Discovery of novel natural anti-biofilm compounds of wide chemical diversity

Master's thesis by

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ABSTRACT

Microorganisms have the tendency to switch from planktonic stage to biofilm and back again depending on the environmental conditions. Microorganisms can attach to all kind of materials and form biofilms. A biofilm develops when microorganisms irreversibly attach to a surface and start producing extracellular polymers. The polymers provide a matrix around the bacteria which protects the microorganisms from environmental factors. A significant part of human bacterial infections is caused by biofilms. Bacterial biofilm infections include lung infections of cystic fibrosis patients, ear infections, wound infections, bacterial endocarditis and infections associated with indwelling medical devices. Biofilms are known to be very resistant to antibiotics and place a significant burden on healthcare system worldwide. *Staphylococcus aureus* is a gram-positive bacterium that has an ability to adhere to surfaces and form biofilms. The antibiotics on the market today are not effective enough against *S. aureus* biofilm-related infections. Furthermore, natural products have since long been used to treat different kind of bacterial infections. The chemical diversity they possess, makes them important in the discovery of lead compounds.

The aim of this work was to identify novel natural compounds with anti-biofilm properties, acting on biofilms formed by *Staphylococcus aureus.* A commercially available Enzo Product Library consisting of 502 natural and naturally derived compounds were at first screened at approximately 40 µM (ranging from 9.4 µM to 130.6 µM), using two *S. aureus* bacterial strains. Bacteria were exposed to the compounds both prior-to and post-biofilm formation. The viable bacterial mass formed in both assay modes was quantified using resazurin staining, a method previously developed in the laboratory. The workflow was a multistep process. First, twenty compounds were found to inhibit biofilm formation over the activity threshold in both prior-to and/or post-exposure tests in at least one of the tested strains. Second, eight compounds from the primary screening were further considered as most selective based on literature searches, as well as retested in a reconfirmation trial. Finally, four compounds, cromomycin A3, mithramycin A, mitomycin C and minocycline were identified as possible anti-biofilm agents due to their activity on both planktonic and biofilm bacteria. Furthermore, the anti-biofilm potencies of all four compounds were high, which makes them good antimicrobials.

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SUPPLEMENTARY TABLE 1

LIST OF ABBREVIATIONS

ADME-tox	Absorption, distribution, metabolism, excretion, toxicity
CF	Cystic fibrosis
CFU	Colony forming unit
CV	Coefficient of variation
CVC	Central venous catheters
DMSO	Dimethyl sulphoxide
eDNA	Extracellular DNA
EPS	Extracellular polymeric substance
HL	Hit limit
HTS	High throughput screening
IC_{50}	50% of maximal inhibitory concentration
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MMOA	Molecular mechanism of action
MPN	Most probable number
PBS	Phosphate buffered saline
PIA	Polysaccharide intercellular adhesin
S/B	Signal-to-background
S/N	Signal-to-noise
TSA	Tryptic soy agar
TSB	Tryptic soy broth
WGA	Wheat germ agglutinin
QS	Quorum sensing
Z' factor	Signal window coefficient

1 INTRODUCTION

Traditional studies of microorganisms have given us information about free living single-cell bacteria which are called planktonic cells. However, additional research has shown that microorganisms are frequently attached to surfaces as multi-cellular communities. A thus-called biofilm can develop when microorganisms irreversibly attach to a surface and start producing extracellular polymers. The polymers provide a structural matrix that has a number of different functions: it is an efficient system for trapping nutrients from the surrounding environment, and it protects the microbes in the biofilm from external factors such as biocides, antibiotics and the host immune response (Donlan, R. M. 2001; Lindsay and Von Holy 2006a).

Bacterial biofilms have been shown to be both tolerant and resistant to antimicrobials since they are involved in a wide range of infections, ranging from chronic wounds to cystic fibrosis and to infections associated with medical devices (Donlan, R. M. 2001). The National Institute of Health has determined that 80% of human bacterial infections are caused by biofilms (Blackledge et al. 2013). *Staphylococcus aureus* (*S. aureus*) is a gram-positive bacterium that is associated with biofilm-mediated diseases (Kiedrowski and Horswill 2011). Very resistant clones of *S. aureus* have caused a myriad of health problems during the last decades (Indrawattana et al. 2013; Lowy 1998).

S. aureus and other pathogenic bacteria put pressure on the medical community to discover new anti-biofilm compounds for the treatment of resistant biofilm infections. Researchers are eager to find new anti-biofilm compounds, as well as discovering new applications for already existing substances. (Worthington et al. 2012). Plants and microbes have gone through centuries of evolution and developed mechanisms to fight environmental infections by using secondary metabolites. Natural products have a significant chemical diversity that has provided us with essential therapeutic agents for different kinds of bacterial infections. Furthermore, natural products are important in the discovery of leads for development of new drugs for the treatment of many diseases, including biofilm-related infections (Cragg and Newman 2013).

2 REVIEW OF THE LITERATURE

2.1 Bacterial biofilms

The theory of biofilms we know today started in the 17th century when Antonie van Leeuwenhoek discovered bacteria from the plaque on his teeth. Today, it is accepted that the cells within biofilms differ from their planktonic counterparts. A biofilm may be defined as a structured community of microorganisms that is most often irreversibly attached to a surface. A biofilm can consist of only one bacterial species or multiple bacterial species. The bacteria in a biofilm function in a co-ordinated manner by forming a complex highly differentiated multi-cultural community where close genetic regulation is required (Lindsay and Von Holy 2006b).

2.1.1 Biofilm structure

Microorganisms, matrix and surface are three key elements regarding the biofilm definition presented above. A biofilm is not able to form or remain intact if one of these elements is missing (Dunne 2002).

Microorganisms

Microorganisms in a biofilm are distinct from planktonic microorganisms because the gene transcription is altered when the microorganisms adhere to a surface (Donlan 2002). Bacteria in the biofilm co-operate and the behavior is in some ways like multi-cellular organisms compared to free living bacteria. The communication between bacteria within the biofilm is called cell-to-cell signalling. Molecular signals diffuse between cells through the cell membranes and the process is also described as quorum sensing (QS). There are several known quorum sensing systems. The most described systems are peptide molecules that are associated with gram-positive bacteria and acyl homoserine lactone systems of gram-negative bacteria (Lindsay and Von Holy 2006b; Parsek and Greenberg 2005).

Only 15% of the whole biofilm volume consists of microbial cells (Donlan and Costerton 2002). The cells are not dispersed uniformly throughout the biofilm, instead they are growing in matrix-

enclosed microcolonies. Microcolonies grow out from the surface as mushroom-shaped structures implicated with less dense regions of matrix (Donlan and Costerton 2002; Dunne 2002). A biofilm is usually heterogeneous, which means that it consists of several bacterial species. The colonization of the surface by one bacterial species will enhance the attachment of other bacteria to the same surface and free-floating planktonic organisms will be entrapped in the matrix (Lindsay and Von Holy 2006b). Biofilms consisting of only one bacterial species are not frequent in nature but common on medical devices and in biofilms associated with infectious diseases (Donlan 2002). Metabolic by-products of one microorganism in the biofilm can provide ligands for the attachment of other bacteria. The competition of nutrients and toxic by-products can also affect the biofilm diversity (Dunne 2002).

Matrix

Matrix, also referred to as the slime layer, consists mostly of water. Extracellular polymeric substances (EPS) form a robust complex and are the main parts of the biofilm matrix next to water. EPS are primarily composed of exopolymers which include polysaccharides, phospholipids and proteins. EPS are produced by the microorganism in the biofilm and they encapsulate the cells. Matrices vary greatly and the composition is difficult to unravel (Allison 2003). *S. aureus,* for example, is known to have a complex matrix consisting of teichoic acids, proteins, polysaccharide intercellular adhesin (PIA) and extracellular DNA (eDNA) (Le et al. 2014). The surrounding environment and internal factors including genotypes of the attached microorganisms will affect the composition of the matrix (Allison 2003).

The biofilm matrix has many essential functions. Firstly, it provides a barrier that anchors and protects the cells in the biofilm. The matrix provides mechanical stability and anchors the microorganisms to the surface. It protects from negative external factors such as antibacterial agents and antibodies from the host immune system and the water content prevents desiccation. The three-dimensional matrix will also entrap nutrients from the surrounding and allow signal molecules to accumulate (Allison 2003; Dunne 2002; Lindsay and Von Holy 2006b).

Surface

Biofilms can basically form on any kind of surface. Investigators have proposed that hydrophobic nonpolar surfaces, such as plastics and Teflon, will more rapidly be covered with biofilm compared to hydrophilic materials such as metals and glass (Donlan 2002). All surfaces that exist in aquatic systems are prone to biofilm formation. Biofilms can therefore be found in soil, rocks and food contact surfaces, but also in all industrial pipelines including ventilation systems and water supply (Dunne 2002; Gilbert et al. 2003). Finally, biofilms are commonly associated with indwelling medical devices and bacterial infections. Implanted medical devices such as artificial joints, catheters and valves pose a great risk of biofilm infections. Biofilms are frequently found in the lungs of patients with Cystic fibrosis (CF) and in chronic wounds (Donlan 2002).

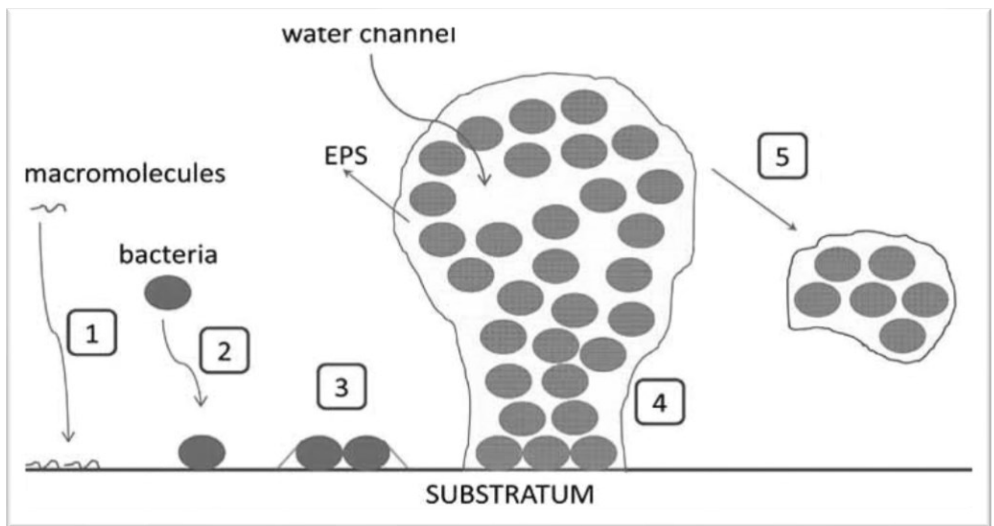
2.2 Biofilm formation

Biofilm formation depends on bacterial species, surface composition and the surrounding environment. There are a number of successive steps in biofilm formation (**Figure 1**) (Cos et al. 2010). The model is generally accepted as a basis for biofilm experiments and includes: 1) surface conditioning and attachment of bacteria, 2) biofilm maturation and extracellular matrix production and 3) detachment of biofilm bacteria and spread of infection. The biofilm formation stages will be described below.

Surface conditioning and attachment of bacteria

The first step is the formation of a conditioning layer on the surface. During this phase, the native surface is modified by the adsorption of organic and inorganic compounds, produced by the host or present in the environment. Substances such as blood, urine and saliva contain proteins which can adhere to biomaterials and influence the attachment of bacteria (Donlan, Rodney M. 2002; Karatan and Watnick 2009).

Figure 1. The biofilm life cycle illustrated in steps: surface conditioning (1), attachment events (2-3), the growth of complex biofilms (4), and detachment by clumps of bacteria (5) (Cos et al. 2010)



The second step in biofilm formation is the bacterial adhesion phase, where bacteria attach to the surface. First, a reversible contact is formed, also referred to as the docking phase. The attractive or repulsive forces between the bacteria and surface will determine bacterial attachment. Van der Waals forces, temperature and hydrophobic interactions are examples of forces that are involved in the docking phase (Dunne 2002).

After the adhesion phase, microorganisms become irreversibly attached to the surface. This phase is called the "locking" phase and bacteria start producing extracellular polymeric substances (EPS) (Donlan, 2002). EPS form a robust complex with receptor-specific ligands on pili or fimbriae and the surface material (Dunne 2002). The irreversible attachment to the surface is dependent on adhesins that are produced by the microorganisms. In a *Staphylococcus aureus* biofilm, for example, surface attachment is associated with polysaccharide intercellular adhesin (PIA), which consists of β-1,6-linked glucosaminylglycans. PIA is the most important polysaccharide in the EPS and promotes the bacterial cell-to-cell adhesion (Cramton et al. 1999).

Different adhesins allow microorganisms to switch from planktonic to sessile depending on the environment. *Vibrio cholera* attaches to epithelium using pilus but hemagglutinin is the adhesin

responsible for its attachment to a surface in an aquatic environment (Dunne 2002). Microcolonies are formed when the microorganisms attach to each other in addition to the surface. Flagellae, for example, are necessary for the attachment of *P. aeruginosa* to a surface but contraction and extension of pili will allow the microorganisms to adhere to each other in microcolonies (Stoodley et al. 2002).

Biofilm maturation and production of extracellular matrix

The third step in the biofilm formation is the maturation phase. The maturation process starts at the end of the locking phase, when the complexity of the biofilm increases and microorganisms start to replicate. Biofilms are dynamic structures that are constantly changing during the maturation phase and where typical water channels are formed to supply and drain nutrients and waste products (Stewart and Franklin 2008).

Material and organisms are entrapped in the complex EPS, that are interacting with the molecules in the environment. Oxygen perfusion, availability of nutrients and internal pH will affect the biofilm maturation (Dunne 2002; Lindsay and Von Holy 2006b). Nutrients and oxygen are not evenly distributed throughout the biofilm. These compounds fail to penetrate the biofilm because they are consumed by bacteria in the upper layers. As a result, there is a lower availability of oxygen and nutrients in the inner layer and a subsequent accumulation of waste products. Consequently, bacteria in these layers will show different functional phenotypes. Bacteria in the biofilm will adapt to their local environment resulting in a considerable heterogeneity. The chemical conditions can change over time and as the development of a biofilm proceeds, the cells within the biofilm will vary from each other in many ways (Stewart and Franklin 2008).

Detachment of bacteria and spread of infection

The final step in biofilm formation is the detachment. Bacteria from the outer layers of the mature biofilm can detach and colonize new surfaces. This process, also called biofilm dispersal, is a particular problem in healthcare. Detached bacteria from an infected surface can infect other parts of the body (Cos et al. 2010; Stewart and Franklin 2008). Fluid shear or starvation has traditionally been explained to cause biofilm dispersal. Furthermore, bacteria seem to be able to detach and

colonize new surfaces before the nutrients become limited, which makes the detachment a more active bacterial behavior. (Hall-Stoodley and Stoodley 2005).

A number of different biofilm dispersal mechanisms have been identified. *S. aureus,* for example, has the tendency to shed clumps of bacteria, also referred to as clumping dispersal (**Figure 2**). Detached clumps containing hundreds of *S. aureus* cells are transported with the fluids to new niches in the organism. The clumps are resistant to anti-microbial treatments which explains the highly infectious metastasis. This process is a particular problem in patients with indwelling medical devices infected with *S. aureus*. Dispersal mechanisms used by other microorganisms include swarming in which individual cells are released from the biofilm, as well as surface dispersal in which the whole biofilm structure is detached (Hall-Stoodley et al. 2004; Hall-Stoodley and Stoodley 2005).

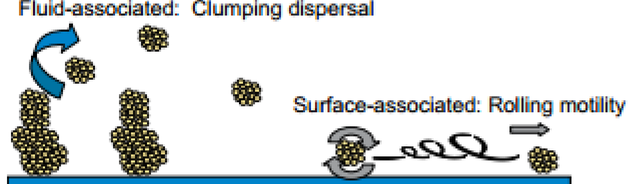


Figure 2. Fluid-driven clumping and rolling dispersal displayed by biofilm-forming *Staphylococcus aureus.* Modified from (Hall-Stoodley and Stoodley 2005).

2.3 Resilience of bacterial biofilms

Bacteria in their planktonic state are more susceptible to antimicrobials than their biofilm counterparts. Bacterial biofilms are both tolerant and resistant to antimicrobials. Firstly, tolerance is defined by the minimum bactericidal concentration (MBC) that will kill 99.9% or more of the cells in a bacterial culture. A tolerant microorganism will survive the presence of one or more antimicrobials but may not grow. Second, resistance is the ability of a microorganism to grow at a concentration of an antimicrobial that normally would inhibit the growth. Resistance is measured using the minimum inhibitory concentration (MIC), which is the lowest concentration of an antimicrobial agent that will inhibit the microbial growth. (Donlan 2001; Hall and Mah 2017).

Biofilms are a strategy for bacteria to protect themselves from antibacterial agents (including antibiotics) (Donlan and Costerton 2002). There are many possible mechanisms representing bacterial tolerance to the antimicrobial therapy, depending on the composition of the biofilm and the antimicrobial agent used (Mah and O'Toole 2001). The first mechanism is the delayed penetration of some antimicrobial agents. Antimicrobial molecules must diffuse through the biofilm matrix to reach the cells within the biofilm. The matrix acts as a diffusional barrier for some antimicrobial molecules, which results in a delayed penetration. Enzymes, that can inactivate antimicrobial agents also accumulate in the biofilm protecting the bacteria (Donlan and Costerton 2002).

The second mechanism for antimicrobial tolerance is the altered metabolic state in a biofilm (**Figure 3**). Bacteria in the inner layers are more susceptible than the ones in the outer layers. They grow slower because of the lack of oxygen and nutrients. Because of the significant heterogeneity within the biofilm, some bacteria are highly resistant to antimicrobial therapy, whereas others are not (Stewart and Franklin 2008).

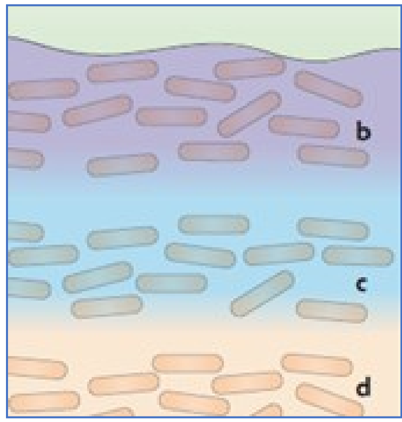


Figure 3. Physiological heterogeneity in a single species mature biofilm. Environments that contain both substrate and oxygen (b), substrate but no oxygen (c) and neither substrate nor oxygen (d) can occur (Modified from Stewart and Franklin 2008).

The persister cells in a biofilm form the third protective mechanism. Because of their dormant state, persister cells are very tolerant to extremely high concentrations of antibiotics and neither grow,

nor die when bactericidal agents are present (Smith 2005). To eradicate a bacterial biofilm, it is essential that the antimicrobial both kills the bacteria and destroys the matrix. If the surrounding protective matrix is not removed, persister cells will re-colonize the remaining matrix. The prospect of using antibiotics alone for treatment of biofilm-related infections is minimal because of the aspects discussed earlier, in addition to the commonly seen bacterial resistance mechanisms, that also operate in planktonic cells (Toté et al. 2009). For instance, the cells within a biofilm can easily exchange plasmids containing antibiotic resistance genes. When they exchange these plasmids, they become more resistant to treatment, in addition to their inherent tolerance (Donlan 2002; Shiau and Wu 1998).

Biofilms are also a challenge for healthcare because of their tolerance to the host's immune system. It has been shown that antibodies cannot reach the surface of the bacterial cells within a biofilm. EPS, produced by *S. epidermidis*, for example, will affect the macrophages by decreasing their phagocytic activity. Detached cells from the biofilm will survive the phagocytic activity in the bloodstream, in order to initiate a bloodstream infection (Donlan 2002; Shiau and Wu 1998)

2.4 Consequences of biofilm formation

Biofilms can form on virtually any surface and depending on where they are located, they have a beneficial or detrimental effect on the organism and/or environment (**Figure 4**). (Gilbert et al. 2003).

Biofilms are "two faced"	
Negative effects	**Positive effects**
Slow down manufacturing processes.	Ecological roles.
Biofouling of pipelines and contamination of food.	Water treatment.
Antibiotic resistant pathogens.	Benefit human large intestine.

Figure 4. The impact of biofilms.

Biofilms are found in soil and in wastewater treatment plants. Bacterial aggregates grow in plants rhizophere where they live in symbiosis with the plant. Living on the human body as commensalism is also a common natural habitat. The human gut is the next environment after the oral cavity, where commensal multispecies biofilms form. Bacteria attached to the surface in the human large intestine, for example, can protect the organism from pathogenic bacteria (Bjarnsholt 2013).

The downside of biofilm formation is their association with major problems in industry and medicine. Biofilms slow down manufacturing processes, damage equipment, contaminate food products and cause biofouling of pipelines. Furthermore, the interior (blood, brain, muscles etc.) of the human body, do not harbor permanent flora but can sometimes be contaminated with bacteria. If the mechanisms of the mucosal membranes fail to remove the bacteria an infection can establish (Bjarnsholt 2013; Gilbert et al. 2003). Biofilms are known to be very resistant to antibiotics and place a significant burden on healthcare system worldwide. Hospital-acquired infections in the US are claimed to be responsible for more annual deaths than emphysema, AIDS, Parkinson's disease and homicides combined (Blackledge et al. 2013). In Europe, during 2011-2012, 3.2 million cases occurred, according to the European Centre for Disease Control and Prevention and 80% of them were catheter-related and urinary tract biofilm infections (Worthington et al. 2012). As much as 80% of human bacterial infections is caused by biofilms

according to the National Institutes of Health (NIH). Typical bacterial biofilm infections include: lung infections of cystic fibrosis patients, ear infections, wound infections, bacterial endocarditis and infections associated with indwelling medical devices (Blackledge et al. 2013).

2.4.1 *Staphylococcus aureus* biofilm infections

Staphylococcus aureus (S. aureus) is a gram-positive, non-spore-forming bacterium that can cause diseases because it is able to adhere to surfaces and form a biofilm. (Kiedrowski and Horswill 2011). *S. aureus* is a bacterium commonly living in symbiosis with the host organism, usually on the skin or on nasal mucosa. *S. aureus* (Class: *Cocci*) is a member of the Staphylococcaceae family. Characteristics for *S. aureus* are positive results of coagulase, mannitol fermentation and positive deoxyribonuclease tests. On microscopical examination, the bacteria appear as spherical *cocci* in clusters and characteristic for *S. aureus* is the gold pigmentation of colonies on agar. *S. aureus* produces many virulence factors, some of them enhance bacterial colonization, whereas others are toxins (Indrawattana et al. 2013; Lowy 1998).

Staphylococcus aureus biofilms are involved in a wide range of infections, ranging from chronic wounds to cystic fibrosis and to infections in medical devices. A wound for example, is an ideal environment for bacteria to grow because of the moist surface and supply of nutrients. Under these conditions, bacteria contaminating the wound may have the ability to form resistant biofilms (Malic et al. 2009). The viscous lung secretions in cystic fibrosis patients provide an environment that protects bacteria from antibiotics and immune cells but favour bacterial growth and persistence. One frequently isolated microorganism from the respiratory tract of young children with cystic fibrosis is *S. aureus* (Baldan et al. 2014). Among indwelling medical devices, central venous catheters (CVC) and mechanical heart valves pose a great risk of infection (Donlan, R. M. 2001). Blood contains proteins that can adhere to biomaterials and influence the attachment of bacteria. It has been shown that biofilm formation occurs within 3 days after catheterization. Commonly isolated species from CVCs are *S. epidermidis* and *S. aureus* (Donlan, R. M. and Costerton 2002).

2.5 Drug discovery from natural products

2.5.1 General aspects

Bacteria have lived on the earth for several billion years and developed resistance to most of the antimicrobial agents that have been used over the past decades (Raja et al. 2010). Natural products are essential in the discovery of leads for development of new drugs. In the area of infectious diseases and cancer, 75% and 60% of new drugs, respectively, were originated from natural products between 1981 and 2002 (Cragg and Newman 2013; Newman et al. 2003). The main area within drug discovery from natural products today is still cancer and infectious diseases. In the area of cancer, 74.8% of the small molecules discovered until the year 2010 were other than synthetic and 48.6% were natural products or directly derived from them (Newman and Cragg 2012). Over 1 million natural compounds have been discovered and 50-60% of them are produced by plants (alkaloids, flavonoids, terpenoids, steroids etc.) and 5% are of microbial origin. Of all the reported natural products, 20-25% show biological activity (Raja et al. 2010). Natural compounds cover a wide range of therapeutic infections. Over 100 new products were in clinical development as anti-infectives and anti-cancer compounds in 2008 and half of the drugs that have been approved since 1994 are derived from natural products (Harvey 2008).

The screening of natural compounds is not a straightforward process. The screening of natural products has rather been considered a troublesome process because it has some limitations compared to screening of synthetic compounds. It is expensive and demanding to maintain high-quality natural product libraries and the purification processes require time-consuming labor-intensive procedures. Natural compounds consist of big complex structures. The complex structures with many functional groups make it more challenging to prepare as many natural product analogs as synthetic chemicals during the same time period (Lam 2007). The drug discovery was sped up in the late 1980s by the development of high throughput screening methods (HTS). The screening processes of natural compounds were not suitable with the new HTS approaches which led to a drop of discovered natural products-based leads in the 1990s (Harvey 2008; Lam 2007). New technological advances, such as improvements in screening programs and isolation techniques, have in recent years shortened the timeline and re-established natural products as a promising source of new lead molecules (Lam 2007).

Drug discovery from natural compounds has been estimated to take 10 years and cost more than 800 million dollars (Balunas and Kinghorn 2005). There are several steps in the drug discovery and development process (**Figure 5**). The process starts with the identification of a lead compound. In order to identify a new compound, an adequate high throughput screening assay must be developed. *In vitro* biochemical and cellular assays are versatile, and it is possible to study a large number of compounds in a time-efficient manner. These types of assays have been used for a long time for HTS and will be discussed more in detail in section 2.5.2. The second step in the drug development process is to optimize the lead compound in order to obtain more potent analogues (Balunas and Kinghorn 2005; Keserű and Makara 2006). One example is the modification of natural products, which leads to new synthetic analogs that possess new biological activities compared to the parent molecule. The modification of complex natural products by organic synthesis has been promising during the past decades due to new synthetic approaches. New approaches in combinational biosynthesis have also made it possible to achieve a wide range of possible structure modifications. New functional groups can be added and further modified, which is a particular characteristic for natural compounds (Keserű and Makara 2006; Lam 2007).

The third step in the drug discovery process is the lead compound development in order to find drug candidates that will be ready for clinical trials. This process will focus on what the drug does to the body by pharmacodynamics studies and pharmacokinetics studies considering ADME-tox (Absorption, Distribution, Metabolism, Excretion and Toxicity) properties. An ideal compound should be stable, not cytotoxic for normal cells and show significant activity in cellular assays. The toxicity is evaluated through animal testing before the drug can be ready for clinical trials on humans (Balunas and Kinghorn 2005; DiMasi et al. 2003). Many of the discovered lead compounds are rejected from the drug development process because they do not meet these strict requirements. It has been estimated that in general only one out of 5000 discovered lead compounds will end up as an approved drug (Balunas and Kinghorn 2005).

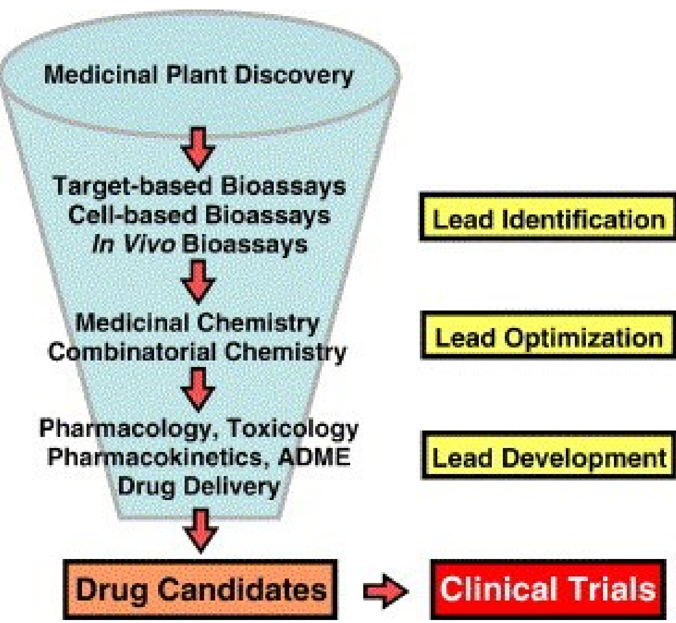


Figure 5. Drug development process from natural compounds is schematically presented (adapted from (Balunas and Kinghorn 2005)).

2.5.2 In vitro screening

There are two approaches in compound screening. One is the phenotypic drug discovery which focuses on screening candidates that alter the cell in a desired manner without the knowledge of the molecular mechanism of action (MMOA). The second is a target-based approach which has been very popular since the 1990s. The target-based approach aims at discovering how compounds affect specific molecular targets, especially proteins implicated in disease (Kotz 2012; Swinney and Anthony 2011).

Phenotypic screening could be considered as more physiologically relevant because the hit compounds can target different types of proteins and various signaling pathways. Many diseases have no identified or validated drug targets, which slows down lead discovery for these understudied diseases and conditions that lack proper treatment. Through phenotypic screening it is also possible to discover novel drug targets in diseases for which no successful drug has been discovered through target-based screening (Zheng et al. 2013). The advantage of target-based screening on the other hand is that the assay throughput is usually high once the target has been

identified and validated, followed by high throughput screening (HTS) of compound libraries. One disadvantage of phenotypic screening is simply that it is more difficult to work with cell-based systems. It is also challenging to optimize the lead compound without prior knowledge of the MMOA (Swinney and Anthony 2011; Zheng et al. 2013). The main limitation with the target-based screening on the other hand is that nothing is known about the cell permeability of the compounds. Furthermore, the ability of the compound to act in a cellular context, with all barriers and cellular components present is unidentified (Macarrón and Hertzberg 2002)

The question that arises is, which one of the two assay approaches is preferable for naturally derived compound screening? The focus since the 1990s has been on drug targets. The modification of certain target activity has been linked to disease pathogenesis and advances have been made in order to develop new tools for target identification (Swinney and Anthony 2011; Zheng et al. 2013). Phenotypic assays were primarily used in drug discovery before the target-based approaches were introduced. However, it has been determined that 28 first-in-class drugs that were discovered during 1998-2008 came from phenotypic screening and 17 drugs was a result from target-based approaches, which indicates that it is important to focus on integrated systems during drug development. Both approaches have their advantages and disadvantages and both assay types can be used in order to discover targets with therapeutic potentials and understand the drug action mechanism (Kotz 2012; Swinney and Anthony 2011).

2.5.3 Statistical aspects of the screening process

The assay screening performance is dependent on various environmental, instrumental and biological factors. The examination of the obtained screening data is one essential step in the screening process. It is possible to determine if the collected data corresponds to the minimal quality requirements by using statistical tools. The data is examined at experiment-, plate- and well-level in order to see if any distinct trend can be detected. Two assay quality parameters in screening settings are signal-to-background (S/B) and signal-to-noise (S/N) ratios. S/B measures the ratio of positive control mean to the background signal. S/N is a similar parameter. These two parameters lack the dynamic signal range in the screening process and are therefore not considered as strong parameters in compound screening (Zhang et al. 1999).

Z' is a widely used statistical parameter in the context of biomolecular screening and assay validation (**Table 1**). The separation between positive and negative controls in a single plate is calculated and it shows the separation of maximal signal values from the background. The parameter has a range from 0-1 and the higher the value the better the assay performance. A 0 value indicates that there is no separation between the maximal and minimal signals. A value of 0.5 is an excellent assay performance with an obvious separation band between the controls (Zhang et al. 1999). Cell-based assays tend to have high signal variability and therefore a Z' ≥0.3-0.4 could be considered as an acceptable value (Merten 2010).

Table 1. Classification of screening assay quality by Z'-factor (Zhang et al. 1999)

Z-factor value	*Structure of assay*	*Related to screening*
1	SD = 0 (no variation), or the dynamic range → ∞	An ideal assay
1 > Z ≥ 0.5	Separation band is large	An excellent assay
0.5 > Z > 0	Separation band is small	A double assay
0	No separation band, the sample signal variation and control signal variation bands touch	A "yes/no" type assay
<0	No separation band, the sample signal variation and control signal variation bands overlap	Screening essentially impossible

The goal of the screening is to narrow down the library to a smaller number of hits with low false discovery rates. It is important to select as many true hits as possible during the elimination process and the selection of hits is performed on plates that have passed the quality criteria discussed above. A hit limit is usually defined as standard derivations (SD) away from the mean of the control signal, 3 X SD. The risk of false positive hits is lower when the mean of the control is further away from the hit limit (**Figure 6**). A high-quality assay will give both clearly positive or clearly negative hits and a minimal amount of points that are found close to the hit limit. The few hits that happen to be located close to the hit limit are confirmed in secondary screening assays (Zhang et al. 1999).

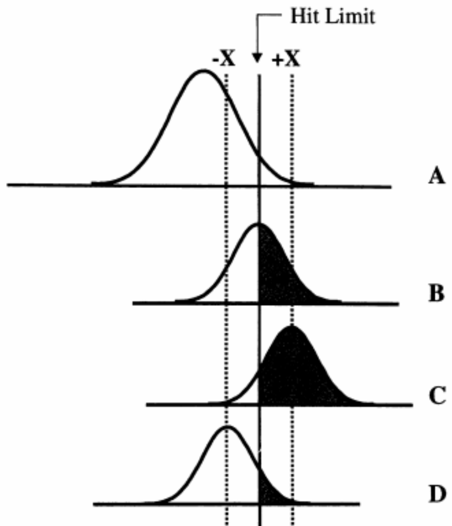


Figure 6. Normal distribution of test samples (A) with sample points on either side of the hit limit. Samples with activity same as hit limit will have 50% chance of being hits. Compounds at hit limit + X or hit limit – X will have much higher or lower chance of being hits (C and D)(Zhang et al. 1999).

2.6 Repositioning as an innovative way of improving drug discovery

2.6.1 Definition and advantages

Hit compounds are found in HTS, however, as already explained in previous section, few of the detected compounds can be developed into drugs. New strategies are introduced to overcome challenges in new drug development (Li et al. 2011). Repurposing and repositioning drugs are strategies to find new uses for already approved medicines, medicines that have not been approved for their initial indication and medicines that are still under clinical development for the primary indication. This approach makes it possible to treat neglected diseases and develop drugs faster and at a lower cost than through traditional approaches (Swamidass 2011).

The first advantage with drug repositioning is the decreased risk of failure. Compounds are usually failing in the Phase III of clinical trials primarily for efficacy reasons. Around 200 compounds that have failed the trials are standing idle in companies and the number is growing. Most of the failed

compounds were safe when administered in the Phase I and Phase II trials, and their safety profiles are well-known. The pharmacodynamic and pharmacokinetic properties of the compounds are usually desirable, which make them potential drug candidates for other indications (Jarvis 2006). The second advantage is that the cost of a repurposing program is cheaper than de novo research and discovery. More than half of the compounds will not pass Phase III, which is the most expensive development phase (Arrowsmith and Harrison 2012). The third advantage of a repositioning approach is the shorter process before the drug can be approved for use. *In vitro* and *in vivo* screenings, toxicology and optimization, formulation development together with further early development can in many cases be bypassed and that speeds up the process with several years (Ashburn and Thor 2004; Jarvis 2006).

There are some desirable properties of a drug-repositioning candidate. In an ideal situation the compound does not need additional chemistry to be optimized for the new indication because it will require further clinical safety and ADME-testing. It is also desirable that the ADME requirements are the same as for the new indication, and the possible reformulation of the medicine will not alter ADME and safety. Reformulation, preclinical and clinical safety studies are costly and an ideal compound would require few, if any steps for optimization (Wilkinson and Pritchard 2015). There are several drugs that have been switched to new indications. One of the best-known example of a successful drug repositioning implementation is the first treatment for erectile dysfunction, Viagra (sildenafil citrate). The drug was originally developed for treatment of angina but then switched to the new indication based on the registered "off-target" effects that were observed during the pre-clinical studies (Barratt and Frail 2012).

2.6.2 Approaches for drug repositioning

Different approaches including cell-based biological*, in vitro, in vivo* and *in silico* experimental platforms have been described for drug repositioning. Phenotypic screening of approved drugs is a versatile approach in drug discovery. The screening makes it possible to achieve approval for new disease indication in a short period of time. Compared to a traditional phenotypic screening approach (described in section 2.5.2) steps such as target identification, lead optimization and preclinical development can be passed (**Figure 7**). A phenotypic repurposing screen also enables

new drug targets. The information obtained from the screening can be used for new drug development once the target has been validated (Zheng et al. 2013).

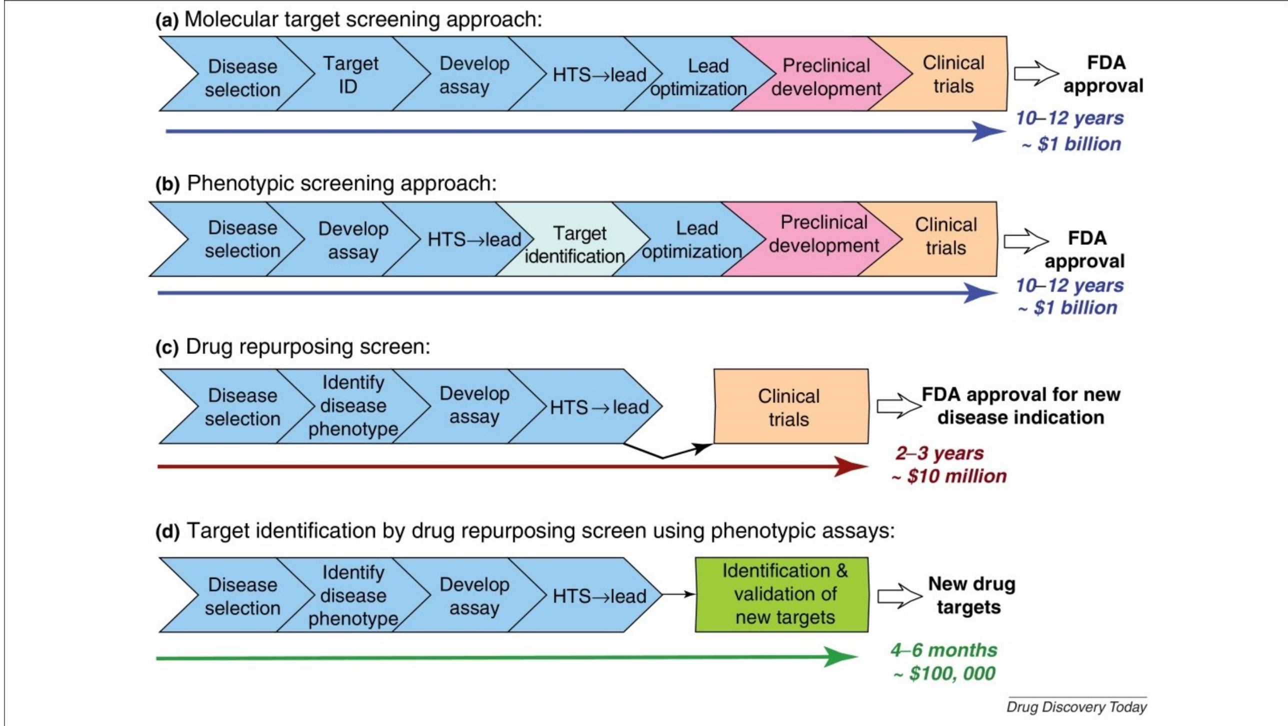


Figure 7. Comparison of different screening approaches in drug discovery and development. **a)** Traditional drug discovery. **b)** Phenotypic based drug discovery. **c)** Drug repurposing screen using phenotypic assays. The development time and cost are much lower compared to traditional drug discovery. **d)** New target identification by phenotypic repurposing screens. Many drugs have known mechanisms and a new target can be a direction for drug discovery. Adapted from (Zheng et al. 2013).

In vitro screens offer several advantages as a starting point for drug repositioning. Multiple compounds with different modes of action can be tested over a full concentration-effect range. The active compounds can then be analyzed using more complex phenotypic assays, be further studied in animal models or added to existing *in silico* models (Wilkinson and Pritchard 2015). One phenotypic repositioning approach is to perform screens with a combination of existing drugs. There are several available molecules on the market that have been regarded as safe but have not been approved based on low efficacy. Synergistic combinations of existing drugs can target important pathways and be repurposed for new indications. The challenge with this approach is to

develop strategies to detect pairwise combinations in the complex and large set of active available molecules (Gonzalez-Angulo et al. 2010; Lee 2012)

Another approach is the *in vivo* settings which are utilized for drug repositioning. The repositioning in animal settings is usually performed in post-approval clinical studies, pre-marketing clinical studies or in preclinical animal models (Saporito et al. 2012). One example is gabapentin which was originally developed to treat epilepsy. The drug was approved for the initial indication but was later found to relieve pain and anxiety in rodent models and was later approved for treatment of neuralgia (Singh et al. 1996). The advantage of compound screening in phenotypic animal models is the broad target screening. It is possible to identify compounds that interact with different physiological and cellular pathways that could not be studied in cell or *in vitro* assays. For example, diabetes is treated with medicines that interact with multiple mechanisms (Saporito et al. 2012).

Since drug repurposing became a more common strategy in drug development, academic researchers have performed high-throughput screens of small molecules. The results of the screenings are nowadays available in public databases (Hergenrother 2006). PubChem administered by the NIH has the largest collection of data obtained by biochemical and phenotypic screens (Wang, Y. et al. 2009). One approach to repurpose and screen lead candidates is *in silico* research. The expensive lab work and clinical trials can be reduced, and drug candidates can be screened more effectively by the utilization of computer programs instead of traditional assays in the lab. Researchers need strategies and tools to mine the resources to systematically identify drug candidates for drug repurposing (Liu, Z. et al. 2013). Mining phenotypic screens seem to be a promising strategy because it makes it possible to identify compounds that work on any target involved in the disease pathogenesis. It is possible to mine thousands of screens in a short period of time. Some compounds need to be experimentally tested but the overall resources that are needed for experiments are reduced. Mining HTS screens is challenging and sometimes sophisticated algorithms are required to incorporate the data. However, careful mining can lead to a discovery of compounds that are suitable for repositioning (Swamidass 2011).

2.6.3 Drug repositioning in antimicrobial drug discovery

There is no selective anti-biofilm drug that has yet been approved. Microorganisms are difficult to treat with available antibiotics and there are an increasing number of dangerous highly resistant bacteria. The introductions of new innovative and effective antibiotics have decreased drastically during the last decades, which makes drug repositioning a new approach also in antimicrobial research (Cassetta et al. 2014). However, there are some limitations in anti-infectious drug repurposing. Firstly, there are many hit compounds in cell-based antibiotic screenings although the targets and the *in vivo* activities and toxicities are relatively unknown. Second, existing chemical genomics databases do not necessarily collect targets of pathogens, which make computational methods for drug repurposing challenging (Ng et al. 2014).

Despite the challenges, attention has recently been focused on repurposing already approved non-antimicrobial drugs as antibacterials. The antiarthritic drug auranofin, for example, has shown bactericidal activity against resistant strains of *S. aureus.* The drug has the advantage of being administered orally, which makes it a promising agent that could be repurposed for the treatment of resistant bacterial infections caused by *S. aureus* (Cassetta et al. 2014). Another example is gallium that is a semi-metallic element that has been used in the treatment of various diseases such as autoimmune diseases and cancers. The compound has the potential to serve as an iron analog and will work as an anti-microbial (Kelson et al. 2013). Gallium seems to also prevent biofilm formation and it is effective against resistant cells that are in the stationary growth phase (Kaneko et al. 2007). There are also examples of antibiotics that have not originally been developed for one particular disease but are repurposed for other infectious diseases. One good success story is fluoroquinolones that have been repurposed to treat multidrug resistant tuberculosis (Palomino and Martin 2013). Linezolid is a compound that also has shown undeniable effect on tuberculosis bacteria but the use is limited due to severe adverse effects such as anemia (Park, I. N. et al. 2006).

The battle between pathogenic micro-organisms and us will continue in the future. The use of repurposed drugs for antibacterial treatment will also be a challenge because the pathogens will continue to change over time. The use of repurposed antimicrobials must further follow strict criteria in order to minimize drug resistance (Palomino and Martin 2013).

3 AIMS

The antibiotics on the market today are not effective enough against *S. aureus* biofilm related infections. It is a challenge for healthcare to treat the infections and they cause inevitable mortality in patients. Biofilm related infections and an increasing drug resistance possess a serious health problem in society and the need for new effective anti-biofilm compounds is therefore enormous. It is also essential to study the anti-biofilm effect of available drugs in order to find new applications for existing substances.

In order to meet these challenges, the specific aims of this work are:

1) to identify natural compounds with anti-biofilm properties, acting on biofilms formed by *Staphyloccus aureus*
2) to select the most promising compounds, based not only on their anti-biofilm activities but also on their selectivity profiles and possible *off*-target effects (based on literature searches)
3) to characterize the best anti-biofilm leads and perform follow-up studies (among others: potency, efficacy)

4 MATERIAL AND METHODS

4.1 Chemical reagents and materials

Tryptic soy broth (TSB), tryptic soy agar (TSA), dimethyl sulphoxide (DMSO) and resazurin sodium salt were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Phosphate buffer saline (PBS) was obtained from Lonza (Verviers, Belgium) and the water used was from a Milli-Q synthesis system (Millipore corporation, US). Penicillin G potassium salt was purchased from Fluka Biochemika (Buchs, Switzerland). Polystyrene 96-well microplates (NunclonTM Δ Surface) were purchased from Nunc (Roskilde, Denmark) and Thermo-Fast 96 Skirted PCR Plates from Thermo Fisher Scientific Oy (Vantaa, Finland). Varioskan Flash Multimode Plate Reader, operated with SkanIt RE for Varioskan Flash 2.4.3 software, was from Thermo Fisher Scientific Oy (Vantaa, Finland).

4.2 Compound library

The commercially available Enzo Product Library consisting of 502 natural and naturally derived compounds was obtained from Enzo Life Sciences Inc., USA. The Enzo library was prepared in DMSO at a concentration of 2 mg/ml. The average molar concentration on the Enzo pre-plated library was 6 mM, ranging from 1 to 19 mM. The library plates were stored at -70 °C and thawed in a water bath at 37 °C. To facilitate the screening process the compounds were diluted to approximately 2 mM stock solutions in DMSO (ranging from 0.47 to 6.53 mM). Stock solutions were kept at +4 °C in sealed Thermo-Fast 96 Skirted PCR Plates for not more than 48 h before screening.

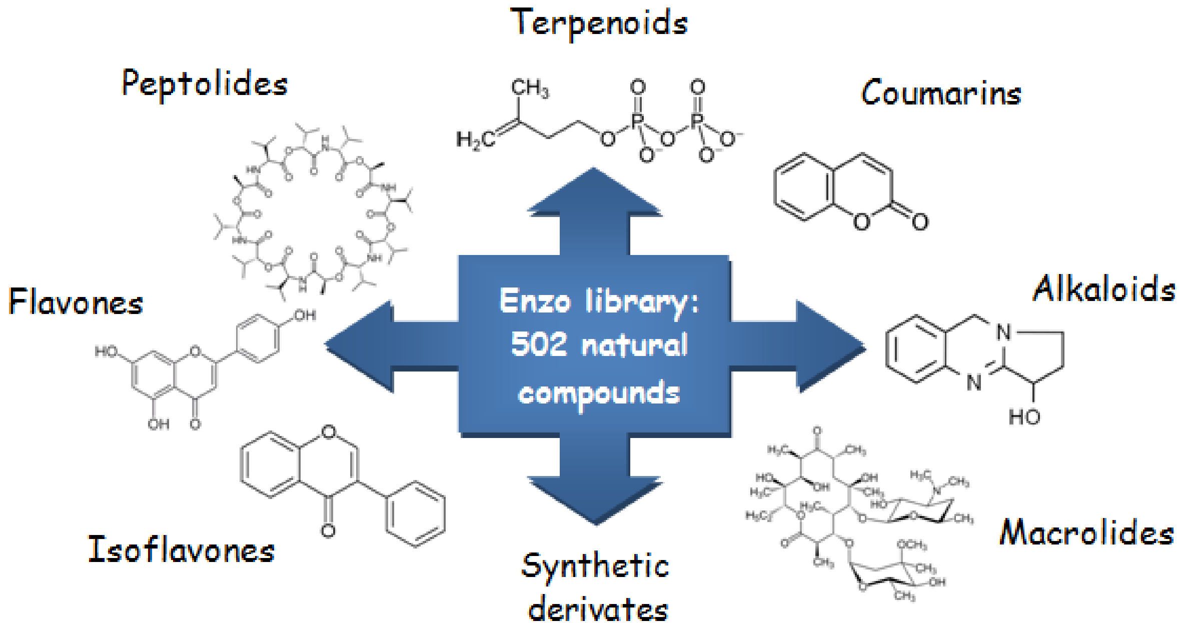


Figure 8. The Enzo library consists of 502 natural compounds including compounds from several classes of products with representative examples shown here.

4.3 Bacterial strains, bacterial culture and biofilm formation conditions

Bacterial strains *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus Newman* were obtained from the HAMBI cultures collection of the University of Helsinki, Finland (http://www.helsinki.fi/hambi/index.html). Both strains were stored in tryptic soy broth (TSB, 30 g/L) containing 20% glycerol at -70 °C. Fresh cultures were prepared on TSA plates from the glycerol stocks. Bacteria were pre-cultured to reach optimal culturing conditions. Colonies were scraped off the TSA plate with a 1 µl inoculation loop in 3 ml of TSB. The pre-culture was incubated at 37 °C, 220 rpm, overnight. 10 µl of the pre-culture was diluted in 10 ml of TSB and incubated at 37 °C, 200 rpm under aerobic conditions approximately 4 h to reach exponential growth. The concentration was estimated by spectrophotometric turbidity measurements at 595 nm using a Varioskan Multimode Plate Reader. A ten-fold serial dilution from the bacterial suspension was prepared (10^{-4} to 10^{-7}) and 5 x 10 µl from each dilution was plated on ¼ of a TSA agar plate using the drop-plate method. The plates were incubated at 37 °C for 24 h and the number of colonies was counted to determine the concentration of bacteria. Biofilms were grown in sterile polystyrene

flat bottom 96-well microplates (NunclonTM Δ Surface). Biofilms were formed from fresh cultures. The exponentially grown bacterial culture was diluted to approximately 10^6 CFU/ml in TSB. Every well was filled with 200 µl of bacterial suspension and non-inoculated TSB was used as control. The plates were incubated at 37 °C, 200 rpm shaking for 18 h.

4.4 Biofilm viability quantification based on the resazurin staining assay

Resazurin (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide) is a blue in itself non-fluorescent dye. Resazurin is reduced by metabolically active cells to resorufin (**Figure 9**), which is highly fluorescent (Guerin et al. 2001). Biofilms formed in the pre-exposure and post-exposure assays were quantified using resazurin staining. The planktonic bacteria were gently removed from the wells after incubation with the compounds. The biofilm was incubated in room temperature and darkness, 200 rpm shaking for 20 min with 200 µl of a 5% solution of resazurin in phosphate buffer saline (PBS) according to Sandberg et al. (2009). The amount of resorufin was detected by measuring the resorufin fluorescence at λ_{ex} 570nm – λ_{em} 590nm with a Varioskan multimode plate reader (Thermo Fisher Scientific, USA) in order to quantify the viable biomass.

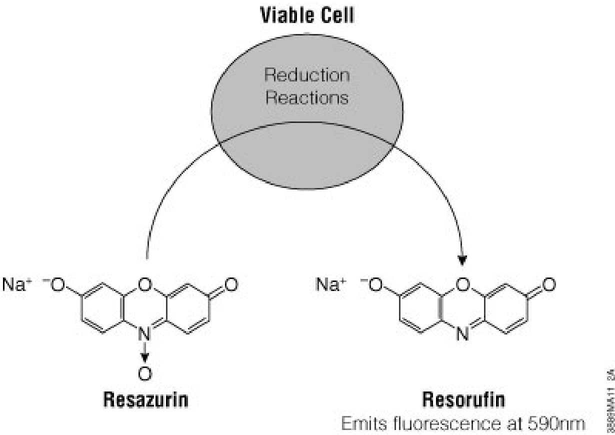


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Figure 9. Conversion of resazurin to resorufin by viable cells results in a fluorescent product. The fluorescence produced is proportional to the number of viable cells (Promega Corporation 2008).

4.5 Exposure to compounds

The screening rationale utilized in this project was based on the strategy previously developed in the laboratory that consists of three phenotypic assays that allow the identification of biofilm inhibitors from natural sources (Skogman et al. 2012). The assays have three different endpoints that quantify: viable biomass with resazurin, total biomass with crystal violet and the poly-N-acetylglucosamine component of the biofilm matrix with wheat germ agglutinin (WGA) staining. Assays are performed in two modes: prior-to-biofilm formation (also referred to as "prevention") and post-biofilm formation tests (also referred to as "destruction"). Given the convenience and higher throughput of the viability staining method (resazurin-based), in this thesis it was selected as the only assay that was applied during the primary screening. Both assay modes were utilized (**Figure 10**).

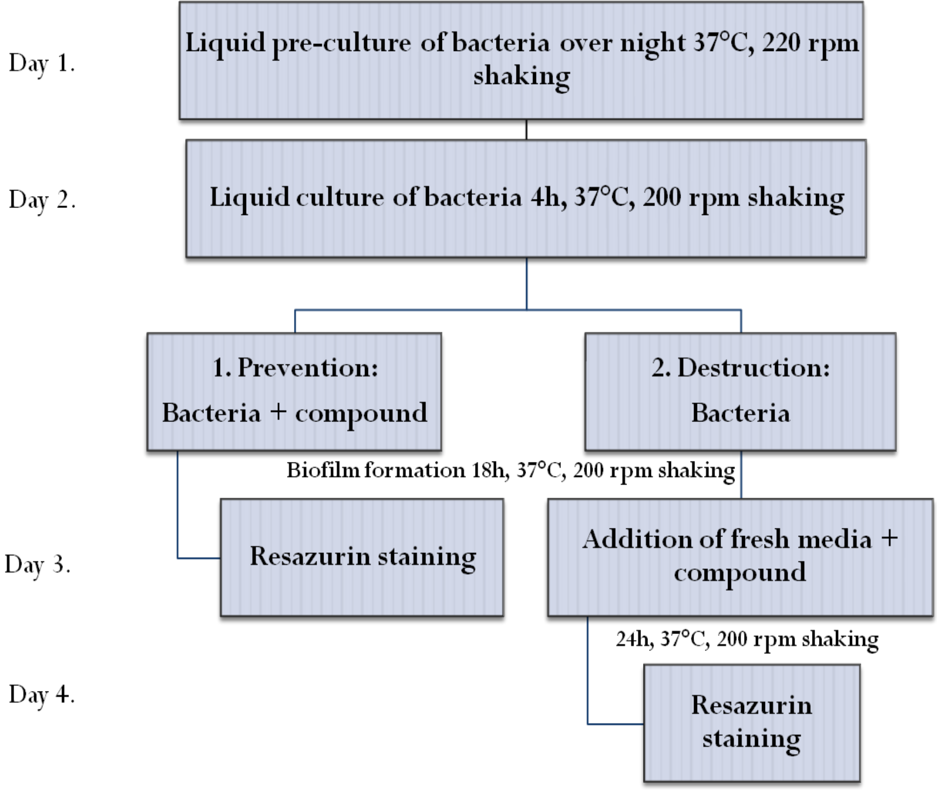


Figure 10. Schematic representation of the workflow for the characterization of anti-biofilm compounds in micro-well plates. Modified from (Skogman et al. 2012)

The natural compounds were tested against *S.aureus* ATCC 25923 and *S.aureus* Newman. Both strains were used in order to see if the activities of the compounds were strain-specific. In the prevention study the compounds were added simultaneously with the bacteria prior-to biofilm formation. The prevention plates were incubated at 37 °C, 200 rpm shaking for 18 h. In the second mode of the assay compounds were added to 18 h old biofilms in order to discover the activity on already formed biofilm. The planktonic bacteria were gently removed and the biofilms were treated with compounds and fresh TSB. The destruction plates were further incubated for 37 °C, 200 rpm shaking for 24 h. Separate control plates for both assay modes were prepared in addition to the experiment plates. As bacterial controls, 200 µl of bacterial suspension was added to the wells (untreated biofilms) while wells containing only sterile TSB were used as negative controls. Penicillin G is an antibiotic with known effects on *S. aureus* biofilms and was used as a positive control at 400 µM in all test runs. The average compound concentration during the primary screening was 40 µM (ranging from 9.4 µM to 130.6 µM) on the reaction plates.

4.6 Hit selection process

Hit compounds from the primary screening were taken further based on careful consideration of the inhibition percentage in prior- and post-exposure tests and based on literature search. Criteria for further selection included confirmed bioactivity over the hit limit, in prior-to- and/or post-exposure tests in at least one of the tested strains, along with sustained evidence (from the literature) of low cytotoxicity in mammalian cells. Other information gathered on the reconfirmed compounds (for instance previous reports of antimicrobial effects) was also weighted for the selection of the most promising actives. The literature search was performed by gathering information about the compounds from PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and PubChem (https://pubchem.ncbi.nlm.nih.gov/).

Furthermore, the activity of eight selected compounds was confirmed in an additional screening with four replicates of each compound at the same concentration (40 µM) as in the initial test. The

compounds were tested against both *Staphylococcus* spp. strains. Only four compounds showed activity in the reconfirmation test and were therefore taken further for the follow-up studies.

4.7 Follow-up studies with the selected compounds

4.7.1 Anti-biofilm potency tests

The anti-biofilm selectivity of four hit compounds was established by anti-biofilm potency tests. 11-19 different concentrations of each compound were tested for anti-biofilm effect in both exposure schemes. The compounds were tested within a wide concentration range of 0.001µM - 400 µM, spanning over 5 log units. A concentration-response curve was established for each selected hit compound based on the potency tests. The potencies, measured by half-inhibitory concentrations (IC_{50}) of the anti-biofilm effects, were calculated from at least 10 concentration points via a non-linear regression analysis, as described in section (4.8).

4.7.2 Bacteriostatic and bactericidal effect on planktonic cells

The antibacterial effects of the four most active compounds were tested on planktonic bacteria. The compounds were tested within a concentration range of 0.001 µM - 80 µM, covering 4 log units using 13-14 different concentrations of each compound. First the minimal inhibitory concentrations (MIC values) were estimated. The minimal inhibitory concentration is defined as the lowest concentration of the compound that will inhibit the visible growth of bacteria. Biofilms were formed in 96-well plates from fresh cultures, as described earlier, and compounds were added simultaneously with the bacteria as in the prevention test. The plates were incubated in same conditions as the plates in prevention test (37 °C, 200 rpm shaking for 18 h). The bacterial suspension was removed from each well and transferred to new sterile 96-microtiter well plates. The optical density of the bacterial suspension was measured at λ= 620 nm with Varioskan plate reader (Fallarero et al. 2013). The minimal bactericidal concentrations (MBC values) were also measured. The minimal bactericidal concentration is the lowest concentration of the compound that

will kill most of the viable bacteria. A 400 µM solution of resazurin in PBS was added to the suspensions in order to reach a final concentration of 20 µM in the reaction wells. Plates were incubated in darkness, 200 rpm shaking in room temperature for 4-5 minutes. The estimation of viable cells was determined by measuring the amount of formed resorufin using Varioskan Flash Multimode Plate Reader at $\lambda_{exc} = 570$ nm; $\lambda_{em} = 590$ nm.

4.8 Data processing and statistical analysis

At least four replicates were included in each well plate and at least two biological replicates were always performed. Positive (bacteria, maximal signal) and negative controls (TSB, minimal signal) were included in order to define the inhibition percentages of the compounds and to evaluate the performance and repeatability of the screening assay. Maximal signal (μ_{max}), and minimal signal (μ_{min}) represent the means of the reduced resazurin in controls. The anti-biofilm and antibacterial effect of each compound was calculated using the formula below.

$$\text{Inhibition } \% = [(\mu_{max} - \mu_{treated\ well})/(\mu_{max} - \mu_{min})] \times 100\%$$

The plate-to-plate variability was determined by comparing the means of maximal signals of the plates run in primary screening. Coefficients of variation (CV) of the maximal signals were calculated. Statistical parameters as signal window coefficient Z'-factor, signal-to-noise (S/N), signal-to-background (S/B), separation band and CV of the signals were calculated according to equations below. SD_{max} and X_{max} represent the standard deviation and the mean of the maximal signal and SD_{min} and X_{min} correspond to the standard deviation and mean of the minimal signal. The hit limits (HL) were established according to equation

$HL = X_{max} - 3 * SD_{max}$.

Signal window coefficient: $$Z' = 1 - \left[\frac{3 * SD_{max} + 3 * SD_{min}}{|X_{max} - X_{min}|}\right]$$

Signal-to-noise (S/N): $$S/N = \frac{X_{max} - X_{min}}{\sqrt{{SD_{max}}^2 + {SD_{min}}^2}}$$

Signal-to-background (S/B): $$S/B = \frac{X_{max}}{X_{min}}$$

$$\text{Separation band} = |X_{max} - X_{min}| - (3 * SD_{max} + 3 * SD_{min})$$

Coefficient of variation (CV): $$CV = 100 * \left[\frac{SD_{max}}{|X_{max} - X_{min}|}\right]$$

Potencies of the anti-biofilm effects (IC_{50}) were calculated from at least 11 concentration points via a non-linear regression analysis (sigmoidal dose-response with variable slope) and the result is presented with 95% confidence intervals. For paired comparisons, unpaired t-test with Welch's correction was utilized. All data processing and statistical analysis was done with Microsoft Excel 2007 software and GraphPad Prism for Mac, GraphPad Software

5 RESULTS AND DISCUSSION

5.1 Assay performance

The reliability of the measured biological activities depends on the robustness of the assay. The measured results should not be affected by minor perturbations in laboratory conditions. Robustness implies also reproducibility of the assay performance on a day-to-day and plate-to-plate basis (Inglese et al. 2007).

Several statistical parameters were used in order to evaluate assay performance. Z’ -factor, coefficient of variation (CV_A), as well as S/N and S/B ratios were calculated according to Zhang et al. (1999) for all the assays performed during the primary screening (**Table 2**). The separation band between minimal and maximal signals was sufficiently large and Z’ was higher than 0.45 in all cases, which is a potent indicator of a well-performing cell-based assay.

Table 2. Statistical parameters from the primary screening

Bacterial strain	Mode of assay	Z' factor	S/N	S/B
S. aureus Newman	Prior-to exposure	0.51±0.08	6.22±0.89	15.38±3.75
	Post-exposure	0.62±0.14	8.53±2.10	22.82±3.05
S. aureus ATCC 25923	Prior-to exposure	0.54±0.12	6.98±1.77	16.11±2.28
	Post-exposure	0.47±0.11	6.10±1.81	15.33±1.86

The variations of the statistical parameters of the assay over plates were also plotted in order to ensure a high screening quality and to detect any possible systematic trends. The plate-to-plate variations of Z’ factor as well as S/N and S/B are presented in **Figure 11**. The assays performed well and no negatively affecting trends could be detected. The lowest Z’ value obtained on one individual plate was around 0.4 which still could be considered as an acceptable value in cell-based assays. For *S. aureus* Newman the average Z’ value was 0.51±0.08 in prior-to exposure test and 0.62±0.14 in post-exposure test. For *S. aureus* ATCC 25923 the average Z’ value in prior-to exposure test was 0.54±0.12 and 0.47±0.11 in post-exposure test. No consistent trend in the behavior of the Z’ factor could be detected according to plate numbers. S/N was in average > 6.10 ± 1.8 and S/B > 15.3 ± 1.9 which are

also acceptable values according to (Zhang et al. 1999). The resazurin assay has previously shown to be a fast, simple and high-quality assay according to Sandberg et al. (2009). Some plate-to-plate variability could be detected but the well-to-well, plate-to-plate and day-to-day variability of the control signals in the assay were in all cases < 13%, while the average calculated *Z'*-factor value, S/N and S/B ratios were 0.66; 9.41 and 10.56, respectively.

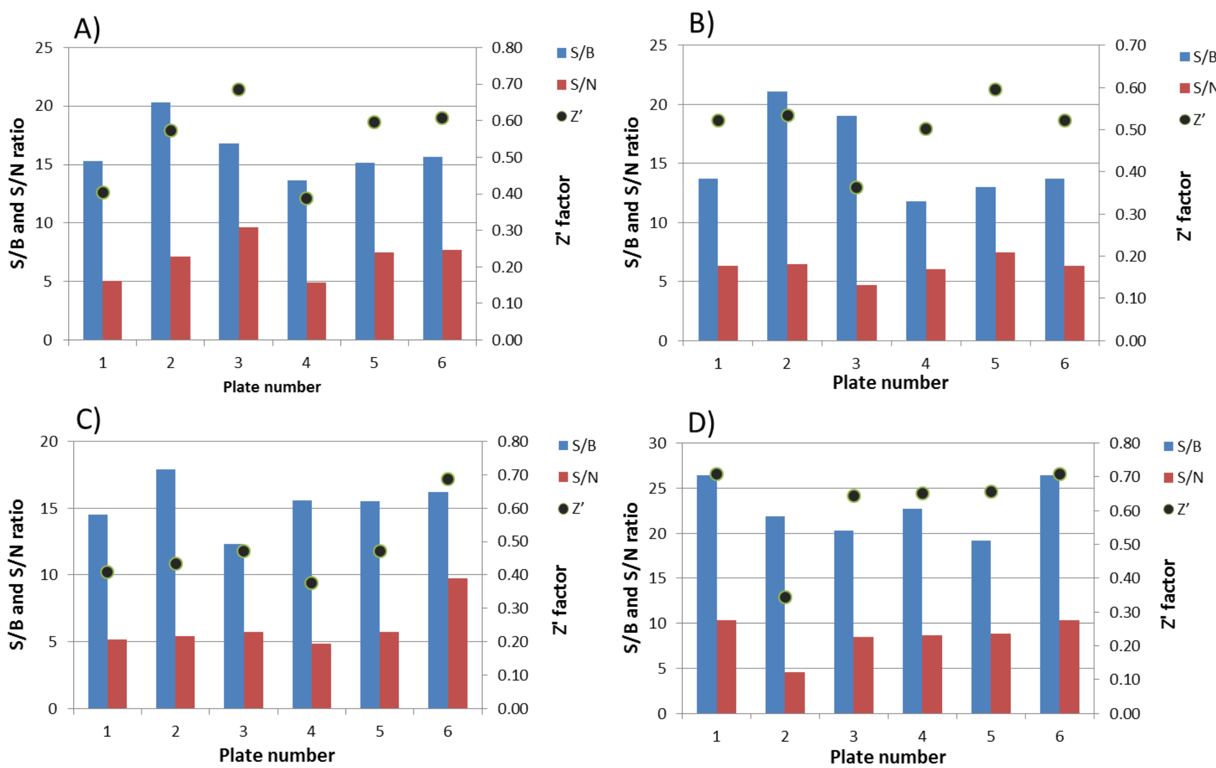


Figure 11. Plate-to-plate variations of statistical parameters during the primary screening: A) prior-to exposure, *S. aureus* ATCC 25923; B) prior-to exposure, *S. aureus* Newman; C) post-exposure, *S. aureus* ATCC 25923; D) post-exposure, *S. aureus* Newman.

The coefficient of variation (CV_A) which is another useful assay performance measure was further calculated according to (Iversen et al. 2006). The advantage of CV_A is that the minimal signal SD does not have to be estimated which means that fewer minimal controls are needed in the assay. A low CV_A value tells us that the assay performed well. Also, the values can be compared to the Z' factor because their statistical properties are similar. Among the 24 tested plates, only three plates gave CV_A values >20% which is not ideal but could be considered as acceptable for cell-based assays (**Table 3**).

Table 3. The coefficient of variation of the assay (CV_A) was calculated for each plate. Strains 1 and 2 correspond to *S. aureus* ATCC 25923 and *S. aureus* Newman, respectively.

	CV_A (%)			
	Prior-to-exposure		Post-exposure	
Plate	Strain 1	Strain 2	Strain 1	Strain 2
1	16	20	10	19
2	16	14	22	19
3	21	10	12	18
4	17	20	12	21
5	13	13	11	18
6	16	13	10	10

As indicated above, there are several advantages with the resazurin assay. Firstly, the method is faster and simpler than the widely used crystal violet staining. The crystal violet method requires more steps, it is more laborious and therefore not as automation friendly (Sandberg et al. 2009). *S. aureus* reduces resazurin to resorufin faster than other bacterial organisms. The incubation period with resazurin is optimized to be 20 minutes for *S. aureus* strains. The resazurin assay is accurate and low standard deviations indicate good assay repeatability and therefore it can be utilized in fast discrimination of promising hits in a large chemical library (Peeters et al. 2008).

Another advantage with the resazurin assay is that it allows differentiating between dead and living cells. It is essential to determine the number of viable cells when testing how susceptible biofilms are to compounds. Resazurin was also the best choice for biofilm quantification when six surrogate assays were compared by Peeters et al. (2008). Additionally, resazurin can be assumed to be more accurate than the measurement of bacterial turbidity alone. Guerin et al. (2001) showed that microorganisms quantified in different environmental samples by turbidity was around 50-70% of the number quantified with resazurin. Furthermore, another dye, methylene blue, did not undergo a reliable color change in the presence of growing contaminant degraders, which indicates that resazurin should be used in the most probable number (MPN) studies instead of turbidity alone or other dyes.

Of note, resazurin can react with organic chemicals that are used in cell-based assays. The disadvantage using the method is that the result could be misleading due to false positives. Guerin et al. (2001) incubated resazurin with 24 different organic chemicals in order to examine the color change of the dye. Out of the tested compounds only four compounds (1,2-dichlorobenzene, 2,4-D,

glycol sulphite and sulphinol) generated false positives. There was no clear correlation between these compounds though. None of these interfering agents are present in the studies performed in this investigation.

Finally, resazurin reduction is both species and strain related (Pantanella et al. 2013). The disadvantage with the resazurin assay is that different bacteria can metabolize differently the probe which means that it is critical that the incubation time is optimized for each bacterial strain (Sandberg et al. 2009). The bacterial concentration will also affect the detection of fluorescent signals. Significant fluorescence signals were detected by Sandberg et al. (2009) when the bacterial concentration of *S. aureus* was higher than $5x10^7$ CFU/ml. This is rarely a problem in the case of biofilm formation in 96-well plates because the bacterial concentrations are high, well over 10^8 CFU/ml.

5.2 The selection process

Screening of known compounds is important for simplifying and speeding up drug discovery. Developing a completely new drug takes tremendous amount of time, money and effort because of the long therapeutic development process. Detailed information such as pharmacology, formulation and potential toxicity is already available for compounds that have previously been tested on humans and are in many cases also in clinical use. By repurposing drugs, new candidate therapies could be ready for clinical trials much sooner than by developing completely novel compounds (https://ncats.nih.gov/preclinical/repurpose).

Drugs typically interfere with biological systems resulting, among others, in phenotypic effects on the human body. The phenotypic effects could be either expected effects, or non-expected side effects. The side effects are usually undesired and are caused by drug interactions with off-targets. However, the off-target effects could also lead to new therapeutic indications and are valuable information in drug repurposing (Iwata et al. 2015). Potential antibiofilm compounds are not only those used as drugs. Compounds already utilized as preservation agents in cosmetics or in food-industry could exhibit activity against *S. aureus* biofilms (Ooi et al. 2015). In terms of polypharmacology, a lot of substances on the market have potential for new indications. The success rate of drug development could be increased by new drug repurposing approaches (Iwata et al. 2015).

In this thesis, the exploration of the natural library consisting of 502 natural compounds was a multistage repurposing process that was divided into 3 stages, as schematically represented below in **Figure 12**. The workflow applied here was adapted from Manner et al. (2013). Primary screening of the compounds performed at an average concentration of 40 µM resulted in 20 active hit compounds. The hit compounds inhibited biofilm formation over the activity threshold described in section 4.6, in both pre- and/or post-exposure tests in at least one of the tested strains. The second stage (the actives-to-hits selection) was conducted based on a literature search of all these 20 compounds. The search focused on: compounds that were non-toxic and novel in biofilm research. In the third step of the selection process, the eight most selective compounds were tested again in the same conditions as in the primary screening for a confirmatory follow-up. Four compounds showed activity in the reconfirmation test and were taken further to follow-up studies. The whole selection process with the three stages is schematically represented in **Figure 12** and discussed in detail in the following chapters.

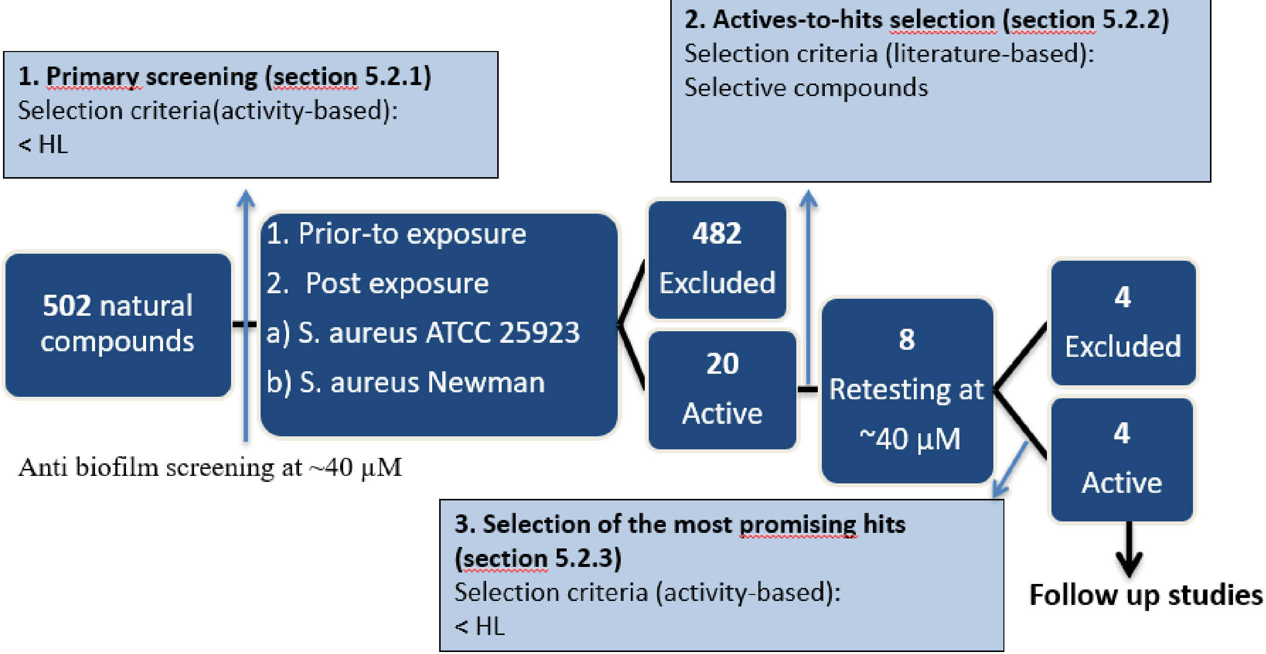


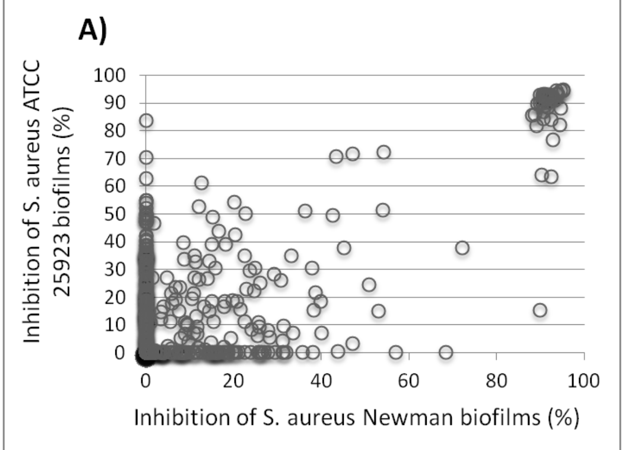
Figure 12. The whole selection process with the three critical stages. HL= hit limit.

5.2.1 Primary screening

The characteristics of this screening were different compared to the other steps of screening because the concentrations on Enzo pre-plated compounds ranged from 1 to 19 mM, as mentioned earlier. The different concentrations made the analysis of the results from the primary screening more challenging. Some compounds were active according to the hit limit criteria but still needed to be compared to each other in order to see which ones were more potent.

The activity of the natural compounds was tested against *S. aureus* ATCC 25923 and Newman in both assay modes to avoid only strain-specific hits. The bioactivity of the library is shown **in Figure 13** and the raw data obtained from the screening is included in **Supplementary table 1**.

When both bacterial strains were considered, the most active compounds are those that are located in the upper right in **Figure 13 A)** and **B)**. The compounds that showed strain-specific activity are located on the X-axis (activity on *S. aureus* Newman) or on the Y-axis (activity on *S. aureus* ATCC 25923). At least 380 of the compounds did not show more activity than 20-30% inhibition. In **Figure 13 A)** the compounds were added prior to biofilm formation and one can see that many compounds are in the upper right corner. **Figure 13 B)** shows the results of the compounds added post-biofilm formation and only a few compounds showed activity on both strains and the inhibition percentages were significantly lower than in the prior-to-exposure test. The results were expected because it is known to be considerably easier to prevent biofilm formation rather than eradicate already formed biofilm (Toté et al. 2009).



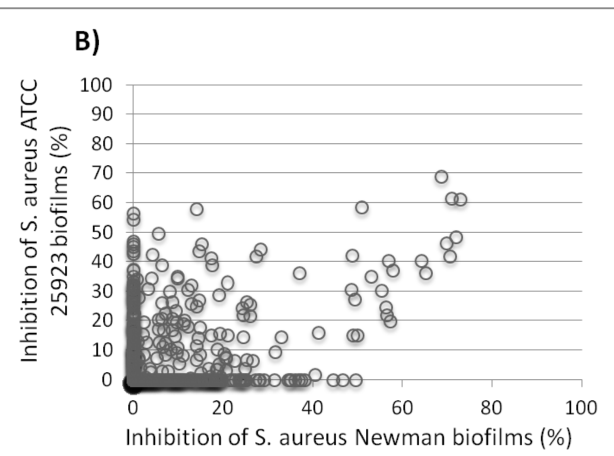


Figure 13. Inhibitory activity of the entire Enzo library represented as a correlation plot. Compounds added prior-to (A) and post (B) biofilm formation in the primary screening.

Several tested compounds showed anti-biofilm characteristics in prior-to biofilm formation mode of the assay (39 compounds caused more than 80% inhibition). However, a smaller number of compounds (20) inhibited biofilm formation over the activity threshold in both prior-to and/or post-exposure tests in at least one of the tested strains. These 20 compounds that were considered as active in primary screening were divided into two groups. The first group of active compounds consisted of eleven compounds that showed activity in only post-biofilm formation test, which was an interesting finding. The second group included nine compounds that were found active in both pre- and post-exposure modes. The whole selection process from 20 active compounds to four most interesting compounds is summarized in **Figure 14**. The list of the 20 initially found actives is summarized in **Table 4**.

The compounds that showed activity in only post exposure test (group 1) were always active on only one of the strains. Among these compounds, two compounds (E215 and E252) were active on *S. aureus* ATCC 25923, and the rest of the 11 compounds were active on *S. aureus* Newman.

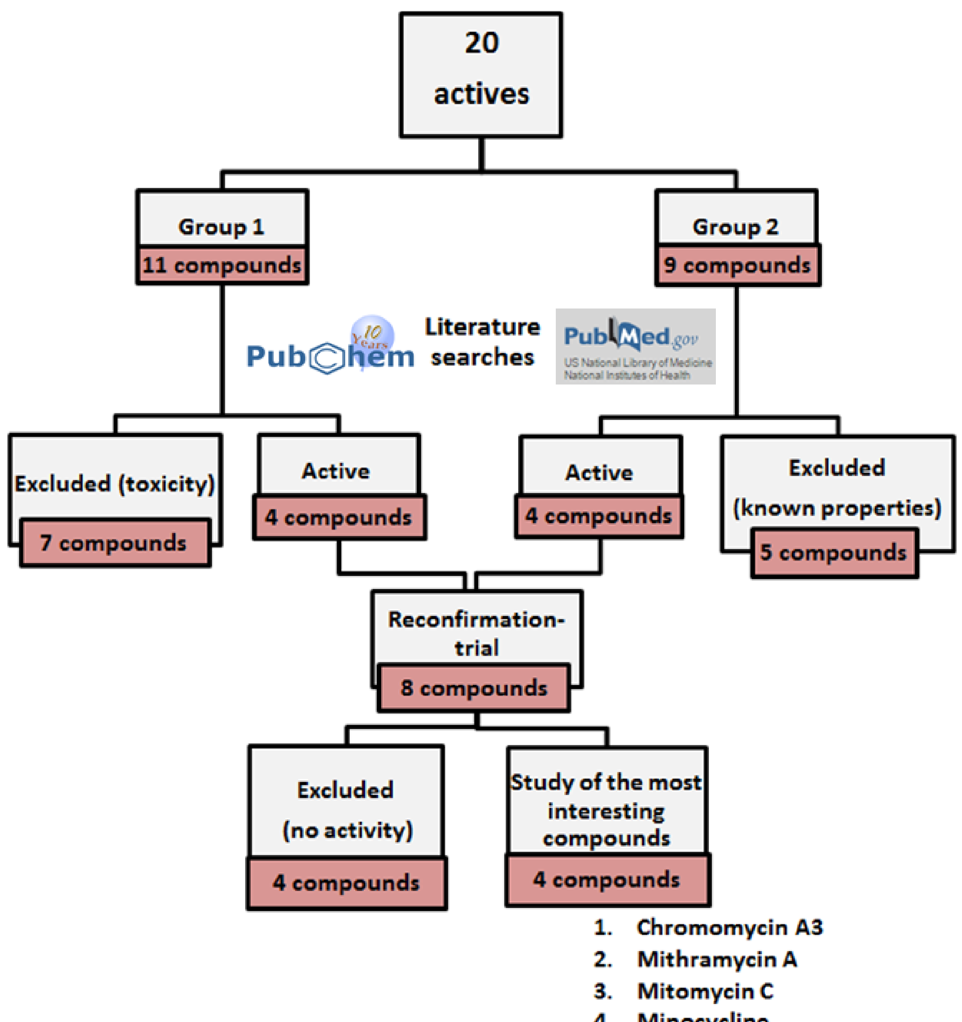


Figure 14. A schematic illustration of the selection process after primary screening. From 20 active hits, 11 compounds (group 1) were active in only post-exposure test and 9 compounds (group 2) showed activity in both pre- and post-exposure tests.

5.2.2 Actives-to-hits selection

The 20 compounds that showed bioactivity according to the hit limit criteria in the primary screening (**Table 4**) were further studied by means of literature searches. Searches were performed in PubChem (www.pubchem.com) and PubMed (www.pubmed.com). PubChem was released in 2004 and is a part of the United States NIH. PubChem is maintained by the National Center for Biotechnology Information (NCBI) and it is a database of chemical molecules and their activities in biological assays. Databases include pure characterized chemical compounds, substances, extracts and bioactivity results from high-throughput screening programs. A broad range of properties such as chemical structure, chemical formula and molecular weight can be searched for in the databases (https://pubchem.ncbi.nlm.nih.gov/sources/sources.cgi;

https://pubchem.ncbi.nlm.nih.gov/about.html). PubMed is another database maintained by the NIH as a part of the Entrez system of information retrieval. PubMed was first released in 1997 for free, home- and office-based MEDLINE searching. Only journals that meet PubMed's scientific standards are included (Lindberg 2000).

The purpose of the literature search was to study the data that have been reported of these known drugs and their activity in different assays. Some compounds have shown activity for example against microorganisms and others have been active against different cancer cell lines. Potential compounds were also the active ones that have not previously shown toxicity against mammalian cells. Some of the compounds are already used as drugs for other indications than bacterial infections, for example cancer, which enables potential drug repurposing. The safety of these compounds is better known than that of completely new compounds and the risk of failure due to adverse toxicology is reduced (Wang, M. et al. 2014). The databases were searched for information about each of the 20 compounds (**Table 4**) that showed activity in primary screening. The compounds will be discussed later in this chapter.

Table 4. Bioactivities of the 20 initially found hits from primary screening.

			Activity (% inhib)			
		Tested at	S. Newman		S. ATCC	
Compound (group 1)	**Code**	**(µM)**	**Pre**	**Post**	**Pre**	**Post**
Embelin	E53	45	0	0	0	37
Apigenin	E141	49	0	0	0	45
Norfluorocurarine	E215	45	0	56	0	0
Strophanthidin acetate	E292	29	0	57	0	0
Deoxyshikonin	E310	49	0	0	0	40
Tetrahydropapaverine	E345	35	0	0	0	57
Kinetin	E359	62	0	0	0	41
Vesicine	E371	71	0	0	0	37
Tropine	E391	94	0	0	0	38
Bis demethoxycurcumin	E485	43	0	0	0	36
Geraldol	E496	44	0	0	0	31
Compound (group 2)	**Code**	**(µM)**	**Pre**	**Post**	**Pre**	**Post**
Chromomycin A3	E25	11	92	72	92	52
Mithramycin A	E86	12	92	49	93	42
Nigericin Na	E91	18	91	57	89	40
Rifampicin	E117	16	94	73	94	61
Rifamycin SV-NA	E325	19	89	71	82	61
Echinomycin	E352	12	91	69	92	68
Salonomycin	E385	18	91	51	93	59
Mitomycin C	E414	40	90	70	93	46
Minocycline	E440	27	90	71	93	42

Firstly, the attention was focused on the first group of compounds that were active only in the post-biofilm formation test, as they could potentially be selective anti-biofilm molecules with minimal risk to cause resistance (**Table 5**). Out of eleven compounds, seven compounds were first excluded based on literature searches in PubChem and PubMed (**Figure 14**). Embelin, deoxyshikonin, bis-demethoxycurcumin and tetrahydropapaverine were discarded because toxicity has been reported in different types of cells. Embelin is a naturally occurring alkyl substituted hydroxyl benzoquinone compound that has shown antitumor activity in various types of cancers by inhibiting the activity of X-linked inhibitors of apoptosis protein (Lu, Jianqin et al. 2013). Deoxyshikonin belongs to the naphthoquinone group of compounds and has antitumor activity against a variety of cancer cells by triggering multiple cell death pathways. The mechanism for its anti-cancer properties remains unclear but Rajasekar et al. (2012) showed that shikonin derivates inhibit the growth of melanoma cells. Bis-demethoxycurcumin belongs to the curcumins that have been shown to be toxic to human A549, HepG2, and MDA-MB-231 cancer cell lines (Lin et al. 2012). Tetrahydropapaverine is a compound

that has shown to be toxic to the nigrostriatal system and was therefore excluded (Koshimura et al. 1997). Geraldol, tropin and vasicine were excluded because they were found as weaker hits with low inhibition percentages on existing biofilms (<50% at 44.4 – 94.4 µM) (**Table 4**).

Two of the remaining molecules were norfluorocurarine, which is an alkaloid, and kinetin, which is a cytokinin. These two compounds were taken further because they seemed to be non-toxic and novel in anti-bacterial research. Strophanthidin acetate was also taken further based on the relatively high activity at a relatively low concentration (57% inhibition at 29 µM). The last interesting compound that was taken further was apigenin that is a flavone. This compound has previously been reported by our laboratory as causing only 5.22% inhibition at 400 µM and was therefore deemed inactive by Manner et al. (2013). In this study, apigenin caused 45% inhibition at 49 µM and was within the established activity threshold. Because of the apparent contradiction between these results, we decided to perform a confirmation test to clarify the true activity. According to literature, an apigenin derivate (4a-4j) has showed some significant activity against several bacterial strains, including *S. aureus* (Liu, R. et al. 2013), but has not been reported as active in biofilm research.

Table 5. Compounds active in only post-exposure tests

Group 1 - active in only post exposure tests			
Compound	**Code**	**Structure**	**Selected for reconfirmation** (yes/no)
Embelin	E53		No
Apigenin	E141		Yes
Norfluorocurarine	E215		Yes
Strophanthidin acetate	E292		Yes

Deoxyshikonin	E310		No
Tetrahydropapaverine	E345		No
Kinetin	E359		Yes
Vasicine	E371		No
Tropine	E391		No
Bis demethoxycurcumin	E485		No
Geraldol	E496		No

The second group of interesting compounds included those that showed anti-biofilm properties in both assay modes and activity on both bacterial strains (**Table 6**). Based on the literature searches, five out of the nine compounds were immediately excluded (**Figure 14**) (rifampicin, rifamycin SV-NA, echinomycin, nigericin Na and salinomycin). Rifampicin, rifamycin SV-NA and echinomycin are known antibiotics in *S. aureus* biofilm studies. Rifampicin and rifamycin SV-NA belong to the rifamycins and it has been observed by Matos et al. (2014) that rifampicin at concentrations equal to or >4.86 µM reduced 50% of *S. aureus* biofilm biomass. Echinomycin has already shown a MIC at 0.03 µM against methicillin-resistant planktonic *S. aureus* (Socha et al. 2009). Park, Y. S. et al. (2008) found out that 50% effective doses of echinomycin were 7-fold lower than those of vancomycin against the same resistant *S. aureus*. Nigericin Na was excluded because of low inhibition percentage on existing biofilms (40% inhibition at 17.9 µM). Salinomycin is an antibiotic belonging to a large group of natural polyether ionophores. It is commercially used in the veterinary medicine as a cocciostatic agent (Antoszczak et al. 2014). This antibiotic has already shown antimicrobial activity against gram-positive bacteria, including *S. aureus* and was therefore not taken further. Even though these compounds were excluded and not selected forward in this work, these results were still very positive, as they correlate well with the previously existing reports, thus supporting the validity of the screening campaign.

Hence, the most promising compounds were the remaining four agents: chromomycin A3, mithramycin A, mitomycin C and minocycline. Chromomycin A3 and mithramycin A and mitomycin C are compounds that have been reported as toxic to human cancer cells but have not been tested on *S. aureus* biofilms, which indicates that this would represent a completely new indication. Minocycline was chosen based on its non-toxic to human cells and known antibacterial properties on *S. aureus* suspensions (Cunha 2013).

Table 6. Compounds active in both pre- and post-exposure tests

Group 2 - active in both pre- and post-exposure tests			
Compound	**Code**	**Structure**	**Selected for reconfirmation** (yes/no)
Chromomycin A3	E25		Yes
Mithramycin A	E86		Yes
Nigericin Na	E91		No
Rifampicin	E117		No
Rifamycin SV-NA	E325		No
Echinomycin	E352		No

Salinomycin	E385		No
Mitomycin C	E414		Yes
Minocycline	E440		Yes

5.2.3 Selection of the most promising hits

In the next step of the selection process, a reconfirmation test was performed with the eight most interesting compounds that were selected in the previous selection stage (5.2.2, compounds numbered E25, E86, E414, E440, E141, E215, E292 and E359). The compounds that had showed some activity in only post-exposure test, were found not active in the second trial, and were therefore excluded. Stromphanthidin (E292) was only <1% units under hit limit in primary screening, which indicated that the chance of being reconfirmed was not high. The three other compounds that were excluded (E141, E215 and E359) had only about a 50% chance of being reconfirmed as they were only ~8% units under hit limit in the primary screening.

One reason for the compounds that seemed to be active in primary screening and not in the second trial, could be that they acted as false positives. Natural compounds can aggregate in biochemical buffers, cause nonspecific inhibition and interfere with optical detection methods (Pohjala and Tammela 2012). Phenolic compounds have the tendency to form aggregates that can cause false positives on *in vitro* bioactivity assays. This could explain the falsely detected activity for compound embelin (E53) and apigenin (E141) in the primary screening. In addition, other false positives could likely be caused by pipetting mistakes. The samples were performed in singletons due to the high number of samples and chances existed of mechanical removing of the biofilms due to the pipetting. In the confirmation test, each sample was performed in four replicates which gave a more accurate result. The result of apigenin being inactive in the second trial corresponded well with the data obtained by Manner et al. (2013). Therefore, only four compounds were reconfirmed as active in the second trial and will be discussed further in this section.

The results of the inhibition of biofilm formation in primary screening and reconfirmation trial are presented in **Table 7**. Four compounds, chromomycin A3 (E25), mithramycin A (E86), mitomycin C (E414) and minocycline (E440), were reconfirmed as active.

Table 7. Reconfirmation trial of the most promising compounds according to the actives-to-hit selection process. Strains 1 and 2 correspond to *S. aureus* Newman and *S. aureus* ATCC 25923, respectively.

		Inhibition of biofilm formation (%)							
		Primary screening at 40 µM				Reconfirmation trial at 40 µM			
		Prior-to-exposure		Post-exposure		Prior-to-exposure		Post-exposure	
Code	**Common name**	**Strain 1**	**Strain 2**	**Strain 1**	**Strain 2**	**Strain 1**	**Strain 2**	**Strain 1**	**Strain 2**
E25	Chromomycin A3	92	92	72	52	91 ± 0.2	92 ± 0.4	82 ± 2.5	39 ± 21.4
E86	Mithramycin A	92	93	49	42	92 ± 0.2	92 ± 0.1	79 ± 4.6	67 ± 20.4
E414	Mitomycin C	90	93	70	46	91 ± 0.2	90 ± 0.8	72 ± 11.0	55 ± 17.5
E440	Minocycline	90	93	71	42	88 ± 0.5	91 ± 0.2	74 ± 4.0	83 ± 15.3
E141	Apigenin	0	0	45	0	0	0	0	0
E215	Norfluorocurarine	0	0	0	56	0	0	0	0
E292	Strophanthidin acetate	0	0	0	58	0	0	0	0
E359	Kinetin	0	0	41	0	0	0	0	0

Mithramycin A, chromomycin A3 and mitomycin C have not previously been reported as active in biofilm research. Both mithramycin A and chromomycin A3 belong to the aureolic acid family, and they are produced by various species of *Streptomyces*. Other examples of compounds in the aureolic acid family are olivomycins, produced by *Streptoverticillum cinnamoneum*; chromocyclomycin, produced by *S. atroolivaceus*; UCH9, produced by *Streptomyces sp*.; and durhamycin A produced by *Actinoplanes durhamensis* (Lombó et al. 2006). All these compounds are glycosylated aromatic polyketides with an intense yellow color, and they are fluorescent under UV light. In all members of the family, two oligosaccharide chains are bound to the aromatic polyketide moiety. The compounds of the aureolic acid family were originally isolated because of their activity against gram-positive bacteria but their main pharmacological interest today is antitumor activity (Lombó et al. 2006). Compounds of the aureolic family interact with the DNA, which will give the compounds significant antitumor activity (Ogawa et al. 1998). Because of permeability problems, compounds of the aureolic acid family (mithramycin A, chromomycin A3 and mitomycin C) are not active against gram-negative bacteria, which makes them more selective against *S. aureus* (Lombó et al. 2006; Waring 1981).

The first member of the aureolic acid family mithramycin was first described in 1959 and it is an anti-cancer compound, which has been used in the treatment of advanced testicular carcinoma. The clinical use is limited because of reported side effects, such as hepatoxicity. Mithramycin binds at the C-fos-depending Sp1 regulatory regions and it generates a global inhibition mechanism by preventing transcription, which could be related to its antibacterial activity (Fernández-Guizán et al. 2014; Lombó et al. 2006).

The structural similarity between chromomycin A3 and mithramycin A is high. The suggested mechanism of action is blocking the RNA synthesis which gives chromomycin antitumor acitivity. Chromomycin has like mithramycin been used in the treatment of some tumor diseases but the clinical use was also limited by side effects (Lu, Jiansheng et al. 2012). Antiviral activity has been described for chromomycin, that also inhibits the binding of the transcription factor Sp1 to its target sequences. The inhibition has shown to inactivate HIV-1 provirus and could probably be related to the mechanism in bacteria as well (Lombó et al. 2006).

Mitomycin C, produced by *Streptomyces caespitosus* and *Streptomyces lavendulaes*, is a naturally occurring compound consisting of a pyrrolo (1, 2-a) indole ring system with an aziridine ring. Mitomycin C is a highly cytotoxic DNA-reactive antibiotic. The US Food and Drug Administration (FDA) approved mitomycin C as an anticancer drug in 1974. The drug is widely used today in the treatment of different types of cancer (bladder, head and neck, cervical, gastric, pancreatic and colon cancers). The compound needs enzymatic activation to become a short-lived quinone that is biologically active and binds to DNA (Danshiitsoodol et al. 2006; Martin et al. 2002). The target in cancer cells can be connected to the antibacterial effect because mitomycin C is a DNA cross-linker compound. It has shown to be effective in killing bacterial cells with a single crosslink per genome (Tomasz 1995). The compound has not really been tested on *S. aureus* or other bacteria which makes it an interesting compound.

Finally, minocycline (7-dimethylamino-6-dimethyl-6-deoxytetracycline) is a second-generation, semi-synthetic tetracycline analogue. The compound is synthesized from natural tetracycline antibiotics and is a broad-spectrum tetracycline antibiotic. The antibiotic has been used for over 30 years for the treatment of infections caused by both gram-positive and gram-negative infections. Minocycline has been approved for the treatment of acne vulgaris and some sexually transmitted diseases (Garrido‐Mesa et al. 2013). Minocycline has been shown to be a promising compound in reducing colonization of *S. aureus* embedded in biofilm on catheter surfaces in an *in vitro* model

(Raad et al. 2003). Raad et al. (2007) further demonstrated that a combination of minocycline and rifampin eliminated MRSA colonization in biofilm for 10 tested bacteremic isolates. Biofilm-associated MRSA infections require long-term oral antibiotics, and the situation could be improved by using new combination therapies. A synergistic effect against MRSA when using a combination of fusidic acid and rifampicin has earlier been reported, although increasing antibiotic resistance requires new approaches. Wu et al. (2013) demonstrated that the effect of minocycline was enhanced when it was used in combination with either fusidic acid or linezolid against biofilm-embedded MRSA clinical isolates.

These four compounds that were chosen further for follow-up studies seemed to be highly active at low concentrations in both preventing bacterial biofilm and reducing already formed biofilm, which makes them good starting points for further research. Furthermore, the safety of these compounds is better known than completely new compounds, which also gives drug repurposing an advantage over traditional drug development.

5.3 Follow-up studies

Follow-up studies were performed in order to determine the antibacterial and anti-biofilm effect of the most promising compounds. The effects on planktonic bacteria, as well as anti-biofilm potencies for the most promising four compounds were determined.

5.3.1 Anti-biofilm potency tests

The anti-biofilm potencies for the four most promising compounds were established. All the antibiotics had the ability to prevent biofilm formation by both bacterial strains. In pre-to-exposure test, the potency (IC_{50}) values were in general lower for *S. aureus* Newman compared to *S. aureus* ATCC which indicated that Newman could be more susceptible to these compounds.

The tested compounds can be considered as highly active according to the low concentrations required to prevent and disrupt existing biofilms. Chromomycin and mithramycin had the highest potencies for inhibiting biofilm formation. The prior-to-exposure IC_{50} value for mithramycin A was 0.07 µM for *S. aureus* Newman and 0.15 µM for *S. aureus* ATCC 25923 compared to chromomycin

A3 which inhibited *S. aureus* Newman biofilm formation at 0.13 μM and *S. aureus* ATCC biofilm formation at 0.16 μM (**Table 8**). These two compounds inhibited biofilm formation more effectively than penicillin, which gave a potency (IC_{50}) value at 0.27 μM for *S. aureus* Newman. Potency measurements showed that effects on biofilm were obtained at only approximately 1.2 – 3.6 times greater concentrations compared to the concentrations needed to prevent biofilm formation. Usually, the concentration must be many folds higher in order to act on already formed biofilms, when compared to the pre-exposure tests (Toté et al. 2009). The chemotolerance of these biofilms to conventional antibiotics is well exemplified by penicillin G, a potent antimicrobial with low IC_{50} values against planktonic cells, but poor activity on existing biofilms. In fact, penicillin G at 400 μM concentration caused only 73% inhibition of the formed biofilms. This result correlates well with the poor activity detected earlier for penicillin G against *S. aureus* biofilms (Fallarero et al. 2013; Manner et al. 2013; Skogman et al. 2012). All four compounds gave post-exposure IC_{50} values under 0.9 μM and 90% inhibition could be reached at 100-150 μM for both chromomycin A3 and mithramycin A. mitomycin C and minocycline caused at least 90% inhibition at 300 μM.

Table 8. Anti-biofilm potencies of the four most active compounds. Strain 1 and 2 correspond to *S. aureus* Newman and S. aureus ATCC 25923, respectively.

		Effects on biofilms (IC_{50}, μM)		
		95% confidence intervals		
Code	**Common name**	**Prior-to-exposure**		**Post exposure**
		Strain 1	**Strain 2**	**Strain 1**
E25	**Chromomycin A3**	**0.13**	**0.16**	**0.17**
		(0.11 to 0.14)	(0.14 to 0.18)	(0.09 to 0.33)
E86	**Mithramycin A**	**0.07**	**0.15**	**0.48**
		(0.06 to 0.08)	(0.13 to 0.17)	(0.17 to 1.36)
E414	**Mitomycin C**	**0.24**	**0.25**	**0.88**
		(0.23 to 0.25)	(0.21 to 0.32)	(0.46 to 1.68)
E440	**Minocycline**	**0.22**	**0.30**	**0.59**
		(0.21 to 0.23)	(0.16 to 0.57)	(0.28 to 1.26)

In the study by Toté et al. (2009) a 15.6 μM concentration of rifampicin did not eliminate more than 50% of viable *S. aureus* biofilm bacteria. Rifampicin was considered as highly active in that study. Among the 12 antibiotics tested by Tote et al. (2009) nine antibiotics did not show significant activity on formed biofilm at concentrations 16X MBC. In our study only 0.17 μM of chromomycin which

also was around the IC_{50} value in prior-to-exposure test was needed to eradicate 50% of the viable biofilm bacteria. Furthermore, the highest concentration required to kill 50% of the viable cells in biofilm was 0.88 µM of mitomycin C that therefore could be considered as the least active compound on *S. aureus* biofilm in this work.

5.3.2 Bacteriostatic and bactericidal effect on planktonic cells

The minimal inhibitory concentration (MIC) values and minimal bactericidal concentrations (MBC) values were further estimated by testing the compounds on planktonic bacteria (**Table 9**). The MIC values were close to the IC_{50} values obtained in the potency tests indicating that all the tested compounds caused significant reduction of planktonic bacteria.

Chromomycin A3 inhibited 50% of biofilm formation at 0.13 µM against *S. aureus* Newman and at a concentration of 0.16 µM against *S. aureus* ATCC 25923, which also corresponds to its MBC values (0.1 µM against *S. aureus* Newman and 0.15 µM against *S. aureus* ATCC 25923). The IC_{50} value for mithramycin A was 0.07 µM against *S. aureus* Newman and 0.15 µM against *S. aureus* ATCC 25923, which were slightly higher than the MIC and MBC values in the case of *S. aureus* (0.075 µM and 0.075 µM), respectively. Mitomycin C inhibited 50% of biofilm formation at 0.24 µM against *S. aureus* Newman and 0.25 µM against *S. aureus* ATCC 25923. MIC values were around 2.5 times higher than the IC_{50} values, which were close to the MBC values. The MIC values for compound minocycline were 0.5 µM against *S. aureus* Newman and 0.75 µM in the case of *S. aureus* ATCC 25923 which were over 2 times higher than the IC_{50} values but around the same as the MBC values.

Table 9. Antibacterial potencies of the four most active compounds

		Effects on suspended bacteria			
		MIC, µM		MBC, µM	
Code	**Common name**	***S. aureus* Newman**	***S. aureus* ATCC**	***S. aureus* Newman**	***S. aureus* ATCC**
E25	Chromomycin A3	0.75	0.75	0.1	0.15
E86	Mithramycin A	0.075	0.075	0.075	0.075
E414	Mitomycin C	0.5	0.75	0.5	0.6
E440	Minocycline	0.5	0.75	0.5	0.5

Lv et al. (2014) have discovered that titanium implants coated with minocycline inhibited 98% of *S. aureus* planktonic bacteria at a 0.2 μM concentration, which agrees with the minocycline results that were obtained above. In our study, a 0.24 – 0.25 μM concentration of minocycline inhibited 50% of biofilm formation and 0.5 μM of minocycline killed 90% of the viable bacteria. All the obtained activity parameters from the follow-up studies (IC_{50}, MIC and MBC) were low, which indicates that all the compounds are potent antimicrobial compounds. Penicillin G prevented biofilm at a concentration of at least 68.7% higher than the concentrations for chromomycin A3 and mithramycin A.

As mentioned earlier, bacteria have the tendency to switch from planktonic stage to biofilm and back again depending on the environmental conditions. In addition, it is crucial to study how compounds affect biofilm formation, including disrupting biofilm as well as killing/inhibition of planktonic bacteria. The mechanisms behind the anti-biofilm activity of chromomycin A3, mithramycin A, mitomycin C and minocycline could be killing of planktonic bacteria, as well as inhibition of bacterial growth according to the viability test results. However, the activities of the compounds in the post-exposure mode of the assay showed that they also disrupt already formed biofilm by affecting biofilm processes at concentrations not much higher than the obtained IC_{50} values in pre-exposure tests.

Moreover, bacterial biofilms are not only dynamic, but also particularly complex structures. Researchers have emphasized the importance of combining the viability with biomass and matrix measurements (Skogman et al. 2012). First, the total biomass is often determined by a crystal violet staining assay. The method is cheap, straightforward, and can be used for a variety of different microorganisms. As discussed earlier, the disadvantage of the assay is the challenge to receive repeatable results. Also, the assay does not detect the difference between dead and living cells. Apparently, this assay is not a first choice, nor the only method for susceptibility testing of biofilms (Peeters et al. 2008). Second, biofilm matrix is an integral part of the biofilm. As biomass and biofilm viability are inhibited by antimicrobials, consequently the biofilm matrix may be overproduced, which could facilitate greater biofilm colonization over time. The *S. aureus* biofilm matrices have successfully been detected by a method called wheat germ agglutinin-Alexa Fluor 488 fluorescent conjugate (WGA) staining (Skogman et al. 2012). To finalize, combined viability studies, total biomass and matrix detection, may result in greater understanding of the tested anti-biofilm compounds.

6 CONCLUSIONS

Firstly, it was demonstrated that the *in vitro* screening method conducted in micro-well plates using resazurin staining was a well-operated and robust method. The bioactivity of natural and naturally derived compounds with anti-biofilm properties was tested. Natural compounds are structurally unique and challenging to screen, however, two exposure modes of the assay made it possible to study not only anti-biofilm, but also anti-microbial properties. Importantly, the compounds were successfully tested on two *Staphylococcus* strains.

Second, this work was a multistage repurposing process. An extensive literature search resulted in the identification of hit compounds, particularly novel in biofilm research. The outcome provides anti-biofilm bioactivity data of natural compounds from several classes of products. The true activity of known compounds was further confirmed and it was in agreement with the results of previous contributors, which verified an adequate assay validation.

Furthermore, four natural compounds were identified as possible anti-biofilm agents due to their activity on both planktonic and biofilm bacteria. Chromomycin A3, mithramycin A and mitomycin C have been of interest in antitumor activity, although novel in *S. aureus* biofilm research, which would represent a new indication. Minocycline has been reported as non-toxic to mammalian cells, as well as possesses known antibacterial properties on planktonic *S. aureus*. Nevertheless, the compound has not previously been reported as active on *S. aureus* biofilms, which makes it an interesting candidate in anti-biofilm research.

To summarize, the potency of all four compounds was high, which makes them good antimicrobials with potent anti-biofilm activity. These compounds have many structurally related derivatives (existing or that can be chemically generated) which would be worth exploring in the future. Moreover, the cytotoxicity of the four compounds needs to be addressed *in vivo* in order to map their safety profiles. Bioavailability studies for all four compounds make it possible to plan future new applications and to design effective and safe formulations for enhancing drug delivery at the target sites.

7 SVENSK SAMMANFATTNING - SWEDISH SUMMARY

Upptäckt av nya naturbaserade substanser med bred kemodiversitet som motverkar bildningen av *Staphylococcus aureus*-biofilmer

7.1 Introduktion

Mikroorganismer tenderar att växla mellan två olika tillstånd beroende på faktorer i deras omgivning. De kan leva som enskilda planktoniska celler, eller i biofilmer som består av cellaggregat. En biofilm bildas när mikroorganismer fastnar på en yta och börjar producera en matris som består av extracellulära polymera substanser (EPS). Ytan som bakterierna fäster sig vid kan bestå av dött eller levande material, och själva biofilmen kan innehålla en eller flera olika sorters bakterier. Matrisen som omger bakterierna i biofilmen har många viktiga funktioner. För det första ger matrisen stabilitet och fäster bakterierna vid underlaget. För det andra skyddar matrisen dess mikroorganismer för yttre faktorer såsom antibakteriella medel och antikroppar som värdorganismen producerar. För det tredje fångar den tredimensionella matrisen upp näringsämnen från omgivningen och ackumulerar signalmolekyler som mikroorganismerna utsöndrar. Biofilmer är dynamiska strukturer som ständigt förändras. Faktorer som syretillförsel, tillgängligheten av näringsämnen och inre pH är inte jämnt fördelade inom biofilmen. De kemiska förhållandena förändras över tiden och när utvecklingen av biofilmen fortskrider, kommer cellerna inom den att skilja sig från varandra på många sätt. När biofilmen mognat kan mikroorganismer från det yttersta lagret slutligen lossna och transporteras till nya ställen där de koloniserar nya ytor.

Nackdelen med biofilmer är att de orsakar allvarliga problem inom industrin och sjukvården. För det första försvåra biofilmer industriella framställningsprocesser samt skadar utrustning. Biofilmer ansamlas i rörsystem och kan också kontaminera livsmedel. För det andra är biofilmer mindre känsliga för antimikrobiella substanser jämfört med mikroorganismer i planktoniskt tillstånd. *Staphylococcus aureus (S. aureus)* är ren gram-positiv bakterie som tenderar att orsaka sjukdomar genom att bilda resistenta biofilmer. Konventionella antibiotika som används för att bekämpa vanliga infektionssjukdomar fungerar dåligt när det gäller behandlingen av sjukdomar orsakade av *S. aureus* biofilmer. Bakteriella infektioner orsakade av *S. aureus*-biofilmer inkluderar: lunginflammation hos patienter med cystisk fibros, öroninflammation, sårinfektioner, bakteriell endokardit samt infektioner i samband med inre medicinska proteser och inplantat. Sjukdomar som är kopplade till *S. aureus*-

biofilmer belastar hälso- och sjukvården globalt och därför är forskare angelägna att hitta nya antibiofilmsubstanser och nya användningsområden för befintliga existerande substanser.

Naturbaserade substanser har sedan länge använts för att behandla olika typer av bakteriella infektioner. De besitter en bred kemisk mångfald som gör dem viktiga i upptäckandet av nya läkemedel. Det är dock en dyr och lång process att ta fram nya läkemedel. Hela processen beräknas ta ca 10 år och den innehåller många kritiska steg. Väldigt få av de nya upptäckta substanserna kommer i slutändan att kunna utvecklas till läkemedel. En strategi är att hitta nya indikationer för redan godkända preparat, alternativt substanser som inte har blivit godkända för den ursprungliga indikationen eller läkemedel som fortfarande genomgår kliniska prövningar för sin primära indikation. Denna strategi gör det möjligt att medicinera försummade sjukdomar och utveckla läkemedel snabbare och till en lägre kostnad än läkemedel som upptäckts i samband med traditionell läkemedelsupptäckt.

7.2 Målsättningar

De läkemedel som finns på marknaden idag är inte tillräckligt effektiva mot biofilmrelaterade infektioner som orsakas av *S. aureus.* Målsättningen med detta arbete var att identifiera olika naturbaserade kemiska föreningar med antibiofilm egenskaper och deras inverkan på *S. aureus*-biofilm. Följande steg var att karakterisera de identifierade antibiofilmföreningarna genom vidare uppföljningsstudier.

7.3 Material och metoder

7.3.1 Primär sållning

Först gjordes en primär sållning av ett kommersiellt substansbibliotek, Enzo Product Library. Totalt 502 naturbaserade/ naturligt härledda substanser testades på *S. aureus*-biofilm vid koncentrationen 40 µM. Två olika bakteriesträngar, *S. aureus* ATCC och *S. aureus* Newman odlades i 96-brunnsplattor och exponerades med substanserna var för sig, både före och efter bildningen av biofilm vilket gjorde sållningen till en tvåstegsprocess. Den levande bakteriemassan bestämdes sedan genom resazurinmetoden som tidigare har validerats i laboratoriet. Resazurin är ett blått icke fluorescerande

ämne. Resazurin reduceras av metaboliskt aktiva bakterier till resorufin som är ett högt fluorescerande ämne. Halterna av resorufin som detekterades fluorometriskt vid λ_{ex} 570nm – λ_{em} 590nm motsvarade mängden levande bakterier som bildats i 96-brunnsplattorna. Kriterier för de substanser som valdes vidare till uppföljningsstudierna var: bekräftad bioaktivitet över den bestämda träffgränsen gällande båda stegen i sållningen, alternativt aktivitet på bildad biofilm. En omfattande litteratursökning bidrog även till uteslutning av vissa substanser. De substanser som endast förhindrade bildningen av biofilm togs inte vidare.

7.3.2 Uppföljningsstudier

Antibiofilmselektiviteten och aktiviteten hos planktoniska bakterier bestämdes under uppföljningsstudierna för de aktivaste substanserna. För det första bestämdes antibiofilmselektiviteten genom att testa substanserna vid olika koncentrationer (0.001µM - 400 µM), både före och efter biofilmbildning. Effekten för 10 olika koncentrationer detekterades med resazurinmetoden och IC_{50}-värdena räknades ut. För det andra testades den antibakteriella effekten hos planktoniska bakterier vid 13 - 14 olika koncentrationer, som varierade mellan 0.001µM - 80 µM. MIC-värdena, det vill säga de längsta koncentrationerna som ökade tillväxten av bakterier, bestämdes för varje substans genom att mäta den optiska densiteten vid λ= 620 nm. Slutligen bestämdes den lägsta koncentrationen av varje substans som dödade de flesta planktoniska bakterier (MBC). Cellsuspensionerna inkuberades med de aktiva substanserna vid en koncentration på 20 µM i respektive brunn och mängden levande celler detekterades genom resazurinmetoden.

Olika statistiska parametrar; Z'-faktor, S/N och S/B togs i beaktande för att säkerställa kvaliteten för de använda metoderna. Penicillin G är en antibiotika som har antibakteriell effekt på *S. aureus* och användes därför som positiv kontroll. Som negativ kontroll användes sterilt TSB.

7.4 Resultat och diskussion

7.4.1 Primär sållning

Den primära sållningen av totalt 502 naturliga substanser resulterade i 20 aktiva substanser. Av de aktiva substanserna ansågs 9 substanser aktiva i båda stegen av sållningen medan 11 substanser visade aktivitet endast på bildad biofilm, vilket var en överraskande upptäckt. Efter litteratursökningen valdes 4 substanser ur varje grupp vidare till en ny bekräftande sållning och då kunde det konstateras att 4 av de 8 substanserna var inaktiva. De fyra substanser som valdes för vidare uppföljningsstudier var slutligen: mithramycin A, chromomycin A3, mitomycin C och minocyklin. Mithramycin A och chromomycin A3 har liknande struktur och är kända inom cancerforskning. Mitomycin C är en cytotoxisk antibiotika som blev godkänd som cancerläkemedel år 1974. Den fjärde substansen, minocycline, är en antibiotika av brett sprektrum och har använts för behandling av infektionssjukdomar i över 30 år. Alla dessa fyra substanser var inte kända antibiofilmsubstanser sedan tidigare.

7.4.2 Uppföljningsstudier

Alla fyra substanser ansågs vara högktiva eftersom det krävdes en låg koncentration för att förhindra men också reducera bildad biofilm. Chromomycin och mithramycin var förhindrade mest effektivt bildningen av biofilm med IC_{50}-värden mellan 0,07 och 0,16 µM, jämfört med penicillin som förhindrade bildningen av biofilm vid 0,27 µM. Mätningarna visade att effekten på redan bildad biofilm var endast 1,2 – 3,6 gånger högre än de koncentrationer som behövdes för att förhindra bildningen av biofilm. Ofta bör koncentrationen vara många gånger högre för att reducera biofilm jämfört med att förhindra bildningen av den. Alla substanser gav IC_{50}-värden under 0,9 µM då det handlade om att förhindra biofilm och 90 procent inhibition kunde uppnås vi 100–150 µM för både chromomycin A3 och mithramycin A. Mitomycin C och minocyklin orsakade minst 90 % inhibering vid 300 µM.

Alla fyra substanser reducerade markant planktoniska bakterier och MIC-värdena motsvarade deras IC_{50}-värden. MBC-värdena var liksom IC_{50} och MIC låga för alla substanser. Den minst aktiva substansen var mitomycin C med ett MBC-värde på 0,6 µM och IC_{50}-värde på 0,88 µM. Mitramycin A hade den största aktiviteten på planktoniska celler, det behövdes endast 0,075 µM av substansen

för att både inhibera tillväxten och döda de flesta bakterier. Penicillin G är känd för sina antibiofilmegenskaper men det behövdes 68,7 % högre koncentrationer av den för att förhindra bildningen av biofilm jämfört med chromomycin A3 och mithramycin A.

Bakterier har en dynamisk livsstil som ständigt förändras och detta arbete gjorde det möjligt att studera hur chromomycin A3, mithramycin A, mitomycin C och minocycline påverkade planktoniska bakterier, biofilmbildning samt effekten på biofilm. Mekanismerna bakom deras antibiofilmegenskaper kunde tänkas vara att de dödar planktoniska bakterier, men också inhiberar tillväxten av bakterier. Substanserna visade sig även ha effekt på biofilm genom att de påverkade biofilmprocesser vid koncentrationer som inte var mycket högre än de koncentrationer som behövdes för att förhindra biofilmbildning.

7.5 Slutsatser

S. aureus bildar biofilmer som orsakar kroniska och resistenta sjukdomar mot vilka konventionella antibiotika inte fungerar. Behovet av nya antibiofilm läkemedel är stort. Detta projekt gav information om flera naturliga substanser med antibiofilm egenskaper. Arbetet var en flerstegsprocess för att återanvända läkemedelsföreningar som var nya inom biofilmforskning. Fyra naturliga substanser identifierades som aktiva både på planktoniska celler och biofilm. Alla substanser var väldigt potenta, vilket gör dem till möjliga kandidater för framtida antibiofilm läkemedel.

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SUPPLEMENTARY TABLE 1. Inhibitory activity of the entire Enzo library in the primary screening

Code	Common name	Activity (% inhibition) prior-to biofilm formation		Activity (% inhibition) post biofilm formation	
		S. aureus ATCC	*S. aureus* Newman	*S. aureus* ATCC	*S. aureus* Newman
E1	Acivicin	42	20	12	0
E2	Actinomycin D	93	92	37	58
E3	Anisomycin	19	40	0	0
E4	Antibiotic A-23187	92	91	25	56
E5	Aristolochic acid A	0	0	0	19
E6	Artesunate	13	12	0	4
E7	Australine HCl	61	13	0	0
E8	Baicalein	3	15	0	19
E9	Betulinic acid	0	7	0	3
E10	Bilobalide	0	15	0	7
E11	Brefeldin A	0	26	0	3
E12	Bromocriptine mesylate	0	0	0	0
E13	C2 Phytoceramide	32	0	8	0
E14	C6 Ceramide	18	0	16	0
E15	Caffeic Acid	21	6	0	7
E16	Camptothecin	64	90	0	10
E17	Cantharidin	7	0	0	10
E18	CAPE	55	0	0	11
E19	Capsaicin	35	0	0	6
E20	Castanospermine	0	0	0	0
E21	Cerulenin	33	14	4	2
E22	Cevadine	18	0	0	3
E23	Chaetomellic acid A	0	0	0	2
E24	Chelerythrine Cl	72	54	45	0
E25	Chromomycin A3	92	92	48	72
E26	Citrinin	0	0	0	0
E27	Colchicine	12	0	27	15
E28	Coumermycin A1	93	91	0	47
E29	Curcumin	10	0	0	5
E30	Cycloheximide	0	0	0	5
E31	Cyclopamine	11	5	0	8
E32	Cyclopiazonic acid	9	0	0	0
E33	Cycloserine, L-	8	0	0	4
E34	Cyclosporin A	0	0	0	14
E35	Cytochalasin B	11	0	0	6
E36	Cytochalasin D	0	4	31	0

E37	Cytochalasin E	5	28	0	0
E38	Daidzein	0	11	0	0
E39	Daunorubicin HCl	92	92	0	0
E40	Decoyinine	22	39	0	14
E41	Deguelin	0	0	0	15
E42	Deoxyphorbol 13-acetate, 12-	11	0	0	11
E43	Deoxyphorbol 13-phenylacetate 20-acetate,12-	38	0	0	1
E44	Dihydroergocristine mesylate	3	0	0	0
E45	Domoic acid	0	0	0	9
E46	Doxorubicin HCl	90	91	0	50
E47	E6 Berbamine	26	0	21	6
E48	E-64	0	4	0	0
E49	E-64-C	11	26	0	0
E50	E-64-D	14	0	0	0
E51	Ebelactone B	49	15	0	0
E52	Ellipticine	93	92	0	24
E53	Embelin	84	93	0	37
E54	Epibatidine, (±)-	33	0	15	0
E55	Epigallocatechin gallate	54	20	0	1
E56	Etoposide	0	68	28	0
E57	Forskolin	0	0	0	2
E58	Fumagillin	35	0	28	1
E59	Fumonisin B2	14	3	6	10
E60	Galanthamine HBr	0	8	0	0
E61	Gambogic acid	77	93	0	0
E62	Genistein	0	0	0	0
E63	Geranylgeranoic acid	0	0	0	17
E64	Gingerol	14	0	0	0
E65	Ginkgolide B	0	3	0	3
E66	Gliotoxin	85	88	27	49
E67	Gossypol	63	93	0	27
E68	Grayanotoxin III	0	0	0	0
E69	Himbacine	42	0	2	0
E70	Huperzine A, (-)-	5	0	32	0
E71	Hydroxycamptothecin, 10-	12	0	0	0
E72	Hypericin	0	4	0	0
E73	Indirubin	0	31	0	0
E74	Ingenol 3,20-dibenzoate	34	0	0	4
E75	Isotetrandrine	23	8	7	1
E76	Jervine	49	0	0	2
E77	Kainic acid	1	0	12	14
E78	Kavain (+/-)	0	0	3	0
E79	Kenpaulone	39	15	0	18

E80	Lapachone, b -	33	0	0	0
E81	Lincomycin	92	92	15	50
E82	Lycorine HCl	0	0	0	2
E83	Mevastatin	0	0	0	14
E84	3-B-Indoleacrylic acid	0	18	0	0
E85	Mimosine, L-	7	34	0	12
E86	Mithramycin A	93	92	42	49
E87	Monensin Na	90	90	36	37
E88	Mycophenolic acid	30	16	0	0
E89	Myriocin	7	40	42	4
E90	Neomycin sulfate	0	44	0	0
E91	Nigericin Na	89	91	40	57
E92	Oligomycin A	4	0	0	8
E93	Ouabain (-)-	0	0	0	9
E94	Parthenolide	0	0	0	0
E95	Perillic acid	35	0	0	0
E96	Phloretin	0	0	44	0
E97	Phorbol 12,13-dibutyrate	0	14	0	0
E98	Phorbol 12,13-dibutyrate, 4-a -	16	0	0	0
E99	Phorbol 12-myristate 13-acetate	0	0	0	0
E100	Phorbol 12-myristate 13-acetate, 4-a -	5	0	4	17
E101	Phytosphingosine	95	95	0	0
E102	Piceatannol	0	16	0	0
E103	Prostaglandin A1	4	0	0	0
E104	Prostaglandin B1	0	29	28	0
E105	Prostaglandin E1	0	0	3	4
E106	Prostaglandin E2	0	0	0	0
E107	Prostaglandin F2a	0	0	3	0
E108	Kahweol acetate	0	0	0	0
E109	Quisqualic acid	0	0	0	16
E110	Radicicol	0	0	35	0
E111	Rapamycin	10	0	13	0
E112	Rauwolscine	0	19	43	0
E113	Resveratrol	5	8	3	0
E114	Retinoic acid (all trans)	94	95	0	0
E115	Retinoic acid, 13-cis-	0	0	0	0
E116	Retinoic acid, 9-cis-	15	90	1	3
E117	Rifampicin	94	94	61	73
E118	Rosmarinic acid	0	0	0	0
E119	Rotenone	0	8	9	0
E120	Rottlerin	82	94	0	35
E121	Ryanodine	0	0	2	0
E122	Shikonin	0	0	4	0
E123	Spectinomycin sulfate	0	0	0	0

E124	Swainsonine	0	0	47	0
E125	Tanshinone IIA	0	0	0	13
E126	Taxol	0	0	23	0
E127	Tetrandrine	0	0	0	0
E128	Thapsigargin	0	0	5	0
E129	Tomatidine	11	0	0	0
E130	Troleandomycin	94	95	25	26
E131	Tunicamycin B	0	26	29	0
E132	Ursolic acid	0	4	0	0
E133	Valinomycin	2	0	33	21
E134	Aconitine	0	11	26	0
E135	Veratridine	0	20	0	0
E136	Vinblastine sulfate	0	31	54	0
E137	Vincristine sulfate	7	11	0	8
E138	Vinpocetine	0	17	4	0
E139	Wedelolactone	0	0	0	0
E140	Wortmannin	0	27	21	0
E141	Apigenin	0	21	0	45
E142	Arecoline HBr	0	0	0	0
E143	Atropine sulfate	0	2	21	0
E144	Berbamine 2HCl	0	19	0	5
E145	Bicuculline, (+)-	0	0	7	0
E146	Bufalin	0	36	1	0
E147	Brucine n-oxide	0	13	5	0
E148	Butein	1	13	46	0
E149	Catalpol	0	0	0	0
E150	Chrysine	0	4	0	0
E151	Desoxypeganine HCl	0	4	3	0
E152	Veratramine	0	0	0	0
E153	Emodin	24	51	15	49
E154	Gramine	15	38	7	0
E155	Harmaline HCl	0	32	31	0
E156	Harmine HCl	0	15	0	0
E157	Hyoscyamine	0	0	0	0
E158	Ivermectin	22	25	0	0
E159	Luteolin	0	2	0	0
E160	Melatonin	0	26	9	0
E161	Morin	1	0	0	1
E162	Myricetin	0	16	18	0
E163	Naringenin	0	0	0	0
E164	Nicotine, (-)-	0	0	19	0
E165	Nonactin	93	94	35	53
E166	Penicillamine, L-	0	20	0	0
E167	Picrotoxinin	0	1	0	1

E168	Pilocarpine HCl	0	0	0	0
E169	Quassin	0	0	0	0
E170	Quercetin	2	5	0	0
E171	Quinidine HCl	0	1	0	0
E172	Quinine HCl	0	28	9	0
E173	Robinetine	4	10	0	0
E174	Menadione	0	0	0	0
E175	Strychnine HCl	0	0	5	0
E176	Tryptanthrin	0	0	0	0
E177	Yohimbine HCl	0	0	17	9
E178	Eburnamonine, (-)-	19	20	10	0
E179	Lysergol	0	0	31	0
E180	Monocrotaline	0	0	0	0
E181	Oxytetracycline, a -apo-	0	0	0	0
E182	Pseudopelletierin HCl	0	0	0	0
E183	Salsolinol HBr	0	0	0	0
E184	Sitosterol, b -	0	0	0	0
E185	Sterigmatocystin	0	0	0	19
E186	Trimethylpsoralen, 4,5',8-	0	0	0	0
E187	Cinobufagin	0	0	0	0
E188	Emetine 2HCl	0	0	0	0
E189	Kaempferol	0	4	0	35
E190	Kanamycin	0	0	0	0
E191	Celastrol	93	94	14	33
E192	Taxifolin (+)	0	0	0	0
E193	Theobromine	11	9	0	0
E194	Baccatin III	0	12	0	0
E195	Carminic acid	0	4	0	0
E196	Cotinine, (-)-	0	0	0	2
E197	Austricin	0	0	0	16
E198	Condelphine	0	0	0	24
E199	Delcorine	34	0	0	2
E200	Deltaline	0	0	0	0
E201	Diacetylkorseveriline	0	0	0	10
E202	Dubinidine	5	0	22	7
E203	Eudesmine	0	0	0	1
E204	Feroline	93	94	0	1
E205	Fillalbin	1	2	0	0
E206	Graveoline	9	0	0	4
E207	Heliotrine	0	4	7	5
E208	Hernandezine	7	9	0	0
E209	Heteratisine	6	0	11	0
E210	Imperialine	0	0	0	0
E211	Karakoline	26	0	0	0

E212	Lapidine	0	0	0	0
E213	Lapiferine	0	0	0	0
E214	Nitrarine 2HCl	29	24	26	0
E215	Norfluorocurarine	19	0	56	0
E216	Peganole	0	0	0	0
E217	Pinocembrin	35	33	0	0
E218	Protopine HCl	18	0	0	19
E219	Remerine HCl	20	0	20	10
E220	Sevedindione	0	4	10	0
E221	Skimmianine	0	9	9	0
E222	Songorine	10	9	0	0
E223	Trichodesmine	33	11	0	0
E224	Tschimganidin	91	93	0	0
E225	Tschimganine	88	95	0	0
E226	Ungerine nitrate	26	12	4	0
E227	Genistin	15	22	0	0
E228	Laudanosine methiodide	0	26	0	0
E229	Apigenin-7-O-glucoside	0	0	0	0
E230	Bavachinin A	51	54	0	0
E231	Decylubiquinone	0	57	0	24
E232	Convolvamine HCl	0	0	7	0
E233	Daidzin	30	0	0	0
E234	Datiscetin	31	25	0	25
E235	Deacetylcolchicine, N-formyl-	34	0	10	0
E236	Oridonin	5	0	0	0
E237	Eriocitrin	0	0	0	0
E238	Eriodictyol	23	23	0	3
E239	Eriodictyol-7-O-glucoside	12	0	0	0
E240	Homobutein	23	7	0	0
E241	Homoeriodictyol (-)	0	0	0	2
E242	Homoorientin	7	0	0	0
E243	Hydroxyflavone, 7-	0	0	0	0
E244	Isorhamnetine-3-O-glucoside	0	4	0	0
E245	Isorhoifoline	0	3	0	28
E246	Isosakuranetin	17	0	0	0
E247	Isovitexin	18	0	10	0
E248	Dihydromethysticin	0	9	0	0
E249	(Beta, Beta-Dimethylacryl) Shikonin	92	93	0	25
E250	Kaempferol-7-neohesperidoside	30	0	0	3
E251	Luteolin-3',7-di-o-glucoside	14	0	22	24
E252	Flavokawain B	0	0	0	0
E253	Marein	0	0	0	0
E254	Maritimein	0	0	0	0
E255	Picropodophyllin	0	0	0	0

E256	Myricitrin	0	1	0	0
E257	Naringenin-7-O-glucoside	0	0	6	0
E258	Narirutin	20	0	0	0
E259	Picrotin	25	26	0	0
E260	Plumbagin	47	0	0	0
E261	Ketopinic acid	0	0	0	24
E262	Scopolomine N-butylbromide	30	0	0	0
E263	Rhamnetine	0	0	44	15
E264	Rhoifolin	0	16	0	1
E265	Sanguinarine	0	0	0	0
E266	Saponarin	0	8	0	0
E267	Manool	0	9	0	0
E268	Citreoviridin	0	0	0	0
E269	Sinensetine	0	18	7	0
E270	Sulfuretine	19	10	0	0
E271	Atropine-N-oxide	0	0	0	0
E272	Tamarixetine	6	0	0	1
E273	Tetrahydroalstonine	0	0	0	0
E274	Diacetoxyscirpenol	16	0	0	0
E275	Vitexin-2"-O-rhamnoside	9	0	28	0
E276	Laudanosoline HBr	0	7	0	0
E277	Bromolaudanosine, (±)-6'-	13	0	0	0
E278	Andrographolide	1	0	0	0
E279	Ajmaline	25	0	0	0
E280	Chelidonine, (+)-	14	0	0	0
E281	Dihydroxyflavone, 6,7-	0	0	0	0
E282	Fisetin	0	0	10	0
E283	Harmalol HCl	12	0	0	0
E284	Harmol HCl	16	0	0	0
E285	Isorhamnetine-3-O-rutinoside	0	6	0	0
E286	Isoscopoletine	25	0	0	0
E287	Methoxyflavone, 5-	0	0	0	5
E288	Pratol	37	0	0	10
E289	Syringetine-3-O-glucoside	50	23	0	11
E290	Conessine	40	0	0	0
E291	Sarsasapogenin	20	0	8	11
E292	Strophanthidin acetate	39	18	58	14
E293	Hordenine sulfate	17	18	0	18
E294	Ferutinin	89	92	3	21
E295	Piperine	0	0	0	0
E296	Quercitrin	18	0	0	2
E297	Scopolamine N-oxide HBr, (-)-	50	0	0	4
E298	Shikimic Acid	32	0	0	18
E299	Stachydrine HCl	28	0	11	0

E300	Ochratoxin A	22	0	0	20
E301	Patulin	0	0	17	6
E302	Zearalenone	47	2	0	19
E303	Isorhamnetine	0	0	9	15
E304	Abscisic acid, (±)-	40	8	46	15
E305	Rifamycin SV-NA	82	89	61	71
E306	Aloe-emodine	0	0	0	0
E307	Antimycin A1	35	11	9	32
E308	Asarinin, (-)-	2	0	14	15
E309	Aucubin	41	0	1	13
E310	Deoxyshikonin	0	16	2	41
E311	Boldine	26	0	5	19
E312	Caryophyllene oxide	22	0	0	0
E313	Catechin hydrate, (+)-	7	12	20	2
E314	Cinchonidine, (-)-	35	0	0	1
E315	Cinchonine, (+)-	47	0	16	0
E316	Cotininecarboxylic acid, trans-4-	49	0	25	14
E317	Demissidine	39	0	4	4
E318	Dipterocarpol	24	0	0	0
E319	Dehydrocostus Lactone	55	0	8	0
E320	Friedelin	10	0	20	11
E321	Indole-3-butyric acid	49	0	9	10
E322	Gibberellic acid, (+)-	15	1	0	17
E323	Gitoxigenin	25	0	0	12
E324	Harmane HCl	0	0	0	0
E325	Hydroxytropinone, 6-	15	14	0	0
E326	Isocorydine HCl	36	0	0	19
E327	Isoreserpine, (-)-	28	0	34	1
E328	Leucomisine	44	17	26	9
E329	Methylergonovine	37	0	34	4
E330	Corydaline	22	0	0	0
E331	Muscarine Cl, (+)-	47	0	17	0
E332	Nalidixic acid	3	47	35	10
E333	Narasin	84	91	40	64
E334	Noreleagnine	0	0	14	25
E335	Norharmane	0	0	6	23
E336	Palmatine Cl	2	12	0	5
E337	Peruvoside	21	11	0	0
E338	Physostigmine	35	0	0	6
E339	6-Acetamido-6-deoxy-castanospermine	36	0	11	7
E340	Podocarpic acid	15	53	50	6
E341	Retrorsine	34	0	18	0
E342	Rhapontin	0	0	17	0
E343	Sclareolide, (3aR)-(+)-	50	0	5	0

E344	Streptonigrin	90	91	30	55
E345	Tetrahydropapaverine	71	43	20	57
E346	Ingenol	0	0	22	0
E347	Syrosingopine	12	3	24	24
E348	Visnagin	11	15	0	17
E349	Wogonin	0	2	0	0
E350	Zearalanol, b -	27	2	0	3
E351	4-Methylumbelliferone	30	38	29	19
E352	Echinomycin	92	91	69	69
E353	Ellagic acid	0	0	0	0
E354	Epicatechin, (-)-	22	0	0	0
E355	Puromycin	53	12	0	0
E356	Glycyrrhetinic acid, 18-b -	11	1	3	8
E357	Griseofulvin, (+)-	42	0	31	3
E358	Isoquercitrine	0	0	3	19
E359	Kinetin	9	12	16	41
E360	Lasalocid A Na	92	91	36	65
E361	Vanillylacetone	0	0	0	1
E362	Sclareol	13	0	0	0
E363	Trigonelline HCl	10	9	7	21
E364	Tubercidin	27	11	27	0
E365	Usnic acid, (+)-	87	91	19	0
E366	Vitexin	0	2	0	0
E367	Acacetine	13	0	0	2
E368	Capreomycin sulfate	14	0	3	6
E369	Carnitine Cl, (±)-	26	0	18	15
E370	Cephradine	0	0	0	15
E371	Vasicine	0	9	0	37
E372	Homatropine HBr	28	29	0	13
E373	Hydrastine, D-b -	0	0	0	16
E374	Khellin	14	0	0	0
E375	Lobeline HCl	52	0	0	13
E376	Osthole	33	0	0	0
E377	Tetrahydrolipstatin	35	22	0	4
E378	Neohesperidin	0	10	0	0
E379	Noscapine, (±)-	36	0	0	0
E380	Oleanolic acid	49	43	0	25
E381	Papaverine HCl	1	6	0	7
E382	Phlorizine	8	28	0	16
E383	Protoveratrine B	8	24	1	17
E384	Reserpine	34	9	0	22
E385	Salinomycin	93	91	59	51
E386	Xanthotoxin	0	18	9	20
E387	Scopoletin	0	9	12	7

E388	Digitoxin	0	24	0	10
E389	Solanine, a -	0	15	0	4
E390	Solasodine	0	0	0	29
E391	Tropine	6	0	0	38
E392	D-Tubocurarine-chloride	0	38	0	10
E393	Myristicin	0	1	0	20
E394	Vincamine	18	15	0	10
E395	Anabasine HCl	0	16	0	0
E396	Cephaeline HBr	2	18	0	22
E397	Dicoumarol	72	47	31	49
E398	Artemisinin	9	31	22	26
E399	Asiatic acid	0	0	15	2
E400	Auraptene	0	0	0	0
E401	Vulpinic acid	19	18	42	27
E402	Berberine HCl	0	2	44	28
E403	Bergenin	8	0	15	21
E404	Biochanin A	0	0	1	19
E405	Bulleyaconitine A	18	0	2	15
E406	Cafestol	35	0	2	0
E407	Cafestol acetate	11	0	0	0
E408	Zerumbone	0	0	0	0
E409	Catharanthine base	19	7	0	8
E410	Cepharanthine	48	0	21	9
E411	Cryptotanshinone	83	0	0	0
E412	Dehydrokawain, 5,6-	0	0	37	0
E413	Demethylepipodophyllotoxin, 4'-	18	6	32	0
E414	Mitomycin C	93	90	46	70
E415	Methysticin	19	0	26	25
E416	Thymoquinone	0	8	23	9
E417	Dihydrotanshinone	86	89	0	0
E418	Azomycin	0	0	0	0
E419	Diosmetine	0	0	32	0
E420	Diosmin	0	14	0	18
E421	Ecdysone	0	0	0	0
E422	Ecdysone, b-	17	4	13	3
E423	Euphorbiasteroid	21	0	0	0
E424	Flavokawain A	0	0	10	0
E425	Lupinine	2	0	37	0
E426	Formononetin	4	0	39	18
E427	Ginkgolide A	0	0	30	12
E428	Harringtonine	6	0	30	0
E429	Hesperetine	0	0	21	6
E430	Hesperidine	0	0	7	14
E431	Honokiol	90	89	8	7

E432	Hypocrellin A	63	0	18	0
E433	Hypocrellin B	70	0	22	0
E434	Lagochiline	16	0	17	7
E435	Lappaconitine	21	0	7	1
E436	Limonin	0	0	0	0
E437	Madecassic acid	0	0	26	6
E438	Magnolol	51	36	16	19
E439	Matrine	0	0	26	13
E440	Minocycline	93	90	42	71
E441	Naringin	0	0	13	6
E442	Indole-3-acetic acid	1	0	0	0
E443	Oxocafestol, 16-	0	0	0	0
E444	Oxokahweol, 16-	0	0	0	5
E445	Panaxadiol	15	0	0	9
E446	Panaxatriol	15	0	23	0
E447	GERI-BP002-A	93	91	22	57
E448	Pimaricin	0	0	14	0
E449	Podophylotoxin	0	0	39	6
E450	Rubescensin A	0	0	17	0
E451	Rutaecarpine	0	0	10	8
E452	Rutin	0	0	24	0
E453	Salsolidine	0	0	0	0
E454	Salsoline	0	6	8	2
E455	Santonin	0	11	7	0
E456	Schisandrin A, R(+)-	16	0	16	0
E457	Schisandrin B, S(-)-	3	0	0	0
E458	Schisantherin A	15	8	19	0
E459	Securinine	21	0	7	0
E460	Sedanolide	0	0	33	0
E461	Silybine	0	0	41	17
E462	Silymarin	0	0	35	10
E463	Sinomenine	0	8	32	13
E464	Solanesol	14	0	18	12
E465	Vindoline	0	0	0	12
E466	Vinorelbine base	0	0	30	8
E467	Yangonin	0	0	0	0
E468	Bergapten	0	1	0	6
E469	Betulin	0	0	0	0
E470	Corynanthine	14	0	0	0
E471	Cytisine, (-)-	27	5	6	0
E472	Spartein sulfate (-)-	2	0	13	1
E473	Brassinin	26	31	28	0
E474	Dihydrorobinetin	22	0	11	10
E475	Flavanomarein	2	0	7	25

E476	Lavendustin B	31	0	8	21
E477	Evodiamine	22	0	0	6
E478	Oxyacanthine sulfate	20	0	15	9
E479	Galangine	38	45	0	9
E480	Lavendustin A	0	27	0	28
E481	Verruculogen	4	0	0	8
E482	Gelsemine HCl	16	13	19	11
E483	Hydrocotarnine HBr	0	17	15	17
E484	Senecionine	18	21	10	19
E485	Bis demethoxycurcumin	27	14	0	36
E486	Dihydrolysergol, 9,10-	4	31	0	16
E487	Amphotericin B	0	23	0	14
E488	Amygdalin	9	26	0	17
E489	Anisodamine	11	23	0	11
E490	Aphidicolin	0	0	4	19
E491	Arbutin	0	20	0	9
E492	Sclerotiorin	0	0	0	0
E493	Bleomycin	29	0	0	0
E494	Chartreusin	38	72	6	27
E495	Chlorogenic acid	5	19	0	9
E496	Geraldol	31	0	0	31
E497	Coumestrol	54	0	0	23
E498	Diindolylmethane	13	0	11	0
E499	Ferulic acid	6	25	4	25
E500	Bakuchiol	91	92	0	35
E501	Hirsutine	0	1	0	17
E502	Indole-3-carbinol	20	0	0	3