Small Molecules as Tools in Biological Chemistry

Effects of Synthetic and Natural Products on the Type III Secretion System

Caroline Zetterström
Small Molecules as Tools in Biological Chemistry

Effects of Synthetic and Natural Products on the Type III Secretion System

Caroline Zetterström

Doctoral Thesis 2013
Department of Chemistry
Umeå University, Sweden
Responsible publisher under Swedish law: the Dean for the Faculty of Science and Technology
This work is protected by the Swedish Copyright Legislation (Act 1960:729)
ISBN (digital version): 978-91-7459-672-4
Cover: Picture of Y. pseudotuberculosis, taken by Per Hörstedt at Umeå Core Facility for Electron Microscopy (UCEM), edited by Bent Christensen
Printed by: VMC-KBC Umeå
Umeå, Sweden 2013
To Per and Rut
Abstract

The increasing use of antibiotics has led to a huge problem for society, as some bacteria have developed resistance towards many of the antibiotics currently available. To help find solutions to this problem we studied small molecules that inhibit bacterial virulence, the ability to cause disease. The type III secretion system (T3SS) is a conserved virulence system found in several gram-negative bacteria, including human and plants pathogens, such as Yersinia spp., Pseudomonas aeruginosa, Chlamydia spp., Salmonella spp., Shigella spp, enteropathogenic Escherichia coli (EPEC), enterohemorrhagic Escherichia coli (EHEC), and Erwinia spp. One class of virulence-blocking compounds is the salicylidene acylhydrazides. They were first identified in a screen towards the T3SS in Yersinia pseudotuberculosis and have since been shown to block the T3SS in a panel of gram-negative bacteria such as Chlamydia spp. Salmonella enterica, Shigella flexneri and EPEC.

We designed and synthesized a library of 58 salicylidene acylhydrazides and evaluated their activity as virulence-blocking compounds in Y. pseudotuberculosis followed by calculations of quantitative structure activity relationships (QSARs). Four QSAR models were calculated, and when used in consensus they correctly classified between five out of eight compounds for Y. pseudotuberculosis as active or inactive and six out of eight compounds for C. trachomatis.

Since the target and mode of action of the salicylidene acylhydrazides were unknown, we used solution and solid phase synthesis to synthesize three different affinity reagents. One of these affinity reagents was used in affinity chromatography experiments, where 19 putative target proteins from an E. coli O157 bacterial lysate were identified. We studied four of the proteins, Tpx, WrbA, FolX, and AdhE, in more detail in Y. pseudotuberculosis and E. coli O157. We believe that the salicylidene acylhydrazides act on multiple targets that together result in down-regulation of T3SS functions. A knockout of AdhE in E. coli O157 showed a similar phenotype as salicylidene acylhydrazide treated E. coli, suggesting that this protein may be particularly interesting as a drug target.

Many of the antibiotics used today originate from natural sources. In contrast, most virulence-blocking compounds towards the T3SS are small synthetic organic molecules. Therefore, a prefractionated natural product library with marine and terrestrial biota samples was screened towards the T3SS in Y. pseudotuberculosis. Neohopeaphenol A was identified as a hit and shown to have micromolar activity towards Y. pseudotuberculosis and P. aeruginosa in cell-based infection models.
Svensk Sammanfattning


I denna avhandling har vi designat och syntetiserat ett bibliotek med 58 salicylidenacylhydrazider och utvärderat deras biologiska aktivitet som virulensblockare i Y. pseudotuberculosis. Vi relaterade den biologiska aktiviteten till de kemiska egenskaperna hos salicyliden cetylhydraziderna i kvantitativa strukturaktivitetsamband. Med hjälp av dessa samband kunde vi prediktera och validera aktiviteten till aktiv eller inaktiv för fem av åtta nya salicylidenacylhydrazider i Y. pseudotuberculosis och sex av åtta i C. trachomatis.


Forskningen i denna avhandling visar att virulensblockare kan hjälpa oss att förstå hur bakterier orsakar sjukdom. Förhoppningsvis kan det i framtiden leda till nya typer av läkemedel mot infektionssjukdomar.
List of Papers

The work presented in this thesis is based on the following papers:


III. Caroline E. Zetterström, Jenny Hasselgren, Rohan A. Davis, Ronald J. Quinn, Charlotta Sundin, and Mikael Elofsson. The resveratrol tetramer neohopeaphenol A inhibits type III secretion in *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. (Manuscript)

Paper I and II have been reprinted with kind permission from the publishers.

# These authors contributed equally to this work.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boc</td>
<td>tert-butyloxycarbonyl</td>
</tr>
<tr>
<td>DIC</td>
<td>N,N’-diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DOOD</td>
<td>determinant optimal onion design</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EHEC</td>
<td>enterohemorrhagic Escherichia coli</td>
</tr>
<tr>
<td>EPEC</td>
<td>enteropathogenic Escherichia coli</td>
</tr>
<tr>
<td>Esp</td>
<td>Escherichia coli secreted protein</td>
</tr>
<tr>
<td>ETEC</td>
<td>enterotoxigenic Escherichia coli</td>
</tr>
<tr>
<td>Fmoc</td>
<td>fluorenylmethyloxycarbonyl</td>
</tr>
<tr>
<td>FRET</td>
<td>förster resonance energy transfer</td>
</tr>
<tr>
<td>h</td>
<td>hour/hours</td>
</tr>
<tr>
<td>Hi-PLS</td>
<td>hierarchical- partial least squares projections to latent structures</td>
</tr>
<tr>
<td>Hi-PLS-DA</td>
<td>hierarchical- partial least squares projections to latent structures-discriminant analysis</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HOAt</td>
<td>1-hydroxy-7-azabenzo triazole</td>
</tr>
<tr>
<td>HPBCD</td>
<td>(2-hydroxypropyl)-β-cyclodextrin</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>HTS</td>
<td>high throughput screen</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LEE</td>
<td>locus of enterocyte effacement</td>
</tr>
<tr>
<td>MIC</td>
<td>minimal inhibitory concentration</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MSNT</td>
<td>1-(2-mesitylenesulfonyl)-3-nitro-1H-1,2,4-triazole</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PLS</td>
<td>partial least squares projections to latent structures</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>QSAR</td>
<td>quantitative structure-activity relationship</td>
</tr>
<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
</tr>
<tr>
<td>SMD</td>
<td>statistical molecular design</td>
</tr>
<tr>
<td>STD</td>
<td>saturation transfer difference</td>
</tr>
<tr>
<td>Syc</td>
<td>specific Yop chaperone</td>
</tr>
<tr>
<td>T3SS</td>
<td>type III secretion system</td>
</tr>
<tr>
<td>TBAF</td>
<td>tetra-n-butyrammonium fluoride</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofurane</td>
</tr>
<tr>
<td>TIR</td>
<td>translocated intimin receptor</td>
</tr>
<tr>
<td>Yop</td>
<td>Yersinia outer protein</td>
</tr>
<tr>
<td>yp</td>
<td>Yersinia pseudotuberculosis</td>
</tr>
<tr>
<td>Ysc</td>
<td>Yersinia secretion</td>
</tr>
<tr>
<td>Å</td>
<td>Ångström</td>
</tr>
</tbody>
</table>
# Table of Content

**Introduction**  
1  
The Type III Secretion System in *Yersinia*  
2  
Inhibitors of Type III Secretion  
5  
Salicylidene Acylhydrazides  
6  
Other Synthetic Compounds  
9  
Natural Products  
14  
Multivariate Methods in Drug Development  
15  
Target Identification  
16

**Objectives**  
19

**QSAR Modeling of Salicylidene Acylhydrazides**  
21  
Statistical Molecular Design  
21  
Synthesis  
22  
Biological Evaluation  
22  
QSAR Models  
24  
Summary  
29

**Identification of Targets of the Salicylidene Acylhydrazides**  
31  
Synthesis of Affinity Reagents  
31  
A Salicylidene Acylhydrazide Connected to a Solid Support  
31  
Biotinylated Salicylidene Acylhydrazides  
33  
Salicylidene Acylhydrazide with Azide and Biotin  
34  
Putative Targets Identified with Affinity Chromatography  
35  
Binding of Salicylidene Acylhydrazides to Putative Targets  
36  
Far Western Blotting  
36  
NMR Spectroscopy  
37
Introduction

A bacterium is a small (0.5-5 \( \mu \text{M} \)) simple unicellular organism. Bacteria are prokaryotes, which means that they do not have a cell nucleus or organelles. An estimated hundred thousand billion bacteria live and coexist in and on the average human. Normally, these bacteria cause no harm, but if the equilibrium is disturbed, people may become susceptible to infection, causing morbidity or in the worse case death. These infections are often treated with antibiotics.

Traditional antibiotics are divided into two subgroups: bacteriocidal compounds, which kill bacteria, and bacteriostatic compounds, which inhibit bacterial growth. However, antibiotic treatment not only kills or inhibits the growth of the pathogenic bacteria but also affect the endogenous gut microflora, causing gastrointestinal problems. The overuse of antibiotics against bacterial infections in both humans and animals has increased worldwide. This has led to the problem of bacteria developing resistance towards the antibiotics. Resistance can occur in two ways: either acquired, mostly from our endogenous bacteria, through horizontal gene transfer, or intrinsic, naturally occurring mutations generated through evolution (survival of the fittest). Bacteria have a very rapid life cycle, and hence evolve relatively rapidly. However, only a few new antibiotic drugs have been developed in the last 40 years\(^1\). This is alarming and means that novel therapies are urgently needed to safeguard human health.

Different bacteria have different virulence systems involved in infection. Virulence systems are novel targets for antimicrobial compounds\(^1\). By attacking such targets, the bacteria would be unarmed rather than killed, and the reaction in the body would likely be similar to vaccination with a live impaired bacterial strain. As the bacteria would still be able to grow and proliferate, the innate and adaptive immune response must clear the infection with minimal effect on the endogenous microflora. It has been hypothesized that the selective pressure to develop resistance towards this kind of compounds is lower compared to conventional antibiotics\(^1-3\). Virulence-blocking compounds could target different systems, e.g., adhesion to the target cell\(^4\), bacterial signaling\(^5\) or effector protein delivery systems, such as the type III secretion system (T3SS)\(^6\).
The Type III Secretion System in *Yersinia*

*Yersinia pseudotuberculosis* is a gram-negative bacterium that belongs to the *Yersinia* genus. The *Yersinia* genus includes eleven species, three of which are pathogenic to man, i.e., *Y. enterocolitica*, *Y. pestis*, and *Y. pseudotuberculosis*. *Y. pestis* is probably the most well-known since it caused the bubonic plague/Black Death in the middle of the 14th century. *Y. enterocolitica* and *Y. pseudotuberculosis* cause inflammation in the gastrointestinal tract of humans and spread through the fecal-oral route, usually from contaminated food or water. The three pathogenic species carry a 70 kb virulence plasmid, pYV, encoding the type III secretion system (T3SS). The T3SS was first discovered in 1994. It is a conserved virulence system utilized by many gram-negative bacteria, including both human and plant pathogens, such as *Yersinia* spp., *Pseudomonas aeruginosa*, *Chlamydia* spp., *Salmonella* spp., *Shigella* spp, enteropathogenic *Escherichia coli* (EPEC), and enterohemorrhagic *Escherichia coli* (EHEC), and *Erwinia* spp. (Figure 1).

![Diagram of bacterial infection sites](image)

**Figure 1.** Schematic over where bacteria cause disease. Drawing by Asa Gylfe.
The T3SS is a protein complex that is important for delivery of effector proteins into the host cell where they cause disease. The protein complex contains more than 20 proteins, forming a needle-like structure spanning the inner and outer bacterial membrane called the *Yersinia* secretion (Ysc) injectisome. *Yersinia* have six effector proteins known as Yops for *Yersinia* outer proteins that are translocated into the eukaryotic cell and three translocator proteins. The effector proteins are not toxic outside of the eukaryotic cell but they work together, inhibiting innate immunity and destroying the cytoskeleton in the host cell, thereby allowing bacteria to avoid phagocytosis, proliferate and spread to a new host. The effector protein YopE functions as a GTPase activating protein (GAP) and it is an essential virulence factor that disrupts actin microfilaments in the infected target cell. YopH is a protein tyrosine phosphatase. It has been shown that YopH inhibits phagocytosis and is an essential virulence factor since YopH single knockout mutants are non-virulent. The effect of YopJ involves the innate immune system and cytokine production. YopM is important for virulence in all *Yersinia* species but its exact function has not yet been determined. The effect of YopT is similar to YopE in that it disrupts the cytoskeleton, but it is not present in all *Yersinia* strains. The *Yersinia* protein kinase A (YpkA), helps the bacteria resist phagocytosis and is essential for virulence. The three translocator proteins, LcrV, YopB, and YopD, form the tip of the needle in the T3S complex and the pore in the eukaryotic cell, facilitating effector translocation. The virulence plasmid also encodes the specific Yop chaperones (Sycs), which are required for some Yops to be secreted.

The regulation of the *Yersinia* T3SS is temperature dependent. When the bacterium senses a temperature shift to 37 °C, the Ysc proteins build up the T3S machinery, and upon bacterial contact with the host cell, the Yops are secreted. At the temperature shift, a transcriptional activator, LcrF, stimulates transcription of the *yop* and *syc* genes. If there is no contact with an eukaryotic cell, a feedback inhibition loop is activated and LcrQ accumulates in the cytosol, suppressing the expression of the Yops. Upon cell contact, LcrQ is released from the cytosol, and Yops are expressed and transported to the Ysc apparatus aided by their Sycs (Figure 2).
How the effectors are translocated is still not completely understood. According to the first proposed model, the effector proteins are translocated through the T3SS apparatus\textsuperscript{10,16}. However, Akopyan et al. has in a recent report suggested that translocation occurs via a two-step process. They showed that Yops were present on the bacterial cell surface, prior to target cell contact. In addition when YopH was added externally it could be translocated into the target cell in a T3SS-dependent manner\textsuperscript{17}. This requires a functional T3SS, a specific translocation domain in the effector protein YopH, the translocators YopB and YopD\textsuperscript{17}.

An in vitro model mimicking the effect of bacterial infection has been developed. By depletion of calcium in the media, at the same time as the temperature is shifted from 26 to 37 °C, secretion is triggered. This phenomenon is called the low calcium response. The low calcium response is a useful tool in the lab, where experiments can be made on bacteria without involving eukaryote cells.

Figure 2. Schematic of the processin the *Yersinia* spp. T3SS at 37 °C. Left: No host cell contact, the T3S machinery is constructed, LcrQ accumulates in the cytosol and suppresses expression of Yops and Syys. Right: When a bacterium senses contact with the host cell, LcrQ is released from the bacterium and the transcriptional activator LcrF stimulates transcription of *yop* and *yse* genes; Yops are expressed and transported by the Syys to the Ysc machinery for secretion.
The T3SS is evolutionarily related to the flagella\textsuperscript{18} with the flagella as the suggested ancestor\textsuperscript{19}. In \textit{E. coli}\textsuperscript{20} and \textit{Y. pseudotuberculosis}, expression of the two systems have shown to be cross regulated to allow either motility or T3SS mediated secretion and translocation. Bacterial protein secretion systems play an important role in interactions between bacteria and their environment. In addition to the T3SS, gram-negative bacteria have five other secretion systems. In the Type I, III, IV, and VI secretion systems, transportation across the inner and outer membrane occurs in one step. In the type II and V secretion systems, and less commonly in the type I and IV secretion systems, secretion occurs first to the periplasm and then further across the outer membrane. Gram-positive bacteria share some of these systems but also have an additional secretion system, known as type VII secretion system. \textit{Y. pseudotuberculosis} and its T3SS is well-studied, especially here at Umeå University, and therefore serves as a good model organism for identification of T3SS inhibitors.

**Inhibitors of Type III Secretion**

The drug discovery process often starts with high throughput screen (HTS) where libraries of many compounds are tested in a robust biological assay. A hit from the biological assay is further validated. To improve the biological activity and establish a structure activity relationship (SAR), analogs are designed, synthesized, and tested. The selection of analogs can preferably be made with multivariate methods. If the target for the compound is unknown target identification and validation can be performed in parallel. One or a couple of the best compounds, leads, are further tested \textit{in vitro} and \textit{in vivo}. At this stage pharmacokinetic and pharmacodynamic properties as well as formulation of the compound needs to be assessed. The last steps in the drug discovery process are clinical trials.

Over the last decade, several screens have been performed to identify virulence-blocking compounds towards the T3SS, and the field has been extensively reviewed in the last year\textsuperscript{21-23}.
Salicylidene Acylhydrazides

In 2003, 9400 compounds were screened by Kauppi et al. for inhibition of the Y. pseudotuberculosis T3SS. Screening was carried out with a phenotype based whole cell assay that was a luciferase reporter gene assay of YopE expression. The screen resulted in identification of three compound classes as potential virulence-blocking compounds, the salicylanilides, arylsulfonylamino-benzanilides and the salicylidene acylhydrazides (Figure 3 and 4). These compounds were shown to target the T3SS and did not significantly inhibit growth. In addition, the salicylidene acylhydrazides were shown to inhibit both the YopE reporter gene signal (Figure 7A) and the bacterial motility. The salicylidene acylhydrazide class was deemed the most promising of the three compound classes and was studied in further detail. Nordfelth et al. showed that one of the salicylidene acylhydrazides INP0007 specifically blocks the secretion of Yops independent of the calcium concentration in the growth medium. It was shown that the effect of the compound was fast and reversible and it does not have a general toxic effect on the bacteria.

The salicylidene acylhydrazides (Figure 3) have also been shown to inhibit virulence in several other gram-negative bacteria. In the obligate intracellular pathogen Chlamydia, there is currently a lack of tools for genetic manipulation. Therefore, the salicylidene acylhydrazides are important chemical tools for studying bacterial functions such as the T3SS. These compounds have been shown low toxicity to the host cell and inhibit the intracellular replication and infectivity of Chlamydia trachomatis in a dose-dependent manner and at different stages of the Chlamydia life cycle. The fact that the effect is reversible is a useful property when using the compounds as chemical probes. Salicylidene acylhydrazides have also been shown to inhibit Chlamydia pneumonia and C. caviae. Layton et al. have shown that addition of exogenous iron attenuates ME0053/INP0403 (Figure 3) in Salmonella enterica serovar Typhimurium. Treatment with the compound resulted in down-regulation of genes involved in T3SS and up-regulation of genes associated with ion acquisition. In addition, most of the flagella genes showed a 1.5-2 fold down-regulation, but these data were not statistically significant. It has also been shown that addition of different iron sources in Chlamydia infected cells, attenuate the effect of the salicylidene acylhydrazides. However, they also showed that the inactive salicylidene acylhydrazide INP0406 retains its ion chelating properties. The underlying mechanism of this is so far unknown, but it is suggested that inhibition of Chlamydia growth may be due to ion chelation. However, Engström showed in his PhD thesis that INP0341 inhibited the generation of
infectious *C. trachomatis* elementary bodies. The elementary body is the environmentally stable and infectious form of *Chlamydiae*. These results indicate that the salicylidene acylhydrazides have an additional mode of action, next to ion chelation.

Another study of the salicylidene acylhydrazides in *Salmonella enterica* by Negrea *et al.* showed that the compounds block the secretion of the translocator protein SipB, a homolog to *Yersinia* YopB, but not the expression. The compound ME0052/INP0010 (Figure 3) also found to inhibit bacterial replication in the intracellular phase of the infection as well as the flagella motility system. The first *in vivo* study of the salicylidene acylhydrazides was made in a bovine intestinal ligated loop model by Hudson *et al.* Ligated loops were inoculated with *Salmonella enterica* pretreated or co-injected with two different salicylidene acylhydrazides and DMSO as a control and the T3SS mediated intestinal secretory and inflammatory response to eukaryotic cells was measured. The studied compounds lowered the responses in the pretreated bacteria cultures but not in the co-injected cultures. It was proposed that this might be due to rapid diffusion of the compound into the intestinal fluid upon co-injection.

![Figure 3. Structures of some salicylidene acylhydrazides. From left to right: common scaffold, ME0052/INP0010, ME0053/INP0403, ME0168, and INP0341.](image)

In *Shigella flexneri*, the secretion of T3SS effector proteins has been shown to be reduced by treatment with salicylidene acylhydrazides. The compounds also inhibit infection of the mouse macrophage cell line J774. The same study Veenendaal *et al.* investigated the assembly of the needle complex. No significant differences in the amount of the two needle complex proteins studied were detected, but electron microscopy analyses revealed a 30-40% decrease in the number of T3S injectisomes.

In *Escherichia coli* O157:H7, four salicylidene acylhydrazides were tested by Tree *et al.* and all were found to inhibit secretion of the virulence factor translocated intimin receptor (Tir) and the *E. coli* secreted protein D (EspD). The compounds reduce expression of the T3SS encoded locus of enterocyte effacement (LEE) genes, and the most effective compound, ME0055, also reduced attaching and effacing lesion formation, which requires the T3SS.

The salicylidene acylhydrazides have not just been used against T3SS. In a recent study, Chu *et al.* showed that the compounds can be used as potential broad-spectrum genital microbicides, believed to be due to iron chelation of the compound. The compounds have been shown to inhibit HIV-1 at low
micromolar concentrations\textsuperscript{37}, \textit{C. trachomatis}, and \textit{Neisseria gonorrhoeae}, a bacteria requiring iron for growth\textsuperscript{38}, but not \textit{Lactobacillus}\textsuperscript{36}, which is a bacteria that naturally occurs in the vagina. In the same study, they showed that a salicylidene acylhydrazide could lower the infection rate of \textit{C. trachomatis} in a vaginal mouse model\textsuperscript{34,36} suggesting that these virulence-blocking compounds have a broad spectrum potential. In addition, the inhibition by the salicylidene acylhydrazides of the two pathogens \textit{C. trachomatis}, and \textit{Neisseria gonorrhoeae} was reversed by the addition of iron\textsuperscript{36}. However, the effect of salicylidene acylhydrazide treatment was not only due to iron chelation, recent data showed that \textit{C. trachomatis} treated with INP0341 exhibit additional effect(s) distinguishable from the iron chelating effect\textsuperscript{34}.

In an additional \textit{in vivo} study of INP0341 (Figure 3) as a potential microbiocide towards \textit{C. trachomatis}, Slepenkin \textit{et al.}\textsuperscript{39} showed that the compound is stable in a vaginal fluid simulant at pH 4.2-7.5. The compound appered to protect mice from vaginal \textit{C. trachomatis} infection for in most cases up to three weeks\textsuperscript{39}.

The 58 salicylidene acylhydrazides synthesized in Paper I have also been evaluated for their effect towards \textit{Chlamydia} spp. infected HeLa cells. Twelve of the 58 compounds were found to have a minimal inhibitory concentration (MIC) between 3 and 50 \(\mu\)M in both \textit{C. trachomatis} and \textit{C. pneumonia}\textsuperscript{40}. Pharmacokinetic analysis in mice with the salicylidene acylhydrazides administered in cassette dosing\textsuperscript{40} showed that ME0177 had the highest maximal plasma concentration and ME0192 (Figure 3) had the longest plasma half-life. ME0192 was also tested in the vaginal mouse model used by Chu \textit{et al.},\textsuperscript{36} where it was shown to inhibit the \textit{C. trachomatis} infection.\textsuperscript{40}

Formulation and administration of compounds needs to be considered before a compound is tested \textit{in vivo}. Six different formulations of the salicylidene acylhydrazide ME0052 were made and studied in a mouse model\textsuperscript{41}. A slow release formulation was made with Poloxamer 407 (P407), which is a thermoreversible gel. The release of ME0052 was confirmed to be slow, but due to the short half-life, therapeutic concentrations in plasma could not be reached. The subcutaneous administration of P407 turned out to be toxic to the mice. Therefore, the gel might be more suitable for topical treatment\textsuperscript{41}. Formulation with the polysaccharide (2-hydroxypropyl)-\(\beta\)-cyclodextrin (HPBCD) made it possible to increase the concentration of ME0052 to 16 mM at physiological pH. This treatment did not affect the mice and gave a maximal plasma concentration of 33 \(\mu\)g/mL (80 \(\mu\)M), which is above the effective concentration for \textit{Y. pseudotuberculosis}\textsuperscript{41}. This indicates that systemic administration of the salicylidene acylhydrazide is difficult. To address the problem of the short half-life of the compounds, the compound and/or the formulation need to be further developed.
Identified Targets of the Salicylidene Acylhydrazides

The targets of the salicylidene acylhydrazides have been unknown for a long time. In the study described in Paper II, we identified 19 potential target proteins for the salicylidene acylhydrazides in *E. coli* O157 and studied three of them in more detail.

Another study aimed at finding the target for the salicylidene acylhydrazides in *Salmonella enterica* serovar Typhimurium was made in parallel by Martinez-Argudo *et al.* In *S. enterica* serovar Typhimurium, treatment with salicylidene acylhydrazides, INP0404 and INP0405 resulted in a phenotype with reduced swimming motility. This result agreed with an earlier study of salicylidene acylhydrazide treated *Y. pseudotuberculosis*. After observing reduced motility upon compound treatment, the authors used a genetic screen to search for mutants with retained swimming abilities in the presence of compound. They identified two mutants that were more motile than the background and applied whole genome sequencing to find the target mutations and potential target proteins. The hypermobile mutant contained two mutations. One mutation was in the protein coding regions of *atpB*, which encodes the inner membrane α-subunit of the F0F1-ATP synthase. The other mutation encoded the γ-subunit of a fatty acid oxidation complex. The salicylidene acylhydrazide insensitive mutant contained a single nucleotide change in *flhA* a gene encoding the flagella inner membrane protein FlhA.

In addition, Engström recently presented in his PhD thesis, potential targets of the salicylidene acylhydrazides in *C. trachomatis*. Selection of *C. trachomatis* populations resistant to INP0341 was made. They performed whole genome sequencing of the mutant population and found a mutation in *hemG*, which they showed mediated the resistance to INP0341.

Other Synthetic Compounds

In addition to the salicylidene acylhydrazides, two other compound classes, salicylanilides and arylsulfonylamino-benzenilides (Figure 4, Table 1), were identified in the initial screen for T3S inhibitors made by Kauppi *et al.* Since *Y. pseudotuberculosis* treated with salicylanilides inhibits the LcrF reporter gene signal, they were suggested to have a regulatory effect upstream of the temperature triggered activator LcrF. The salicylanilides did not affect bacterial motility, which suggests that they affect regulation of the Ysc T3SS. One of the studied unacetylated salicylanilides showed strong growth inhibition for *Y. pseudotuberculosis*. Interestingly, the acetylated counterpart only showed a slight growth effect. These results indicate that the acetylated and unacetylated compounds have different modes of action. In the same study, 50 salicylanilide
analogs were synthesized and biologically tested in the original screening assay as well as tested for growth inhibition. Quantitative structure-activity relationship (QSAR) models were calculated to correlating the biological response with structural changes. The model showed good correlation between predicted and experimental data and was able to correctly rank new compounds according to their biological activity.

The arylsulfonylamino-benzanilides (Figure 4, Table 1) were shown to inhibit T3SS and Yop secretion. However, the growth and motility were unaffected at low concentrations, and due to limited solubility, the effect of higher concentrations was unclear. In an attempt to identify other putative T3S inhibitors, seven analogs were synthesized and a structure activity relationship (SAR) was established. With the SAR results in hand, a statistical molecular design (SMD) was calculated with all possible combinations, yielding 612 virtual compounds. Out of these, 19 were synthesized, biologically evaluated and a QSAR model was calculated which showed that the hydrophobicity and halogenation patterns were important for activity.

Several groups working with different organisms have used phenotypic screening to find inhibitors of the T3SS. Gauthier et al. screened 20000 compounds towards EPEC, for their ability to affect the secretion of one of the effector proteins, EspB, but not affect growth. One hit compound, a salicylideneaniline (Figure 4, Table 1), was studied in further detail and was shown to reduce the amount of secreted proteins through a regulatory mechanism but did not directly affect the T3SS apparatus. In 2007 Pan et al. developed a screen for T3SS inhibitors in a luminescent Y. pestis strain. They screened over 70000 compounds in an assay in which they assumed that a T3SS inhibitor would suppress the low calcium response and thereby facilitate bacteria to grow in the absence of calcium. Eight compounds from different compound classes were selected and evaluated in a secondary assay, where four inhibited Yop secretion and turned out as promising leads for SAR studies. One of the compounds was cytotoxic, but the other three compounds protected infected HeLa cells. Treatment of Y. pestis with the three different compounds gave rise to three different secretion profiles of Yop M, H, and D. This result together with the result that only two of the compounds, compound 1 and compound 4 (Figure 4), inhibited secretion of the effector protein Tir in EPEC KC14, indicates that the compounds have different targets.
The 2-imino-5-arylidene thiazolidin-4-one, TTS29, (Figure 4, Table 1) was first identified by Felise et al. as a hit in a screen of 92000 natural and synthetic compounds for inhibitors towards the T3SS in Salmonella enterica serovar Typhimurium. In their assay, they used the phospholipase YpA of Y. enterocolitica as reporter to the effector protein SipA. Inhibition of secretion of the reporter protein, and hence the T3SS, was measured by using a fluorescent substrate for the phospholipase. TTS29 was the only hit that did not affect growth, general or T3SS specific transcription, but was suggested to target the assembly of the T3SS in S. typhimurium. In addition, the compound was shown to be a potent virulence-blocking compound in several species. It inhibited the T3SS in Y. enterocolitica and the plant pathogen Pseudomonas syringae, the type II secretion system and the type IV pilus assembly in P. aeruginosa, as well as secretion of Francisella novicida virulence proteins. SAR studies of TTS29 showed that the only position tolerant for modification was the 3-amido nitrogen. Modifications in this position improved the biological activity for inhibition of SipA to low micromolar ranges. Further development of TTS29 by modifying the 3-amido position with peptide chains or as a dimer, resulted in compounds with higher potency for SipA compared to the initial compound. The authors speculated that the common target for the compound in these species may be involved in the secretion or assembly process of the T3S apparatus. A more direct target could be the secretin, a protein that is a part of the type II, type III secretion systems as well as type IV pilus assembly.

The company Microbiotix published results of a screen to identify inhibitors towards the T3SS in Pseudomonas aeruginosa. Two whole cell reporter
assays yielded in five inhibitors, which belong to three compound classes, with IC\textsubscript{50} values in low micromolar range. The most promising compound was the phenoxyacetamide MBX1641 (Figure 4, Table 1), which was also shown to inhibit the T3SS of C. trachomatis and Y. pestis\textsuperscript{53}. Based on SAR studies, it was found that the R-enantiomer was the active enantiomer.

Harmon \textit{et al.} performed a screen for small molecules that specifically inhibit translocation but not Yop synthesis or secretion of the effector proteins\textsuperscript{52}. Six of the eight hits from the screen were shown to cause leakage but not internalization of Yops from \textit{Y. pseudotuberculosis} infected HEp-2 cells. One compound, \textbf{compound 20} (Figure 4), also reduced \textit{Y. pseudotuberculosis} adherence to HEp-2 cells. The effect of the compounds was also tested in \textit{P. aeruginosa}. Five of the eight compounds (Table 1) inhibited ExoS-mediated cell rounding in \textit{P. aeruginosa} infected HEp-2 cells\textsuperscript{52}. The effector protein ExoS in \textit{P. aeruginosa} is highly similar to YopE in \textit{Yersinia} and disrupts actin microfilaments in the host cell. The inhibitory effect on translocation indicates a common conserved target between the species.

Świetnicki \textit{et al.}\textsuperscript{53} showed that a \textit{Y. pestis} strain where the catalytic domain of the \textit{yscN} gene was deleted was non-virulent in mice. Furthermore, the authors performed a computational screen of commercially available drug-like molecules towards the ATPase YscN in \textit{Y. pestis}. The potential inhibitors were validated in an \textit{in vitro} ATPase assay. Compound 7146 (Figure 4, Table 1), were shown to inhibit the homolog, BsaS from \textit{Burkholderia mallei} and the secretion of YopE in \textit{Y. pestis}.

In another study, previously known \textit{Chlamydia} T3SS inhibitors were subjected to structural analysis, experimental screening, chemical improvement, and synthesis in order to find a new inhibitor. A thiadiazine, \textbf{compound 17}, (Figure 4, Table 1) was considered one of the best compounds found and shown to inhibit the reproduction of three \textit{Chlamydia} species, \textit{C. trachomatis}, \textit{C. pneumonia}, and \textit{C. muridarum} in cell culture models\textsuperscript{54}.

Engquist \textit{et al.} reported on SAR studies performed on derivatives of 8-hydroxyquinoline found in an additional screen using the YopE reporter-gene assay in \textit{Y. pseudotuberculosis}. The 8-hydroxyquinoline was shown to be a virulence-blocking compound that inhibits the T3SS in \textit{Y. pseudotuberculosis} and replication of \textit{C. trachomatis}\textsuperscript{65}. The best compound, \textbf{INP1855} (Figure 4, Table 1), inhibited T3SS with an EC\textsubscript{50} of 6.2 µM for \textit{Y. pseudotuberculosis} and the minimal inhibitory concentration (MIC) at 3.2 µM for \textit{C. trachomatis}\textsuperscript{65}.

In addition, to the more general T3SS inhibitors described above and found by screening, several groups have investigated T3SS inhibitors and many of them affect the T3SS regulation\textsuperscript{56-60}. A considerable effort is also being put into finding inhibitors towards different effector proteins\textsuperscript{61}, particularly the \textit{Yersinia} effector protein YopH\textsuperscript{62-73}.
<table>
<thead>
<tr>
<th>Compounds</th>
<th>HTS</th>
<th>Possible target or mode of action</th>
<th>Effective against</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylanilide</td>
<td>YopE reporter luciferase</td>
<td>Unknown, possibly a regulatory mechanism</td>
<td><em>Y. pseudotuberculosis</em></td>
<td>6,43,74</td>
</tr>
<tr>
<td>Arylsulfonylamino-benzanilides</td>
<td>YopE reporter luciferase</td>
<td>Unknown</td>
<td><em>Y. pseudotuberculosis</em></td>
<td>6,44</td>
</tr>
<tr>
<td>Salicylideneanilide</td>
<td>EspB secretion and growth</td>
<td>Unknown, suggested to be a regulatory mechanism</td>
<td><em>E. coli, P. aeruginosa</em></td>
<td>45</td>
</tr>
<tr>
<td>Compound 1 and 4</td>
<td>Monitored luminescent bacteria growth using the low calcium response</td>
<td></td>
<td><em>Y. pestis, EPEC</em></td>
<td>47</td>
</tr>
<tr>
<td>2-imini-5-arylidene thiazolidin-4-one, TTS29</td>
<td>SipA-YplA fusion protein secretion</td>
<td>Possibly secretion or assembly of the T3SS</td>
<td><em>S. typhimurium, P. aeruginosa, P. Syringae, F. mellitor</em></td>
<td>48-50</td>
</tr>
<tr>
<td>Phenoxacetamide MBX1641</td>
<td>ExoT luciferase transcriptional fusion</td>
<td></td>
<td><em>P. aeruginosa, C. trachomatis, Y. pestis</em></td>
<td>51</td>
</tr>
<tr>
<td>Compound 15, 19, 20, 22, 24, 34, and 38</td>
<td>Yop translocation, monitored by fluorescence</td>
<td>Unknown</td>
<td><em>Y. pseudotuberculosis, P. aeruginosa</em></td>
<td>52</td>
</tr>
<tr>
<td>7176</td>
<td>Lowering YscN ATPase activity</td>
<td>YscN ATPase</td>
<td><em>Y. pestis</em></td>
<td>53</td>
</tr>
<tr>
<td>Thiadiazine, Compound 17</td>
<td>In silico design</td>
<td>Unknown</td>
<td><em>C. trachomatis, C. pneumonia, C. muridarum</em></td>
<td>54</td>
</tr>
<tr>
<td>8-hydroxyquinoline, INP1855</td>
<td>YopE reporter luciferase</td>
<td>Unknown</td>
<td><em>Y. pseudotuberculosis, C. trachomatis</em></td>
<td>55</td>
</tr>
</tbody>
</table>
Natural Products

From a chemical perspective, a natural product is a compound found in nature produced by a living organism, which often has biological activity. Many of the antibiotics used today originated from plants, microorganisms, or invertebrates. The natural products have coevolved with their host and are produced with the purpose to fit into a protein that is beneficial for the host. In contrast, most T3SS inhibitors currently described in the literature are small synthetic organic drug-like compounds. However, there are some studies of natural products as T3SS inhibitors.

In 2002, a screen was performed by Linnington et al. with the aim of finding T3SS inhibitors extracted from marine invertebrates. In this screen, the natural product caminoside A (Figure 5) was found to inhibit the secretion of effector proteins, EspS, in EPEC. Later caminocides B, C, and D were also shown to be T3SS inhibitors. Interestingly caminocides A, B, and D also showed antimicrobial activity against vancomycin resistant Enterococcus as well as methicillin resistant Staphylococcus aureus (Table 2).

In another study performed by Iwatsuki et al. searching for T3SS inhibitors, six guadinimines were isolated from a Streptomyces culture broth prepared from a soil sample. These six guadinimines were shown to inhibit EPEC T3SS induced hemolysis of erythrocytes. Guadinamines A, B and D showed dose-dependent inhibition, with guadinamine B (Figure 5) having an IC50 of 14 nM. These data suggests that natural products can be potent inhibitors of the EPEC T3SS (Table 2).

![Figure 5. Structures of natural products inhibiting the type III secretion](image)

Aurodox (Figure 5) is another natural product found in culture broth extract from actinobacteria. The compound specifically inhibits EPEC T3SS mediated hemolysis with an IC50 of 1.8 µM. In an in vivo model, mice were infected with a lethal dose of Citrobacter rodentium, a rodent homolog to EPEC.
with the T3SS (Table 2). The mice were administrated with aurodox once a day for the first four days (25 or 100 mg/kg/day). All the treated mice survived to day 18, in contrast to DMSO-treated control mice, which died between day 11 and 17. The mechanism whereby aurodox inhibits the T3SS is still unknown, but it was suggested to inhibit the transcription of virulence genes similarly to how salicylidene acylhydrazides inhibit the T3SS in EPEC. Aurodox is also known as the antibiotic X-5108, goldinodox, or goldinomycin, which has been shown to inhibit protein biosynthesis by acting on the bacterial elongation factor Tu.

In a screening campaign for *Y. pseudotuberculosis* T3SS inhibitors made at Eskitis Institute, Griffith University, Brisbane, Australia, a prefractionated natural product library constructed of extracts from marine and terrestrial biota samples was screened. In this screening, pseudoceramine B (Figure 5), spermatinamine, and neohopeaphenol A (Paper III) were found as hits. In addition to inhibition of the T3SS, Pseudoceramine B and spermatinamine also showed growth inhibition, indicating general antibacterial activity (Table 2).

<table>
<thead>
<tr>
<th>Compound</th>
<th>HTS</th>
<th>Source</th>
<th>Effective against</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caminosides</td>
<td>Esp secretion</td>
<td>Marine sponge</td>
<td>EPEC</td>
<td>75,76</td>
</tr>
<tr>
<td>Guadinimines</td>
<td>Measured the hemolytic activity of EPEC</td>
<td><em>Streptomyces</em> spp. K01-0509</td>
<td>EPEC</td>
<td>77</td>
</tr>
<tr>
<td>Aurodox</td>
<td>Measured the hemolytic activity of EPEC</td>
<td><em>Streptomyces</em> spp.</td>
<td>EPEC, <em>Citrobacter rodentium</em></td>
<td>79</td>
</tr>
<tr>
<td>Pseudoceramines and spermatinamine</td>
<td>YopE reporter luciferase</td>
<td>Marine sponge</td>
<td><em>Y. pseudotuberculosis</em></td>
<td>81</td>
</tr>
</tbody>
</table>

Since a number of synthetic organic molecules inhibiting the T3SS it is not surprising that natural products also have been found as potent inhibitors of the T3SS.

**Multivariate Methods in Drug Development**

Different strategies are used to improve the biological activity of a compound in the field of drug development. If the target for a compound is known, a variety of methods can be used, such as molecular docking and structure-based design. However, if the target for a compound is unknown, as in our case (Paper I),
some kind of ligand-based strategy can be used to improve biological activity. One computational strategy is to use SMD together with QSAR modeling.

SMD is used to ensure structural diversity among a virtual set of molecules, spanning the chemical space in both structural and physicochemical properties, while at the same time minimizing the number of compounds that need to be synthesized and tested. For a specific compound class, the SMD can be made at the building block or product level. As long as an adequate number of building blocks are selected, SMD at the building block level has several advantages over design of final products. In SMD, physicochemical properties describing the building blocks are usually compressed by a principal component analysis (PCA) before further selection. In a PCA, the main variation in the data is extracted to identify similarities and differences between the building blocks (or molecules), which makes the data less complex and easier to interpret. Using SMD, a selection of a small subset of building blocks can be extracted from a large collection of building blocks without losing too much physicochemical diversity. The building blocks are subsequently combined to make products, and these molecules are then bought or synthesized and further tested experimentally for a certain biological response. The response can be the output of any robust biological assay that can be used to rank the activity of the tested compounds.

The biological response may be related to the properties of the constituting building blocks of a compound using QSAR models. In the work described in Paper I, we used partial least squares projections to latent structures (PLS) to generate QSAR models. PLS is a linear regression method that can be used to correlates the descriptive data (e.g., descriptors of compounds) to response data (e.g., a biological readout). SMD offers several benefits for obtaining a good quality QSAR model with a high predictive capacity. Since SMD ensures diversity among the compounds, it increases the chance of sufficient variation in the response, yielding a good QSAR model. If SMD is applied to the building blocks of the compound, it contains local information of the compounds as opposed to global information. This makes it easier to interpret the QSAR models and make decisions on how to change the compounds to increase the biological response.

Target Identification

The two most common ways to identify biologically active compounds are phenotypic- and target-based screening. The main advantage of phenotypic-based screening is that the complex biological system can be taken into account. The phenotypic-based screening is therefore more popular and has resulted in more drugs on the market between 1999 and 2008 than target-based screening. However, identification of target/targets of biologically active compounds...
identified by phenotypic-based screening it is often challenging. Many small organic compounds also tend to have more than one target\textsuperscript{87}. In a target-based screening, as the name indicates, the target is known. For instance, this type of screening may examine inhibition of an enzyme \textit{in vitro}.

There are several ways to identify a target for a molecule. We successfully used affinity chromatography (\textbf{Paper II}) followed by identification with proteomics to identify the putative targets for the salicylidene acylhydrazides. This technique aims to “fish out” the target of interest based on its binding to the bioactive molecule attached to a solid support. After the affinity molecule has bound to the potential target proteins, extensive washing is performed followed by final elution with free molecule or harsh washing. The eluted proteins are separated by gel electrophoresis and identified by mass spectrometry\textsuperscript{88-91} (Figure 6A).

Another similar technique with almost the same workflow is activity-based protein profiling (Figure 6B). Here, the activity-based probe consists of three parts: 1) a reactive electrophile, a “clickable” or photoreactive group, e.g., alkyne or azide, which is used to facilitate a covalent interaction between the molecule and the target. 2) a linker and/or affinity group, which minimizes steric hindrance and enables the molecule to bind to specific part of the target; 3) a reporter or tag, such as biotin, used for the detection and separation of targets\textsuperscript{89,92-94}.

The main advantage of these techniques is that they can be performed in any organism and on any cell type lysate. In addition, there is no limitation to identify proteins in a panel of recombinant proteins. Instead, the whole proteome is available. However, two potential problems are the analysis method does not detect each protein equally well and that membrane proteins can be troublesome due limited solubility. The SAR of the bioactive compound needs to be taken into account when designing the compounds. To increase the chances of identifying the actual target, several compounds should be designed with linkers in different places and with different lengths.

In addition, label-free techniques are also possible, such as drug affinity responsive target stability (DARTS) (Figure 6C). This technique has the advantage that the bioactive compound need not to be chemically modified. The method is based on the higher stability of a protein compound complex compared to the free protein. The main principle behind DARTS is that proteases added to the protein compound mixture will hydrolyze free protein to small peptides but the compound-protein complex remains intact. After gel electrophoresis, the protein in the compound-target complex can be identified with mass spectrometry\textsuperscript{89,95,96}.

Selection of compound resistant mutants is another method used in drug target deconvolution. By sequencing the resistant mutants, either by whole genome sequencing or RNA/transcriptome sequencing, followed by comparison
between wild-type and mutant sequences, the target(s) can be identified. The advantage with this method, as for DARTS, is that there is no need for chemical modification of the compound. However, to ensure a reliable result, resistance must occur at a high frequency in order to generate several resistant clones containing the same mutations. In cases where more than one target is involved, this can be hard to achieve.94,97 There is also a risk for detecting false targets due to a mutation that has occurred to compensate for the compound effect but is not directly connected with the target.

The rapid development of techniques used to determine potential targets for a compound of interest makes it hard to recommend any technique over another. All have advantages and disadvantages, and to increase the chance of successfully identifying target protein(s), a combination of techniques and target validation is often the best approach.

![Diagram of target identification methods](image)

**Figure 6.** Schematic showing the principle behind different target identification methods, (A) Affinity-based target identification, (B) activity-based protein profiling, (C) drug affinity responsive target stability (DARTS).
Objectives

The aim of this thesis was to synthesize a library of new salicylidene acylhydrazides based on statistical molecular design (SMD) and evaluate their biological activity towards the type III secretion system (T3SS) in *Yersinia pseudotuberculosis*. The physicochemical properties of the compounds were correlated to the biological activity using quantitative structure activity relationship (QSAR) models. Using the models, we attempted to identify the main properties important for activity. The resulting models was also used to predict the activity of new salicylidene acylhydrazides for *Y. pseudotuberculosis* as well as other gram-negative bacteria utilizing the T3SS.

We also attempted to identify the target and mode of action for the salicylidene acylhydrazides and their role in inhibiting the T3SS. To identify potential targets, different affinity-based strategies were used, such as radiolabeling, cross-linking, and biotinylation. The aim was to synthesize several affinity compounds, focusing particularly on the aromatic azide as a photo-inducible cross-linking group together with biotinylated compounds. Putative target protein(s) were then “fished out” of a bacterial lysate and detected with the biotin-labeled compounds.

We also searched for new virulence-blocking compounds towards the T3SS. Since many antibiotics originated from nature, a screen of a prefractionated natural product library was conducted to identify compounds that could inhibit the T3SS in several gram-negative bacterial species. The potential hits were then further characterized in *Y. pseudotuberculosis* and other species.
QSAR Modeling of Salicylidene Acylhydrazides

A set of salicylidene acylhydrazides had previously been synthesized and biologically evaluated by Nordfelth et al.\textsuperscript{24}. From inspection of the biological data, it was difficult to discern a clear SAR from the compounds’ substitution pattern. The salicylic phenol was shown to be crucial for retaining the biological activity, but the other positions on the salicylic aldehyde are able to tolerate both polar and hydrophobic substituents. The hydrazide part is more flexible when it comes to retaining the biological activity. It tolerates both heteroaromatic and fused aromatic rings. In the work described in \textit{Paper I}, we investigated salicylidene acylhydrazides and established SAR/QSAR models. SMD in building block space was used to choose compounds to synthesize.

Statistical Molecular Design

From commercial sources, 48 salicylic aldehydes and 92 hydrazides were selected. In total, 70 descriptors describing size, pK\textsubscript{a}, hydrophobicity, partial charges etc. were calculated on the low energy conformation of the 48 salicylic aldehydes. These descriptors were grouped and combined block-wise using PCA. The PCA model of the salicylic aldehyde building blocks contained five principal components ($R^2 = 0.85$, $Q^2 = 0.53$). For the 92 hydrazides PCA was calculated on 50 interpretable descriptors that contained five principal components ($R^2 = 0.88$, $Q^2 = 0.77$). The five principal components from each model were used separately in a two-layer determinant optimal onion design (DOOD)\textsuperscript{98}. The DOOD selected 17 salicylic aldehydes and 18 hydrazides based on physicochemical diversity. To obtain two building block sets that were equal in size, one salicylic aldehyde was manually selected, 5-bromo-salicylic aldehyde. The final 18 salicylic aldehydes and 18 hydrazides were combined so that each building block was represented three times. The combination generated 54 potential salicylidene acylhydrazides, which were subsequently synthesized.
Synthesis

One equivalent salicylic aldehyde and 1.2-1.4 equivalents of hydrazide were subjected to conventional heating or microwave irradiation in ethanol (Scheme 1). Out of the 54 salicylidene acylhydrazides, 50 were successfully synthesized in a one step procedure (Paper I, Table 1). One of the hydrazide building blocks, butyric acid hydrazide, did not react under the chosen conditions and was therefore omitted from the study. We did not manage to obtain the salicylidene acylhydrazide 4-methyl-[1,2,3]-thiadiazole-5-carboxylic acid (2-hydroxy-3-methoxy-5-nitro-benzylidene)-hydrazide in greater than 80 % purity, and therefore it was also omitted from the study. The resulting 50 compounds were obtained in at least 95 % purity and most were greater than 98 % pure. They were all analyzed with LC-MS and 1H NMR spectroscopy using DMSO as a solvent.

![Scheme 1. One step procedure for synthesis of salicylidene acylhydrazides.](image)

Biological Evaluation

The 50 successfully synthesized salicylidene acylhydrazides were investigated for their activity as T3SS inhibitors in Y. pseudotuberculosis in the YopE reporter gene assay together with the YopH assay, as described below (Figure 7). Both assays are phenotypic-based, and the YopE reporter gene assay was previously used in the screen where the salicylidene acylhydrazides were first identified. In the assays, the amounts of expressed YopE and secreted effector protein YopH were measured. A luciferase-encoding hybrid gene (luxAB from Vibrio Harveyi) was cloned under the same promoter as the gene coding for the effector proteins YopE in Y. pseudotuberculosis. Upon eukaryotic cell contact or in calcium-depleted media, Y. pseudotuberculosis activates the T3SS and secretes the effector proteins. A corresponding amount of LuxAB protein is also expressed and after addition of the enzyme substrate n-decanal, light is emitted. The amount of emitted light is directly proportional to the amount of expressed YopE (Figure 7). To ensure that the compounds did not interfere with the assay or quenching readout, the YopE reporter gene assay was run in combination with the enzymatic assay measuring phosphatase activity of secreted YopH. YopH dephosphorylates p-
nitrophenylphosphate to give p-nitrophenol. The conversion of substrate is directly proportional to the amount of YopH secreted and can be easily measured from the absorbance (Figure 7).

The 48 salicylidene acylhydrazides were tested at eight different concentrations, ranging from 1.6 to 200 µM. The remaining two compounds (ME0151 and ME0158) were tested at seven concentrations (1.6-100 µM) due to limited solubility. Out of the 50 compounds, 23 were classed as active, exhibiting more than 40 % inhibition at 50 µM in the YopE reporter gene assay. Five of the 23 compounds did not show a dose-response pattern in the YopH assay and were therefore omitted from the model calculations. The 50 compounds showed no or a modest effect on Y. pseudotuberculosis growth at two concentrations (50 and 100 µM), which indicates that the observed activity was not due to general toxicity. Based on the experimental evaluation, 18 salicylidene acylhydrazides were classed as T3SS inhibitors showing a dose-response pattern and were further investigated by QSAR modeling.

Figure 7. Schematic of the assays used. (A) YopE reporter gene assay; (B) YopH assay.

In addition, one inactive (ME0181) and four active (ME0165, ME0166, ME0168, and ME0174) compounds were evaluated in a Yersinia infection model, in which a mouse macrophage-like cell line (J774A) was infected with wild-type Y. pseudotuberculosis, YPIII(pIB102). The T3SS translocates effector proteins into the cytosol of J774A cells, resulting in reduced viability or cell death. As a control, J774A cells were infected with a translocation deficient mutant of Y. pseudotuberculosis, YPIII(pIB604) ΔYopB, lacking the translocator protein YopB. The YopB mutant has previously been shown to be non-virulent.99 The effect on J774 infection was monitored by CalceinAM, which inside living cells will be hydrolyzed to the green fluorescent calcein. Three of the afore-mentioned active compounds ME0165, ME0166, and ME0168, were active in this assay since they rescued the infected cells. ME0168 (Figure 3) completely blocked the toxic effect from the infection at 50 µM concentration (Figure 8). ME0174 and ME0181 were both inactive and did not rescue the infected J774A cells. To confirm selectivity for the T3SS, the J774A cells were infected with another gram-negative gastrointestinal pathogen, enterotoxigenic Escherichia coli (ETEC). ETEC lacks the T3SS and instead uses the type II secretion system for toxin secretion. ME0168
rescued the J774A cells from the *Y. pseudotuberculosis* infection but could not rescue the ETEC infected cells (Figure 8). This indicates that the inhibitors are selective for the T3SS and not for general secretion of toxins. The compounds were also found to be non-toxic and active in a complex cell-based infection model.

Figure 8. Viability of *Y. pseudotuberculosis* infected macrophages treated with ME0168. Macrophages (J774A) were infected with wild-type *Y. pseudotuberculosis*, YPIII(pIⅢB102), and a translocation deficient mutant, YPIII(pIB604) ΔyopB or enterotoxigenic *Escherichia coli* (ETEC). The cells were treated with three different concentrations of ME0168. Whereas YPIII(pIB604) ΔyopB infected and uninfected were largely cells unaffected, the viability of cells infected with YPIII(pIB102) was improved considerably with 50 and 100 µM ME0168. ME0168 could not rescue ETEC infected cells.

QSAR Models

QSAR modeling of the salicylidene acylhydrazides proved to be difficult. All the models showed a nonlinear relationship between the building block descriptors and the biological response, which is problematic when using linear regression models such as PLS. To compensate for the non-linearity, both quadratic and interaction terms were included in all PLS models. We chose to do all the modeling with the biological response from the YopE reporter gene assay at 50 µM compound concentration because the highest diversity in the response was obtained at this concentration. The addition of quadratic and interaction terms resulted in a huge set of variables, which made it difficult to overview and interpret the data. We used hierarchical PLS (Hi-PLS) to overcome this problem.
In the Hi-PLS method, the data describing the compounds are first divided into sub-blocks and PCA or PLS is then used on each sub-block. The PCA or PLS components are then correlated to the biological response in a Hi-PLS model. Another extension of the PLS is the discriminant analysis (DA) method. Here each compound is given a predefined score depending on whether it is, for example, active (1) or inactive (0). The PLS-DA model then finds the variation among the descriptors separating the two classes.

Hi-PLS-1 and Hi-PLS-DA-1 were calculated using an earlier published method. PLS score vectors for each building block set were first calculated from the descriptors and the biological response. The extracted PLS score vectors were then used together with quadratic and interaction terms to calculate the final Hi-PLS-1 and Hi-PLS-DA-1 models. The Hi-PLS-1 model showed a good correlation between the predicted and observed luciferase signal (Figure 9), whereas the Hi-PLS-DA-1 showed separation between active and inactive compounds (Figure 10). These two models were unfortunately very hard to interpret. Therefore, two additional models were calculated. Before calculating the new models, descriptors for each building block describing the same properties were grouped. A PCA model was calculated on each group and the score vectors put together with the descriptors that did not fit into any group. The data for the two building blocks were combined and quadratic and interaction terms were added. PLS regression resulted in the two new models, Hi-PLS-2 and Hi-PLS-DA-2. Hi-PLS-2 showed better correlation between the predicted and observed luciferase signal for highly active and inactive compounds compared to moderately active compounds. The predicted values for the moderately active compounds were less scattered than the observed values (Figure 9). Hi-PLS-DA-2, on the other hand, does not distinguish between active and inactive compounds as Hi-PLS-DA-1. Three of the models, Hi-PLS-1, Hi-PLS-2 and Hi-PLS-DA-1, had similar and acceptable statistics ($R^2_Y$ above 0.65 and $Q^2$ above 0.50) and were therefore used in consensus to predict the activity of new compounds (Table 3).

<table>
<thead>
<tr>
<th>Model</th>
<th>$R^2_X$</th>
<th>$R^2_Y$</th>
<th>$Q^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hi-PLS-1</td>
<td>0.19</td>
<td>0.67</td>
<td>0.51</td>
</tr>
<tr>
<td>Hi-PLS-DA-1</td>
<td>0.12</td>
<td>0.67</td>
<td>0.55</td>
</tr>
<tr>
<td>Hi-PLS-2</td>
<td>0.29</td>
<td>0.69</td>
<td>0.53</td>
</tr>
<tr>
<td>Hi-PLS-DA-2</td>
<td>0.21</td>
<td>0.43</td>
<td>0.35</td>
</tr>
</tbody>
</table>
Many properties of the salicylidene acylhydrazides contribute to their biological activity. The Hi-PLS-2 model was used for majority of the interpretation because the descriptors were grouped and easier to interpret. The salicylidene acylhydrazides clearly had a complex SAR that could not be easily identified by eye. The QSAR models did reveal some information. Overall, the properties of the salicylic aldehyde were more important than the hydrazide properties. The pKa of the salicylic phenol in the 2-position was found to be the most important property involved in seven interaction terms. Other important properties were the electrostatic potential charges of the aromatic carbons and the size of the salicylic aldehyde. In conclusion, these complex models suggest that the biological response is highly dependent on the interaction terms between the building blocks. Thus, the salicylic aldehyde and hydrazide should not be varied independently.

**Figure 9.** Observed vs. predicted plots for Hi-PLS-1 and Hi-PLS-2. The ID numbers shown in the plots are the last two numbers of the compound IDs.

**Figure 10.** Hi-PLS-DA-1 score plot. The model separated between active (black boxes) and inactive (grey triangles) compounds. The ID numbers shown in the plot are the last two numbers of the compound IDs.
The virtual library of all possible combinations of the 48 salicylic acids and 92 hydrazides consisted of 4416 compounds. Among these, 327 were predicted as active by all three models. To validate the models, a validation set of eight compounds was chosen manually. Five of them were predicted as active by all three models and three were predicted as inactive. The eight compounds were synthesized and tested in the YopE reporter gene assay to evaluate how good the models were at predicting compound activity. The models correctly classified five out of the eight compounds (Table 4). The three compounds predicted as inactive were all found to be inactive, whereas three out of the five predicted were actually active. ME0259 was predicted as active but was actually borderline active.

In addition to the biological evaluation in Y. pseudotuberculosis, the validation set (ME0257-ME0264) was also evaluated in Chlamydia trachomatis. The compounds were added at HeLa cells in five different concentrations (3.2-50 µM) one hour after C. trachomatis infection. The response to treatment by the compound was measured by the MIC, which is the lowest compound concentration resulting in complete inhibition of C. trachomatis infection, as determined by microscopy. Five of the eight tested compounds (ME0259-ME0261 and ME0263-ME0264) had MIC values of 50 µM or lower. The models correctly classified six (ME0258-ME0262 and ME0264) out of the eight compounds in their ability to inhibit C. trachomatis. The two compounds that were not correctly classified in C. trachomatis were correctly classified for Y. pseudotuberculosis. This could be due to many reasons, e.g., the permeability of the compounds through the eukaryotic and bacterial cell membranes may differ, which may result in that potentially active compounds turn out to be inactive.

Overall, the results demonstrated that although the models are complicated, they can be used in consensus to predict the activity of new salicylidene acylhydrazides in complex biological systems.
Table 4. Percent inhibition of the luciferase signal for *Y. pseudotuberculosis* strain YPIII(pIB102) yopE-luxAB and minimal inhibitory concentration for *C. trachomatis*.

<table>
<thead>
<tr>
<th>ID</th>
<th>Structure</th>
<th>50 µM*</th>
<th>Hi-PLS-1</th>
<th>Hi-PLS-2</th>
<th>Hi-PLS-DA-1</th>
<th>Chlamydia MIC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME0257</td>
<td><img src="image" alt="Structure" /></td>
<td>84 ± 2</td>
<td>49</td>
<td>47</td>
<td>Borderline active</td>
<td>Inactive</td>
</tr>
<tr>
<td>ME0258</td>
<td><img src="image" alt="Structure" /></td>
<td>26 ± 3</td>
<td>25</td>
<td>34</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>ME0259</td>
<td><img src="image" alt="Structure" /></td>
<td>38 ± 6</td>
<td>73</td>
<td>48</td>
<td>Active</td>
<td>50</td>
</tr>
<tr>
<td>ME0260</td>
<td><img src="image" alt="Structure" /></td>
<td>44 ± 2</td>
<td>64</td>
<td>46</td>
<td>Active</td>
<td>25</td>
</tr>
<tr>
<td>ME0261</td>
<td><img src="image" alt="Structure" /></td>
<td>26 ± 4</td>
<td>68</td>
<td>51</td>
<td>Active</td>
<td>50</td>
</tr>
<tr>
<td>ME0262</td>
<td><img src="image" alt="Structure" /></td>
<td>22 ± 3</td>
<td>20</td>
<td>31</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>ME0263</td>
<td><img src="image" alt="Structure" /></td>
<td>17 ± 15</td>
<td>26</td>
<td>34</td>
<td>Inactive</td>
<td>50</td>
</tr>
<tr>
<td>ME0264</td>
<td><img src="image" alt="Structure" /></td>
<td>28 ± 10</td>
<td>53</td>
<td>62</td>
<td>Borderline active</td>
<td>50</td>
</tr>
</tbody>
</table>

*YopE reporter gene inhibition at 50 µM. Compound were classified as active if inhibition was above 40%.
Summary

We successfully designed and synthesized 58 salicylidene acylhydrazides with a purity of more than 95%. The compounds were evaluated for their ability to inhibit the T3SS in *Y. pseudotuberculosis*. The physicochemical properties of the compounds were correlated to their biological activity in four QSAR models. These models were then used in consensus to predict the biological activity of eight new compounds. The models correctly classified five out of eight possible compounds as active or inactive in *Y. pseudotuberculosis*. The models also correctly classified six out of eight compounds for their ability to inhibit *C. trachomatis*. Although the models are complicated, they can be used in consensus to predict the activity of new salicylidene acylhydrazides against both *Y. pseudotuberculosis* and *C. trachomatis*. If additional salicylidene acylhydrazides would be synthesized and studied for their inhibitory activity towards the T3SS these models serve as a good starting point.
Identification of Targets of the Salicylidene Acylhydrazides

Synthesis of Affinity Reagents

Since the target and mode of action of the salicylidene acylhydrazides was unknown, we used three affinity-based strategies to identify potential target proteins. The compounds synthesized for the two first strategies contained a linker and handle attached to a salicylidene acylhydrazide (Paper II). The linker was used as a spacer to decrease steric hindrance in the experiments. The handle was used to “fish out” potential target proteins from a bacterial lysate. It has previously been shown that the phenol on the salicylic part of the salicylidene acylhydrazide is crucial for activity. The hydrazide part is more tolerant for changes compared to the salicylic part. (Paper I) Therefore the linker and handle were attached to the hydrazide part of the salicylidene acylhydrazide. We chose to synthesize these derivatives from ME0052 and ME0055 as starting points. ME0052 was chosen since it has been extensively studied in Y. pseudotuberculosis as well as other pathogens. ME0055 was chosen because it is the most active compound in E. coli O157. Three different affinity reagents were synthesized by a combination of solid phase and solution phase synthesis. The modifications to the salicylidene acylhydrazides to generate the different affinity reagents were all made with the same aim: to identify potential target proteins but with different strategies. The first compound was attached directly to a solid support, while the second was attached to a biotin that was further bound to streptavidin coated magnetic particles, while the last compound was attached to an azide for photo-crosslinking to the target protein and a biotin for detection. Most of the work in this part of the project was published in Paper II.

A Salicylidene Acylhydrazide Connected to a Solid Support

The compound synthesized for the first strategy was a salicylidene acylhydrazide connected to a solid support, as outlined in Scheme 2. The first part of the synthesis was carried out in the solid phase, followed by solution phase synthesis. As starting material, a polystyrene resin, Argogel-Wang (I), was used to enable cleavage to a carboxylic acid. In the first step, 4-(Fmoc-aminomethyl) benzoic
acid was coupled with 1-(2-mesitylenesulfonyl)-3-nitro-1H-1,2,4-triazole (MSNT) and 1-methylimidazole to form an ester bond to the benzylic alcohol on the solid support. Deprotection to 2 was made with 20 % piperidine in dimethylformamide (DMF). A spacer was introduced to increase the distance between the Affigel and the salicylidene acylhydrazide and to avoid steric clashes. We chose to use Affigel, an agarose based solid support, for this compound since it was been used in similar affinity-chromatography experiments as the one we prepared. Fmoc-6-aminohexanoic acid was used as the spacer and coupled to the benzyl amine using 1-hydroxy-7-azabenzotriazole (HOAt) and N,N'-disopropylcarbodiimide (DIC) as coupling reagents. The reaction was monitored using bromophenol blue. After recoupling with Fmoc-6-aminohexanoic acid, cleavage of 3 from the solid phase was made with 90 % TFA in water, giving 4 in 61 % yield based on the initial loading capacity of the resin. To generate the salicylidene acylhydrazide part of the compound, the acid 4 was first transformed into a hydrazide. Instead of using highly toxic hydrazine hydrate, tert-butyl carbazate was coupled to the acid with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) and 4-(N,N-dimethylamino)pyridine (DMAP). The Boc-protected hydrazide, 5 was deprotected with TFA to obtain hydrazide 6 in 50 % yield. Product 6 was stirred with 2,4-dihydroxybensaldehyde to give 7 in 36 % yield. The last step in the synthesis of ME0055-Aff was carried in one pot, where the Fmoc group was removed with tetra-N-butylammonium fluoride (TBAF) in THF followed by addition of Affigel-10. Experimental procedure and analytical data can be found in supporting information to Paper II. This compound was used to “fish out” potential target proteins from an E. coli lysate as described below.

Scheme 2. Synthesis of ME0055-Aff. a) i. 4-(Fmoc-aminomethyl) benzoic acid, MSNT, 1-methylimidazole, DMF:CH$_2$Cl$_2$ ii. 20 % piperidine in DMF. b) Fmoc-6-aminohexanoic acid HOAt, DIC, DMF. c) TFA/H$_2$O. d) tert-butyl carbazate, EDCI, DMAP, DMF/THF. e) TFA/CH$_2$Cl$_2$. f) 2,4-dihydroxybensaldehyde, EtOH. g) i. TBAF in THF, DMF; ii. Affigel-10
Biotinylated Salicylidene Acylhydrazides

The compound synthesized for the second strategy was designed to identify targets interacting with the salicylidene acylhydrazide part of ME0052-Bio and ME0055-Bio. Experiments where streptavidin coated magnetic particles and ME0052-Bio or ME0055-Bio were incubated together, followed by addition of bacterial lysate were performed. Owing to unspecific binding of proteins to the magnetic particles, the strategy was abandoned and the compounds were instead used in far western analysis. In far western blotting proteins in a blot are detected using labeled small molecules instead of the primary antibody used in conventional western blotting.

The synthesis of the compounds ME0052-Bio and ME0055-Bio was mostly carried out by solid phase synthesis. The resin used for this synthesis was TentaGel-OH, a copolymer composed of polyethylene glycol linked to polystyrene. Initially, 4-(Fmoc-aminomethyl) benzoic acid was coupled to Tentagel-OH (8) using MSNT and 1-methylimidazole and was then deprotected with 20 % piperidine in DMF yielding 9 (Scheme 3). The same spacer, Fmoc-6-aminohexanoic acid, as for the synthesis of ME0055-Aff was used and coupled onto the solid phase with HOAt and DIC. Deprotection of Fmoc with 20 % piperidine in DMF gave amine 10, before coupling with biotin resulting in 11. The reactions were monitored with bromophenol blue. Cleavage from the solid phase was achieved with hydrazine hydrate to give 12 (37 % yield based on the initial loading capacity of the resin). From 12, two different biotinylated compounds were synthesized: ME0052-Bio and ME0055-Bio. The final products were purified on a pre-packed reversed phase column to more than 95 % purity (22 % and 14 % yield, respectively).

Scheme 3. Synthesis of ME0052-Bio and ME0055-Bio. a) i. 4-(Fmoc-aminomethyl) benzoic acid, MSNT, 1-methylimidazole, DMF-CH₂Cl₂; ii. 20 % piperidine in DMF. b) i. Fmoc-6-aminohexanoic acid HOAt, DIC, DMF; ii. 20 % piperidine in DMF. c) Biotin, HOAt, DIC, DMF. d) Hydrazine hydrate. e) 3,5-dibromo salicylaldehyde, EtOH. f) 2,4-dihydroxybensaldehyde, EtOH.
Salicylidene Acylhydrazide with Azide and Biotin

The compound synthesized for the third strategy was a salicylidene acylhydrazide containing two functionalities: an azide and a biotin. The azide was attached for its cross-linking properties. The biotin was attached to detect proteins bound to the salicylidene acylhydrazide. Irradiating an aryl azide with UV-light generates a nitrene group, which can initiate addition reactions with double bonds, C-H, N-H, or form a ring expansion that can react with primary amines, resulting in formation of a covalent bond with the surrounding e.g. a protein. To utilize this property, we planned to mix the compound with a bacterial lysate from Y. pseudotuberculosis.

The first steps in the synthesis of the azide and biotinylated salicylidene acylhydrazide were carried out in the solid phase. The two first steps to yield were identical to the synthesis of ME0052-Bio and ME0055-Bio. To add a second functionality in a subsequent synthetic step, Fmoc-protected lysine-biotin was used. Fmoc-Lys-(biotin)-OH was coupled with HOAt and DIC. After deprotection to 13, 4-azidobenzoic acid was coupled onto the solid phase (14). Coupling reactions were monitored using bromophenol blue. Hydrazine hydrate was used to cleave the compound from the resin to give the hydrazide 15 in 36 % yield. The last step involved reaction with 3,5-dibromo salicylaldehyde. The reaction gave low yield, mostly due to loss of the azide functionality, which was confirmed by comparing the IR spectra of the starting material and compound 16.

Scheme 4. Synthesis of an azide and biotin functionalized salicylidene acylhydrazide. a) i. 4-(Fmoc-aminomethyl) benzoic acid, MSNT, 3-methylimidazole, DMF:CH2Cl2; ii. 20 % piperidine in DMF. b) i. Fmoc-6-aminohexanoic acid HOAt, DIC, DMF; ii. 20 % piperidine in DMF. c) Fmoc-lysine-biotin, HOAt, DIC, DMF. d) 4-azidobenzoic acid, HOAt, DIC, DMF. e) Hydrazine hydrate. f) 3,5-dibromo salicylaldehyde, EtOH, DMSO.
The low yield was also due to the fact that product 16 is a large hydrophobic compound, which caused problems with solubility during purification. Experimental procedures and analytical data are provided in the appendix.

This compound was used in attempts to identify potential targets from a *Y. pseudotuberculosis* bacterial lysate. The experiments carried in the presence of competing free ME0052. Cross-linking compound 16 and different concentrations of free ME0052 was added to bacterial lysate and exposed to UV light at 360 nm for 30 min. Cross-linked lysates were analyzed with western blots, probing the cross-linked biotinylated proteins with streptavidin-HRP. The western blot analyses showed high background and none of the protein bands could be competed out by the addition of free ME0052. This could possibly be due to insufficient or weak cross-linking and/or binding to targets, as well as detection of naturally occurring biotinylated proteins. We therefore decided to concentrate on the two other strategies described above.

**Putative Targets Identified with Affinity Chromatography**

To identify potential target proteins for the salicylidene acylhydrazides, ME0055-Aff was mixed with a bacterial lysate from *Escherichia coli* O157:H7. The beads were washed extensively in PBS before elution with 20 and 200 µM free ME0055. As a final elution step, the beads were treated with 1 % acetic acid. The different elutions were separated with SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and the gel was stained with colloidal blue

![Figure 11. Commassie stained gel from the affinity chromatography experiment. Protein bands cut out and analyzed are numbered 1-16. (Paper II)](image-url)
staining. The proteins appearing in elutions with 20 and 200 µM ME0055 and not as abundant in the stripping of the affigel with 1 % acetic acid, were excised form the gel and subjected to in gel trypsin digestion. The peptide fragments were analyzed by liquid chromatography electrospray ionization tandem mass spectrometry. A total of 16 excised protein bands resulted in 19 identified proteins as putative targets for the salicylidene acylhydrazides.

**Binding of Salicylidene Acylhydrazides to Putative Targets**

To study the binding of the salicylidene acylhydrazides to the putative target proteins, an attempt was made to clone and overexpress them (Paper II). Twelve genes were successfully cloned and seven were overexpressed. The later seven proteins were Tpx, FolX, z2714, z3974, WrbA, SurA and SteE. To verify the interaction between the proteins and the salicylidene acylhydrazide far western blotting was performed.

**Far Western Blotting**

Instead of using an antibody to detect proteins, we used a salicylidene acylhydrazide connected to a biotin, ME0052-Bio, as a probe and streptavidin-HRP conjugate for compound detection. Far western analysis confirmed that three of the proteins, Tpx, FolX and WrbA, bound to the salicylidene acylhydrazides.

Tpx is a peroxiredoxin that reduces alkyl hydroperoxides. It is upregulated in *E. coli* exposed to oxidative stress and thereby is a part of the bacteria defense system against reactive oxygen species (ROS). WrbA is a tryptophan repressor binding protein and a NAD(P)H quinine oxidoreductase that belongs to the family of flavoproteins. FolX is a dihydronicotinamide mononucleotide epimerase.

The three potential targets were identified in *E. coli* O157. Since salicylidene acylhydrazides are known to inhibit the T3SS in many gram-negative bacteria, their targets are most likely conserved. To confirm that the targets were conserved, we attempted to study the binding of salicylidene acylhydrazides to these three proteins in other pathogens. In addition to *E. coli* O157, cloning and overexpression of genes coding for Tpx and WrbA were made from *Y. pseudotuberculosis*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Shigella flexneri*. However, the gene encoding for FolX is less conserved and only encoded by *E. coli* O157, *P. aeruginosa*, and *S. flexneri*. Instead of carrying the gene encoded by
FolX, *Y. pseudotuberculosis* and *S. typhimurium* carry the homolog FolB. Therefore, FolB was overexpressed from *Y. pseudotuberculosis*, *P. aeruginosa* and *S. typhimurium*. Far western analyses showed binding of the salicylidene acylhydrazides to Tpx, WrbA and FolX for all species (Figure 12). Binding to the FolX structural homolog FolB could not be confirmed in any of the species using ME0052-Bio as the chemical probe. A far western competition experiment, where 200 μM free ME0052 was added in combination with 20 μM ME0052-Bio, showed a clear reduction in binding for all species. This indicates that the compounds bind to the targets and these potential targets are conserved in several gram-negative species utilizing the T3SS.

![Far western blotting confirmed binding of the salicylidene acylhydrazides to Tpx, FolX, WrbA, AdhE and C-terminal AdhE.](image)

**Figure 12.** Far western blotting confirmed binding of the salicylidene acylhydrazides to Tpx, FolX, WrbA, AdhE and C-terminal AdhE. *E. coli* O157 (EC), *Y. pseudotuberculosis* (YP), *S. typhimurium* (ST), *P. aeruginosa* (PA), and *S. flexneri* (SF)

### NMR Spectroscopy

To validate the binding of the salicylidene acylhydrazides to *Y. pseudotuberculosis* Tpx (ypTpx), we also used nuclear magnetic resonance (NMR) spectroscopy. We performed a 1H saturation transfer difference (STD) experiment with ME0052 and ypTpx. The STD experiment generates a spectrum, where only the signals from the bound part of the ligand are shown. The spectrum is obtained by subtracting the spectrum for the saturated protein from a spectrum without protein saturation.\(^{108}\) In an STD experiment, it is possible to detect binding...
between a macromolecule, in our case a protein, and a ligand. It can also be used to determine which part of the ligand binds the protein\textsuperscript{106}. From our experiment, we concluded that ME0052 binds to \( \gamma \beta \)Tpx and that it is the salicylic part of the compound that interacts with the protein (Figure 13). These data are in line with previous data that the salicylic part is more sensitive to variation.

To determine a binding constant between ME0052 and \( \gamma \beta \)Tpx, we performed \(^1\)H NMR titration experiments with increasing concentrations of ME0052. During the titration experiments, we observed ME0052 concentration dependent phenomena resulting in peaks shift and changes in intensity (data not shown). When \(^1\)H NMR experiments was run on just the salicylidene acylhydrazides alone dissolved in DMSO, the peak shifts could be minimized by heating the sample, which suggests that the shifts are due to intramolecular rotation. Because of this problem, calculations of the binding affinity were not possible. Thus, a point mutation was made in \( \beta \)X, where cysteine 61 known to be essential for Tpx function\textsuperscript{105} was exchanged for a serine, C61S, resulting in a forced reduced mutant. The NMR experiments were repeated with this point-mutated protein, \( \gamma \beta \)TpxC61S, together with ME0052. Intensity differences in the peaks were observed, indicating binding of ME0052 to oxidized \( \gamma \beta \)Tpx. The STD results also indicate weaker binding of ME0052 to \( \gamma \beta \)TpxC61S compared to oxidized \( \gamma \beta \)Tpx (data not shown).

![Figure 13: ME0052 binds to \( \gamma \beta \)Tpx. Overlay of two \(^1\)H NMR spectra: STD experiment with ME0052 and \( \gamma \beta \)Tpx (Blue spectrum), and \(^1\)H NMR on ME0052 (Red spectrum).](image)

To obtain information about where on the protein ME0052 binds, \( \gamma \beta \)Tpx was labeled with \(^{15}\)N and heteronuclear single quantum coherence (HSQC) experiments were recorded in the absence and presence of 100 \( \mu \)M ME0052 (Paper II). From a \(^{15}\)N,\(^1\)H HSQC experiment, it is possible to detect binding of a
ligand to a protein and further identify the amino acids in the binding site\textsuperscript{108}. Our NMR spectra showed chemical shift changes for a couple of backbone amino acids. After assigning 81\% of the backbone, the spectrum indicated that ME0052 binds to a specific site in the dimer interface in \textit{ypTp}, giving rise to minor conformational changes. The amino acid residue with the largest shift was Val60 located next to the catalytically active residue Cys61. Due to problems of poor solubility and possible intramolecular rotation with ME0052, we could not use the NMR data to calculate the dissociation constant, \(K_d\). Therefore, we also tried surface plasmon resonance and isothermal titration calorimetry, but unfortunately, the same problem of solubility was also encountered with these methods (data not shown).

**Crystallization**

Crystals were obtained of purified \textit{ypTp} (Figure 14A) and catalytically inactive \textit{ypTpC61S} for X-ray crystallography. The resolution of the diffraction pattern was less than 2.5 Å\textsuperscript{109}. The crystal structures for \textit{ypTp} were solved for both the oxidized and reduced state (Figure 14B). The oxidized state was shown to have an intramolecular disulfide bond between Cys61 and Cys95, as expected (colored yellow in Figure 14B)\textsuperscript{110}. The forced reduced structure of \textit{ypTpC61S} was identical to the wild-type reduced \textit{ypTp}. \textit{ypTp} was shown to exist as a homodimer, where the dimer interface was mostly formed by hydrophobic interactions. The dimer interface was found to be of similar size in the reduced and oxidized structures, but some conformational changes were detected, particularly in the active site of \textit{ypTp}, i.e., partial unfolding of the first alpha helix and a shift of the two cysteines 61 and 95 (Figure 14B).
Molecular docking of ME0052 and ME0055 to both the oxidized and reduced forms showed that both compounds bound to the same pocket in the oxidized form of the protein\textsuperscript{110}. The amino acids that gave rise to the chemical shift in the NMR experiments belonged to both subunits (\textit{Paper II}, Figure 3 and 4), and one of the amino acids was found to neighbor an amino acid involved in hydrogen bonding to ME0052 identified from the docking study\textsuperscript{110}. These data demonstrated the importance of the dimer interface for compound binding. Unfortunately, despite extensive efforts using various methods, we were not able to obtain a co-crystal between Tpx and a salicylidene acylhydrazide. This was probably due to the poor solubility of the compound except with high concentrations of DMSO, which makes crystallization of Tpx unfavorable.

\section*{Binding Affinity of a Salicylidene Acylhydrazide to Tpx}

To determine the binding affinity and stoichiometry of ME0052 to \(\text{ypTpx}\), we used analytical ultracentrifugation. Analytical ultracentrifugation is a method that has been used for many years to study macromolecular interactions. The concentration dependent distribution between Tpx and different concentrations of ME0052 was measured at several times by monitoring absorbance at 395 nm. The collected data were used to calculate the dissociation constant, \(K_d\) and was determined as 51 µM for binding of ME0052 to oxidized \(\text{ypTpx}\). The \(K_d\) for binding to reduced \(\text{ypTpx}\) was 71 µM. For the mutant protein \(\text{ypTpxC61S}\), \(K_d\) was 93 µM, which is almost two-fold higher than that for oxidized \(\text{ypTpx}\). The stoichiometry of the binding was calculated as one molecule of ME0052 per \(\text{ypTpx}\) dimer.

\section*{The Role of Putative Target Proteins in Bacterial Virulence}

To further study the role of the putative target proteins in the T3SS, the genes coding for Tpx, WrbA, and FolX were knocked out in \textit{E. coli} O157:H7 and \textit{Y. pseudotuberculosis} YPIII(pIB102). The \textit{Y. pseudotuberculosis} analog of \textit{folX}, i.e., \textit{folB}, was found to be essential for survival and could not be knocked out.
**Tpx, WrbA and FolX**

To evaluate the phenotype of the knockouts in *Y. pseudotuberculosis*, we initially analyzed the secretion of the effector proteins. Surprisingly we observed a slight increase of the secreted effectors in the Tpx and WrbA deleted strains compared with wild type. Addition of 50 µM ME0052 to both knockout strains decreased the amount secreted proteins. In addition, we evaluated the mutants in the macrophage infection assay used in the study described in Paper 1. Bacterial infection of the mouse macrophage-like cells (J774A) reduced the cell viability to the same extent for the two mutants and the wild type. The viability of the infected cells was 46 ± 18 % for wild type, 42 ± 12 % for the Δtpx strain and 54 ± 4 % for the Δwrba strain. Addition of 25 µM of ME0052 rescued the macrophages from infection, restoring the viability for wild type and both mutants.

The gene expression levels of the *E. coli* O157 tpx, wrbA, and folX knockouts were analyzed by microarray experiments. The transcriptomic profiles showed that genes associated with T3SS were up-regulated and genes associated with the flagella were down-regulated in all three mutants compared to wild type. The opposite trends were previously reported for treatment with salicylidene acylhydrazides: gene expression profiles of *E. coli* O157 cultures treated with salicylidene acylhydrazides showed that addition of compound decreased the expression of T3SS associated genes and increased the expression of flagella genes.

We believe that bacterial treatment with salicylidene acylhydrazide induces complex regulation involving multiple proteins, resulting in inhibition of the T3SS. The microarray data for *E. coli* O157 indicates that the compounds stabilize or activate targets rather than inhibiting them or they possibly inhibit binding of a repressor to the targets. Addition of a compound to a biological system is often expected to result in a loss of function, studied in a knockout, but it can also result in gain of functions, which is more difficult to study. The interaction between these potential target proteins and the T3SS is however not clear.

**AdhE in Y. pseudotuberculosis**

In addition to the analysis of Tpx, WrbA, and FolX, we decided to investigate another putative target, AdhE. AdhE is a bifunctional enzyme with alcohol dehydrogenase and acetaldehyde-CoA dehydrogenase activity. The binding of salicylidene acylhydrazides to *E. coli* AdhE was verified by far western analysis.
Figure 12. Far western blot showed that the compound binds to whole AdhE and the C-terminal part but not to the N-terminal part. To understand the role of AdhE in T3SS inhibition, knockouts were made in *Y. pseudotuberculosis* and *E. coli* O157.

The *Y. pseudotuberculosis* phenotype was studied in three different assays. In the first assay, we measured the amount of secreted effector proteins. There were no differences in the amount of secreted Yops when wild type and ΔadbE strains were compared (data not shown). To investigate the effect on effector protein translocation, and thereby virulence of the AdhE knockout in *Y. pseudotuberculosis*, a β-lactamase reporter system was used. In addition to the *adbE* deletion, the bacterial strains used had the effector protein YopE fused to β-lactamase. HeLa cells were infected with YPIII(pIB102) *yopE-bla* and YPIII(pIB102) ΔadbE *yopE-bla* with a multiplicity of infection (MOI) of 50. As a control experiment, HeLa cells were also infected with a translocation deficient mutant, YPIII(pIB604) ΔyopB *yopE-bla*. The infected HeLa cells were then analyzed with fluorescent microscopy (Figure 15A-D). Bacteria with a deletion of *adbE* were able to translocate effector proteins as effectively as the wild type. Thus, the mutant was clearly as virulent as the wild type.

To confirm these results, a viable count was performed, where the number of HeLa cell engulfed bacteria were counted according to previously published procedures. HeLa cells were infected for 1 h with wild-type, ΔadbE strain or YPIIIc (a bacteria strain without the virulence plasmid) bacteria, after which gentamicin was added to kill all the extracellular bacteria. The HeLa cells were permeabilized with triton and then scraped off the bottom each well. The bacteria were plated in different concentrations and counted. The number of engulfed mutant bacteria was found to be of the same range as the number of engulfed wild type bacteria (data not shown). These data agree with the findings from the translocation data that the *Y. pseudotuberculosis* ΔadbE strain is as virulent as the wild type.
AdhE in *E. coli*

To evaluate the role of AdhE in *E. coli virulence*, a bovine epithelial cell line was incubated with *E. coli* O157 and *E. coli* O157 Δ*adhE* strains. After 4 h bacteria and host cells were fixed using paraformaldehyde and stained for condensed actin performed using TRITC-phalloidin, as described previously. Condensed areas of actin adjacent to the bacteria, indicative of attaching and effacing lesions were observed using fluorescent microscopy. The *E. coli* O157 Δ*adhE* strain still had flagella and was poorly attached to the host cells. In comparison, the wild type bacteria attached and condensed host cell actin with far greater efficiency (data not shown).

The secretion profile of *E. coli* effector proteins was determined to further characterize the phenotype of the *E. coli* O157 Δ*adhE* strain. Western blot analyzes of supernatants from *E. coli* O157 and *E. coli* O157 Δ*adhE* were performed to compare the amount secreted effector proteins Tir and EspA. EspA, the needle-tip protein in *E. coli*, is a homolog to *Yersinia* LcrV, whereas Tir is a key virulence associated protein. To investigate whether flagella, which are closely related to the T3SS, are affected, the flagella protein FliC was also analyzed. To determine if the *E. coli* O157 Δ*adhE* affects general protein expression or stability, intracellular levels of the housekeeping protein GroEL were also examined in the western blot analysis as a control. The western blot analysis showed that the level of the control protein GroEL was unaltered, indicating no effect on general protein expression. However, the significantly lower levels of Tir, and EspA were observed in the Δ*adhE* strain than in wild type (Figure 16), which indicates that *E. coli* Δ*adhE* has a down-regulated T3SS compared to wild type, in contrast to *Y. pseudotuberculosis* Δ*adhE*. The level of FliC increased in *E. coli* O157 Δ*adhE* compared to wild type. This phenotype is similar to that arising when *E. coli* is treated with a salicylidene acylhydrazide like

![Figure 16. *E. coli* O157 Δ*adhE* does not secrete the effector proteins Tir and EspA. Western analysis of *E. coli* O157 wild type and Δ*adhE* strain. The analysis showed a decreased secretion of the effector proteins Tir and EspA and increased production of the flagella associated protein FliC. There was no difference in the amount of the housekeeping protein GroEL.](image-url)
ME0055\textsuperscript{35}, suggesting that the bacteria are prepared for flight instead of fight. This is in contrast to the Y. pseudotuberculosis secretion profile, where the secretion was unaffected, which could possibly indicate gene redundancy.

To elucidate whether the *E. coli* O157 ΔadhE affects the T3SS at a transcriptional level, cDNA-sequencing using Illumina platform was performed. The data showed that the ΔadhE strain had increased expression of all flagella genes and decreased expression of T3SS associated genes. This correlates well with the secretion profile that showed increased flagellin and decreased secreted virulence proteins in the ΔadhE strain. When AdhE is knocked out, we postulate that changes in the pathway consisting of acetyl-coenzyme A (acetyl-CoA) to acetaldehyde and further to ethanol are likely. The acetyl-CoA is converted by phosphotransacetylase (Pta) to acetylphosphate and then further by acetate kinase to acetate (Figure 17A). To assess this, we sought to determine acetate levels. Acetate diffuse freely across the membrane, levels of extracellular acetate reflect the intracellular level\textsuperscript{115}. By measuring the extracellular levels of acetate with NMR, the concentration was measured as 4.4 ± 0.03 mM for the wild type and 5.5 ± 0.05 mM for the ΔadhE strain. Increased acetate concentration increases the activity of the transcriptional regulator CRP as well as increases transcription of genes and translation of proteins associated with the flagella\textsuperscript{116} (Figure 17B).

When motility studies of *E. coli* O157 ΔadhE were conducted, it was found that the bacteria were paralyzed. This was surprising since both expression of flagella genes and the amount FliC protein were increased in the ΔadhE strain, and therefore we expected it to be more motile than wild type. Chemotaxis proteins control the flagella rotation direction, one of which is CheY. CheY is phosphorylated by acetyl kinase via acethylphosphatase (Figure 17A). When CheY is phosphorylated it activates clockwise rotation of the flagella and the bacteria starts to tumble\textsuperscript{117}, swim in all directions resulting in a paralyzed phenotype. CheY is also acetylated by acetyl coenzyme A synthetase (Acs) (Figure 17A). This promotes the clockwise rotation of the flagella, resulting in tumbling.\textsuperscript{118} We hypothesize that the increased acetate concentration, results in post-translational modification of CheY and that this mechanism explain the paralyzed phenotype. *E. coli* O157 ΔadhE show similar phenotype as treatment with salicylidene acylhydrazide, which makes *E. coli* AdhE a particularly interesting potential target for virulence-blocking compounds.
Disulfiram a Known Inhibitor of AdhE

The human homolog to alcohol dehydrogenase/acetaldehyde-CoA dehydrogenase, AdhE has a known inhibitor disulfiram (also known as antabuse), which is used as treatment for alcoholism. It acts by inhibiting human acetaldehyde dehydrogenase, increasing the amounts of acetaldehyde causing hangover symptoms. Therefore, the potential inhibitory effect of disulfiram towards the T3SS in Y. pseudotuberculosis was investigated using assays described in Paper I. disulfiram showed inhibitory effect in both YopE reporter gene and YopH assays, with IC$_{50}$ at 49 µM and 34 µM, respectively (Figure 18). Analysis of the secreted Yops with western blot showed that disulfiram treated Y. pseudotuberculosis have a decreased Yop secretion (data not shown). However disulfiram also had some impact on Y. pseudotuberculosis growth. Even though our preliminary results cannot tell if the effect of disulfiram of the T3SS is due to growth inhibition or if it is two separate effects, it further supports that AdhE is an interesting target for inhibition of the T3SS.

Figure 17. Pathways affected by a deletion of the bifunctional enzyme AdhE. (A) AdhE catalyzes the formation of acetyl coenzyme A (acetyl-CoA) from ethanol via acetaldehyde. There are two pathways of acetate formation from acetyl-CoA with links to CheY. Phosphotransacetylase (Pta), acetyl coenzyme A synthetase (Acs). (B) Deletion of AdhE results in increased concentrations of acetate in the bacteria, which in turn increases CRP-activity and flagella gene transcription and protein translation.
Next we investigated the effect of disulfiram of Chlamydiae infection. HeLa cells were infected with *C. trachomatis* and *C. pneumoniae* for 1 h, before treatment with disulfiram, as described previously ([Paper I](#)). After 19 h of infection with *C. trachomatis*, preliminary data showed dose-dependent decrease in *C. trachomatis* growth with a MIC around 25 µM. In addition, 24 h post infection of *C. pneumoniae* and treatment with disulfiram, preliminary data indicate MIC=6 µM (data not shown).

**Summary**

We identified 19 potential target proteins for the salicylidene acylhydrazides. How many of these proteins are associated with the T3SS inhibition phenotype that the compounds cause in treated bacteria is not known. We investigated four of the proteins in more detail, Tpx, WrbA, FolX and AdhE, with a focus on Tpx and AdhE. As the salicylidene acylhydrazides are known to affect the T3SS in several gram-negative bacteria, we also investigated homologs of the putative target proteins from different species. We showed that the salicylidene acylhydrazides bind to Tpx and WrbA from five species and to the less conserved FolX from three species.

Based on NMR studies, we concluded that the salicylidene acylhydrazides to bound to ϕTpx. Binding also gave rise to chemical shift perturbation in HSQC NMR experiments and the changes were attributed to a few amino acids close to the active site of Tpx. Using analytical ultracentrifugation, we calculated the affinity of the compound to ϕTpx; for binding of ME0052 to the oxidized form, $K_d=51$ µM. In addition, we managed to crystallize ϕTpx in three different forms, i.e., reduced, oxidized and the force reduced C61S mutant.

Surprisingly the *Y. pseudotuberculosis Δtpx, Δwrba, or ΔadhE* strains showed similar virulence to the wild type. Microarray data from *E. coli* O157 strains treated with salicylidene acylhydrazides showed down-regulation of T3SS associated genes and up-regulation of flagella genes. In contrast, *E. coli* O157 with a deleted gene encoding the target protein of Tpx, WrbA, or FolX showed...
up-regulation of genes associated with the T3SS. Interactions between the salicylidene acylhydrazides and these three targets could possibly result in a gain of function. Based on these data, we believe that the compounds interact with multiple targets affecting the regulation of the T3SS. The salicylidene acylhydrazides may activate or stabilize the potential targets or inhibit binding from a repressor. The binding of salicylidene acylhydrazides to Tpx, WrbA and FolX does not necessarily need to be related to the T3SS. The result of salicylidene acylhydrazides binding to these potential target proteins could also be due to a side effect, next to the virulence-blocking effect.

The phenotype and gene expression profile of the *E. coli* O157 ΔadhE strain was found to be similar to that occurring after treatment with salicylidene acylhydrazide, the T3SS was down-regulated and the flagella up-regulated. These results make *E. coli* AdhE a particularly interesting target. However, the mechanism of how this is regulated is still not completely understood. One hypothesis is that increased acetate concentration in *E. coli* O157 ΔadhE up-regulates the flagella, followed by indirect down-regulation of the T3SS. The similar virulence of the *Y. pseudotuberculosis* ΔadhE strain to the wild type can be explained by, the potential targets are identified in *E. coli* and not regulating the T3SS in *Y. pseudotuberculosis* or by redundancy in *Y. pseudotuberculosis*. In *Y. enterocolitica* it has been shown that the three different pathways for T3S (the virulence plasmid encoded, the chromosomal encoded systems and the flagella) can secrete YplA independent of each other. It has also been shown that more than one T3SS could operate simultaneously.120 These data supports the hypothesis that *Y. pseudotuberculosis* ΔadhE strain is non-virulent due to redundancy.

Our data for *E. coli* AdhE showed a connection between decreased motility and acetate metabolism. Therefore, we propose that the mechanism involves indirect down-regulation of the T3SS through up-regulation of the flagella. The human homolog to AdhE has a known inhibitor, disulfiram Since the effect of disulfiram by inhibiting the human AdhE homolog is known, treatment with a virulence-blocking compound that inhibits AdhE is likely to be safe with few side effects in humans.

This together with the *E. coli* data that show the similar phenotype from treatment with salicylidene acylhydrazide as the AdhE knockout makes AdhE a particularly interesting as a target for virulence-blocking compounds.

Taken together, we believe that the phenotype arising from salicylidene acylhydrazide treatment is due to interactions with multiple target proteins and that the targets might differ between species. Some of the potential targets act in concert where some targets have higher input on T3SS regulation rendering non-virulent bacteria.

47
Neohopeaphenol A as a Type III Secretion Inhibitor

Many of the drugs used today originated from nature or are based on a natural product. Throughout history, natural products have been used as active ingredients in medicines, e.g., the analgesic drug morphine and the anticancer agent paclitaxel, more known as taxol. Natural products come from plants, microorganisms (e.g., penicillin), marine organisms or animals. A wide diversity of chemical structures is available in nature, and therefore natural product libraries serve as useful starting points in HTS. In our search for novel T3SS inhibitors, a prefractionated natural product library with over 18,000 marine and terrestrial biota samples were screened in a previously described assay. From this screen it has previously been reported on identification and total synthesis of one hit extract containing pseudoceramine A-D and spermatidinamine in a previously described assay. In the same screen neohopeaphenol A was found as a hit and its biological effects of the T3SS in Y. pseudotuberculosis and P. aeruginosa were studied and described in Paper III.

Neohopeaphenol A Inhibits the T3SS of Y. pseudotuberculosis

Using bioassay guided purification and structure determination, neohopeaphenol A was identified as a T3SS inhibitor towards Y. pseudotuberculosis in an extract from the stem bark of Hopea hainanensis, a tree growing in the Southeast Asian rainforest. Neohopeaphenol A is one of many hopeaphenols that are resveratrol tetramers belonging to the class of polyphenols. Resveratrol (Figure 19) is a well-studied trans-stilbene, a compound containing two phenyls connected with a two-carbon methylene bridge (Figure 19). This moiety is prone to form oligomers, presumably via oxidative coupling reactions, resulting in a large variety of natural products with varying complexity and stereochemistry. The first report of the biological activity of resveratrol was described in 1992. Since then, it has been shown to have anticancer, antifungal, anti-inflammatory and antimicrobial properties. The total synthesis of a few resveratrol-based natural products has been achieved.
Neohopeaphenol A is a large compound, with a molecular weight at 907 g/mol. It is built up by 56 carbons, 10 phenols, and contains eight stereocentra, distinguishing it from the other hopeaphenols. As mentioned above resveratrol can form a large amount of oligomers with different stereochemistry and two of the resveratrol dimers are epsilon-viniferin and amelopsin. Amelopsin B is a ringclosed epsilon-viniferin and neohopeaphenol A is a dimer of the two enantiomers of amelopsin B.

![Figure 19. Structures of resveratrol and three of its oligomers.](image)

Neohopeaphenol A showed a dose-response inhibition in both the YopE reporter-gene assay and YopH phosphatase assay with IC\textsubscript{50} values of 6.6 µM and 3.3 µM, respectively (Figure 20A). It had no or limited effect on bacterial growth at concentrations up to 100 µM (Figure 20B). To establish if addition of neohopeaphenol A at different stages of \textit{Y. pseudotuberculosis} infection potentiates the compound effect, neohopeaphenol A was added at seven time points during infectious conditions. The compound (50 µM) was added at the time points of \( t = 0, 30, 60, 90, 120, 150, \) and 180 min. A temperature shift from 26 to 37 °C at \( t = 60 \) min was used to mimic bacterial contact with the eukaryotic cell. Addition of neohopeaphenol A up to \( t = 90 \) substantially reduced the YopE reporter gene signal, whereas addition at later time points had no significant effect (Figure 20C). This indicates that the effect of neohopeaphenol A is rapid, similar to data reported for salicylidene acylhydrazides by Nordfelth et al. and in Paper I.

To investigate if neohopeaphenol A affects the expression and secretion of effector proteins, we performed a western blot analysis on total bacteria culture and supernatant. Wild-type bacteria, YPIII(pIB102), was incubated with seven different concentrations of neohopeaphenol A for 1 h at 26 °C followed by 3 h at 37 °C. Analysis of the translocator protein YopD showed a dose-dependent response for both expression and secretion (Figure 20D). The expression of YopD was reduced but continued at all concentrations, while concentrations above 13 µM neohopeaphenol A completely blocked the secretion of YopD. These findings are in line with the data obtained from the YopE reporter gene and YopH phosphatase assays, and suggest that neohopeaphenol A targets the T3SS directly rather than by general transcription of the T3SS genes as observed for the salicylidene acylhydrazides in \textit{E. coli}.
Nordfelth et al. reported that the effect of the salicylidene acylhydrazides was reversible. Therefore, we investigated if the effect of neohopeaphenol A also was reversible. ME0052 was used as a control during the whole experiment (data not shown). Overnight cultures of YPIII(pIB102) were diluted and divided into two cultures that were treated with either neohopeaphenol A (40 µM) or DMSO alone. The T3SS was induced without triggering effector protein secretion by growing the cultures in media containing calcium for 30 min at 26 °C followed by 2 h at 37 °C. The cultures were then divided into eight tubes and washed to remove calcium. The eight cultures were resuspended in media with or without calcium and with or without 40 µM neohopeaphenol A. To trigger the effector protein secretion the cultures were incubated at 37 °C for an additional 45 min. Western blot analysis performed on total culture samples all showed Yop expression, confirming that neohopeaphenol A inhibits secretion without inhibition of Yop expression (Figure 21A, lane 1-8). Western blot analysis of the supernatants showed that none of the cultures grown in the presence of calcium secreted any Yops regardless of the pretreatment with neohopeaphenol A or DMSO (Figure 21B, lane 1, 3, 5, and 7). The culture pretreated with DMSO grown under calcium depletion secretes Yops into the growth media as expected.
When neohopeaphenol A was added to the culture pretreated with DMSO and grown in the absence of calcium, secretion was completely blocked (Figure 21B, lane 3). This result shows that the effect of neohopeaphenol A on the T3SS is rapid. The cultures pretreated with neohopeaphenol A were unable to secrete effector proteins both in the DMSO control and when treated with neohopeaphenol A independent of the presence or absence of calcium (Figure 21B, lane 5-8). This suggests that the effect of neohopeaphenol A is not reversible and indicate that the compound might bind covalently to its target(s) (Figure 4B, lane 8).

Figure 21. The effect of neohopeaphenol A treated *Y. pseudotuberculosis* is irreversible. Western blot analysis of the reversibility of neohopeaphenol A treatment of *Y. pseudotuberculosis*. Samples in lane 1-4 were pretreated with DMSO and lane 5-8 with neohopeaphenol A. After pretreatment, the two cultures were divided into four: two were diluted with medium supplemented with calcium and the other two with calcium-depleted media. Neohopeaphenol A (40 µM final concentration) or DMSO were then added to the different conditions. (A) Total culture. (B) Supernatant.

Figure 20D shows that *Y. pseudotuberculosis* reduces the expressed and secreted amounts of the translocator YopD. We also wanted to elucidate the effects on translocation. Thus, we used the same β-lactamase reporter systems17,112,113 as we used for the *Y. pseudotuberculosis* ΔAdhE strain (previous chapter: AdhE in *Y. pseudotuberculosis*). HeLa cells were infected with YPIII(pIB102) *yopE-bla* at MOI = 50, together with addition of five different concentrations neohopeaphenol A. After 1 h infection, the HeLa cells were analyzed with fluorescent microscopy. In addition, the fluorescent signal was measured in a microplate reader and the percentage of translocation was calculated and normalized to the signal for DMSO-treated bacteria (set to 100 % translocation). The infected HeLa cells treated with neohopeaphenol A showed a dose-dependent response (Figure 22K). Translocation was completely inhibited.
by addition of 13, 25 and 50 µM neohopeaphenol A (Figure 22A-C), whereas at 3.3 and 6.5 µM the bacteria were able to translocate the effector (Figure 22D-E). Wild type infected cells with or without treatment with DMSO exhibited the same amount of translocated YopE-Bla, indicating that the effect was not due to the solvent (Figure 22F and 22H). As controls cells were infected with the translocation deficient mutant YPIII(pIB604) ΔyopB and were left uninfected with or without 50 µM neohopeaphenol A. The uninfected treated control is not affected by neohopeaphenol A, indicating that the compound does not exert an toxic effect during the time of the experiment. These results show that neohopeaphenol A is an irreversible, nontoxic T3SS inhibitor for Y. 

*pseudotuberculosis.*
Figure 22. Neohopeaphenol A inhibits translocation of effector proteins. (A-F) Y. pseudotuberculosis infected HeLa cells treated with five different concentrations neohopeaphenol A and DMSO. (G-J) As controls HeLa cells were infected with a translocation deficient mutant, YPIII(pIB604) ΔyopB, YPIII(pIB102) wild type and left uninfected with or without 50 μM neohopeaphenol A. (K) % translocation compared to DMSO-treated bacteria.
Neohopeaphenol A Inhibits the T3SS of *P. aeruginosa*

The fact that there is a high similarity between the T3SS of *Y. pseudotuberculosis* and *Pseudomonas aeruginosa* and salicylidene acylhydrazides inhibit the T3SS in several gram-negative bacteria makes it particularly interesting to study the effect of neohopeaphenol A in *P. aeruginosa*. *P. aeruginosa* is a gram-negative opportunistic pathogen that infects immunocompromised, cystic fibrosis and leukemia-patients, and wounds e.g., burn wounds. It belongs to the six ESKAPE pathogens (Enterooccus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter species, *P. aeruginosa*, and Enterobacter species). These six pathogens are together with *Mycobacterium tuberculosis* considered as the most important and emerging infection threats of this century. *P. aeruginosa* has a unique capacity for developing intrinsic and acquired resistance; it is resistant to practically all antimicrobial drugs on the market and is therefore known as a “superbug”. We should not underestimate the threat that these multi-resistant bacterial strains pose to our society, making the need for new antimicrobial drugs and/or novel therapies extremely urgent.

The effector protein exoenzyme S (ExoS) in *P. aeruginosa* is highly similar to *Y. pseudotuberculosis* YopE. To investigate the effect of neohopeaphenol A on the *P. aeruginosa* T3SS, western blot analysis was performed on expressed and secreted ExoS under T3SS inducing conditions. As for *Yersinia*, the T3SS in *P. aeruginosa* can be triggered by growth at 37 °C and removal of calcium. *P. aeruginosa* cultures were treated with neohopeaphenol A or DMSO for 3 h at 37 °C. Treatment with different concentrations of neohopeaphenol A decreased the ExoS expression in a dose-dependent manner (Figure 23). Treatment with concentrations of 50 and 100 µM neohopeaphenol A essentially completely inhibited secretion of ExoS, while treatment with 10 and 20 µM decreased the ExoS secretion dose-dependently (Figure 23). As controls a T3SS mutant (PAKexsA) and calcium-treated *P. aeruginosa* were used, and as expected they did not express or secrete any ExoS.
HeLa cells were infected with the *P. aeruginosa* PAK strain to investigate effect of neohopeaphenol A in a cell-based infection model. HeLa cells were infected with PAK for 5 h in the presence of neohopeaphenol A at four concentrations ranging from 20 to 150 µM. Cells treated with 100 and 150 µM neohopeaphenol A were fully rescued from the infection (Figure 24 A-B), whereas cells treated with 50 µM were only partly rescued from the infection. Treatment with 20 µM neohopeaphenol A did not inhibit the *P. aeruginosa* infection (Figure 24D) and were equally affected as the PAK wild type infection (Figure 24E). As controls, cells were left uninfected with and without 150 µM neohopeaphenol A (Figure 24F-G). The neohopeaphenol A treated uninfected cells did not show any signs of toxicity during the experiment (Figure 24G).

Figure 23. Western blot analysis of expressed and secreted *P. aeruginosa* ExoS. The T3SS was induced in *P. aeruginosa* treated with 100 and 50 µM treatment. As controls, a T3SS mutant (PAKexsA) and calcium-treated *P. aeruginosa* were used.

![Figure 23. Western blot analysis of expressed and secreted *P. aeruginosa* ExoS.](image)

Figure 24. *P. aeruginosa* infected HeLa cells treated with neohopeaphenol A. (A-D) HeLa cells infected with wild-type *P. aeruginosa*, strain PAK for 5 h with Neohopeaphenol A concentrations ranging from 150 to 20 µM. (E-G) Controls with untreated infection, uninfected cells and uninfected cells with addition of 150 µM neohopeaphenol A.

![Figure 24. *P. aeruginosa* infected HeLa cells treated with neohopeaphenol A.](image)
These results correspond well to the results obtained from the above-mentioned experiments with *Y. pseudotuberculosis*, suggesting that neohopeaphenol A could function as virulence-blocking compound in different gram-negative bacterial species.

In addition, a panel of five gram-positive and five gram-negative bacterial species were tested for growth inhibition in the presence of neohopeaphenol A. The gram-positive bacteria tested were *Micrococcus luteus, Staphylococcus epidermis, Staphylococcus aureus, Bacillus subtilis*, and *Enterococcus faecalis*, whereas the gram-negative bacteria (in addition to *Y. pseudotuberculosis* and *P. aeruginosa*) were *Proteus mirabilis, Klebsiella pneumonia*, and *E. coli K12*. None of the strains showed growth inhibition when grown together with 25, 50 or 100 µM neohopeaphenol A for 24 h. Thus, neohopeaphenol A appears to be a selective inhibitor of the T3SSs in *Y. pseudotuberculosis* and *P. aeruginosa*.

**Summary**

A prefractionated natural product library was screened, whereby neohopeaphenol A was identified as a T3SS specific inhibitor. According to the YopE reporter-gene assay, neohopeaphenol A has an IC$_{50}$ value of 6.6 µM in *Y. pseudotuberculosis*. Neohopeaphenol A showed a dose-dependent response in the YopE reporter-gene and YopH assays as well as in the western blot secretion profile analysis, whereas the Yop western blot expression profile only showed minor reduction. Concentrations of neohopeaphenol A that completely blocked Yop secretion had no effect on the growth, indicating a specific T3SS activity. In addition, neohopeaphenol A did not significantly inhibit growth in a panel of gram-positive and gram-negative bacteria. Pretreatment and washout experiments with neohopeaphenol A showed its effect was irreversible, suggesting that it binds covalently to its target. Neohopeaphenol A is large and heavy with a molecular weight of 907 g/mol and therefore it is likely to have low cell permeability, further suggesting that the compound might interacts directly with the extracellular part of the T3SS or with a target on the surface of the bacteria. We also showed that neohopeaphenol A inhibits the T3SS of the multiresistant “suberbug” *P. aeruginosa*. At concentrations above 50 µM, neohopeaphenol A completely blocked secretion of the effector protein ExoS and prevented cytotoxicity in infected HeLa cells.

One of many oligomerizations of resveratrol results in Neohopeaphenol A, with a complex structure and many stereocentra. It would be of high interest to in the future test analogs to and fragments of neohopeaphenol A for their inhibitory effect on the T3SS, this is not possible at the moment since the high complexity of the structure makes them difficult to synthesize.
Neohopeaphenol A is the first T3SS inhibitor, active against human pathogens, originated from a plant. Since the T3SS is present in plant pathogens e.g. *Erwinia* spp. it is likely that nature have produced inhibitors to defend themselves against infection from these pathogens. Therefore we hypothesize that more natural products that inhibit the T3SS in human pathogens would be identified in the future.

In conclusion we have identified the first natural product originating from a plant, neohopeaphenol A, as a selective and irreversible T3SS inhibitor with an IC$_{50}$ value in eukaryotic whole cell assays of about 6 µM for *Y. pseudotuberculosis* and 60 µM for *P. aeruginosa*. 
Concluding Remarks

Bacterial resistance to many of the current antibiotics is now of global concern. New strategies to deal with this problem are urgently needed. One such strategy is the development of virulence-blocking compounds, which render bacteria unable to cause disease. The salicylidene acylhydrazides belong to a class of virulence-blocking compounds that inhibits the T3SS in a panel of gram-negative bacteria such as Yersinia spp., Pseudomonas aeruginosa, Chlamydia spp., Salmonella spp., Shigella spp., and EHEC. The compound class is well-studied in all the above-mentioned bacteria, where they can be utilized as chemical tools, particularly in Chlamydia for which traditional genetics is difficult. The salicylidene acylhydrazides have moderate potency, are acid sensitive and hydrophobic. The pharmacokinetic property of a short half-life, create difficulties with systemic administration of the salicylidene acylhydrazides. The compounds would probably work better with topical/local administration. This has been demonstrated in a mouse model by Chu et al. for Chlamydia – by administering the compound directly into the vagina, Chlamydia infection was inhibited. The salicylidene acylhydrazides therefore have been shown to be promising anti-chlamydial compounds. Further, this example highlights that the salicylidene acylhydrazides have shown a proof of concept as a virulence-blocking compound and that the strategy is promising.

The activity of the salicylidene acylhydrazides in different species has led researchers to propose several mechanisms. In Shigella, Veenendaal et al. suggested a direct effect of the salicylidene acylhydrazides on the T3SS basal apparatus. In E. coli, the compounds were thought to affect proteins that govern the T3SS expression and function. In Chlamydia, treatment with salicylidene acylhydrazide was proposed to change the availability of iron due to chelation and in Salmonella, ion-related genes were shown to be up-regulated when treated with salicylidene acylhydrazides. This was in contrast to the microarray data for salicylidene acylhydrazide treatment in E. coli, where iron-related genes were found to be unaltered. In addition it has recently been shown that the salicylidene acylhydrazides have an effect on C. trachomatis infection next to the chelation of iron. In another study aimed at finding the target for salicylidene acylhydrazides in Salmonella, the authors suggested that the inhibition of the T3SS was due to general disruption of basic bacterial physiology through complex regulation. Together with our findings reported in Paper II,
these results indicate that the salicylidene acylhydrazides have more than one potential target and the targets might differ between species.

SMD was used to select salicylidene acylhydrazides for a focused library. We synthesized 58 compounds that were evaluated for inhibition of the T3SS in *Y. pseudotuberculosis*. Furthermore, the QSAR between the salicylidene acylhydrazides and their biological activity in *Y. pseudotuberculosis* was shown to be complicated, which indicates that the mode of action that results in inhibition of the T3SS is highly complex. The QSAR models were validated in two gram-negative bacterial pathogens, suggesting a conserved mode of action. Difficulties with QSAR modeling could be due to that the salicylidene acylhydrazides interact with multiple targets. Certain physicochemical properties may predispose compounds to be active against one target but less active against another, resulting in a complex QSAR. The fact that many small organic compounds tend to have more than one target supports our hypothesis of multiple targets for the salicylidene acylhydrazides.

Although many gram-negative bacteria have the conserved T3SS the regulation of the system differ between species. The salicylidene acylhydrazides are inhibitors of the T3SS, but from all target identification studies made, the targets likely differ between species and the targets are related to regulation. It is however unclear how the salicylidene acylhydrazides can function as virulence-blocking compounds in all these pathogens.

The identification of target protein(s) and mode of action for the salicylidene acylhydrazides proved far from simple. We identified 19 potential target proteins for the salicylidene acylhydrazides in *E. coli* and studied four of them in more detail in both *E. coli* and *Y. pseudotuberculosis*. The most interesting protein was *E. coli* AdhE. The *E. coli* ΔAdhE strain exhibited the same phenotype as *E. coli* treated with salicylidene acylhydrazides. In addition disulfiram is a known drug that inhibits the AdhE homolog in humans. The fact that the side effects that will arise in humans when treated with a novel virulence-blocking compound targeting bacterial AdhE are known would make clinical trials for such compound easier.

The fact that several drugs on the market e.g., antibiotics, originated from nature made us screen a prefractionated natural product library towards the T3SS in *Y. pseudotuberculosis*. Neohopeaphenol A was found and we showed that it was a virulence-blocking compound towards both *Y. pseudotuberculosis* and *P. aeruginosa*. In addition we showed that treatment of *Y. pseudotuberculosis* with neohopeaphenol A was irreversible and since the compound is large and heavy we hypothesize that it binds to a target on the bacterial surface. In the future, when synthetic procedures have been developed it would be interesting to test analogs to neohopeaphenol A. A couple natural products as T3SS inhibitors have been identified but Neohopeaphenol A is the first T3SS inhibitor originating
from a plant an active against human pathogens. Since the T3SS is present among plant pathogens we hypothesize that the plants have developed an endogenous defense towards these pathogens and that nature have more T3SS inhibitors to provide.

When the field of virulence-blocking compounds towards the T3SS is summarized one realize that several research groups are working with this strategy. Even though the salicylidene acylhydrazides are not going to be a drug on the market towards the T3SS, I believe in this strategy and think virulence-blocking drugs will be developed in the future, and they might originate from nature.
Appendix

Synthetic Procedure for Salicylidene Acylhydrazide with Azide and Biotin

Synthesis of salicylidene acylhydrazide is described in Scheme 4. Tentagel-OH (8) (1 g, 0.53 mmol) was swelled 1 h in CH₂Cl₂ rinsed with CH₂Cl₂ (5 x 5 ml) before a solution of 4-(Fmoc-aminomethyl) benzoic acid, (0.797 g, 2.13 mmol), MSNT (0.710 g, 2.4 mmol) and 1-methylimidazole (0.17 ml, 2.13 mmol) in CH₂Cl₂:DMF (1:8 5 ml) was added. The reaction was shaken at room temperature (rt.) for 65 h, washed with CH₂Cl₂ (3 x 5 ml) followed by DMF (5 x 5 ml). Deprotection was performed with 20% piperidine in DMF, a constant flow for 3 min followed by shaking for 7 min and rinsed with DMF (5 x 5 ml).

To the benzyl amine (9) (0.53 mmol) a solution of Fmoc 6-aminohexanoic acid (0.80 g, 2.26 mmol), DIC (0.33 ml, 2.12 mmol), HOAt (0.304 g, 2.23 mmol) and BFB (0.26 ml, 2 M in DMF) in DMF (3 ml) was added. The resin was shaken for 70 h in rt. The resin was washed with DMF (5 x 5 ml) and Fmoc deprotected with 20% piperidine in DMF a constant flow for 3 min followed by shaking for 7 min and rinsed with DMF (5 x 5 ml).

To amine (10) (0.53 mmol) a mixture of Fmoc-lysine-biotin (0.378 g, 0.64 mmol), DIC (0.3 ml, 2.12 mmol), HOAt (0.303 g, 2.23 mmol) and BFB (0.265 ml, 2M in DMF) in DMF was added. The resin was shaken 7 h in rt., rinsed and put under vacuum.

Compound 13, 957 mg (0.47 mmol) was deprotected with 20% piperidine in DMF a constant flow for 3 min followed by shaking for 7 min and rinsed with DMF (5 x 5 ml). A solution of 4-azidobenzoic acid (0.339 g, 2.08 mmol), DIC (0.3 ml, 1.93 mmol), HOAt (0.260 g, 1.91 mmol) and BFB (0.34 ml, 2 M in DMF) in DMF (1.2 ml) was added. The resin was shaken for 12 h before a recoupling with 4-azidobenzoic acid (0.330 g, 2.07 mmol), DIC (0.3 ml, 1.93 mmol), HOAt (0.260 g, 1.91 mmol) and BFB (0.34 ml, 2 M in DMF) in DMF (1.2 ml) was made with shaking for 1 h. The resin was washed with DMF (5 x 5 ml) followed by MeOH (2 x 5 ml).

The resin (14) was transferred to a round bottom flask and MeOH (2 ml) was added followed by hydrazine hydrate (2 ml, 41.15 mmol). The reaction was shaken under nitrogen for 4.5 h. The resin was removed by filtration and rinsed...
with dioxane and DMSO. The solution was concentrated to give the crude 15. The crude was purified by reversed phase HPLC to give more than 95% pure 15 (8 mg, 36% yield).

To 15 (8 mg, 0.010 mmol) dissolved in d\textsuperscript{6}DMSO 3,5-dibromo salicylaldehyde (3.1 mg, 0.011 mmol) was added and put on a shaker for 4 h in the dark. \textsuperscript{1}H NMR showed product 16 used without further purification. \textsuperscript{1}H NMR (400 MHz; DMSO): \(\delta 12.75\) (s, 1H), 12.5 (s, 1H), 8.53 (s, 1H), 8.32-8.42 (m, 2H), 7.89-7.98 (m, 5H), 7.78-7.64 (m, 2H), 7.73 (t, \(J = 5.8\) Hz, 1H), 7.4 (d, \(J = 8.2\) Hz, 2H), 7.19 (d, \(J = 8.5\) Hz, 2H), 6.36-6.45 (bs, 2H), 4.31-4.36 (m, 3H), 4.29 (dd, \(J = 8.0, 4.8\) Hz, 1H), 4.1 (dd, \(J = 7.8, 4.5\) Hz, 1H), 2.96-3.12 (m, 5H), 2.81 (dd, \(J = 12.4, 5.1\) Hz, 1H), 2.57(d, \(J = 12.5\) Hz, 1H), 2.15 (t, \(J = 7.5\) Hz, 2H), 2.01 (t, \(J = 7.5\) Hz, 2H), 1.2-1.76 (m, 18H). LC-MS \(m/z\) calc for C\textsubscript{44}H\textsubscript{53}Br\textsubscript{2}N\textsubscript{11}O\textsubscript{7}S: 1040.83 [M+H] found: 1040.8.
Acknowledgment

Jag vill passa på att Tacka alla som gjord min doktorandtid lyckad och möjlig! Tack...


Hans, min biträdande handledare, för idéer och kommentarer kring min forskning. Hans, Andreas och Bent för att Ni ställt upp och bidragit med kloka råd på mina uppföljningsmöten. Bent tack för hjälpen med framsidan!

I would also like to Thank our collaborators in Glasgow, Andrew for your never ending enthusiasm and a big thanks to You, Dai and Mads for all the work with our papers and for taking such good care of me during my lab time in Glasgow. Kate thanks for a nice collaboration, especially with all the AdhE work. Alette for letting me stay at your place and for shoe shopping!

Creative Antibiotics, nya och gamla personer för samarbete genom åren. Speciellt tack till Charlotte, Jenny, Pia L och Pia K, för er hjälp och engagemang.

Alla medförfattare till artiklarna och alla som hjälpt mig med experiment här och var. Speciellt tack till Mattias för NMR, Thomas och Roland för bakterier och hjälpen med mikroskåpet och Christin för proteinrening.

Ni som får allt runt om kring att fungera, speciellt tack till Pelle, Carina, Barbro, Rosita, L-G, Erik, Thomas, Martin, Cathrine och Kemiförädet.


Seniorerna på avdelningen för att ni bidrar med kunskap och en bra stämning. Frippe för din visslande glädje och för att jag fick göra
kandidatexjobbet i din grupp. **Anna** för bra samarbete med både forskning och undervisning. Tack för att jag fått vara på dina gruppmöten under delar av doktorandtiden.

**Hanna/Babe/PCR woman!** Tusen tack för att du hjälpt mig i labbet och alltid vänligt och pedagogisk svarat på alla mina frågor, som du nu på slutet fått chansen att ger igen för 🥰 Du är en klippe! Även om jag har ett par ”tantiga jävla loafers” så tackar du aldrig nej till en vinrelaterad aktivitet, det uppskattar jag!

**Lunchgänget** runt bordet i fikarummet! Hur många kan vi rymmas vid ett och samma bord? 😊 Tack **David** för korrekturläsning av beräkningsdelen, dina Word kunskaper och trevlig fikaraster.

Tack mina vänner! **Ida** för att du är så vänlig och för att du ringer upp när man skickar ett meddelande. **Sara** för alla trevliga pratstunder om allt möjligt, promenader och simning. **Maria** för din genuina omtänksamhet, och tack till dig och **Daniel** för hjälp med barnpassning, goda middagar och schlagersällskap!

**Hanna** och **Johanna** i nuvarande borneommiddagsgänget, tack för alla avslappnade middagar. Det är så skönt att efter jobbet mitt i veckan vara bortbjuden på middag inkl. efterrätt till er. 😊 Vi saknar dig **Lisa**!

**Syjuntagänget**, för att ni alla, alltid är så glada och peppande i alla syprojekt. Det är alltid lika trevligt att träffa er och sy, eller bara prata om allt mellan himmel och jord.

**Ume-tjejerna!** Inte trodde ni väl att de var jag som skulle vara den som blev kvar här i stan!? Tack för att ni är ni, även fast ni flyttat och inte ses så ofta, så är allt som ”vanligt” när vi väl ses. De måste vara dags för en träff snart!

**Greta** för allt som vi upplevt tillsammans, för att du hjälpte till på vårt bröllop. Jag saknar dig i stan! Tack **Lisa** för att du alltid har tid att lyssna och komma med kloka ord! **Kristin** och **Marie** för ert glada sinne!

**Christopher, Maria och Nils** våra bästa vänner som är som familj! Tack för alla trevliga, goda och långa middagar, för härliga utflykter året runt och för er hjälp. För att ni alltid finns till hands även om jag bor långt bort. **Susanne, Pernilla** och er familjer för att ni är mina vänner. Tack **Mormor** för din omtanke och för att du försöker förstå min forskning. Tack hela familjen **Zetterström** för er enorma gastfrihet.

**Per**, för din kärlek och att du alltid finns där för mig! Tack för att du engagerat dig i min forskning, hjälpt mig med bilder, redigering och för att du ställt upp extra mycket för mig under skrivandet. Tack **Rut** för att du får mig att släppa jobbet och tänka på annat när jag kommer hem. Jag ålskar er, min egen lilla familj, så himla himla mycket!
References


58. Yamazaki A, Li J, Zeng Q, Khokhani D, Hutchins WC, Yost AC, Biddle E, Toone EJ, Chen X, Yang CH (2011) Derivatives of plant phenolic compound affect the

70
type III secretion system of *Pseudomonas aeruginosa* via a GacS-GacA two-component signal transduction system. Antimicrob Agents Ch 56:36–43.


Caroline Zetterström was born and grew up in Dyltabruk, outside of Örebro, in 1982. She graduated from high school in 2001 in Örebro. She completed a Bachelor in Chemistry at the Department of Chemistry, Umeå University. The title of her Diploma project was “Synthesis of highly substituted ring fused 2-pyridones”. In 2007 she received her Master of Science in Biotechnology and Engineering Genomics at Umeå University. The Diploma project “Haplotype analysis of the KNS2 gene in relation to Alzheimer’s diseases diagnosis and CSF biomarkers” was performed at the Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg.