 8. NMR spectra from three CPMG experiments (spectra are shifted for clarity). Initial spectrum of ligand (blue). Spectrum recorded after addition of protein to ligand and the decrease indicates binding (red). Displacement with a stronger ligand, where recovery of signal indicates competition between the two ligands (green).

One of the most frequently used experiments is heteronuclear single-quantum correlation (HSQC), where a two-dimensional spectrum is obtained for the correlation between ^{1}H and the directly bound ^{15}N (or ^{13}C) giving one peak for each amide N-H. Titrating a ligand to a sample containing the target and measuring ^{15}N HSQC after each titration point allows the determination of the ligand binding site because of changes in chemical shifts upon binding. The shift changes may also arise due to binding at a distant site or dimerization through induced conformational changes. In addition to structural information, the binding constants for the ligand target interactions can be determined. The nature of the chemical shifts may differ depending on the type of binding which might exchange fast (gradual shift in signal with increasing ligand concentration) or slow (free protein signal gradually disappears and bound protein signal appears with increasing ligand concentration).

4.3.2 Fluorescence resonance energy transfer (FRET)

Fluorescence resonance energy transfer (FRET) based assays are often used in HTS for the initial screening of compounds and has previously also been used in screening of SrtA inhibitors.[47] FRET is a nonradiative process that occurs between a donor molecule in the exited state and an acceptor molecule in the ground state. The energy is transferred via long range dipole-dipole interactions between donor and acceptor which makes the transfer highly distance dependent. The rate of energy transfer further depends on the extent of spectral overlap of the emission spectrum of the donor and the absorption spectrum of the acceptor as well as the relative orientation of the donor acceptor transition dipoles.[125]

In the presented work, a FRET-based assay using an internally quenched fluorescent (IQF)[126] substrate was used in the screening of SrtA inhibitors by monitoring SrtA activity (Figure 9).[127] It is based on SrtA cleaving a substrate analogue of the LPXTG motif, Abz-

Leu-Pro-Glu-Thr-Gly-Lys(Dnp)-NH_2 where Abz (2-aminobenzoyl) is the fluorophore (donor) and Dnp (2,4-dinitrophenyl) is the quencher (acceptor). The emission from the fluorophore is quenched while the substrate is intact but upon cleavage FRET can no longer occur which gives rise to a fluorescence signal that can be detected. This allows for the detection of SrtA activity and thereby its inhibition.

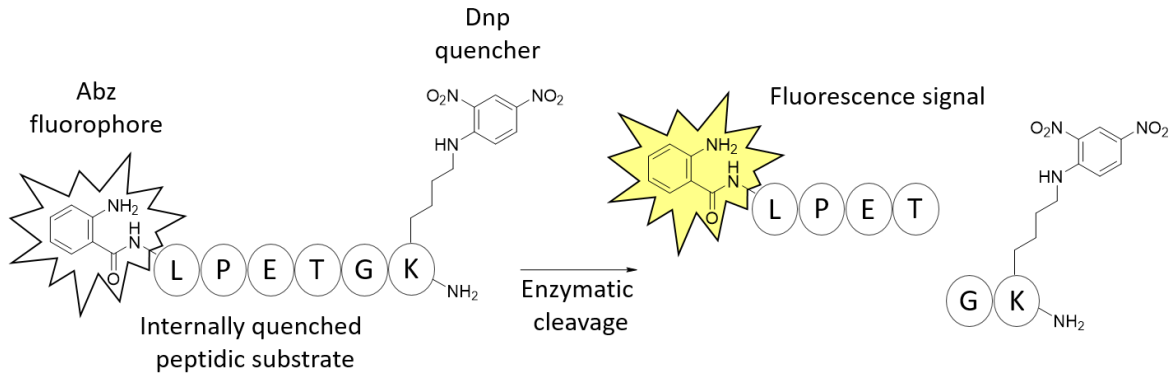


Figure 9. FRET based assay by IQF. SrtA cleaving a substrate analogue containing the LPXTG motif, Abz-LPETG-K(Dnp)-NH_2 where Abz is the fluorophore and Dnp is the quencher. The emission from the fluorophore is quenched when the substrate is intact. Upon enzymatic cleavage the fluorophore is no longer quenched and a fluorescence signal can be detected.

4.3.3 High-performance liquid chromatography

In this study, a high-performance liquid chromatography (HPLC) assay published by Kruger *et al.*[128] was used in addition to the FRET based assay utilizing the same inhibition reaction but quantified by HPLC using UV detection. The ratio product/substrate was calculated by integrating the areas under the HPLC trace in the chromatogram (Figure 10). This was done to confirm the FRET assay results and to get data for compounds disturbing the FRET signal.

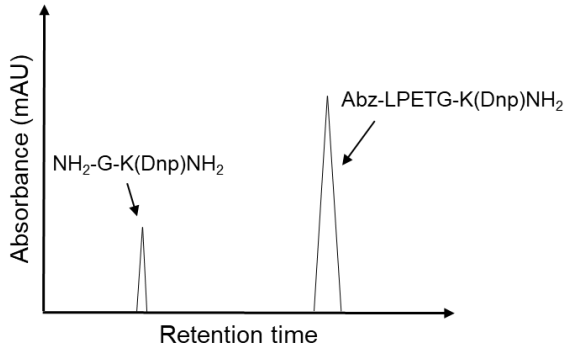


Figure 10. HPLC assay using Abz-LPETG-K(Dnp)-NH_2. SrtA-catalyzed reaction showing the HPLC trace of the substrate Abz-LPETG-K(Dnp)-NH_2 and the cleaved product NH_2-G-K(Dnp)-NH_2 at 355 nm.

4.4 HIGH THROUGHPUT SCREENING OF SORTASE A INHIBITORS (PAPER II)

In paper II, a small-molecule compound library was screened with the aim to identify new SrtA inhibitors. After evaluation, one of the most promising and structurally novel hits was synthetically modified for increased potency.

4.4.1 HTS results and compound selection

A library of ~28,500 compounds (originating from ChemBridge and Biovitrum AB) was screened for inhibition of SrtA using the FRET-based assay described above. The HTS resulted in 110 primary hits that reduced the readout signal more than 60% at 10 μM concentration, and 60 of these showed a concentration dependent inhibition. The hits were evaluated to identify unfavorable structures such as PAINS. A number of compounds were identified to potentially cause assay interference because of reactivity, chelation, or color (Figure 11). Some of these classes of compounds, e.g. rhodanines and substituted benzothiazolinones, have previously been explored as SrtA inhibitors.[51, 73]

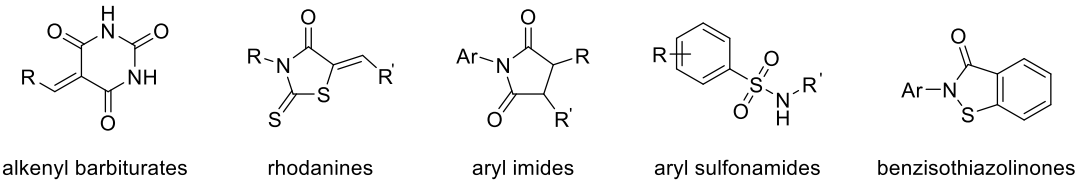


Figure 11. Potential assay interference compound classes found among the HTS primary hits.

In the evaluation process, hits were excluded when identified as PAINS and when structurally similar compounds in the screen showed no activity. This resulted in the identification of four hits (**17c**, **23a**, **24**, and **25**, Figure 12) which were confirmed as SrtA binders by ligand detected binding studies (1D ^{1}H CPMG NMR experiments).

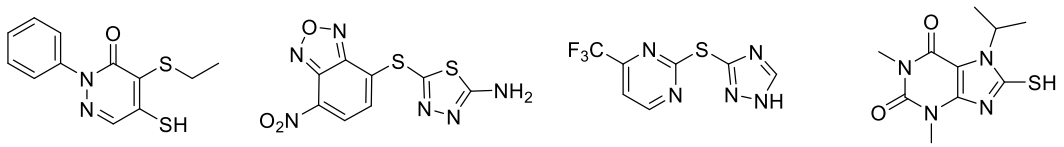
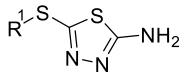


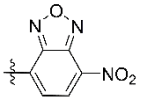
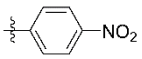
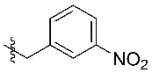
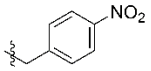
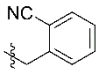
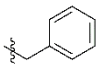
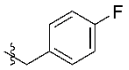
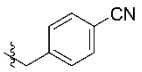
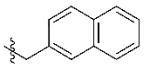
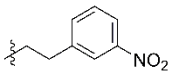
Figure 12. Top hit structures from HTS screen.

Hits **23a** and **24** are believed to share a common pharmacophore. Compound **17c**[129] was earlier discovered as a covalent SrtA inhibitor with an IC_{50}-value of 1.4 μM which is in agreement with our inhibitory activity of 3 μM. NMR studies further showed that the caffeine analogue **25** is a non-reversible probably covalent binder. Compound **23a** displayed reversible binding properties and was confirmed in both ligand- and protein-

detected NMR experiments. In addition, it contains multiple structural features for possible optimization of its affinity to SrtA. Therefore, **23a** was selected as the starting point for structural hit-to-lead optimization.

Table 2. Evaluation results of compounds **23a–c**, **26a–g**, and **27** in FRET assay, and 1D CPMG NMR experiments.



Cmpd	R^1	FRET Inhib (%)[a]	FRET IC_{50} (μM)[d]	NMR CPMG
23a		101 ± 0.7[b,c]	6.2 ± 0.2	active
23b		35 ± 2.3[c]	n.a.	non-active
23c		25 ± 3.7	n.d.	non-active
26a		11 ± 4.3	521 ± 52	active
26b		101 ± 1.6	26 ± 0.7	active
26c		1 ± 3.8	1745 ± 129	active
26d		0 ± 3.8	n.d.	non-active
26e		5 ± 3.9	n.d.	non-active
26f		2 ± 3.6	n.d.	non-active
26g		13 ± 2.8	n.d.	sol. issue
27		13 ± 3.3	n.d.	non-active

[a]Inhibition ± standard deviation (SD) (n = 3) at 200 μM with 15 min incubation time. [b]Inhibition ± SD (n = 2) at 167 μM with 15 min incubation time. [c]Fluorescence quenching properties. [d]IC_{50} ± SD (n = 3). n.a. = not applicable due to FRET assay interference. n.d. = not determined. sol. issue = solubility issue.

4.4.2 Structure-activity relationships

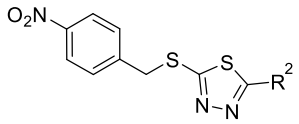
A series of structural analogues of **23a** were synthesized and tested (Table 2). The structural modifications included removal of the oxadiazole moiety, because this structural feature has shown to cause cytotoxicity.[130] Further, introduction of phenyl moieties with different electronic properties, and elongation of the sulfide substituent. The removal of the oxadiazole moiety from **23a** resulted in a significant decrease in activity and binding

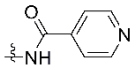
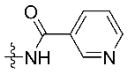
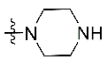
capacity (**23b–c**) whereas an elongation of the sulfide substituent by introducing a methylene spacer between the sulfur and the phenyl (or naphthyl) ring (**26a–g**) restored the binding capacity and increased inhibitory activity in some cases (**26a–c**). Introducing an ethylene spacer (**27**) showed similar activity as **26a** but loss of binding.

The most potent compounds were **26a** and **26b** (Table 2) containing a nitro substituent on the phenyl ring in either meta or para position. It seems as if the activity is due to the nitro-group rather than depending on the electronic nature of the substituent (**26c**, **26e**, **26f**). Fluorescence quenching properties was observed for only two compounds, the para substituted nitrophenyl thioethers, **23a** and **23b**.

Test results of derivatives of **26b** with modifications in the amino function are listed in Table 3. The compounds inhibit SrtA and show IC_{50}-values between 9-127 μM. From these results it seems as the exocyclic primary amino group of **26b** is not required for binding.

Table 3. Evaluation results of compounds **26b**, **28a–c**, and 2**9a–b**, in the FRET assay, and 1D CPMG NMR experiments.



Cmpd	R^2	FRET Inhib (%)[a]	FRET IC_{50} (μM)[b]	NMR CPMG
26b		101 ± 1.6	26 ± 0.7	active
28a		94 ± 0.8	127 ± 19	active
28b		99 ± 0.5	42 ± 2.0	non-active
28c		100 ± 0.4	3.8 ± 0.3	non-active
29a		104 ± 0.7	9 ± 0.6	active
29b		80 ± 1.3	71 ± 2.0	active

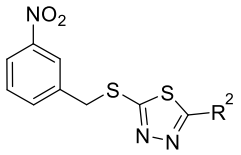
[a]Inhibition ± standard deviation (SD) (n = 3) at 200 μM with 15 min preincubation time. [b]IC_{50} ± SD (n = 3).

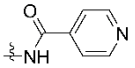
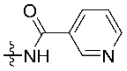
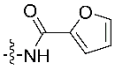
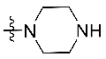
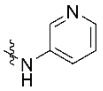
The size of the substituents in **28b–c** may indicate that there is an unfilled volume in the binding pocket. The morpholine and piperazine derivatives **29a** and **29b** both showed binding, with **29a** being more potent. Compound **28c**, containing the nicotinamide moiety, emerged as the most potent inhibitor from this series (IC_{50} = 3.8 μM). Ligand detected 1D ^{1}H CPMG NMR experiments confirmed binding for all compounds except **28b–c**. This

could however be a false negative result due to strong binding or slow exchange rate. Compound **28c** was retested using protein detected 2D NMR experiment (^{1}H-^{15}N HMQC) which confirmed its activity as a binder.

The compound series in Table 4 are derivatives of **26a** and include similar modifications as those for **26b** but with no significant effects on the activity. Longer incubation time did not affect the potency, only a slightly increased potency of **31b** (27% to 38% inhibition at 200 μM) was observed.

Table 4. Evaluation results of compounds **26a**, **30a–d**, **31a–b**, **32** and **33** in FRET assay and 1D CPMG NMR experiments.



Cmpd	R^2	FRET Inhib (%)[a]	NMR CPMG
26a		11 ± 4.3	active
30a		18 ± 3.8	sol. issue
30b		8 ± 2.9	active
30c		13 ± 3.6	sol. issue
30d		6 ± 2.7	sol. issue
31a		29 ± 3.7	active
31b		27 ± 2.7	non-active
32		37 ± 2.3	sol. issue
33		19 ± 2.2	sol. issue

[a]Inhibition ± standard deviation (SD) (n = 3) at 200 μM with 15 min preincubation time. sol. issue = solubility issue.

4.4.3 Mode of action

Because SrtA contains an active site cysteine residue (Cys184) there is a possibility of inactivation by sulfur oxidation. This was investigated in a study by Melvin *et al.*[131] where SrtA was found to be highly resistant to oxidative inhibition. They hypothesize that SrtA

is able to maintain its high reduction potential of Cys184 because of its unusual active site which employs the reverse protonation mechanism for transpeptidation (Figure 2).

Nevertheless, we wanted to test whether adding a reducing agent to the buffer would have any effect on the inhibitory activity of **23a**, **26b**, and **28c**. When having ditiotreitol (DTT) present the SrtA activity was recovered for all three compounds. We found that DTT actually interacts with **23a**, explaining the loss of its inhibitory activity. Compounds **26b** and **28c** did not show to react with DTT. This might indicate that inhibition of SrtA in this case is acquired through sulfur oxidation or disulfide bond formation, which both can be reduced by DTT.

To explore this further, oxidation states and other modifications were determined for the trypsin digested protein with and without **26b** and **28c** present, and analyzed by liquid chromatography mass spectrometry (LCMS). All oxidations states of the cysteine, i.e. mono-, di-, and trioxidation, were observed in all samples with no significant difference between samples. Interestingly, also other modifications were observed involving covalently bound fragments to the active site cysteine. Addition of a nitrobenzyl thio (+167.00410 Da, $+C_7H_5NO_2S$) fragment to Cys was present in both **26b** and **28c** containing samples. Additionally, **26b** contained the amino-thiadiazolyl thio (+130.96119 Da, $+C_2HN_3S_2$) modification, and **28c** nicotinamido-thiadiazolyl thio (+235.98265 Da, $+C_8H_4N_4OS_2$). This suggests that the mode of action involves a cysteine thiol reaction resulting in a disulfide bond formation.

4.4.4 Molecular modeling and docking

Dockings were performed (in 2KID and 1T2W) using both standard flexible docking (non-covalent and covalent docking) and induced fit docking (IDF) because of the large flexibility and difference in binding sites of the available PDB structures.

For both docking approaches different orientations of **28c** were identified (Figure 13A). This positioning of the ligand might facilitate disulfide bond formation. Both approaches show that **28c** mainly occupies the binding pocket of the substrate but the orientation of the ligand differed 180° between IFD and non-covalent docking. Covalent docking of the thiadiazol fragment of **28c** (Figure 13B) resulted in a conformation well aligned with the conformation of the bound substrate in 2KID (Figure 13C). In both docking approaches, Arg197 and His120 interact with the *p*-nitrophenyl and pyridine rings by hydrophobic interactions. In IFD π-π interactions are observed between His120 and *p*-nitrophenyl while hydrogen bonds are observed between Arg197 and the amide and between Thr164 and the pyridine nitrogen.

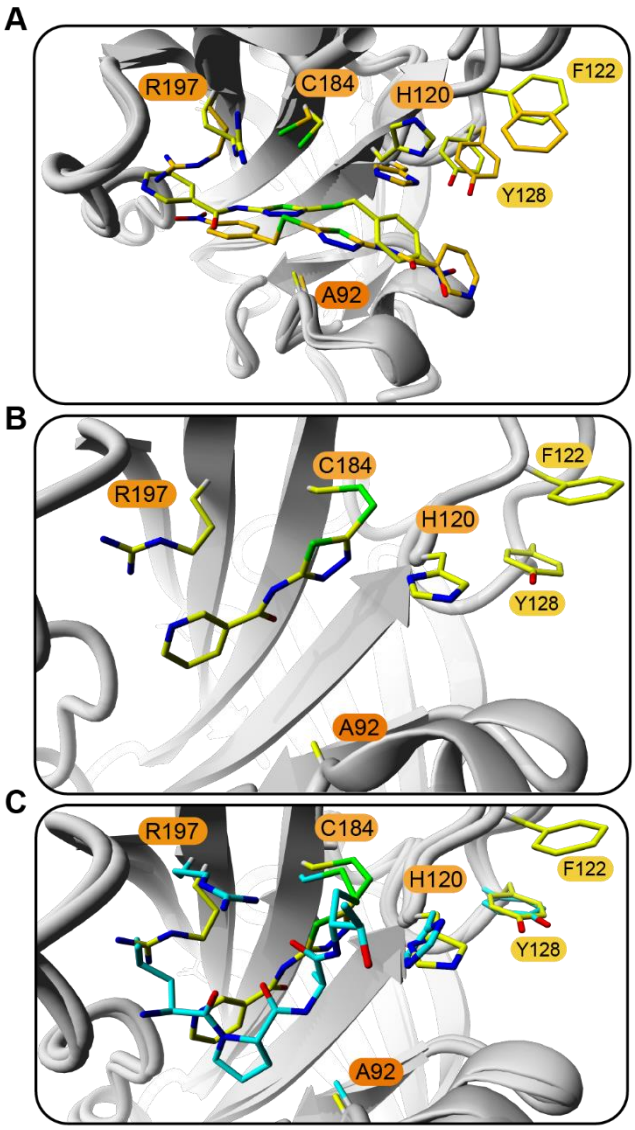


Figure 13. A) Docking results of **28c** using induced fit docking (carbon atoms colored yellow) and non-covalent docking (carbon atoms colored orange), general coloring scheme: nitrogen – blue, oxygen – red, sulfur – green, protein backbone is colored in gray; B) Covalent docking of the nicotinamide-thiadiazole thiol fragment of **28c**; and C) **28c** superimposed with the covalently bound substrate (LPAT, carbon atoms colored cyan) of SrtA (PDB ID: 2KID).

4.4.5 Inhibition of bacterial growth

Deletion of the SrtA gene has shown to reduce pathogenicity without affecting bacterial growth.[41] SrtA inhibitors should have the therapeutic effect without affecting bacterial growth, which would otherwise indicate off target effects. The Minimum inhibitory activity (MIC) was therefore determined for **23a-c** showing that the compounds are not intrinsically toxic to the two bacteria strains *S. aureus* and *E. coli*.

4.4.6 Summary Paper II

In this study, small-molecule SrtA inhibitors with IC_{50} values in the low micromolar range have been developed by optimization of an HTS hit, the new compounds are among the most potent SrtA inhibitors published until now. LCMS studies showed modifications corresponding to compound fragments covalently bound to the active site cysteine through a disulfide bond. Even though these compounds inhibit SrtA by covalent inactivation, they are selective inhibitors as they do not affect bacterial growth.

4.5 FRAGMENT BASED LEAD DISCOVERY OF SORTASE A INHIBITORS (PAPER III)

A fragment based screen (FBS) of SrtA inhibitors was performed and hits were evaluated using 1D and 2D NMR experiments, and FRET- and HPLC-based assays. One of the hit compounds was selected for further investigation to explore fragment growing or fragment linking opportunities. It was eventually grown into a hybrid compound with the HTS hit **23a**.

4.5.1 Fragment screening

A fragment library (Maybridge diversity library Ro3, Thermo Fischer Scientific Inc., 2006) consisting of 1000 fragments was screened for SrtA binding, using CPMG NMR experiments to detect weak binding. The fragments were screened in cocktails of 10 and the preliminary hits were retested as individual fragments. The FBS resulted in the identification of 14 fragment hits (Table 5).

4.5.2 Evaluation and hit selection

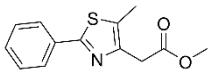
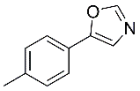
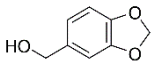
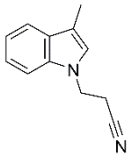
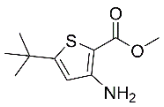
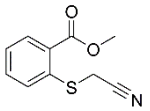
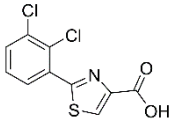
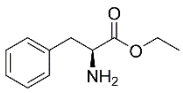
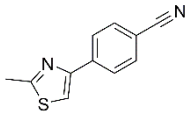
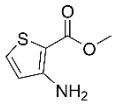
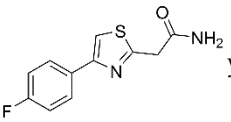
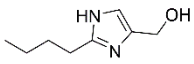
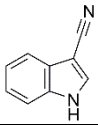
The reversibility of the fragments was investigated by 1D ^{1}H CPMG NMR displacement experiments using the Abz-LPETG-K(Dnp)-NH_2 substrate. Seven of the 14 fragments were displaced by the substrate and are therefore considered as reversible binders. Protein-detected NMR experiments (^{1}H-^{15}N 2D HMQC) confirmed binding for five fragments (**39**, **42**, **43**, **46**, and **47**, in Table 5).

The inhibitory activity was assessed using the same FRET assay as earlier and resulted in 4–69% inhibition at 200 μM concentration. Fragment **39** showed strongest inhibition, however it likely binds covalently to the protein. Since several fragments (**34**, **36**, **37**, **42**, and **45**) interfered with the FRET signal a second biochemical assay was applied using the same inhibition reaction but quantified by HPLC using UV detection. The HPLC assay gave comparable results to the FRET assay and complemented the inhibitory information

for the fragments that exhibited FRET signal interference. In this case, fragment **47** showed strongest inhibition using the HPLC method with 63% inhibition at 200 μM concentration.

The most promising hits after evaluation were **42**, **43**, **46**, and **47** because of being reversible, show binding in the HMQC assay, and are confirmed in at least one biochemical assay.

Table 5. Evaluation results of fragment hits **34–47**, in 1D ^{1}H CPMG NMR experiments, 2D ^{1}H-^{15}N HMQC NMR assay, FRET assay, and HPLC assay.

ID	Structure	rev[a]	HMQC[b]	FRET Inhib (%)[c]	HPLC Inhib (%)[c]	ID	Structure	rev[a]	HMQC[b]	FRET Inhib (%)[c]	HPLC Inhib (%)[c]
34		no	no	n.a.	8.4 ± 9.4	**41**		yes	no	8.9 ± 4.6	25 ± 7
35		yes	no	3.7 ± 0.7	14 ± 6	**42**		yes	yes	n.a.	18 ± 5
36		no	no	n.a.	17 ± 6	**43**		yes	yes	10 ± 5.9	19 ± 1
37		yes	no	n.a.	8.2 ± 6.2	**44**		no	no	41 ± 8	21 ± 17
38		no	no	5.3 + 1.2	13 ± 6	**45**		no	no	n.a.	2.6 ± 3.5
39		no	yes	69 ± 3	2.4 ± 11	**46**		yes	yes	5.9 ± 2.8	12 ± 4
40		no	no	no inhib.	6.8 ± 11	**47**		yes	yes	24 ± 2.5	63 ± 5

[a]Reversible binding, displacement by substrate. [b]Binding; [c]Inhibition ± standard deviation (SD) (n = 3) at 200 μM. n.a. = not applicable due to FRET assay interference.

1D ^{1}H CPMG NMR displacement experiments were performed between pairs of fragments to investigate the possibility of fragment linking. All four fragments were competing with each other, which indicates that the fragments all bind in the same region of the active site or in close proximity. Therefore, fragment linking was not considered as an option. Indole derivatives similar to **42** and **47** have previously been studied (Figure 3) and were therefore not selected for further investigation. Fragment **43** was eventually selected for further exploration because of having several possible sites for fragment

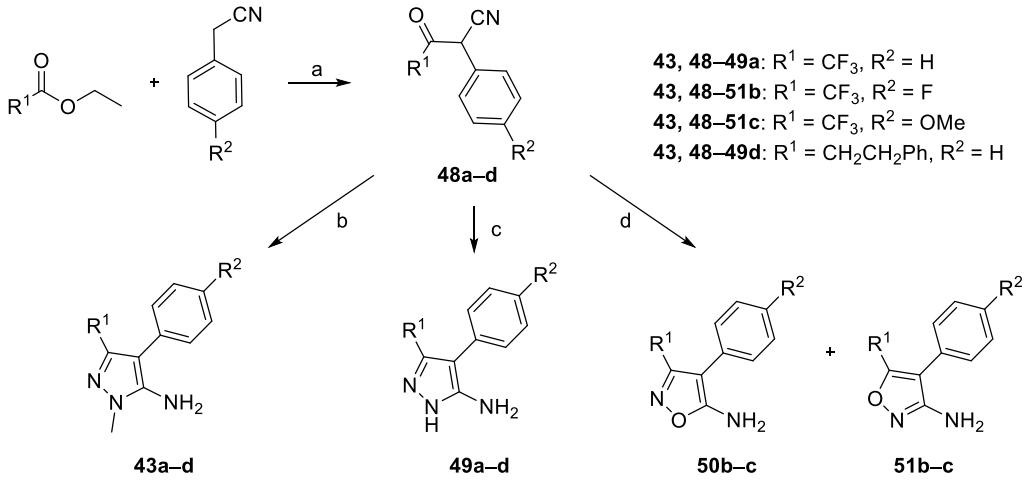
growing. It also shows structural similarity to our HTS analogues (**26b**) described in section 4.4.

Small structural modifications of **43** were first made to explore electrostatics of the phenyl moiety and ring effects, and subsequent attempts to grow **43** into a hybrid with **26b** were performed.

4.5.3 Synthesis of analogues

Commercially available esters were reacted with various benzyl cyanides to yield the corresponding keto-nitriles (**48a–d**) used in the subsequent reactions (Scheme 1). Reacting **48a–d** with methyl hydrazine under acidic conditions gave the methylated pyrazoles **43a–d** in good yields (69–97%). The non-methylated pyrazoles **49a–d** were prepared under similar conditions by reacting **48a–d** with hydrazine monohydrate to obtain **49a–d** in moderate to good yields (33–88%). The isomers of the isoxazoles **50b–c** and **51b–c** were prepared from the reaction of **48b–c** with hydroxylamine hydrochloride under basic conditions yielding the products as isomeric mixtures (30–52%).

Scheme 1. General Methods for the Syntheses of Compounds **43a-d**, **49a-d**, **50b-c, and 51b-c**.[a]



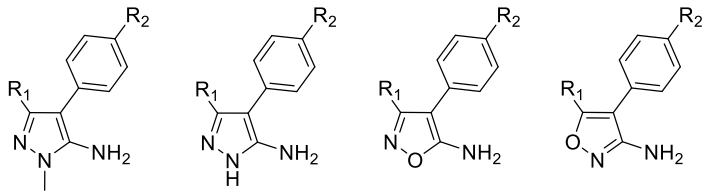
[a]Reagents and conditions: (a) appropriate ester and nitrile, THF, NaH, reflux, 3–24 h, 48–94%; (b) methyl hydrazine, EtOH, HCl, reflux, 3–24 h, 69–97%; (c) hydrazine monohydrate, EtOH, acetic acid, 90 °C, 1 h, 33–88%; (d) hydroxylamine hydrochloride, water, NaOH, 40 °C→reflux, 24 h, 30–52%.

4.5.4 Substituent effects on activity

Substituent effects were investigated around fragment **43** by synthesizing analogues with different electronic properties at the para-position of the phenyl ring (**43a–c**), Table 6. All compounds show similar range of inhibitory activity against SrtA (19–25% inhibition at 400 μM concentration), suggesting that the electronic properties of the phenyl ring are not important for binding or activity. The role of the methylated pyrazole nitrogen was

explored by removal of the methyl, introducing a hydrogen bonding opportunity instead (**49a–c**). Non-methylated pyrazoles (**49a–c**) showed both binding and inhibitory activity in the same range as **43a–c** (17–26% inhibition at 400 μM). The heterocycle was further evaluated by replacing one of the nitrogens with oxygen (**50b–c** and **51b–c**). The isoxazoles gave similar results as the pyrazoles. The *p*-OMe substituted isoxazoles **50c** and **51c** showed slightly better inhibition than the *p*-F substituted analogues.

Table 6. Evaluation of compounds **43a-c**, **49a-c**, **50b-c** and **51b-c** in CPMG and HSQC NMR experiments, and FRET assay.



Cmpd (isomer ratio)	R^1	R^2	FRET Inhib (%)[a]	NMR HSQC	NMR CPMG
43a	CF_3	H	22 ± 2	binder	binder
43b	CF_3	F	25 ± 3	binder	binder
43c	CF_3	OMe	19 ± 2	binder	binder
49a	CF_3	H	26 ± 3	binder	binder
49b	CF_3	F	22 ± 3	binder	binder
49c	CF_3	OMe	17 ± 2	binder	binder
50b	CF_3	F	20 ± 3	binder	binder
50b/51b (1:7)	CF_3	F	18 ± 4	n.d.	binder
50c/51c (7:1)	CF_3	OMe	31 ± 2	binder	binder
50c/51c (4:1)	CF_3	OMe	28 ± 1	n.d.	binder

[a]Inhibition ± standard deviation (SD) (n = 3) at 400 μM. n.d. = not determined.

4.5.5 FBS and HTS merging

From evaluating the fragment hits and the HTS compounds in section 4.4, the idea of merging fragment **43** with **26b** arose (Figure 14). To test our hypothesis all fragments, HTS compounds, and the hybrid (**52**) were docked using induced fit docking and the docking poses were evaluated using Glide XP score. Fragment **43** showed to dock in the lower part of the pocket (Figure 15A) aligning well with the docked pose of the nicotinamide part of **28c**, one of the most potent compounds from the HTS series. The best docking pose of the hybrid compound **52** aligns nicely with **28c** (Figure 15B). This could indicate that the

initial binding mode of the two compounds is similar while **28c** reacts further with the cysteine. From the scoring, the hybrid (**52**) ranks highest in this set of compounds. The hybrid was therefore synthesized with the aim to obtain a higher affinity compound which lacks the possibility of covalent inhibition by disulfide bond formation.

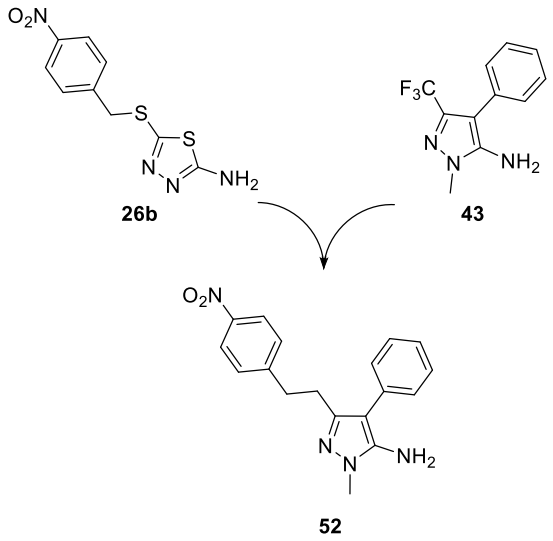


Figure 14. Hybrid compound between HTS analogue **26b** and FBS hit **43**.

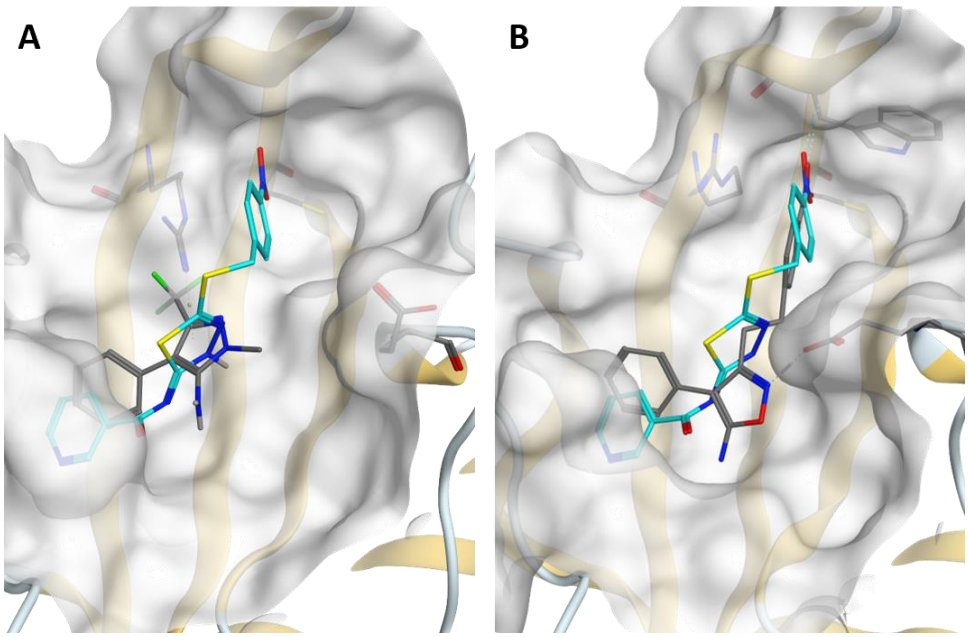


Figure 15. A) Induced fit docking of **28c** (cyan), overlaid with the induced fit docking pose of **43** (gray). B) Induced fit docking of **52** (gray) overlaid with **28c** (cyan).

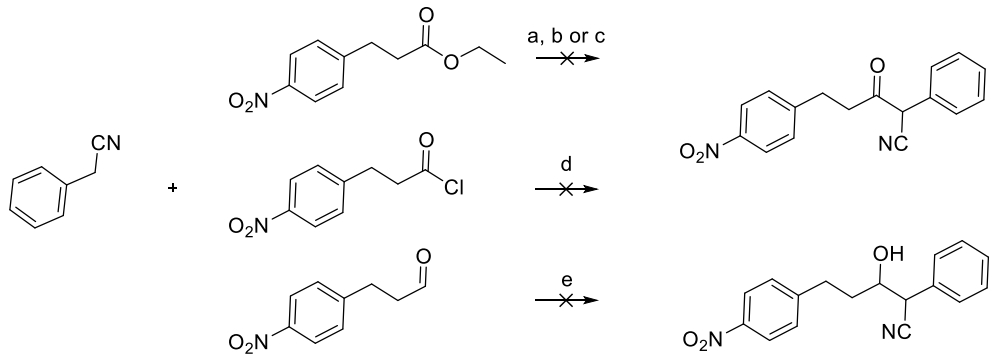
4.5.6 Synthesis and evaluation of the FBS-HTS hybrid

As a first step to further expand our fragment hit and grow it into the hybrid compound **52**, the trifluoromethyl group was replaced with a 2-phenylethyl substituent to obtain **43d**. It showed no additional gain in activity compared to the trifluoromethylated analogues

(~20% inhibition at 400 μM in the FRET assay). The next step was to introduce a *p*-nitro substituent on the phenyl ring (**52**).

Several synthetic attempts to synthesize **52** were performed using the benzyl cyanide and the corresponding ester, acid chloride, or aldehyde, under different conditions (Scheme 2) without success.

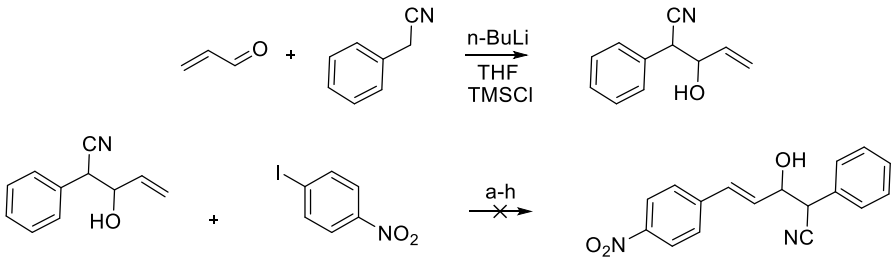
Scheme 2. Attempted synthesis of **52**.



[a]Reagents and conditions. (a) NaH, THF, 0 °C; (b) $KN(SiMe_3)_2$, THF, -78 °C→r.t.; (c) *t*-BuOK, THF, r.t; (d) LDA, THF, -78 °C; (e) *t*-BuOK, THF, r.t.

Another approach was attempted (Scheme 3), synthesis of an alkene for use in a Heck reaction with iodonitrobenzene. However, only traces of the desired product were observed independent of conditions used.

Scheme 3. Attempted synthesis of **52** using the Heck reaction.

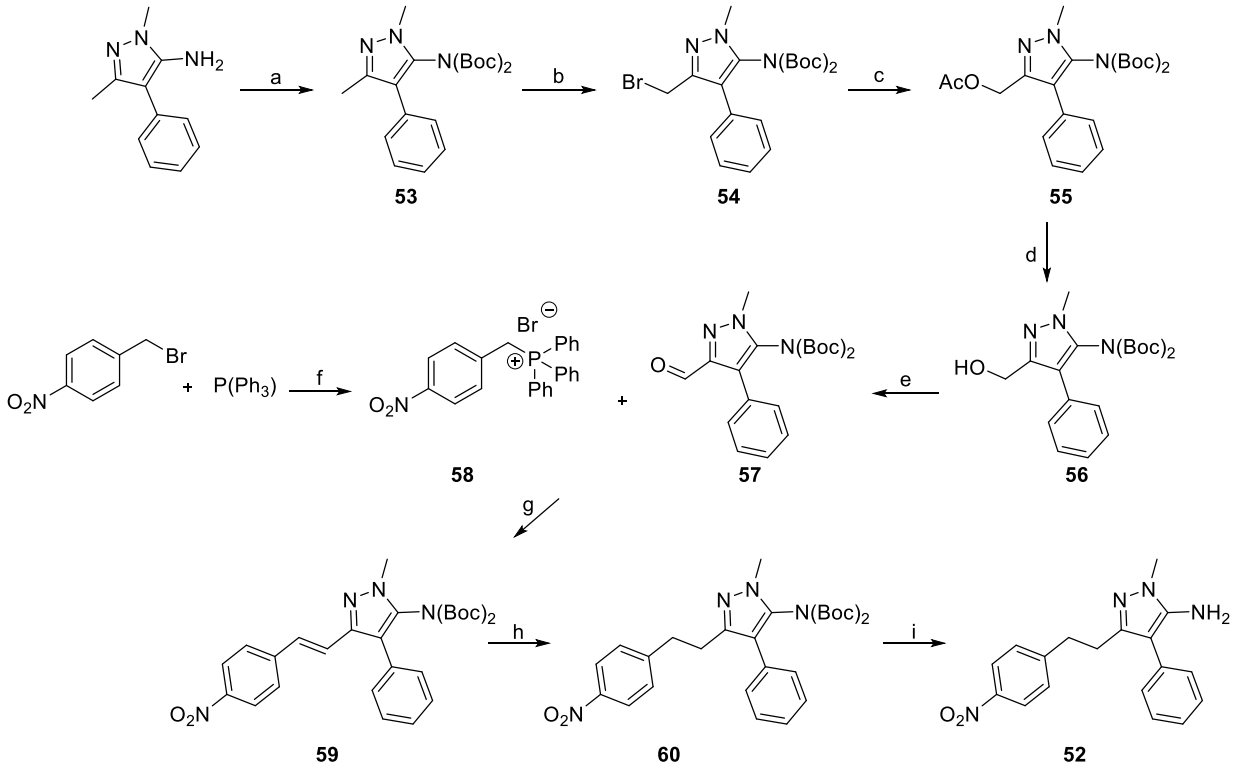


a. $Pd(OAc)_2$, Et_3N, ACN
b. $Pd(OAc)_2$, NBu_4Cl, $NaHCO_3$, DMF
c. $Pd(OAc)_2$, NBu_4Cl, K_2CO_3, DMF
d. $Pd(OAc)_2$, NBu_4Cl, K_2CO_3, DMF, H_2O
e. $Pd(OAc)_2$, PPh_3, $NaHCO_3$
f. $PdCl_2(PPh_3)_2$, $NaHCO_3$, DMF
g. $Pd(OAc)_2$, NBu_4Cl, Et_3N, DMF
h. Pd(OAc)2, NBu_4Cl, Bu_3N, DMF

The hybrid compound **52** could be successfully synthesized following the synthetic strategy shown in Scheme 4. The strategy involves bis-Boc protection of the free amine (**53**), followed by bromination of the methyl group to obtain **54** in moderate yield (56%). The dibrominated product was formed as a byproduct (6%). The isolated monobrominated product was used in the subsequent acetylation of **54** to obtain the acetate **55** in moderate yield (60%). Hydrolysis of ester **55** gave the corresponding free alcohol **56** (94%) which was oxidized to the corresponding aldehyde **57** using MnO_2 (77%). A phosphonium salt

(**58**) was synthesized from *p*-nitrobenzylbromide and triphenylphosphine in 87% yield. **57** and **58** were used in the subsequent Wittig reaction yielding the unsaturated product **59** (77%) as a mixture of cis and trans isomers. Catalytic hydrogenation of **59** was performed using Wilkinson´s catalyst (10%) in dry THF, reducing only the double bond and leaving the nitro group intact. The reaction was stopped after 25% conversion (24 h). The saturated product **60** was deprotected using 4N HCl in dioxane to yield the final product **52** (47%).

Scheme 4. Synthesis of **52**.[a]



[a]Reagents and conditions: (a) Boc_2O, DMAP, THF, 40 °C, 1 h, 93%; (b) NBS, AIBN, CCl_4, 80 °C, 17 h, 56%; (c) AcOK, KI, DMF, 80 °C, 20 h, 60%; (d) K_2CO_3, MeOH, 80 °C, 3 h, 94%; (e) MnO_2, DCM, r.t., 16 h, 77%; (f) $P(Ph_3)$, toluene, r.t., 15 h, 87%; (g) *t*-BuOK, 30 °C, 48 h, 77%, cis/trans mixture; (h) Wilkinson's catalyst (10%), THF, 25% conv., 24 h; (i) 4N HCl/dioxane, r.t., 3 h, 47%.

When a *p*-nitro substituent was introduced on the phenyl ring to obtain **52** a significant increase in inhibitory activity was obtained, IC_{50} = 188 μM. In addition, when having DTT present, **52** retained its activity as oppose to **28c** which lost its inhibitory activity completely in the presence of a reducing agent. Even though **52** and **28c** may bind SrtA in a similar fashion, as seen from the docking, the higher activity of **28c** (IC_{50} = 3.8 μM) could simply be because it further reacts with the cysteine.

4.5.7 Summary Paper III

Promising small-molecule leads for SrtA inhibition have been discovered using FBS. Evaluation of hits by HMQC NMR experiments, FRET- and HPLC-based assays resulted

in the discovery of a substituted pyrazole as a promising starting point for further optimization. A hybrid compound between the HTS discovered **26b** and fragment hit **43** was supported by induced fit docking. Hybrid **52** was synthesized using an 8-step procedure. The hybrid showed to inhibit SrtA (IC_{50} = 188 μM) and is believed to inhibit through non-covalent binding.

5 MORIN AND SORTASE A DIMERIZATION (PAPER IV)

All SrtA screening campaigns, so far, have been focused on inhibition by targeting the catalytic site of SrtA. What if modulation of SrtA can be achieved through allosteric binding?

The inhibitory activity of flavones on SrtA has previously been determined using FRET-based assays usually without any orthogonal assays or explanation of their MoA. Herein, biophysical and biochemical assays have been used to explore the SrtA binding of morin (Figure 3) and to further understand the dimerization of SrtA. Additionally molecular dynamics simulations were used to simulate these binding events and the influence of morin on dimerization.

5.1 ANTIBACTERIAL ACTIVITY OF FLAVONOIDS

Flavonoids (Figure 16) are a large class of natural products with many biological and pharmacological effects including antibacterial, antiinflammatory, anticancer and antioxidative activity.[132-133] The nature of their antimicrobial activity has been questioned, whether they are bacteriostatic or bactericidal.[132, 134-135] It may be that flavonoids induce bacterial aggregation which enables the host's defense system to remove potential pathogens because large aggregates are more easily detected compared to bacteria in biofilm or planktonic forms.[135]

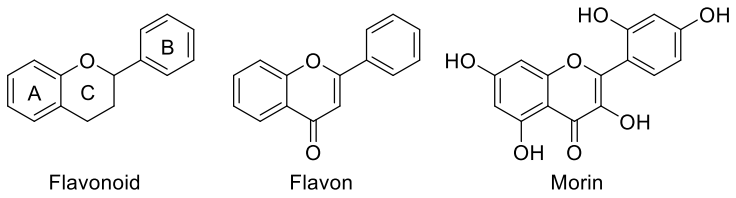


Figure 16. Flavonoid backbone with its three rings A, B, and C. Flavon containing the chromone scaffold and the flavonol morin.

5.1.1 Flavonoids as SrtA inhibitors

The flavonoids morin,[61, 136] myricetin,[62] isovitexin,[63] acacetin,[64] and quercetin[65] all show strong inhibitory activity against SrtA. They reduce bacterial adhesion to host cells and biofilm formation without affecting the bacterial viability.[61-62, 136]

5.2 METHODS USED FOR BINDING EVALUATION

The previously described assays using FRET, and ligand- and protein-detected NMR experiments were also implemented herein. Also surface plasmon resonance (SPR) and thermal shift assay (TSA) were used to gain additional information and these techniques are described in the following sections.

5.2.1 Surface plasmon resonance (SPR) assay

The surface plasmon resonance (SPR) assay was used to confirm binding and to determine affinities and kinetics for the protein ligand binding. This is done by immobilizing the protein onto a gold surface of a sensor chip while the ligands, in buffer solution, are flowed over the sensor surface enabling the ligands to interact with the bound protein (Figure 17). When the surface is illuminated with polarized light, the light will be reflected and passed onto the detector. The light is absorbed by the electrons in the gold film at a certain angle (resonance angle) causing them to resonate (surface plasmons). This absorption leads to a decrease in intensity of the reflected light and gives rise to a minimum in the SPR reflection intensity curve and reflective light intensity at a particular angle. As molecules bind to the surface, the refractive index near the sensor surface changes, altering the angle of minimum reflective intensity.

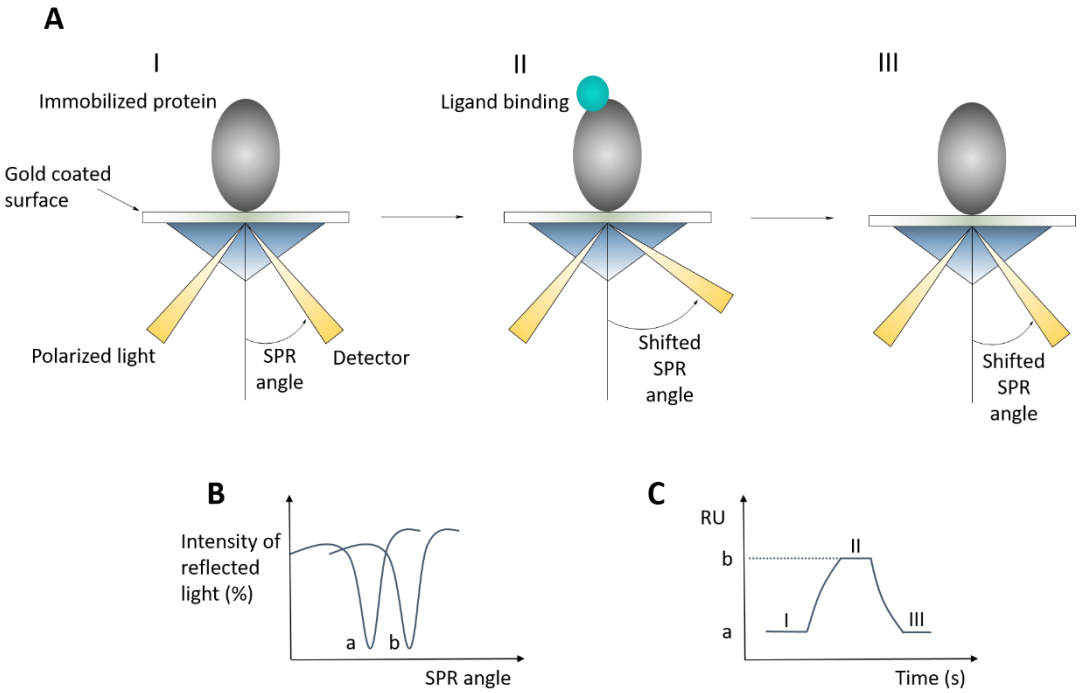


Figure 17. Illustration of SPR. A) I. The SPR angle is measured of the immobilized protein (gray) in absence of ligand (cyan). II. SPR angle increases upon ligand binding. III. Ligand is removed by subsequent washing with running buffer result in a decrease of the SPR angle. B) Changes in SPR angle a and b upon ligand binding. C) Sensogram of a typical SPR measurement for one concentration (RU = resonance units).

The change in SPR angle is further proportional to the mass of ligand bound, which allows measuring the association (on-rate, K_{on}) and dissociation (off-rate K_{off}) of the binding and calculating the dissociation constant (binding constant, K_d).[137-138]

5.2.2 Thermal shift assay (TSA)

TSA can be used for studying protein dynamics, to screen for changes in the melting temperature of a protein under varying conditions, or screening for ligand-protein interactions.[139] In this study, it was applied for studying the dimer dissociation of SrtA.

The technique utilizes the properties of a fluorescent dye (usually Sypro orange) which is sensitive to its environment. The fluorescent dye is quenched in water but upon protein unfolding the dye binds to the hydrophobic surface of the protein, giving rise to fluorescence which is monitored over increasing temperatures (Figure 18). Melting temperatures T_m, or thermal denaturation, can in this way be determined.

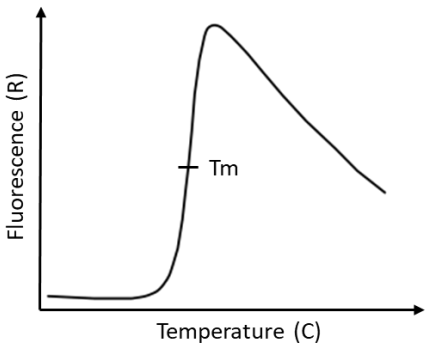


Figure 18. Thermal shift assay showing the melting curve of a protein. As the temperature rises, the protein unfolds resulting in increased fluoresces caused by binding of the dye (Sypro orange) to the hydrophobic surface of the protein. Tm is the melting temperature of the protein.

5.3 MORIN INHIBITION OF SRTA (PAPER IV)

5.3.1 Binding and inhibitory activity of morin

The FRET-based assay used in Chapter 4 was implemented herein to confirm the activity of morin (IC_{50} = 15 μM), which is in agreement with published data.[61] Addition of DTT gave no indications of oxidation.

Ligand-detected (1D ^{1}H CPMG) NMR experiments and displacement experiments, using the substrate-derived peptide (Abz-LPETG-K(Dnp)-NH_2), were performed to confirm morin binding and to analyze possible reversibility and specificity of morin. The experiments confirmed morin binding to SrtA and when adding the substrate to the morin-

SrtA mixture the signal intensity was partially recovered, indicating at least partial binding to the catalytic site.

Displacement experiments were also performed between morin and fragments **42**, **43**, **46**, and **47** from section 4.5.2 to test if morin could be used for displacement as replacement for the substrate which is consumed over time. The fragments were not able to displace morin, probably because they are weaker binders. Interestingly, when trying to displace the fragment by adding morin to each individual fragment a decrease in the NMR signal was observed in all four cases. This could indicate an increased affinity of the fragments for the protein in the presence of morin. Control experiments were performed without SrtA present to confirm that the fragments do not precipitate upon addition of morin, but showed no such effects.

These findings prompted further experiments to determine how and where morin binds to SrtA. The 1D NMR experiments were followed up by 2D ^{1}H-^{15}N HMQC-SOFAST NMR experiments. When titrating morin to SrtA, chemical shift changes were observed for amino acid residues in the dimerization site of SrtA, including Ile76 and Lys84, as well as in loops surrounding the active site (Ala104, Glu105, Tyr187, and Trp194, in Figure 19). The question arose, whether morin might interfere with the homo-dimer formation or allosterically influence SrtA by binding at the dimer interface.

The NMR experiments were complemented with a direct binding assay using SPR which also confirmed that morin binds to SrtA. Its dissociation constant (K_d) was determined to 11 μM. The response curves of various morin concentrations (see Paper IV) was fitted to a two-state reaction model which indicates an initial binding followed by a conformational change that gradually leads to a more stable complex.[140-141]

5.3.2 SrtA dimerization

The oligomeric organization of many enzymes adds to their complexity and is important for numerous biological processes. The dissociation of oligomers often affects enzyme activity.[142]

It has been shown that dimerization of the truncated $SrtA_{\Delta 59}$ can easily be disrupted by a single mutation at the dimerization interface,[143] while the full length SrtA forms a stronger dimer at the cell membrane and is not as easily disrupted. The role of the dimer has been debated. Lu *et al.*[144] proposed that the SrtA homodimer has increased activity in comparison to the monomeric enzyme. A few years later, the same group published that *S. aureus* which has a monomer SrtA mutant is more invasive than the wild-type SrtA (in monomer-dimer equilibrium) which would suggest that the monomer is the more active form.[145]

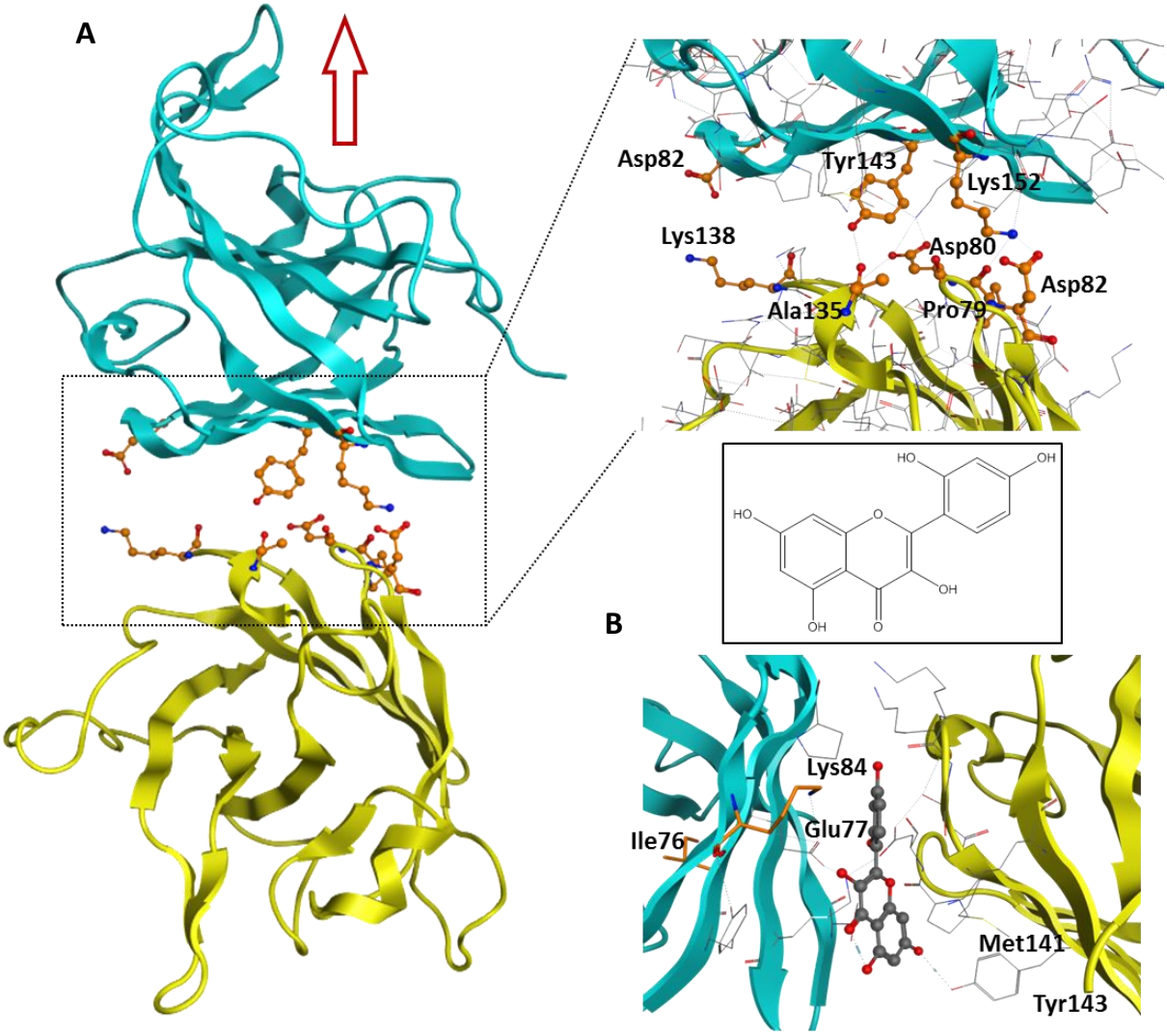


Figure 19. A) Dimer interaction site between monomer C (yellow) and A (blue) showing the important residues for stabilizing the SrtA dimer (labeling from crystal structure PDB ID: 1T2P). The pulling direction from the SMD simulations is illustrated with the red arrow. B) Morin (gray) binding at the dimer interface.

In this study, the dimerization of SrtA was investigated using TSA. Higher concentrations of SrtA (10 and 100 μM) showed a transition in the melting curve at 54 °C while the monomer (0.1 – 10 μM) did not undergo a thermal transition until ~70 °C. The dissociation constant for the dimer was determined to K_d = 2.3 μM by a concentration dependent response of SrtA.

Could the SrtA dimerization affect the screening of inhibitors? For the NMR based assays used in this work, the SrtA concentration was around the dimerization K_d meaning that both monomer and dimer of SrtA would be present. In the FRET and SPR assays the monomer would be dominating, in FRET because of the low concentration (200 nM). The dissociation of the dimer may result in conformational changes that influence the activity and therefore the outcome of assays.

5.3.3 Docking of morin and steered MD

The influence of morin binding at the SrtA dimer interface was further investigated using docking and steered molecular dynamics (SMD) simulations. When docking morin at the dimer interface, hydrogen bonds were seen with several amino acid residues in both monomer A and C (Figure 19A) and additional vdW interactions were observed to monomer A. The dimer is not symmetric which explains the difference in interactions.

During the SMD, monomer A is pulled away from monomer C and finally all inter-monomeric interactions were broken separating the dimer into the two monomers (Figure 19B) The interaction energies of the two systems, with and without morin present, were then calculated. The dimer in the dimer-morin system showed to have a higher energy minimum than without morin present. Morin thus reduces the overall strength of the interaction of the dimer and facilitates the dimer dissociation.

5.3.4 Summary Paper IV

This study shows that morin inhibits SrtA by binding in the active site but also that it binds at the dimer interface. Morin seems to induce a conformational change in the protein allowing various fragments to bind more efficiently in the active site. Morin further seems to break the SrtA dimer into the monomers. The monomer, being the more active form, would bind the fragments with higher affinity. In support to this, SMD studies show that morin accelerates the dissociation process of the dimer by interfering with the hydrogen bond network between the two monomers.

These findings indicate that morin might not only work as an inhibitor, but also as an activator. This effect could plausibly also be transferable to other flavonols similar to morin and might furthermore provide an additional explanation for the difficulties observed in virtual screening of SrtA inhibitors. This study further sheds light on the importance of investigating the inhibitory mechanism of morin and other SrtA inhibitors to fully understand how they operate which will aid in the development of more potent inhibitors.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The antibiotic resistance crisis has spurred the development of innovative therapeutic strategies for treatment of bacterial infections. Anti-virulence strategies being one of them is gaining more attention where targeting SrtA has resulted in several promising SrtA inhibitors. Much effort has been put into the early discovery of new hits using several different strategies. Now more effort is needed in developing these hits further into promising leads and clinical candidates. In this thesis some progress has been made in that direction, not only by generating new inhibitors but also by providing insights into their mechanism of action. The complexity of SrtA as a target has also become evident.

Further investigations of special interest include additional studies on the SrtA structure and its dynamic active site. Extending the structural investigation of SrtA by investigating how different ways of selecting MD snapshots, actives, and libraries for docking may influence VS performance. Unraveling of binding modes and MoA of already existing sortase A inhibitors and inhibitor classes and to investigate flavons as allosteric modulators or the possibility of inhibiting sortase A by targeting the dimerization interface. Further development of fragments and HTS-fragment hybrid compound would also be of interest.

SrtA has shown to be a more complex target for developing anti-virulence drugs than was first expected, but this challenge also makes it more interesting.

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