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Target-based and phenotypic studies towards the discovery of antimicrobial adjuvants

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Author's Declaration

Under the terms of “Decreto-Lei no 216/92, de 13 outubro”, it is hereby declared that the author afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results, and manuscript preparation of the original articles included in this thesis.

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*The most important thing in life is to stop saying 'I wish' and start saying 'I will'.
Consider nothing impossible, then treat possibilities as probabilities.*

Charles Dickens

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Abstract

Antimicrobial resistance is one of the greatest public health issues of current times. The discovery of new antibiotics seems to have come to a halt, with very few antibiotics having approval in the 21st century. This leads to a necessity of finding new strategies, and one of them is to act in the core of the problem, that is, overcoming antimicrobial resistance.

One of the most important resistance mechanisms is the overexpression of efflux pumps; as even when this is not the main resistance mechanism present, it can also contribute to resistance by decreasing the concentration of antibiotic within the bacterial cell and allowing the bacteria to survive, while developing resistance. The inhibition of bacterial efflux pumps arises as a strategy to act as both reversion and prevention of antimicrobial resistance. Therefore, the objective of this thesis was to find new compounds that could be potential efflux pump inhibitors by target-based approaches and phenotypic studies.

Considering privileged moieties present in known bacterial efflux pump inhibitors, and the fact that aminated thioxanthenes have been proven to be P-glycoprotein modulators, a virtual library of 1000 aminated thioxanthenes, bearing guanidine, urea, and sulfonamide moieties, was designed. Then, they underwent docking studies in the AcrB portion of the AcrAB-TolC efflux pump and in a homology model of P-glycoprotein, as a way to seek for selectivity. The virtual hit compounds were synthesized, and seven thioxanthone derivatives and four unexpected tetracyclic thioxanthenes were obtained. These derivatives were tested for their antibacterial activity against four reference strains of relevant bacteria, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*, and for their potential to potentiate antibiotics in resistant strains of *E. coli* and *E. faecalis*. The new derivatives and an existing in-house library of five thioxanthenes were tested for their potential to inhibit efflux pumps in a clinical strain of *S. aureus* and a mutant strain of *Salmonella enterica* serovar Typhimurium, using the accumulation of ethidium bromide, a known efflux pump substrate, as a marker of efflux inhibition. Related mechanisms such as the inhibition of biofilm formation in a reference and a clinical strain of *S. aureus* were also studied, as well as inhibition of quorum-sensing on models of *Chromobacterium violaceum* and *Serratia marcescens*. Lastly, these compounds were evaluated for their cytotoxicity in NIH/3T3, and Caco-2 cell lines, and modulation of P-glycoprotein was also assessed, showing that thioxanthenes could act as both bacterial and human efflux pump inhibitors.

A phenotypic screening of a library of 175 structurally diverse compounds, which includes xanthenes, xanthenes, gallic acid derivatives, deoxycholic acid derivatives, chalcones, diarylpentanoids, fiscalins, and metabolites from marine-derived fungi was also performed to discover potential antimicrobial adjuvants. These compounds were evaluated for their antibacterial activity, potentiation of antibiotics, and efflux pump inhibition. The most promising derivatives were evaluated for the inhibition of biofilm formation and quorum-

sensing, and cytotoxicity in NIH/3T3 cell line. Within these classes, a few hit compounds were discovered. Particularly, a deoxycholic acid derivative showed broad spectrum antibacterial activity, as well as efflux pump inhibition in the Gram-negative strain studied, inhibition of biofilm formation, and quorum-sensing.

A library of eight deoxycholic amides was then synthesized, and tested against 21 strains of reference, clinical and animal strains of bacteria, as well as against fish pathogens and a wide variety of fungi, showing potential antimicrobial activities. However, the previously obtained hit compound was shown to have the broadest spectrum. These compounds showed potential as bacterial efflux pump inhibitors, although they had low potential for the inhibition of biofilm formation.

Lastly, and since the interest in impairing the functioning of bacterial efflux pumps is not limited to a clinical setting, a small library of 15 compounds was tested against *Synechocystis* sp. PCC 6803, as a model of cyanobacteria responsible for blooms in aquatic environments. These bacteria also have a secretion system composed of the AcrAB-TolC efflux pump. Herein, using a TolC-deletion mutant available it was discovered that these compounds do not inhibit these efflux pumps; instead, they appear to be substrates of this secretion system.

Phenotypic screenings disclosed several hits and scaffolds as promising not only for the inhibition of bacterial efflux pumps but also for the circumvention of antimicrobial resistance.

Keywords: antimicrobial; deoxycholic acid derivatives; efflux pump inhibitors; phenotypic screening; target-based design; thioxanthenes.

Resumo

A resistência aos antimicrobianos é um dos maiores problemas de saúde pública da atualidade. A descoberta de novos antibióticos parece ter abrandado, com muito poucos antibióticos aprovados no século XXI. Isto conduz a uma necessidade de procurar novas estratégias, sendo uma delas atuar na causa do problema, isto é, combater a resistência aos antimicrobianos.

Um dos mecanismos de resistência mais importantes é a sobre-expressão de bombas de efluxo, uma vez que mesmo não sendo o principal mecanismo de resistência presente, pode contribuir para a resistência ao diminuir a concentração de antibiótico no interior da bactéria e permitir a sua sobrevivência, enquanto este desenvolve resistência. A inibição de bombas de efluxo surge como uma estratégia que pode em simultâneo reverter e prevenir a resistência aos antimicrobianos. Assim, o objetivo desta tese é a descoberta de novos compostos que possam ser potenciais inibidores de bombas de efluxo através de abordagens baseadas no alvo e estudos fenotípicos.

Considerando fragmentos privilegiados presentes em inibidores de bombas de efluxo bacterianas conhecidos, e no facto de que tioxantonas aminadas demonstrarem ser moduladoras da glicoproteína-P, uma biblioteca virtual de 1000 tioxantonas aminadas, contendo porções guanidina, ureia e sulfonamida, foi planeada. Posteriormente, foi submetida a estudos de *docking* na porção AcrB da bomba de efluxo AcrAB-TolC e num modelo de homologia da glicoproteína-P, numa tentativa de conseguir seletividade. Os compostos *hit* virtuais foram sintetizados, e sete derivados tioxantónicos e quatro tioxantenos tetracíclicos foram obtidos. A atividade antibacteriana destes compostos foi testada em quatro estirpes de referência de *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, e *Enterococcus faecalis*, e para o seu potencial na potenciação de antibióticos numa estirpe resistente de *E. coli* e *E. faecalis*. Posteriormente, os novos derivados e uma biblioteca de cinco tioxantonas *in-house* foram testadas para aferir o seu potencial em inibir bombas de efluxo bacterianas numa estirpe clínica de *S. aureus* e numa estirpe mutante de *Salmonella enterica* serovar Typhimurim, usando a acumulação de brometo de etídio, um substrato conhecido de bombas de efluxo, como um marcador de inibição de efluxo. Mecanismos relacionados, tais como a inibição da formação de biofilme numa estirpe de referência e numa estirpe clínica de *S. aureus* também foram estudados, tal como a inibição de *quorum-sensing* em modelos de *Chromobacterium violaceum* e *Serratia marcescens*. Por último, a citotoxicidade destes compostos foi avaliada nas linhas celulares NIH/3T3 e Caco-2, e a modulação da glicoproteína-P também foi estudada, mostrando que as tioxantonas podem atuar cumulativamente como inibidoras de bombas de efluxo humanas e bacterianas.

A parte seguinte aborda o *screening* de uma biblioteca de 175 compostos estruturalmente diversos, que inclui xantonas, xantenos, derivados do ácido gálico, derivados do ácido desoxicólico, calconas, diarilpentanóides, fiscalinas, e metabolitos de fungos marinhos. Estes compostos foram avaliados em relação ao seu potencial antibacteriano, potenciador de antibióticos, e inibidor de bombas de efluxo. Os derivados mais promissores foram ainda estudados em relação à inibição de formação de biofilme e *quorum-sensing*, e citotoxicidade na linha celular NIH/3T3. Dentro destas classes, alguns compostos *hit* foram descobertos. Em particular, um derivado do ácido desoxicólico demonstrou largo espectro de atividade antibacteriana, bem como na inibição de bombas de efluxo na estirpe Gram-negativo estudada, inibição de formação de biofilme e *quorum-sensing*.

Uma biblioteca de oito amidas desoxicólicas foi seguidamente sintetizada, e testada contra 21 estirpes de referência, clínicas e animais, bem como bactérias patogénicas de peixes e uma larga variedade de fungos, mostrando potencial antimicrobiano. No entanto, o composto *hit* previamente obtido mostrou ter o mais largo espectro de atividade. Os compostos mostraram ainda potencial como inibidores de bombas de efluxo bacterianas, ainda que tivessem baixo potencial na inibição de biofilme.

Por fim, e como o interesse em diminuir o funcionamento das bombas de efluxo não limitam ao contexto clínico, uma pequena biblioteca de 15 compostos foi testada contra a *Synechocystis* sp. PCC6803, enquanto modelo de cianobactérias responsáveis pela proliferação de algas em ambientes aquáticos. Estas bactérias também possuem um sistema de secreção composto pela bomba de efluxo AcrAB-TolC. Assim, usando um mutante com deleção da TolC, foi descoberto que estes compostos não inibem estas bombas de efluxo; pelo contrário, parecem ser substratos deste sistema de secreção.

Os estudos fenotípicos revelaram muitos compostos *hit* e estruturas promissoras, não só como inibidores de bombas de efluxo, mas também contra outros mecanismos de resistência aos antimicrobianos.

Palavras-chave: antimicrobianos; derivados do ácido desoxicólico; design baseado no alvo; inibidores de bombas de efluxo; *screening* de fenótipo; tioxantonas.

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Abbreviations and acronyms

ABC – ATP-binding cassette

AbgT – *p*-Aminobenzoyl-glutamate transporter

ADMET – Absorption, distribution, metabolism, excretion, and toxicity

ADP – Adenosine diphosphate

AHL – Acyl-homoserine lactones

AMP – Adenosine monophosphate

AMR – Antimicrobial resistance

ATCC – American Type Culture Collection

ATP – Adenosine triphosphate

Boc – *tert*-Buthyloxycarbonyl

CADD – Computer-aided drug design

CECT – Colección Española de Cultivos Tipo

CCCP – Carbonyl cyanide *m*-chlorophenylhydrazine

CCDC – Cambridge Crystallographic Data Centre

CLSI – Clinical and Laboratory Standard Institute

CMA – Corn meal agar

COMU – (1-Cyano-2-ethoxy-2-oxoethylidenaminoxy)dimethylamino-morpholino-carbenium

hexafluorophosphate

CTX – Cefotaxime

CV – Crystal violet

CV026 – *Chromobacterium violaceum* 026

DIEA – *N, N*-Diisopropylethylamine

DMEM – Dulbecco's modified Eagle medium

DMSO – Dimethyl sulfoxide

EDTA – Ethylenediaminetetraacetic acid

EP – Efflux pump

EtBr – Ethidium bromide

ESBL – Extended spectrum β -lactamase

ESI – Electrospray ionization

EZF – *Sphingomonas paucimobilis* Ezf 10-17

FDA – Food and Drug Administration

FI – Fluorescence intensity

FMM – *Flexibacter maritimus* medium

GC-MS – Gas chromatography-mass spectrometry

HMBC – Heteronuclear multiple bond correlation

HRMS – High resolution mass spectrometry

HSQC – Heteronuclear single quantum coherence

IC₅₀ – Half maximal inhibitory effect

IUPAC – International Union of Pure and Applied Chemistry

LB*-A – Modified Luria-Bertani agar

LB-B – Luria-Bertani broth

MATE – Multidrug and toxic compound extrusion

MBC – Minimum bactericidal concentration

MDR – Multidrug resistance

MFC – Minimum fungicidal concentration

MFS – Major facilitator superfamily

MHB II – Cation-adjusted Müller-Hinton broth

MIC – Minimum inhibitory concentration

MOPS – 3-(<i>N</i> -morpholino)propanesulfonic acid	RFI – Relative fluorescence index
MRSA – Methicillin-resistant <i>Staphylococcus aureus</i>	RND – Resistance-nodulation-division
mp – Melting point	SAR – Structure-activity relationship
MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	SBDD – Structure-based drug design
NMR – Nuclear magnetic resonance	SD – Standard deviation
NR – Neutral red	SDA – Saburaud Dextrose agar
OD – Optical density	SDS – Sodium dodecyl sulfate
ORTEP – Oak Ridge thermal ellipsoid plot	SMR – Small multidrug resistance
PAβN – Phenylalanine-arginine β-naphthylamide	SRB – Sulforhodamine B
PACE – Proteobacterial antimicrobial compound efflux	TFA – Trifluoroacetic acid
PBS – Phosphate-buffered saline	TLC – Thin-layer chromatography
P-gp – P-glycoprotein	TMS – Tetramethylsilane
PMZ – Promethazine	TSA – Tryptic Soy agar
QS – Quorum-sensing	TSB – Tryptic Soy broth
RF – Relative fluorescence	UV – Ultraviolet
	VAN – Vancomycin
	VRE – Vancomycin-resistant enterococci
	wt85 – <i>Chromobacterium violaceum</i> wild-type 85

Outline of the thesis

The present thesis is organized in seven main parts:

Chapter 1 – General introduction

In this chapter, a brief introduction about bacteria and antimicrobial resistance is given. This section comprises a short overview on the history of the discovery of antibiotics, as well as on the milestones achieved, and their downfall with the appearance of resistance of different origins. Particular emphasis is put onto the overexpression of efflux pumps, and what is being done to overcome multidrug resistance.

Chapter 2 – Aims

In this chapter, the main aims that were set for this work are presented.

Chapter 3 – Thioxanthenes as potential bacterial efflux pump inhibitors

The design and virtual screening on a relevant bacterial efflux pump of a library of 1000 aminated thioxanthenes is described. The virtual hit compounds were synthesized and structurally elucidated, and were evaluated for their potential as antimicrobial agents, and for the reversal of antibacterial resistance mechanisms, specifically the potentiation of antibiotics, inhibition of bacterial efflux pumps, biofilm formation and quorum-sensing. The safety of these compounds was evaluated by cytotoxicity assays in a mammalian and human cell lines, and their selectivity was evaluated through the assessment of the modulation of the human efflux pump P- glycoprotein.

Chapter 4 – Screening for antimicrobial activity and docking studies on structurally-diverse compounds with potential to circumvent antimicrobial resistance mechanisms

Throughout this chapter, a library of 175 structurally diverse compounds was screened for antimicrobial activity, and for the same reversal of antibacterial resistance mechanisms, namely the potentiation of antibiotics, inhibition of bacterial efflux pumps, biofilm formation and quorum-sensing, is described. Docking studies were performed to propose binding sites for the different molecules, and the respective compounds were tested against a mammalian cell line, as a way to assess cytotoxicity.

Chapter 5 – Deoxycholic amides: synthesis and antimicrobial activities

In this chapter, a deoxycholic amide was found as a hit compound for antibacterial activity, while still showing inhibition of bacterial efflux pumps. Following, the synthesis of

derivatives with the same scaffold is described, as well as their structure elucidation. The compounds were tested against a broad spectrum of bacteria and fungi and evaluated for their potential in inhibiting bacterial efflux pumps and biofilm formation. Their cytotoxicity was evaluated in a mammalian cell line.

Chapter 6 – Inhibitory studies in cyanobacteria

In this chapter, a screening of a small library of 15 compounds against *Synechocystis* sp. PCC 6803 and their TolC-deletion mutant is described, as a way to evaluate the interaction of the compounds with Slr1270, a TolC-like secretion system that cyanobacteria express as a detoxification pathway.

Chapter 7 – Conclusions

The main remarks and findings of this project are summarized in this chapter. Future perspectives and possible directions of this work are also presented.

References

The references used are present in the end of each chapter and follow the Vancouver style guide. The bibliographic search engines used were PubMed, Science Direct and Google Scholar.

Chapter 1 – General introduction

1.1 Bacteria and antibiotics

Bacteria are prokaryotic microorganisms that are present ubiquitously, including on the surface and inside the human body. They play important and essential roles, including the defense against other pathogens, the metabolism of natural products and the interaction and improvement of the immune system, done by bacteria present in skin and mucous membranes (1-3). Furthermore, other important roles played by bacteria happen in the digestive system, where digestion, endocrine regulation, toxin elimination, and drug metabolism are paramount features (4, 5).

Despite all these advantages associated with these microorganisms, a small percentage of bacteria are also pathogens, being able to invade the host and cause damage, leading to the development of diverse diseases. For this to happen, bacteria must have virulence, meaning they must be able to invade the host and successfully spread, as well as display toxicity (6, 7). These factors combined with the susceptibility of the host to the infection, determine the degree of the disease process (7).

If, on the one hand, the host's immune system has evolved and adapted to become less susceptible to bacterial infections, on the other hand bacteria have also found ways to modify their virulence to penetrate the host's defenses. This bacterial adaptation has also been triggered by the use of antibiotics, which has significantly halted the spread of pathogens (8).

Another source of bacterial infections is related to opportunistic pathogens, which are microorganisms that would not ordinarily cause disease, but take advantage of immunocompromised hosts or underlying conditions in order to cause a disease. These are more prevalent in hospital settings, and are commonly caused by the commensal bacteria, the ones responsible of carrying out vital functions for the host's proper function, and even survival (9-12).

It is, therefore, vital to employ drugs that can successfully fight against pathogenic and opportunistic bacteria when necessary. In this scope, antibacterial drugs arise as a useful and essential strategy. These drugs made up the greatest medical breakthrough of the 20th century, making therapeutics, as it is nowadays, possible. In fact, it has been documented that pre-historic civilizations used plants and molds to treat infections, with traces of tetracycline of unknown origin being discovered in ancient skeletons (13, 14).

However, it was not until the late 19th century that a causal relationship was established between bacteria and pathology, which led to an increase in treatment experimentation. As a consequence, salvarsan was discovered by Paul Ehrlich in the 20th century, and approved as the first antibacterial, for the treatment of syphilis, caused by *Treponema pallidum*.

However, it was not until the 21st century that it was disclosed as a mixture of **1.1** and **1.2** (**Figure 1.1**) (15, 16).

The next breakthrough in antibacterial therapy was the discovery of pyocyanase, which was the first antibacterial to be produced industrially used in a hospital setting. This was isolated from a macerate of *Pseudomonas aeruginosa* and proved to have better efficacy at infections caused by Gram-positive bacteria. Even though its activity was attributed to an enzyme, it has later been proved that a mixture of the pigment and quorum-sensing (QS) molecule pyocyanin (**1.3**, **Figure 1.1**) and 2-alkyl-3-hydroxy-4-quinolone (**1.4**, **Figure 1.1**) were the active ingredients in this formulation. However, since the supernatant was used, and not the pure compounds, the efficacy was often variable (17-19).

Through a systematic screening approach, sulfamide drugs were discovered. In fact, sulfonamidochrysoidine (**1.5**, **Figure 1.1**), commonly called Prontosil, was the first synthetic antibacterial, and possessed a sulfa moiety. It was also noticed that **1.5** was just a precursor to the actual therapeutic structure, sulfanilamide (**1.6**, **Figure 1.1**), which had been used as a dye before its pharmaceutical potential for the treatment of infections caused by *Streptococcus* sp. was uncovered. The fact that **1.6** was easily synthesized and modified led to the discovery of several sulfamide derivatives (20-22).

Despite these efforts and the fact that sulfamides were considered “miracle drugs”, the true antibacterial “golden age”, one of the most profitable periods for chemotherapy, began with the serendipitous discovery of penicillin. In fact, the lack of growth of *Staphylococcus aureus* in a Petri dish contaminated with *Penicillium rubrum* mold led to the conclusion that this fungus was able to produce antibacterial compounds (22). Efforts were placed into discovering the compound responsible of this activity, and the structure of penicillin G (**1.7**, **Figure 1.1**), which possessed a β -lactam moiety, was elucidated and, not long after, mass produced (23).

Henceforth, medicine and surgery went through a revolution, as antibacterial drugs were proven successful in treating infections in patients with comorbidities, as well as kept sepsis in settings where needed. They have also been responsible for increasing the lifespan of people, in developed and developing countries, especially when it comes to food- and water-borne diseases, as well as poverty-related infections (24).

The discovery of penicillin has opened the doors not only to new medical practices, but also to research into natural matrices as a source of antibacterial compounds, yielding new scaffolds (13, 21, 24). Another reason that favored research into this field, leading to the synthesis of a plethora of derivatives, was the appearance of strains resistant to antibiotics (25).

At this point, microorganisms capable of producing compounds that could destroy other microorganisms were given a spotlight. It was within this scope that the filamentous

soil-dwelling bacteria Actinomycetes were discovered as a very prolific source of antibacterial compounds. In fact, the first active compound against tuberculosis, the aminoglycoside streptomycin (**1.8, Figure 1.1**), was first discovered in a colony of *Streptomyces* sp., actinomycetes responsible for the production of compounds that can be used against infections, cancer, or immunosuppression (26-28).

The following years saw the unveiling of over half the drugs currently in use for the treatment of bacterial infections, most of them produced by *Streptomyces* sp.. Tetracycline (**1.9, Figure 1.1**) and chloramphenicol (**1.10, Figure 1.1**), inhibitors of bacterial protein synthesis were major breakthroughs when they were isolated from *Streptomyces aureofaciens* and *Streptomyces venezuelae*, respectively (29, 30). Then the macrolide erythromycin (**1.11, Figure 1.1**) was isolated from *Saccharopolyspora erythraea* (formerly *Streptomyces erythraea*) and vancomycin (**1.12, Figure 1.1**), a glycopeptide, from *Amycolaptosis orientalis* (formerly known as *Streptomyces orientalis*) (31, 32). Other bacteria were also important, such as *Paenibacillus polymyxa*, which yielded the antibacterial colistin (**1.13, Figure 1.1**) (33).

The explanation for the production of these antibacterial compounds by actinomycetes lies in the fact that bacteria need to produce chemical defenses or attacks, in order to thrive in an environment such as soil. These specialized metabolites can also serve as signaling molecules to interact with hosts or could have arisen as an interaction provided by the colonization of plant roots (34-36).

But soil-living organisms were not the only prolific source of antibacterial drugs found in the 20th century. In fact, the fungus *Cephalospora acremonium*, present in sewage waters, was found to be the producer of cephalosporins, a class of antibacterial drugs that now counts with five different generations according to their antimicrobial activity (37). Three active compounds were found in this fungus, being cephalosporin C (**1.14, Figure 1.1**) the one with broader spectrum, which ended up being a lead compound for the synthesis of more potent derivatives such as cephalotin (38).

Even though these natural compounds were a major contribution to the definition of health in the 20th century, these were not the only ones. In fact, other microorganisms also served as a source of templates for the semi-synthesis of antibacterial drugs. One example is the carbapenem meropenem (**1.15, Figure 1.1**), obtained by the modification of a compound isolated from *Streptomyces cattleya*, or the monobactam aztreonam (**1.16, Figure 1.1**), based on a compound isolated from *Chromobacterium violaceum* (21, 39).

Another strategy employed to obtain new antibacterial compounds was chemical synthesis. This strategy was first employed with the arsphenamines and sulfonamides but took a back seat when there was the boom of antimicrobials with great potential isolated from microorganisms. In this scope, the most relevant class of antibacterial drugs

discovered by synthesis is the quinolones. While manufacturing the antimalarial chloroquine, one impurity was noticed to present a moderate activity against Gram-negative bacteria. Studies put into place led to the synthesis of the structurally related nalidixic acid (**1.17**, **Figure 1.1**), which presented a narrow spectrum, being mostly used in the treatment of urinary tract infections caused by enteric bacteria. However, this drug paved the way for the breakthrough development of the improved, broad-spectrum fluoroquinolones, such as ciprofloxacin (**1.18**, **Figure 1.1**). Fluoroquinolones were active against both Gram-negative and Gram-positive pathogens, as well as *Mycobacterium tuberculosis*, making them one of the blockbusters for the treatment of infectious diseases (40, 41).

In the beginning of the 21st century, linezolid (**1.19**, **Figure 1.1**), a synthetic oxazolidinone, was introduced in therapeutics after a screening program. This constituted a completely new chemical class of antibacterial drugs, with no intrinsic resistance and no rapid *in vitro* resistance development. Linezolid arose as an option to treat MDR infections caused by Gram-positive strains (42-44). It binds to the 50S subunit of the bacterial ribosome and blocks its complexation with the 30S subunit. This leads to the assembly of an unfunctional complex, causing defective translation, as the peptide elongation is compromised. Consequently, virulence factors are not synthesized, which is particularly useful in *Staphylococcus* sp. and *Streptococcus* sp.. Furthermore, the target is different than that of macrolides, which makes linezolid evade the resistance mechanisms developed against them (45).

Synthetic efforts have successfully yielded many antibacterial compounds and have successfully circumvented problems that had appeared. For instance, methicillin (**1.20**, **Figure 1.1**) was synthesized as a way for β -lactams to resist the action of penicillinases, achieved by the introduction of bulky substituents (46). Omadacycline (**1.21**, **Figure 1.1**), a recently approved aminomethylcycline antibacterial, was designed to prevent both efflux and ribosomal protection (47).

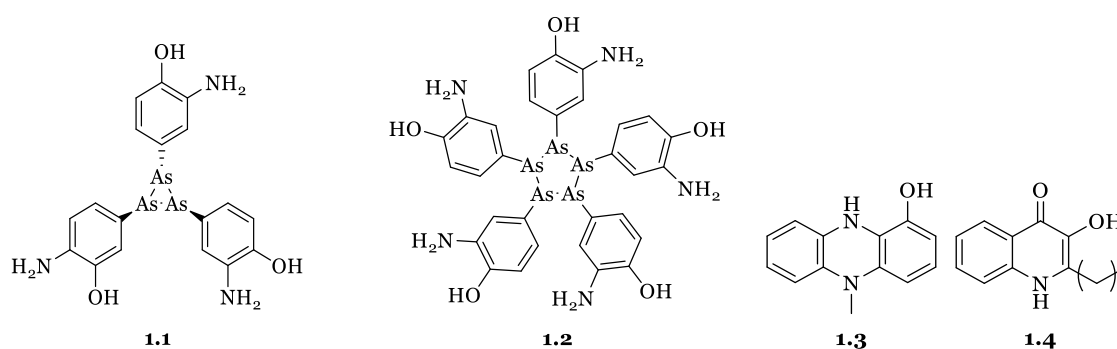


Figure 1.1 Structures of the most relevant antibacterial drugs.

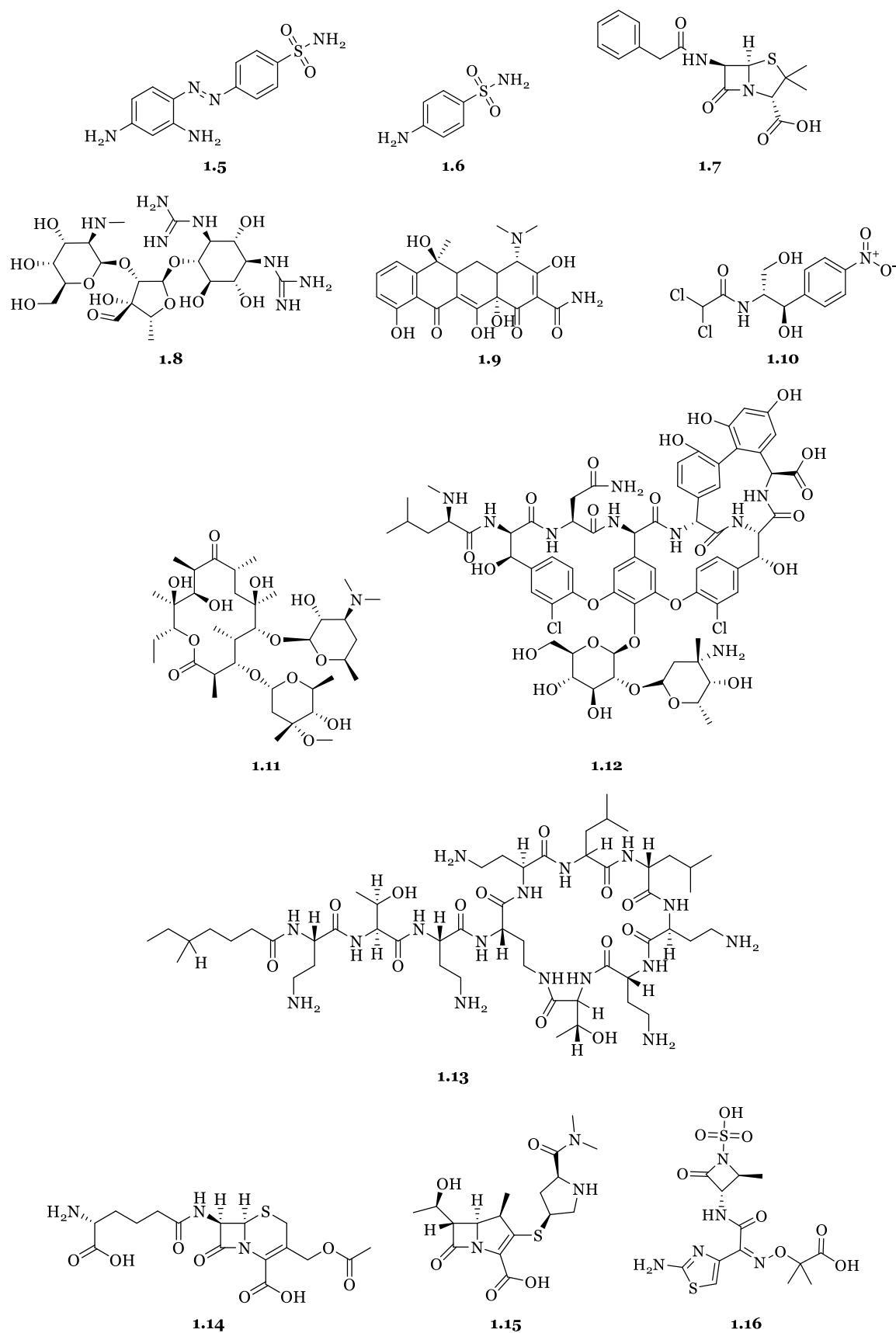


Figure 1.1 Structures of the most relevant antibacterial drugs (cont.).

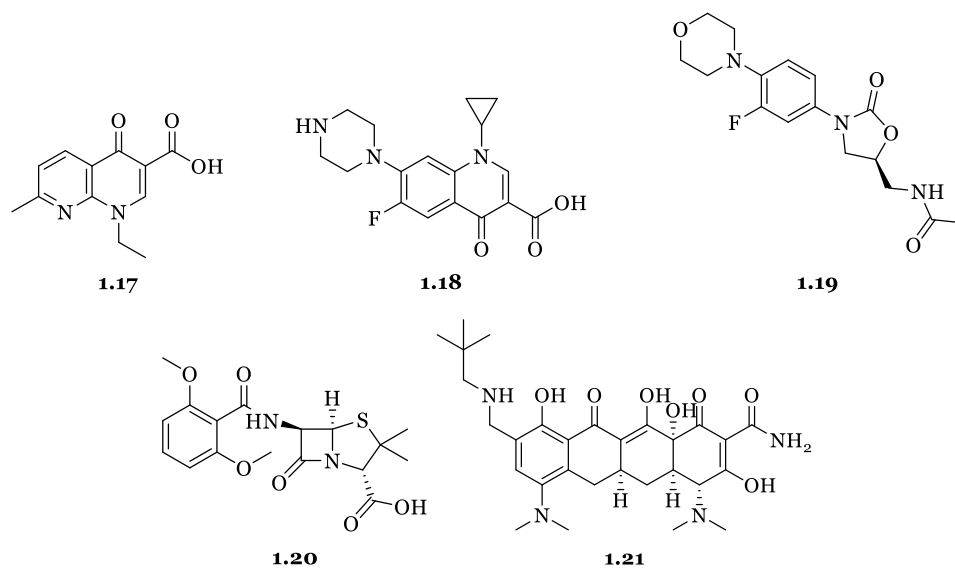


Figure 1.1 Structures of the most relevant antibacterial drugs (cont.).

In current days, the search for new and effective antibacterial compounds is a pressing demand, given that the current state of antibacterial resistance is turning all antibacterials in therapeutics ineffective (48). However, several hurdles still hinder the discovery of antibacterial drugs, such as the lack of attractiveness of this field for potential investments (49). In fact, in 2021 no new antibacterials were approved by the U.S. Food and Drug Administration (FDA) (50).

New platforms are needed to disclose new molecular entities with antibacterial activity, so that antibacterial resistance is no longer a threat. High-throughput screening (HTS) and the discovery of new targets are examples of those platforms. A better understanding of the physicochemical properties essential to antibiotics is also necessary, as antibacterial drugs are generally very diverse from each other, as can be seen in **Figure 1.1** (49, 51). Drug repurposing can also pose as a solution for this challenge, having the extra advantage of being an accelerated process, as their pharmacological and toxicological profiles are already well defined. In this context, repositioned drugs can act as antibacterials *per se*, or as antibacterial adjuvants (52, 53).

One other approach would be the restoration of the activity of antibacterials that are currently used therapeutically, via the blockage of antibacterial resistance mechanisms (54). The following chapters will focus on those mechanisms and ways to avoid/inhibit them.

1.2 Antimicrobial resistance

Antimicrobial resistance (AMR) is characterized by the capacity of microorganisms to resist the effect of antimicrobial agents, consequently leading to loss of efficiency (55). It is predicted to be more deadly than all the cancers combined by 2050 (56).

AMR can be intrinsic. This comes as no surprise since bacteria produce antibacterial compounds themselves and must be able to survive them. These mechanisms are usually part of the core genetic material of the microorganism and are encoded by chromosomes. Furthermore, some antibiotics simply do not exert any action in specific bacteria, as is the example of the impermeability of Gram-negative bacteria to large glycopeptides such as vancomycin (**1.12, Figure 1.1**), provided by the outer membrane. Intrinsic resistance usually confers a lower level of resistance, which can be problematic in commensal bacteria that become opportunistic pathogens in patients whose immune system has been compromised (57, 58).

However, the major problem regarding AMR comes when it is acquired. As previously mentioned, antibacterial drugs completely revolutionized therapeutics, bringing several advantages such as increased life expectancy and increased survival odds for people with comorbidities. Therefore, there was an increase in the public demand for antibacterials, leading to abuse. In fact, before penicillin was introduced in therapeutics, there had already been descriptions of *Staphylococcus* strains that could resist to this drug. This happens because bacteria possess remarkable genetic plasticity, and the overuse of these drugs led to the emergence and dissemination of resistant bacterial strains (24, 59).

There are several ways by which bacteria can evolve towards antibacterial resistance. The most common way is to inherit resistance genes from reproduction, but a spontaneous mutation can happen, favoring the ability of bacteria to evade the activity of an antibacterial. They can also transfer genes horizontally, between different species, and mobile genetic elements, such as plasmids. Noteworthy is also the fact that the overuse of antibacterials will remove susceptible bacteria and, therefore, remove competitors for resistant bacteria, allowing them to reproduce freely and without competition (60, 61).

The excessive and inappropriate use of antibacterial drugs are also factors responsible for selecting resistant strains. Currently, most antibiotics are prescribed empirically, without taking the susceptibility of the bacteria into account. Moreover, they are often incorrectly prescribed for viral infections, due to the similarity of symptoms observed. In the light of the current SARS-CoV-2 pandemic, antimicrobial resistance can also be favored due to the drug practices and incorrect and ineffective antibacterial prescription (62-64). Social reasons can also be used to justify the proliferation of resistant microorganisms. With globalization and urbanization, the entire global population has turned into a single biological entity, making the transmission and dissemination of infectious diseases easier (64, 65).

Another source of AMR is the increasing extensive use of antibacterials in agriculture and in the food industry. It has become a common practice to use antibiotics as profilactic agents in livestock, which results in products of higher quality and better yields, and the

overall improvement of their health. However, residues of these drugs are then ingested by humans, resulting in the transfer of resistant bacterial strains. This also has consequences concerning the environmental dissemination of resistant bacterial strains, which will be discussed in **Chapter 6** (66). The use of antiseptics for hygiene and cleaning could also be a contributor for the acquisition of resistant bacteria, not only for the reasons stated previously, but also due to the limitations they impose on the development of immunity to environmental pathogens (67).

There are several mechanisms through which AMR is manifested, which can be categorized into three main groups: inactivation of the drug, either by hydrolysis or chemical modification; target modification, both by genetic mutation or post-translational modification; and minimization of the concentration of the antibacterial, which can happen by decreased penetration or increased efflux (68). These mechanisms are illustrated in **Figure 1.2**.

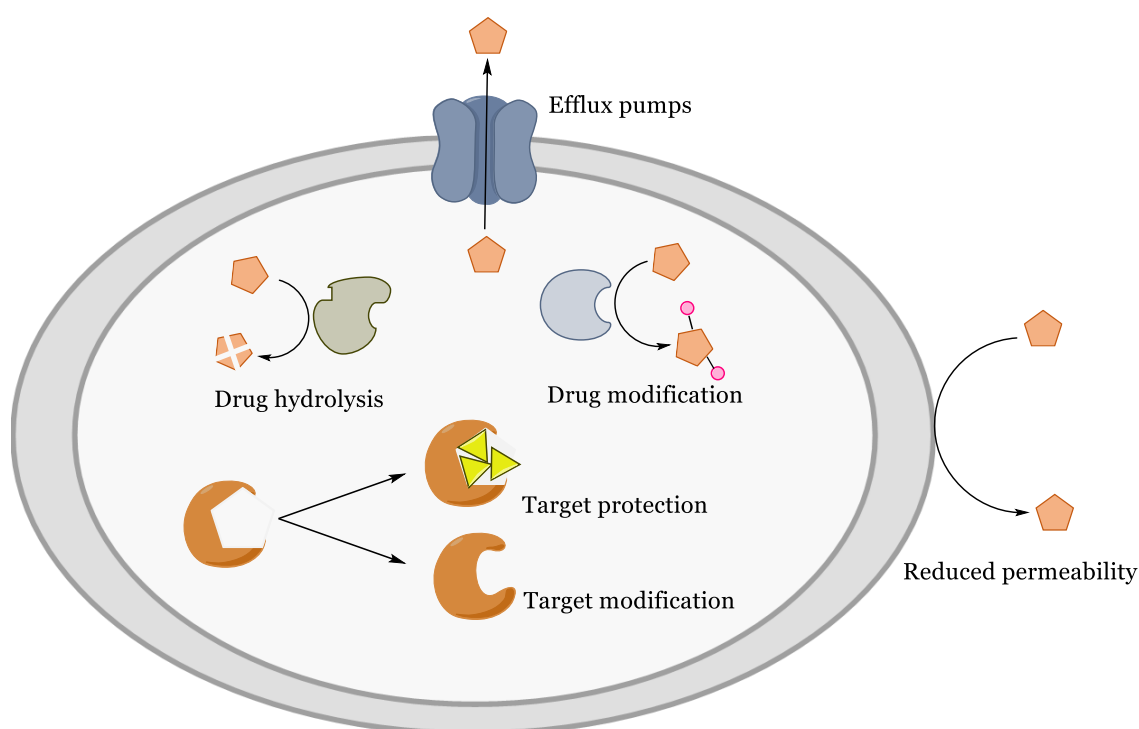


Figure 1.2 Mechanisms leading to AMR in bacteria [adapted from (68, 69)].

1.2.1 Drug inactivation

The inactivation of the antibacterial drug was one of the first mechanisms to be discovered and studied (70). It is also one of the most successful strategies used by bacteria to handle the presence of threatening drugs. This can happen either by modification of the drug molecule, or by its destruction, which in both cases makes the drug unable to interact with its target and exert its action (68, 71).

Inactivating modifications are more prevalent in antibacterials whose mechanism of action is the inhibition of protein synthesis, at the ribosome. The most common chemical modifications are acetylation, phosphorylation and adenylation, which increase steric hindrance and prevent the drug from effectively interacting with its target. Aminoglycoside modifying enzymes are the most common example of this phenomenon, being the most predominant resistance mechanism to this class of antibacterial drugs and can modify both the hydroxy and the amino groups in the molecular structure (72, 73).

One notable example of antibacterial destructing enzymes is that of β -lactamases, enzymes produced by Gram-positive and Gram-negative bacteria, capable of hydrolyzing the amide moiety in the β -lactam scaffold, thus rendering the antibacterial ineffective. These enzymes can be found in the periplasm of Gram-negative bacteria, and in the surroundings of Gram-positive bacteria, as the latter secretes β -lactams to their exterior (74, 75). When these enzymes are capable of inactivating third generation cephalosporins, they are called extended-spectrum β -lactamases (ESBL) (76).

1.2.2 Target modification

Bacteria have also developed strategies to change their target, in a way that can prevent the specific binding of antibacterials while maintaining their function. However, this is only true for RNA polymerase and DNA gyrase, as mutations in other targets must be accompanied by further changes in the bacterial cell, in order to adapt to the changes made in the target. These can happen via direct target modification or by target protection, thus preventing the antibacterial reaching its binding site, both resulting in a decreased affinity to the antibacterial drug (68, 71, 77).

Target modifications can be manifested through alterations of the binding site through the action of enzymes, single-nucleotide mutations to the genes encoding the target and/or target bypass or replacement. These are responsible for resistance to β -lactams, quinolones, macrolides, among others (58, 71, 77).

On the other hand, the protection of the target happens most often post-translationally and consists in the chemical modification of residues, mainly by acetylation, phosphorylation, methylation, and hydroxylation, the attachment of molecules such as glucose, adenosine monophosphate (AMP) or adenosine diphosphate (ADP), the covalent binding of small proteins, or the modification of side-chain residues. This is a particularly important resistance mechanism to macrolides, chloramphenicol, aminoglycosides, and quinolones, among others (68, 78, 79).

1.2.3 Permeability changes

Another resistance mechanism relates to the decrease of the intracellular concentration of antibacterial, either by a reduction in the influx or an increase in the efflux. Decreased permeability causes a clear problem, as the majority of antibacterial drugs currently used need to access their targets intracellularly. This acquires an even greater difficulty in Gram-negative bacteria, where the drug must pass through both the outer membrane and the cytoplasmic membrane (80).

Changes in permeability affect mostly hydrophilic drugs, as is the case of β -lactams and some fluoroquinolones, since these compounds use porins, water-filled diffusion channels, as a means to cross the outer membrane. This is evidenced by the fact that *Pseudomonas* sp. and *Acinetobacter baumannii*, which have a lower susceptibility for β -lactams and fluoroquinolones, also express a reduced number of porins (81-83).

Porins can be modified, which leads to the development of AMR. This can happen through changes in the expression of porins, in the type of porins expressed and in the decrease of their function. One notable example is OmpF, a non-specific outer membrane channel, whose mutation impairs the activity of β -lactams in several Gram-negative strains, such as *Escherichia coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, among others (84). However, modifications in these proteins often leads to low AMR, being this phenomenon usually paired with other resistance mechanisms, such as the overexpression of efflux pumps (EP) (85).

EP are membrane proteins, responsible for the transport of several substrates to the exterior of the bacterial cell, being antibiotics prone to this extrusion. These structures are ubiquitous to bacteria, as it is a mechanism of detoxification. However, these can also lead to multidrug resistance (MDR), especially when overexpressed, making them one of the most dangerous resistance mechanisms. In fact, antimicrobial resistance is only possible due to the existence of these structures, as they remove most compounds non-specifically, allowing the microorganism to gain time and develop more specific resistance mechanisms. Furthermore, EP can also be a means for bacteria to communicate and increase their virulence, as well as to produce biofilms (8, 68, 86-89).

All bacteria carry genes that code for EP, and some of those genes have been mobilized onto plasmids that can be transferred between bacteria. This field has been continuously studied, and new EPs are still being discovered. Some pumps are more specific regarding substrates, as is the case of the TetA pump, which confers resistance to tetracycline but not to tigecycline, while others can take a multiplicity of structurally-diverse substrates, as is the case of the AcrAB-TolC efflux system, that comprises several large and poorly structured binding sites, that serve as accommodation for a wide range of unrelated compounds (51, 90, 91).

The most important bacterial EP can be divided into six families, according to their sequence pattern and structural features, substrate specificity, amount of transmembrane spanning regions and components, and energy sources (92). These are the adenosine triphosphate (ATP)-binding cassette (ABC), the major facilitator superfamily (MFS), the resistance-nodulation-division (RND), the small multidrug resistance (SMR), the multidrug and toxic compound extrusion (MATE), and the proteobacterial antimicrobial compound efflux (PACE) (93, 94). **Figure 1.3** illustrates the classes of bacterial efflux pumps. These pumps are not exclusive to bacteria, and can be transversal to multiple living beings (95). Recently, a new class of antimetabolite transporters has been described, the *p*-aminobenzoyl-glutamate transporter (AbgT), which has been demonstrating its relevance as a sulfonamide transporter (96).

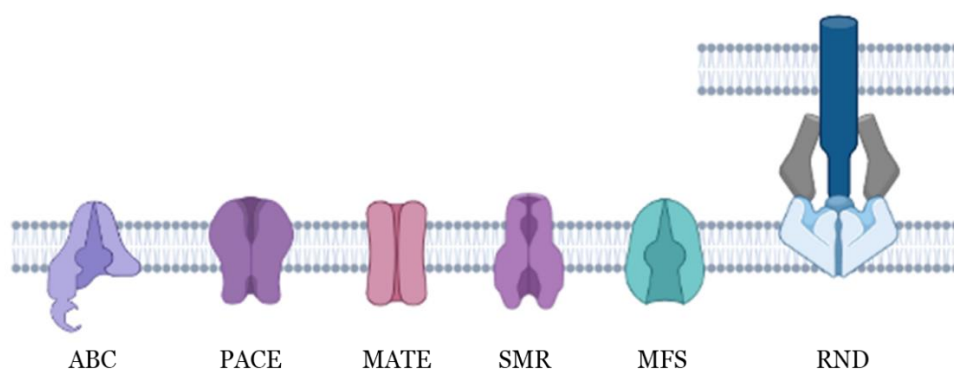


Figure 1.3 Classes of bacterial efflux pumps.

The ABC are considered primary transporters, as they use ATP as energy source. This family is also relevant in chemotherapy failure in humans, particularly the EP P-glycoprotein (P-gp), or ABCB1. ABC transporters are not exclusively exporters, and in fact can have other functions such as importing micronutrients inside the bacteria, and also taking part in the translation of mRNA and in the repairing of DNA (97, 98). This family is of great importance in Gram-positive bacteria, albeit they are not as relevant as other families of EP. Actually, even though ABC transporters have led to resistance to aminoglycosides, tetracyclines, macrolides and quinolones, to name a few, they seem to be better at promoting bacterial growth in nutrient-deficient conditions, due to their ability to transport nutrients inwards (98). The same is true for Gram-negative bacteria, where the studies on ABC transporters are even sparser. It is known, however, that these transporters have a tripartite structure, and can be responsible for the efflux of norfloxacin and tetracycline in some strains (99). On the other hand, the other EP families use a chemical gradient as a source of energy, being therefore considered secondary transporters. Their

distinction lies, hence, on sequence conservation and/or similarities in their functions (100).

The PACE family has only recently been recognized as MDR transporter, with no tertiary structure available to date. This family has been able to avoid being detected due to its very narrow substrate specificity, of which chlorohexidine and acriflavine belong to. The first description of a PACE pump was the AceI, from *Acinetobacter baumannii*, assembled by four transmembrane helices, similarly to other families, but with a distinct sequence, and highly conserved sequences at the cytoplasmic boundary of each helix. After that, AceI homologues have been discovered in other Gram-negative bacteria, such as *P. aeruginosa*. Concerning energy source, it is thought that these pumps use the proton gradient to ensure their function (101-103).

The MATE family is ubiquitous, spanning across humans, plants, and bacteria. These transporters belong to the multidrug/oligosaccharidyl-lipid/polysaccharide flippase superfamily, being MATE the only one family within this to have so far demonstrated drug exporting capability. They use an electrochemical gradient as energy source, with ions such as H⁺ or Na⁺ having an important role, and can recognize and extrude mainly organic cations. Structurally, they contain twelve transmembrane helices and short hydrophilic loops, and the most relevant members of this family are the YdhE pump in *E. coli* and the NorM pump in *Vibrio parahemolyticus* and *Neisseria gonorrhoea* (94, 104, 105).

The SMR family comprises the smallest efflux pumps, as its name indicates. They are the major family within the drug/metabolite transporter superfamily, capable of exporting metal ions, drugs, and endogenous metabolites, particularly small cationic molecules. Regarding their constitution, they typically consist of a little over 100 amino acid residues, grouped into four transmembrane helices. Remarkable efflux pumps from the SMR family are, for instance, the EmrE of *E. coli* and the QacC of *S. aureus* (106-108).

Even though these families play a role in MDR, the most important are the MFS and the RND. The MFS pumps are ubiquitous active transporters that use the proton-motive force as a source of energy. They can operate as uniporters, antiporters, or symporters, and are known to extrude a wide variety of substrates, including drugs, organic and inorganic ions, sugars, metabolites, amino acids, peptides, and nucleosides. Usually, MFS transporters are hydrophobic and helical, with twelve transmembrane domains. This family constitutes the most relevant efflux system in Gram-positive bacteria, with the NorA pump, present in the core genome of *S. aureus*, being considered the most efficient efflux pump, even though its tertiary structure has not been elucidated yet (109, 110). This is different in Gram-negative bacteria, where MFS pumps have tripartite structure, similar to RND and ABC pumps, formed by the inner membrane component, which does not encompass the periplasm, an outer membrane channel, and an adaptor protein that seals the former components

together (111). One notable example of a Gram-negative MFS pump is the EmrD, present in *E. coli* (112).

On the other hand, when it comes to MDR in Gram-negative bacteria, the RND family acquires a determinant role. These ubiquitous efflux systems are substrate/H⁺ antiporters and are made up by a tripartite protein assembly that spans the Gram-negative double membrane. There is an inner membrane protein, responsible for drug selectivity, where the drug/H⁺ binding occurs, an outer membrane channel and a periplasmic adaptor, which connects the other two portions together. One example is the AcrA-AcrB-TolC efflux system (**Figure 1.4**), also known as AcrAB-TolC, where AcrA refers to the dimeric periplasmic adaptor, AcrB to the trimeric inner membrane efflux pump and TolC to the trimeric outer membrane channel (89, 113, 114).

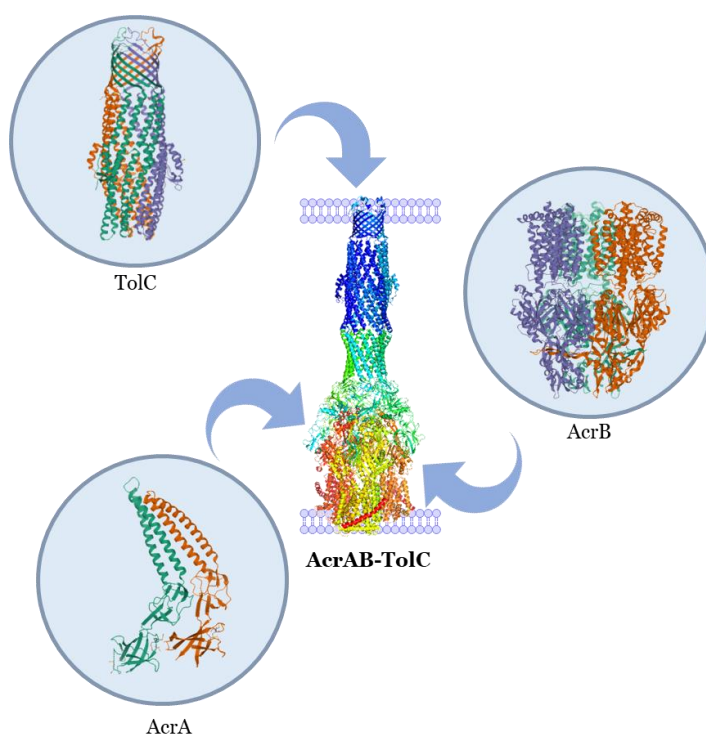


Figure 1.4 Structure of the AcrAB-TolC efflux system (PDB 5O66) and the different portions that constitute this efflux complex (AcrA – PDB 2F1M; AcrB – PDB 1IWG; TolC – PDB 1EK9).

These EPs have the broadest specificity for their substrates, being able to preferably promote the efflux of amphiphilic and hydrophobic antibiotics, dyes, antiseptics, heavy metals, ethidium bromide, and other compounds (115-117). Although these efflux pumps acquire a great importance in Gram-negative bacteria, they are also present in Gram-positive microorganisms. Recently, there have been descriptions of an RND pump in *S. aureus*, FarE, thought to be involved in the transport of fatty acids (118, 119). Similarly, in *Mycobacterium tuberculosis*, there is an RND-like EP, MmpL3, which has been described as a mycolic acid exporter (120, 121).

Furthermore, EPs play an important role in other resistance mechanisms. In fact, it is likely that EPs are essential to the formation of biofilm, mainly through the efflux of extracellular polymeric substances, which will constitute the biofilm itself. They can also indirectly regulate the expression of genes related to biofilm formation and modulate the adhesion to other cells or surfaces. Additionally, EPs can modulate QS, a chemical communication mechanism in bacteria, which is not only important in the regulation of biofilm but can also be regarded as crucial for various virulence mechanisms (86, 122, 123).

Considering the resistance mechanisms that are known so far, it comes as no surprise that efforts have been put towards the discovery of new compounds that can circumvent these antimicrobial resistance mechanisms. This kind of compounds, called resistance breakers or antimicrobial adjuvants, have been reviewed over the past years, with natural products, synthetic compounds, or drug repurposing at the center of this matter (52, 54, 124-126). Another way to fight AMR is to focus on prevention, added to the mitigation that needs to happen for current resistant microorganisms. This happens through the development of vaccines for specific bacteria, prone to become resistant (127-129).

The focus of this work was the development of bacterial EP inhibitors (EPI), and therefore this is the class of resistant breakers that will be discussed throughout the next chapter.

1.2.3.1 Efflux pump inhibitors

Finding an effective EPI is an arduous task, as some requisites must be met. Apart from being safe and possessing a good absorption, distribution, metabolism, excretion, and toxicity (ADMET) profile, an EPI should not display antibacterial activity, as it would lead to the selection of resistant mutants and, ultimately, to its inutility. Moreover, and since EPs are transversal to every life form, a successful bacterial EPI must only target bacterial EPs and have no action against other EPs. Lastly, it should be easily synthesized and economically feasible (100, 130, 131).

In order to guide the design of an effective EPI, it must be considered that the inhibition of EPs can happen at different levels. Tampering with the expression of the pump is one of them, and the use of antisense nucleic acids or oligonucleotides has been proved to inhibit the expression of *acrAB* in *E. coli* and *cmeABC* in *Campylobacter jejuni*, respectively. The usefulness of this, however, remains to be proven (132, 133).

Another mechanism of inhibition is at the level of the assembly, since the performance of the pump will be affected should it not be correctly assembled or fused into the membrane. There is still scarce information about this, as there is still much to be learned about the assembly of efflux pumps, particularly the tripartite ones. It was shown that the

interaction between AcrA and AcrB could be inhibited by specifically designed ankyrin repeat proteins (89, 134).

Interference with the energy source can also pose as an effective mechanism for EP inhibition. For instance, vanadate (**1.22**, **Figure 1.5**) can inhibit ABC-type pumps, likely by replacing inorganic phosphate, and carbonyl cyanide *m*-chlorophenylhydrazine (CCCP, **1.23**, **Figure 1.5**), a proton motive force inhibitor, is an inhibitor of RND pumps (135, 136).

Another strategy to avoid efflux is the modification of antibiotics in a way that makes them unrecognizable by EPs. Considering that EPs take such a wide variety of substrates, this task is not an easy one. However, one example of a success is omadacycline (**1.21**, **Figure 1.1**), an approved drug that can overcome efflux-mediated resistance (47, 89, 137).

Finally, EPIs can inhibit EPs by blocking them. This is particularly important in the RND family, due to their tripartite structure. In this scope, inhibitors can be designed to bind competitively to the substrate pocket, which prevents antibacterial drugs from binding there and, therefore, be expelled. Phenylalanine-arginine β -naphthylamide (PA β N, **1.24**, **Figure 1.5**), regarded as the standard for EP inhibition assays, is thought to competitively displace the substrates from their binding sites in the AcrB portion of the AcrAB-TolC efflux system, while increasing membrane permeabilization. Although PA β N has been shown to inhibit the efflux of some drugs, such as fluoroquinolones, the same could not be noted for β -lactams, which can be explained by the existence of more than one substrate-binding site (138-140).

Another remarkable example is the pyranopyridine MBX2319 (**1.25**, **Figure 1.5**), which acts similarly to PA β N and can potentiate the action not only of fluoroquinolones, but also of β -lactams (141, 142).

The development of conjugates of antibiotics and EPIs can also pose as an attractive choice for EP inhibition, particularly in the case of Gram-negative bacteria. One such example is the use of a tobramycin vector coupled with a known EPI against MDR *P. aeruginosa*. The choice of tobramycin as a vector reduces intrinsic resistance, as it can cross the outer membrane via self-promoted uptake of aminoglycosides. It also evades efflux, as aminoglycosides are only substrates of the RND efflux system MexAB-OprM and can disrupt the proton motive force due to their amphiphilic nature. Remarkably, a conjugate of tobramycin and paroxetine (**1.26**, **Figure 1.5**), an antidepressant and known RND EPI, was able to decrease the MIC of minocycline by 32-fold in *P. aeruginosa* PAO1, a strain predicted to overexpress RND EPs. Noteworthy is the fact that compound **1.26** alone has a MIC 32 times higher than tobramycin alone (143, 144).

Regarding the sources of EPIs, they can be divided into three major groups: natural products, (semi)synthetic compounds, and drug repurposing. In terms of natural products, several distinct classes have been described, ranging from terpenes (145-159), to flavonoids

(160-177), glycosides (156, 168, 178-180), polysaccharides (181, 182), polyphenols (183-187), quinones (183), curcumin (188), coumarins (189-191), and alkaloids (192-195). Among the latter, reserpine (**1.27**, **Figure 1.5**), an indole alkaloid, acquires a great importance. Reserpine can also be considered a case of drug repurposing, which is the identification of new uses for approved drugs that fall out of their original scope, as it had previously been approved by the FDA as an antihypertensive agent, which also displayed antipsychotic properties (196). Considering EP inhibition, reserpine has been on the spotlight since the end of the 20th century, where it was described as an inhibitor of the Bmr pump present in *Bacillus subtilis*, belonging to the MFS. Further studies also showed its potential as a reverser of tetracycline resistance in *S. aureus* previously resistant to this drug, as well as a NorA inhibitor. In fact, reserpine is one of the most used standards against which new compounds with EPI activity are tested in Gram-positive bacteria. Recently, reserpine also showed potential at inhibiting AcrB, opening this drug's potential as an RND EPI. However, reserpine displays neurotoxicity at the concentrations needed to exert EPI activity, making it ineffective for human use (197-200).

In addition, reserpine also proved to be effective against the formation of biofilm in *S. aureus* and *P. aeruginosa* strains, as well as an inhibitor of virulence, through the blocking of QS. These mechanisms can relate to EPs, as these membrane structures are responsible for the efflux of extracellular polymeric substances, needed for the formation of bacterial biofilm, and QS molecules. They are also responsible for transporting molecules that will induce genes that ultimately will lead to biofilm formation, and EPs can also promote or inhibit cellular aggregation (86, 201, 202).

Some natural products have been the template for the synthesis of new EPIs by semisynthesis. The alkaloid piperine (**1.28**, **Figure 1.5**) has shown potent NorA inhibition. The synthesis of derivatives showed a greater inhibition than the natural product itself, with compound **1.29** (**Figure 1.5**) being eight times more potent than **1.28** (203, 204). Another alkaloid, berberine (**1.30**, **Figure 1.5**), a known antibacterial, was conjugated with an EPI, yielding compound **1.31**, which was much more effective than berberine alone as an antibacterial and was able to inhibit the MFS NorA pump at a very low concentration (205). Recently, berberine was described as an EPI for the RND pump MexAB-OprM in *P. aeruginosa* (206, 207).

Plenty of synthetic efforts have been placed in finding compounds that revert the excessive efflux of antibacterials. PA β N (**1.24**) and MBX2319 (**1.25**) are examples of compounds that have been synthesized and proven effective as RND EPIs (138-142). Considering the MFS, a series of 1,8-naphthyridines sulfonamides was synthesized with the aim of inhibiting NorA and potentiating norfloxacin in a resistant strain of *S. aureus*. Among the compounds obtained, compound **1.32** (**Figure 1.5**) showed remarkable

potential, being able to decrease the MIC of norfloxacin by 128-fold in a wild-type strain, while displaying a 16-fold MIC reduction in a mutant strain overexpressing the *norA* gene (208).

Drug repurposing is a strategy which presents a wide array of advantages in multiple levels. First, the pharmacological and toxicological profile of the reference drug has already been established and approved, as well as its efficacy. Second, it poses a faster strategy in terms of drug development. It is also a cheaper strategy, as less money is invested in clinical trials, and can lead to the discovery of new targets and pathways (209).

Antipsychotic drugs such as (*E*)-flupentixol (**1.33**, **Figure 1.5**) are also NorA inhibitors. Although these compounds presented mild antibacterial activity, they have shown EP inhibition in strains overexpressing efflux pumps (210). Chlorpromazine (**1.34**, **Figure 1.5**), another antipsychotic, has demonstrated the same inhibitory activity against AcrB, of the RND efflux system AcrAB-TolC (211).

The search for inhibitors of bacterial EPs has been ongoing for a long time, as is evidenced by the number of reviews that have been published regarding this topic in the last few years (100, 124, 130, 133, 212-221). Considering that EPs are both a MDR mechanism and lead to MDR by promoting the appearance of other mechanisms, finding safe and effective inhibitors may be a promising strategy against antimicrobial resistance. However, no EPIs have been approved for human use up to this time point.

The lack of structural coherence when it comes to EPIs can pose a problem, as no clear structural features or moieties can be identified. It has been noted that small lipophilic compounds can cross the bacterial membranes and inhibit EPs, as well as hydrophilic compounds that can be transported through porins, since the bottleneck for EP inhibition has been proved to be cellular penetration. Furthermore, compounds containing atoms which are not usually present in natural compounds appear to penetrate and to inhibit EPs, likely due to the lack of exposure of EPs to them (51).

Another problem that has been found was the lack of selectivity of the EPIs. This is particularly true if natural products are considered, due to their multitarget properties. Likewise, the repositioning of existing drugs has this obvious issue, and the concentration required for the compound to present efflux pump inhibition may also lead to the display of other undesired effects (124).

Nevertheless, bacterial EPs are still regarded as attractive targets for drug discovery, and the search for a safe, selective, and effective EPI that can not only help restore the efficacy of antibacterial drugs, as well as circumvent other resistance and virulence phenomena is a very current topic in medicinal chemistry and the fight against MDR bacteria.

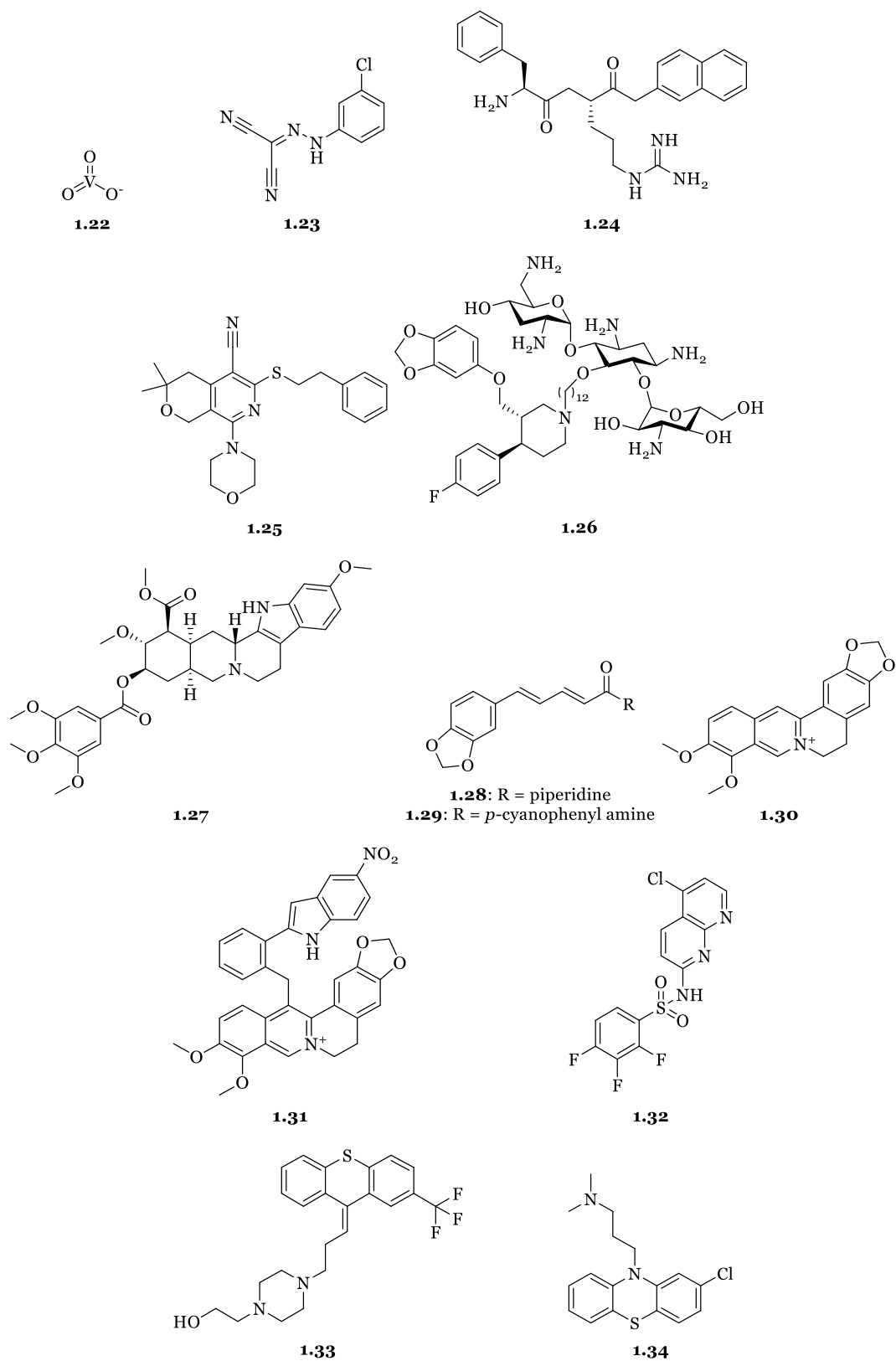


Figure 1.5 Structures of bacterial efflux pump inhibitors.

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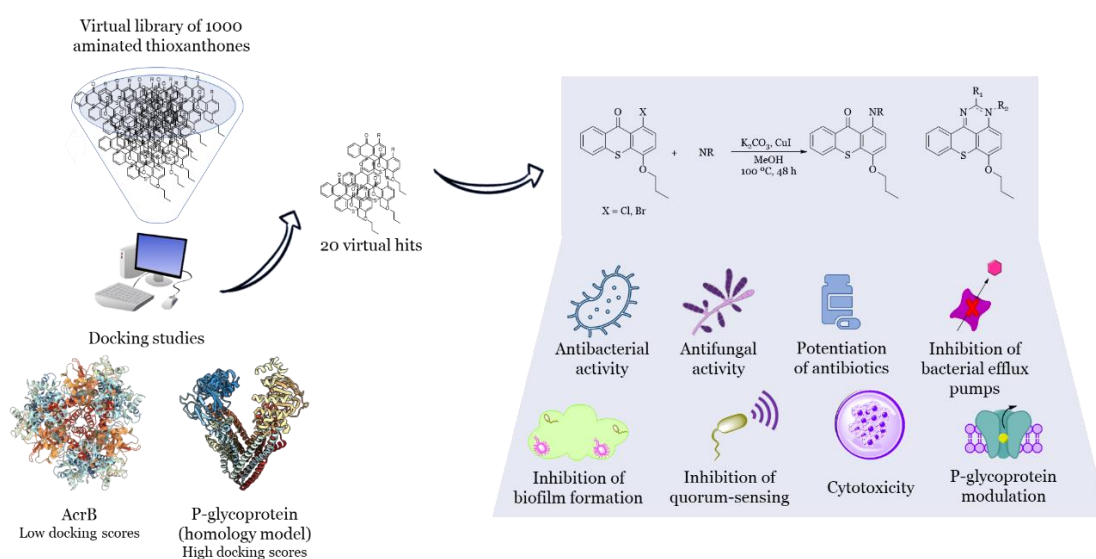
Chapter 2 – Aims

The main aim of this thesis was the design and synthesis of new compounds with the ability of inhibiting EPs in Gram-positive and Gram-negative bacteria, as well as interfering with related mechanisms such as biofilm formation and quorum-sensing.

The specific goals of this thesis were:

- i) To design a virtual library of 1000 thioxanthone derivatives and to perform docking studies against relevant bacterial EPs, as well as a homology model of the human efflux pump P-gp;
- ii) To synthesize the virtual hit compounds, corresponding to the molecules that displayed lower docking scores for bacterial efflux pumps than for P-gp;
- iii) To test the compounds for relevant biological activity: antibacterial, synergy with antibiotics, antifungal, bacterial EP inhibition, inhibition of biofilm formation, and quorum-sensing inhibition;
- iv) To study the effect of the compounds on P-gp;
- v) An additionally main goal was to perform a screening of a library of 175 structurally diverse compounds for the activities mentioned in iii) and extract useful data from structure activity relationship (SAR) studies;
- vi) To assess the cytotoxicity of the most promising compounds in mouse embryonic fibroblasts;
- vii) After the discovery of a broad-spectrum antimicrobial hit compound, a new library of analogues was synthesized to establish SAR;
- viii) To additionally test the most promising derivatives against bacteria and fungi pathogenic to fish;
- ix) To perform a screening of 15 selected compounds against a wild-type and a TolC-deleted strain of *Synechocystis* sp., a model used to study cyanobacteria which may be responsible for the formation of toxic algal blooms.

Chapter 3 – Thioxanthenes as potential bacterial efflux pump inhibitors ¹



¹ Durães, F., et al. *Pharmaceuticals* **2021**, *14*, 572.

Cardoso, J., et al. *Antibiotics* **2022**, *11*, 1488.

Author contributions: The design, docking, synthesis, and characterization of compounds **3.8**, **3.22**, **3.33**, **3.34**, **3.36**, **3.37**, and **3.39 – 3.44** were performed by Fernando Durães, under the supervision of Prof. Emília Sousa and Prof. Madalena Pinto. The synthesis and characterization of compounds **3.5 – 3.7**, **3.11**, and **3.45**, and the preparation of the P-gp homology model were performed by Dr. Andreia Palmeira, under the supervision of Prof. Emília Sousa and Prof. Madalena Pinto. The antibacterial and potentiation of antibiotics assays were performed by Fernando Durães, under the supervision of Prof. Paulo Martins da Costa. The efflux pump inhibition, inhibition of biofilm formation, quorum-sensing inhibition, and cytotoxicity in mouse embryonic fibroblasts assays were performed by Fernando Durães and Nikoletta Szemerédi, under the supervision of Dr. Gabriella Spengler. The antifungal assays were performed by Joana Freitas da Silva, under the supervision of Prof. Eugénia Pinto. The effects on P-gp expression and activity, and cytotoxicity in human cell lines were assayed by Bárbara Cruz, under the supervision of Dr. Renata Silva and Prof. Fernando Remião. The characterization of the compounds by X-ray crystallography was performed by Prof. Luís Gales.

3.1 Introduction

3.1.1 Thioxanthenes

Thioxanthenes (**3.1**, **Figure 3.1**) are synthetic sulfur heterocycles that possess a tricyclic dibenzo- γ -thiopyrone scaffold and are isosteres of xanthenes. They are privileged structures, meaning they contain geometrical features suitable for the insertion of varied side chains that would bind to different targets. These structures are often inspired or derived from natural products, which in this case it is true, as xanthenes have been isolated from natural sources. They are also attractive scaffolds for the design of screening libraries, both as hit compounds or as chemical substrates for molecular modifications (1, 2).

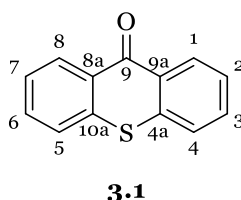


Figure 3.1 Structure of thioxanthone (**3.1**) and International Union of Pure and Applied Chemistry (IUPAC) numbering.

The interest in the thioxanthone scaffold began with the discovery of lucanthone (**3.2**, **Figure 3.2**) and hycanthone (**3.3**, **Figure 3.2**) as antischistosomal agents, in a systematic program. Even though these compounds were approved, they were later withdrawn due to an adverse mutagenicity, possibly caused by the intercalation between base pairs through the interaction with the side chain in C1 and phosphate residues, and the heterocyclic scaffold and DNA base residues (3, 4). However, this did not discourage the synthesis of thioxanthone derivatives, and currently, compounds with this scaffold have been studied for their potential as antiallergic (5), neurotropic (6), and antitumor agents (7-18). Moreover, some thioxanthenes were described as modulators of ABC transporters such as P-gp, a mammalian EP, which is important in MDR in tumor cells (12, 19-23).

It was found that substitution with an isopropyl group in C2 or propoxyl group in C4, as seen in compound **3.4**, did not cause genotoxicity, as thioxanthenes with this kind of substitution have been described as photoinitiators, used in ultraviolet (UV) curable inks for food packaging (4). In this scope, using a C4 propoxy-substituted thioxanthone as template, a series of C1-amino thioxanthenes were synthesized, and the most interesting compounds were **3.5**, which demonstrated promising results at inhibiting the growth of the chronic myelogenous leukemia cell line K562, and **3.6**, which was able to non-competitively inhibit P-gp. These results shed a light on the potential of these compounds as mammalian EPIs, which had not previously been reported (12).

Finally, a series of thioxanthone derivatives has been tested for their antibacterial activity and synergy with antibiotics in resistant bacterial strains. Compound **3.5** (**Figure 3.2**) was active against *S. aureus* American Type Culture Collection (ATCC) 25923 and *B. subtilis* ATCC 6633, with a minimum inhibitory concentration (MIC) of 64 µg/mL. Additionally, this compound was active against methicillin-resistant *S. aureus* (MRSA) B1, displaying the same MIC of 64 µg/mL. Interestingly, a hydrochloride salt of **3.5** was more potent and displayed a broader spectrum, showing MICs of 16 µg/mL for *S. aureus* ATCC 25923 and *S. aureus* B1, 8 µg/mL against *B. subtilis* ATCC 6633, and 32 µg/mL against *Enterococcus faecalis* W1, a vancomycin-resistant enterococci (VRE). Furthermore, compound **3.5** presented synergy with oxacillin and ampicillin against *S. aureus* B1, and its hydrochloride was able to potentiate oxacillin, ampicillin, and ciprofloxacin (24).

Another derivative that presented promising results regarding antibacterial activity was compound **3.7**, with mild activity against *S. aureus* ATCC 25923 (256 µg/mL). Its hydrochloride salt presented a broader spectrum, with the same activity against *S. aureus* ATCC 25923 and a MIC of 16 µg/mL against *B. subtilis* ATCC 6633. When it comes to synergy with antimicrobials, compound **3.7** was able to potentiate the activity of oxacillin, ampicillin, and ciprofloxacin against *S. aureus* B1, while its hydrochloride showed no interaction with the antimicrobials used (24).

Compound **3.8** (**Figure 3.2**) was the only thioxanthone described with synergic activity with an antibacterial against a Gram-negative strain. In fact, it showed ability to potentiate the activity of streptomycin against the MDR *E. coli* G1, as well as ampicillin against the VRE *E. faecalis* W1 (24, 25).

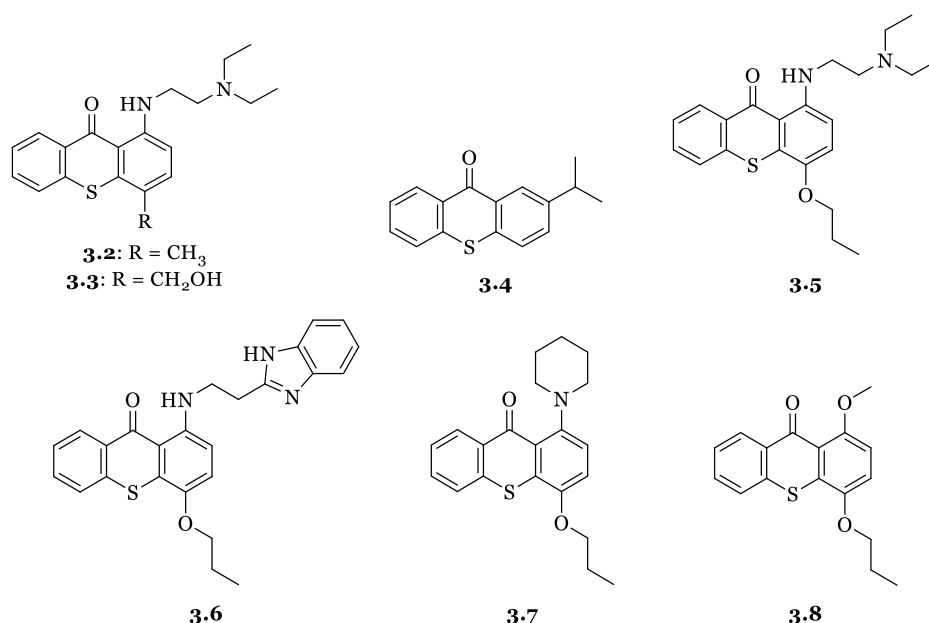


Figure 3.2 Structures of relevant thioxanthones.

These studies show the potential of thioxanthenes both as antibacterials and as antibacterial adjuvants since they can also increase the activity of antimicrobials in resistant strains. However, there has been no previous effort to unveil the specific mechanism through which the antimicrobial potentiation occurs. As such, there is room to explore not only which resistance mechanism is being affected by the thioxanthenes, but also to design and synthesize new thioxanthone derivatives to help circumvent antimicrobial resistance.

3.1.2 Computer-aided drug design

Typically, drug discovery is a slow and time-consuming process, which involves arduous work and large investments. As a way to improve this process, computer-aided drug discovery (CADD) can be used as a strategy, by resorting to computational methods with the aim of discovering and designing new compounds, that could be developed into new drugs, taking the chemical and biological spaces into account (26, 27).

CADD employs molecular modeling procedures, which allow the analysis of a large number of compounds in a short period of time, predicting the way they interact with the biological target, allowing a rational and timesaving strategy for choosing compounds to synthesize and test (26).

Within this, virtual screening is an important tool, which allows the assessment of large libraries or databases of chemical structures to identify the ones with the highest likelihood of binding to a target. In order for a virtual screening to happen, the three-dimensional structure of the target macromolecule must be available in platforms such as the Protein Data Bank (28). In situations where the macromolecule is not available, a homology model can be built, as this is the main alternative to the absence of crystal structures. Briefly, homology modeling is based on the relationship between the sequence of the target macromolecule and a known structure, which may serve as a template. Once this is identified and the sequences of target and template are aligned, a model is generated based on the three-dimensional structure of the template and the sequence alignment. The model is then refined and validated (29, 30).

When the target is chosen and available, structure-based drug design (SBDD) can be performed. Docking studies are useful in this scope, as they place the small molecules into the target macromolecule in different poses, as a way to discover which of the poses in the most favorable, translating into the compounds that are predicted to display better activity (31-33).

In situations where the structure of the target is not available, ligand-based drug design can be used. Based on the hypothesis that a set of compounds that are active against a target will bind similarly, a pharmacophore can be built, based on similar structural features (34).

Virtual screening comes as a faster, simpler, and cheaper way to filter large libraries of compounds without the need to perform expensive experiments, which makes it a good starting point for drug discovery projects.

3.2 Design of a virtual library of bacterial efflux pump inhibitors

The inspiration towards the design of new bacterial EPIs was based on compounds that have proven their potential in this scope (35). The selection of the thioxanthone scaffold came from the previous potential demonstrated as mammalian EPI, as well as antibacterial and potentiator of antibiotics (12, 24). The fact that antipsychotic drugs such as (*E*)-flupentixol (**3.9**, **Figure 3.3**) and chlorpromazine (**3.10**, **Figure 3.3**), MFS and RND inhibitors, respectively, also possess a tricyclic scaffold with a sulfur heteroatom reinforced this selection (36, 37).

Furthermore, unpublished results with aminated thioxanthenes showed that compound **3.7** (**Figure 3.2**) was effective at inhibiting the efflux of ethidium bromide (EtBr), a known EP substrate, in the wild-type *E. coli* K-12 AG100 strain, which expresses the AcrAB-TolC efflux system at basal level, and the AcrAB-TolC-deleted mutant *E. coli* AG100A. In the same study, thioxanthone **3.11** (**Figure 3.3**) was effective at inhibiting EtBr efflux in *E. coli* K-12 AG100, but not in the mutant strain, suggesting AcrAB-TolC inhibition.

The thioxanthone precursor chosen for the design of the virtual library was 1-chloro-4-propoxy-9*H*-thioxanthen-9-one (**3.12**, **Figure 3.3**), which allowed by a cross-coupling reaction, amine substituents at C1 (12). Within the pool of amines, guanidine (**3.13**, **Figure 3.3**) derivatives stood out, as this moiety is present in PAβN (**3.14**, **Figure 3.3**), an RND inhibitor (38). Guanidine-containing compounds show a wide variety of biological activities, made possible by the way guanidine interacts with other targets and drugs. Chemically, guanidine is a strong organic base, meaning it can establish tight bonds with carboxylates, phosphates, and metals. Furthermore, the protons in the nitrogen atoms act as hydrogen bond donors, which increase the capability of providing hydrogen interactions with other atoms (39, 40). Urea (**3.15**, **Figure 3.3**) is structurally similar to guanidine, but has an oxygen atom, which makes it a hydrogen bond acceptor. This moiety is present in drugs that span a wide range of biological activities (41). It is also present in compound **3.16** (**Figure 3.3**), an antibacterial/EPI hybrid comprising a bis-aryl urea motif and an ofloxacin core, able to inhibit MFS and MATE EPs (42). The last moiety chosen for the design of the virtual library was the sulfonamide (**3.17**, **Figure 3.3**), as this moiety has been described as antibacterial and privileged for other activities, but is also included in AE-848/42434549 (**3.18**, **Figure 3.3**) a hit that proved to be effective at decreasing NorA-mediated efflux (43, 44).

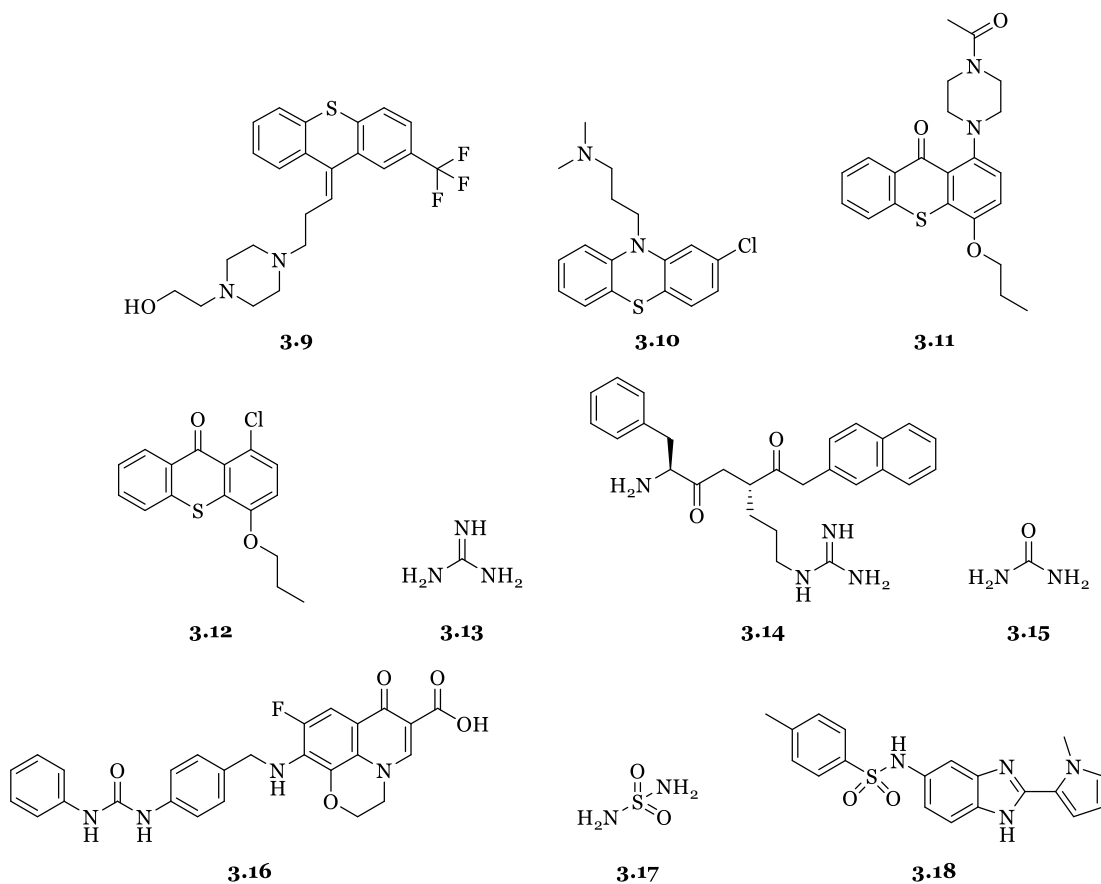


Figure 3.3 Structure of EPIs and moieties with features considered for the design of a virtual library.

Figure 3.4 summarizes the general structure of the virtual library of bacterial EPIs designed, considering the features chosen.

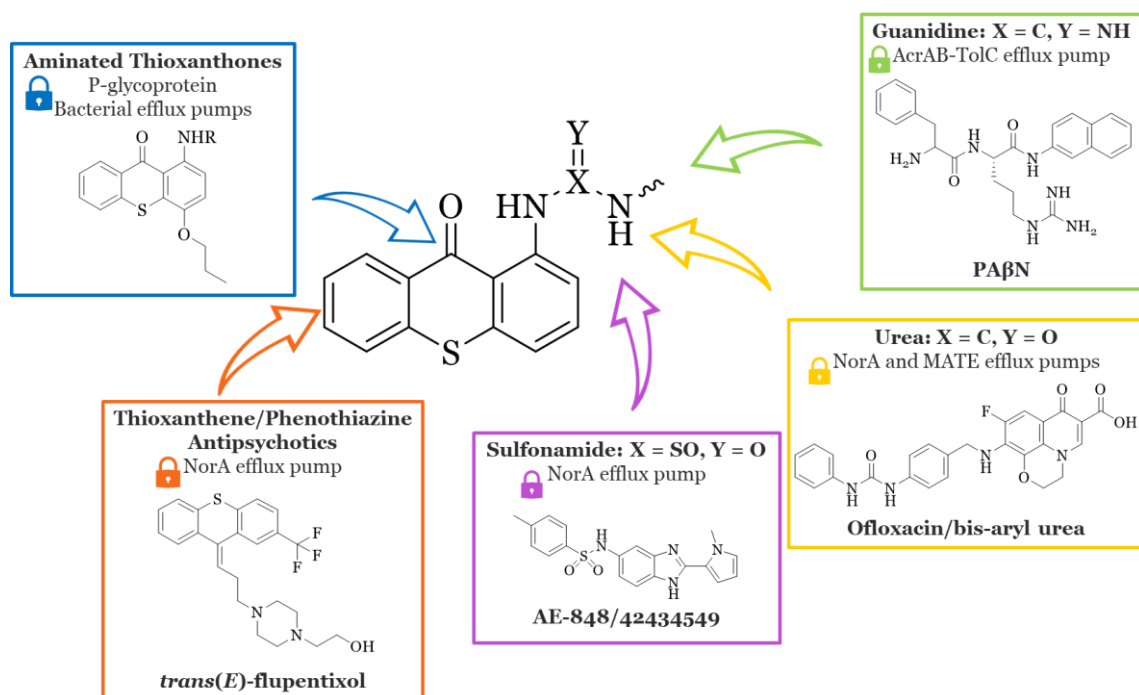


Figure 3.4 General structure of the virtual library of thioxanthenes designed.

Then, a virtual library of 1000 C1-aminosubstituted thioxanthenes were drawn using ChemDraw ®, based on the guanidine, urea and sulfonamide derivatives provided by suppliers (Merck, TCI Chemicals, Alfa Aesar, and Fisher Scientific), as well as in-house guanidine, urea and sulfonamide derivatives and amines that had not previously been synthesized in the context of EP inhibition.

The virtual compounds and positive controls used for docking were minimized using ArgusLab and were then ready for the docking studies.

3.3 Virtual screening of a library of thioxanthenes

After the preparation of the ligands, docking studies were performed. The target chosen was the AcrB portion (PDB: 4DX5) of the AcrAB-TolC efflux system. Within this target, two sites were chosen according to what had been previously described: the substrate binding site and the hydrophobic trap (45).

The substrate binding site is a multi-specific pocket, able to recognize a plethora of chemicals, making the design of specific EPIs a hard task. It is a large cavity, constituted by hydrophobic residues such as phenylalanine (Phe-136, Phe-178, Phe-610, Phe-615, Phe-617, and Phe-628) and polar residues, such as asparagine (Asn-274) and glutamine (Gln-176). Studies with the drugs minocycline and doxorubicin revealed that the most important interactions were van der Waals and π - π stacking interactions with the hydrophobic residues, and hydrogen interactions with the polar ones. However, it was shown that both drugs have their unique set of residues with which they interact, thus emphasizing the lack of specificity of AcrB (45-47).

The hydrophobic trap is a unique narrow cleft, composed by hydrophobic residues (Phe-136, Phe-178, Phe-610, Phe-615, and Phe-628), which extends into the substrate binding site. The presence of a water filled pocket near the exit of the hydrophobic trap contributes to the existence of polar interactions. A few inhibition mechanisms have been proposed regarding this binding site. It could be related to the possibility that EPIs display better affinity for the hydrophobic trap than the substrates, therefore causing steric hindrance and blocking the substrate from binding. The high affinity to the hydrophobic trap can also lead to the hypothesis that an EPI can freeze AcrB in an inactive conformation or saturate the binding sites and disrupting the functional conformation of the AcrB trimer. Finally, it was also postulated that conformational changes needed for the activity of the pump are prevented by binding to the hydrophobic trap (45, 48-50).

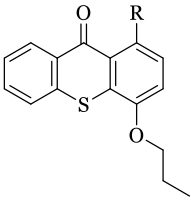
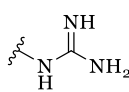
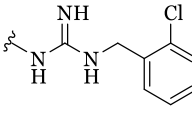
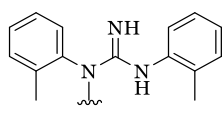
The design of an effective EPI can be tricky, considering that the hydrophobic nature of the AcrB binding sites limits EPIs to either water-insoluble or amphiphilic compounds, with all the pharmacokinetic and toxicity issues that come with it (45).

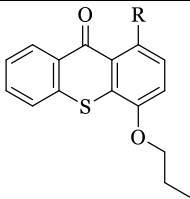
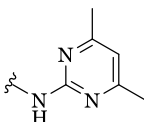
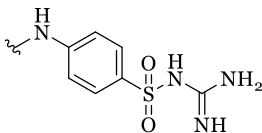
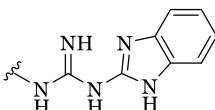
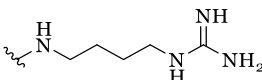
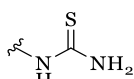
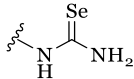
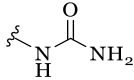
The other target chosen for the docking studies was a homology model of P-gp, as the objective was the design of EPIs that were selective to bacteria. The sites chosen for docking were the ATP-binding site on the nucleotide binding domain and the drug binding pocket on the interface of the transmembrane domain (12).

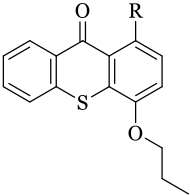
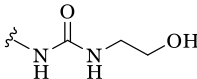
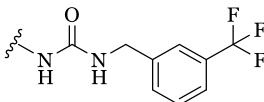
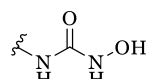
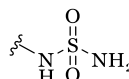
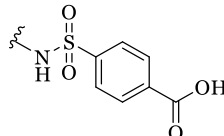
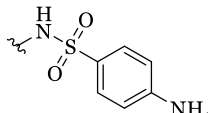
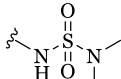
The docking studies were performed using AutoDock Vina (51), and the pose with the lowest docking score was the one considered.

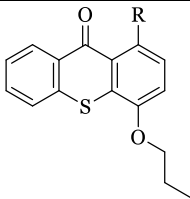
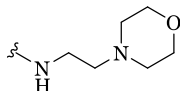
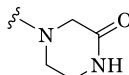
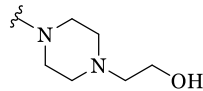
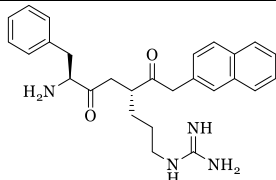
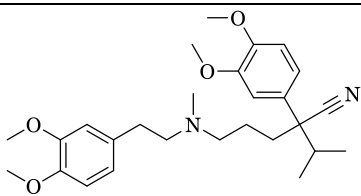
Upon a first analysis of the docking scores, it could be noted that most compounds had similar scores towards AcrB and P-gp, which could be problematic for the proposed aim of selecting compounds that would display selectivity. For that reason, the virtual hits were considered the compounds whose docking scores were lower than P-gp and the ones that were up to a unit higher. After this selection, all the structures were analyzed, and considering factors such as the Lipinski rules and the feasibility of the synthesis, 20 compounds were selected. The structures of the compounds and scores obtained are present in **Table 3.1**.

Table 3.1 Virtual hits and respective docking scores.

 3.19 - 3.38				
Guanidine derivatives				
Compound	R	Docking scores (kcal/mol)		
3.19		AcrB	HT	-6.3
			SBS	-6.7
		P-gp	NBD	-7.3
			TMD	-7.3
3.20		AcrB	HT	-5.2
			SBS	-8.6
		P-gp	NBD	-7.1
			TMD	-9.1
3.21		AcrB	HT	-2.2
			SBS	-7.5
		P-gp	NBD	-7.2
			TMD	-7.8

 3.19 - 3.38				
Compound	R	Docking scores (kcal/mol)		
3.22		AcrB	HT	-2.5
			SBS	-8.1
		P-gp	NBD	-7.6
			TMD	-8.0
3.23		AcrB	HT	-4.9
			SBS	-7.5
		P-gp	NBD	-7.1
			TMD	-7.7
3.24		AcrB	HT	-5.2
			SBS	-8.4
		P-gp	NBD	-7.3
			TMD	-9.2
3.25		AcrB	HT	-5.9
			SBS	-7.5
		P-gp	NBD	-6.6
			TMD	-7.4
3.26		AcrB	HT	-5.7
			SBS	-6.3
		P-gp	NBD	-6.5
			TMD	-6.9
3.27		AcrB	HT	-6.6
			SBS	-7.5
		P-gp	NBD	-6.7
			TMD	-7.4
Urea derivatives				
3.28		AcrB	HT	-6.6
			SBS	-6.7
		P-gp	NBD	-6.5
			TMD	-7.4

 3.19 - 3.38				
Compound	R	Docking scores (kcal/mol)		
3.29		AcrB	HT	-6.1
			SBS	-6.8
		P-gp	NBD	-6.1
			TMD	-7.3
3.30		AcrB	HT	-6.9
			SBS	-9.2
		P-gp	NBD	-8.1
			TMD	-9.7
3.31		AcrB	HT	-6.6
			SBS	-7.6
		P-gp	NBD	-5.8
			TMD	-8.2
Sulfonamide derivatives				
3.32		AcrB	HT	-5.9
			SBS	-6.8
		P-gp	NBD	-5.7
			TMD	-7.7
3.33		AcrB	HT	-5.7
			SBS	-8.2
		P-gp	NBD	-6.9
			TMD	-8.2
3.34		AcrB	HT	-4.8
			SBS	-7.8
		P-gp	NBD	-5.7
			TMD	-5.5
3.35		AcrB	HT	-5.9
			SBS	-7.3
		P-gp	NBD	-6.1
			TMD	-7.6

 3.19 - 3.38				
Compound	R	Docking scores (kcal/mol)		
Amines				
3.36		AcrB	HT	1.6
			SBS	-7.5
		P-gp	NBD	-5.8
			TMD	-7.7
3.37		AcrB	HT	-3.6
			SBS	-7.3
		P-gp	NBD	-5.5
			TMD	-8.1
3.38		AcrB	HT	-5.8
			SBS	-8.0
		P-gp	NBD	-5.6
			TMD	-7.4
Positive controls				
PAβN		AcrB	HT	-4.7
			SBS	-7.1
		P-gp	NBD	-
			TMD	-
Verapamil		AcrB	HT	-
			SBS	-
		P-gp	NBD	-7.0
			TMD	-8.5

P-gp: P-glycoprotein; **HT:** Hydrophobic trap; **SBS:** Substrate binding site; **NBD:** Nucleotide binding domain; **TMD:** Transmembrane domain.

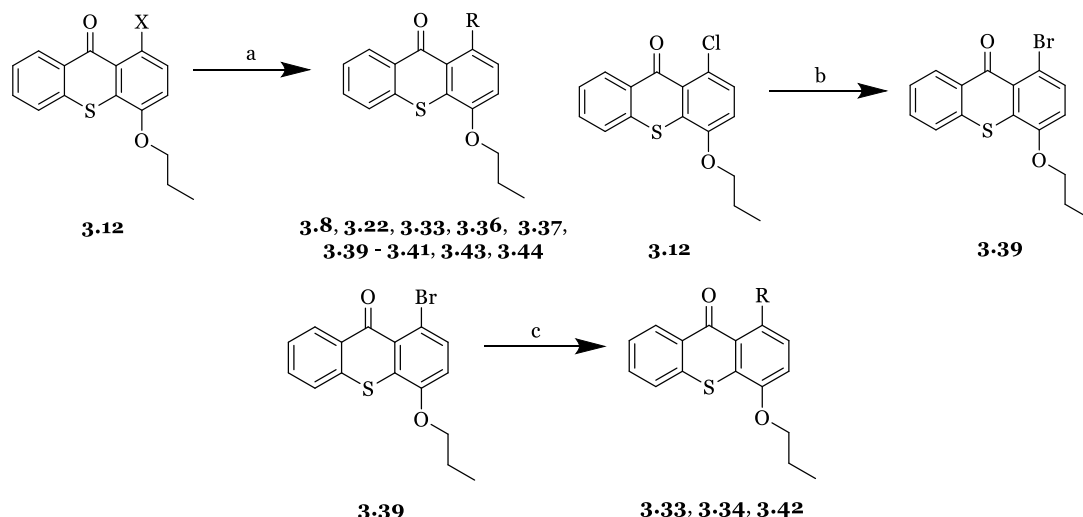
Through the analysis of **Table 3.1**, some information can be drawn. Apart from the aforementioned fact that the scores between the two efflux pumps studied are very close, it can be inferred that the compounds are predicted to bind more effectively in the substrate binding site of AcrB and in the transmembrane domain of P-gp. Guanidine derivatives are predicted to be the class with the most effective compounds.

With the knowledge obtained from this virtual screening, the next step was the synthesis of compounds correspondent to the virtual hits.

3.3 Synthesis of thioxanthone derivatives

The synthesis of thioxanthone derivatives was achieved through a copper-catalyzed C – N Ullmann type coupling reaction, with compound **3.12** as starting material, using potassium carbonate as base and copper(I) iodide, and methanol as a solvent, at 100 °C in a sealed flask (procedure **a**, **Scheme 3.1**), similarly to what has been described (12).

A 1-bromine derivative (**3.39**) was also synthesized, according to the reaction described in (52), according to procedure **b** (**Scheme 3.1**). This derivative was then also used for the synthesis of aminated thioxanthenes (procedure **c**, **Scheme 3.2**).



Scheme 3.1 Synthetic pathway employed for the synthesis of thioxanthone derivatives. Reagents and conditions (**a**) X = Cl (**3.12**), amine, K₂CO₃, CuI, MeOH, 100 °C, 48 hours, sealed flask; (**b**) X = Cl (**3.12**), KBr, H₃PO₄, CuI, nitrobenzene, 48 hours, reflux, yields compound **3.39**; (**c**) X = Br (**3.39**), amine, K₂CO₃, CuI, MeOH, 100 °C, 48 hours, sealed flask.

Using this reaction, thioxanthone derivatives **3.22**, **3.33**, **3.34**, **3.36** and **3.37** (**Table 3.1**) were synthesized for the first time. The methoxy derivative **3.8** (**Figure 3.2**) was obtained in every reaction performed, as it is a side product from the reaction of **3.12** with methanol.

When it comes to the guanidine and urea derivatives, the reaction used did not yield the expected compounds. Interestingly, a dehydrative cyclization occurs in a concerted manner, furnishing a tetracyclic scaffold (53). Therefore, instead of thioxanthenes **3.19**, **3.20**, **3.21**, and **3.28**, tetracyclic thioxanthenes **3.40** – **3.43** (**Figure 3.5**) were obtained through procedures **a** (**3.40**, **3.41**, and **3.43**) and **c** (**3.42**).

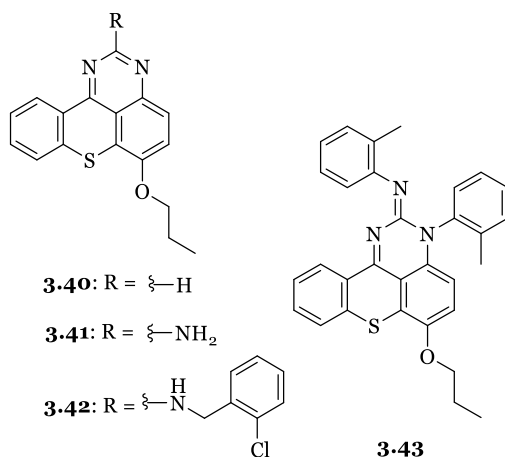
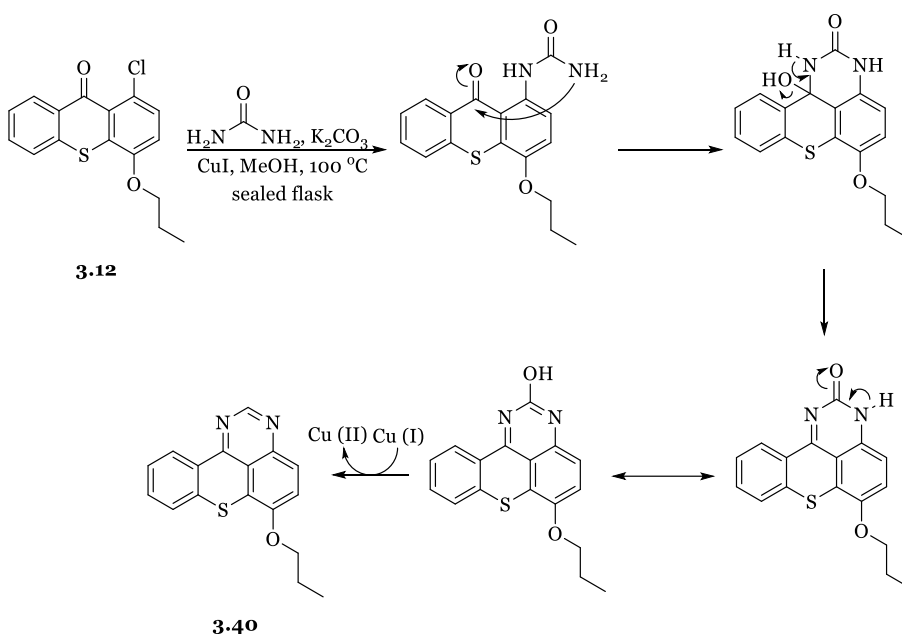


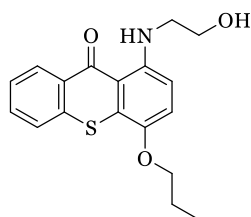
Figure 3.5 Structure of tetracyclic thioxanthenes obtained.

It should be noted that compound **3.40** results from the reaction of **3.12** with urea. This can be explained by the occurrence of a reduction, following the dehydrative cyclization. A proposed mechanism for this reaction can be found in **Scheme 3.2**.



Scheme 3.2 Proposed reaction mechanism for the synthesis of compound **3.40**.

The last compound synthesized was thioxanthone **3.44** (**Figure 3.6**), instead of the expected **3.29**, due to a formamide loss. The synthesis of the other hit compounds was attempted, but through these synthetic strategies only traces were obtained.



3.44

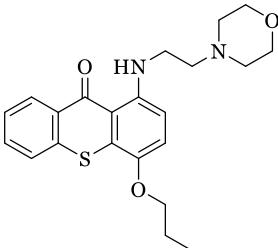
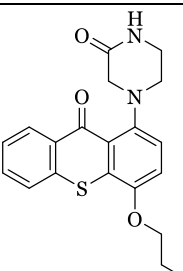
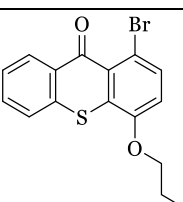
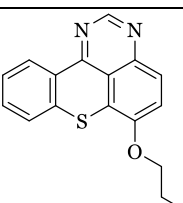
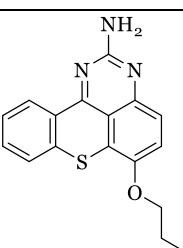
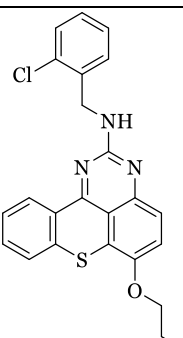
Figure 3.6 Structure of compound **3.44**.

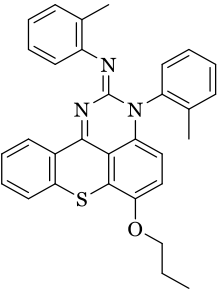
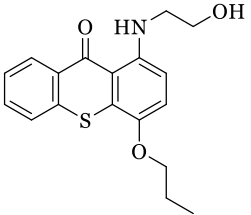
The compounds were obtained in diverse yields, which are present in **Table 3.2**. It is worth mentioning that compound **3.39** was used as starting material for the synthesis of compounds **3.33**, **3.34**, and **3.42**.

Out of the 20 virtual hit compounds obtained from the virtual screening (**Table 3.1**), ten were successfully synthesized and purified. The synthesized compounds, along with a library of thioxanthenes previously synthesized (12) were tested for their biological activity in this scope. This included compounds **3.5** – **3.8** (**Figure 3.2**), compound **3.11** (**Figure 3.3**), and compound **3.45** (**Figure 3.7**).

Table 3.2 Structure of the synthesized compounds and yields.

Compound	Structure	Yield (procedure)
3.22		3% (a)
3.33		3% (a) 48% (c)
3.34		28% (c)

Compound	Structure	Yield (procedure)
3.36		45% (a)
3.37		12% (a)
3.39		54% (b)
3.40		11% (a)
3.41		25% (a)
3.42		45% (c)

Compound	Structure	Yield (procedure)
3.43		13% (a)
3.44		7% (a)

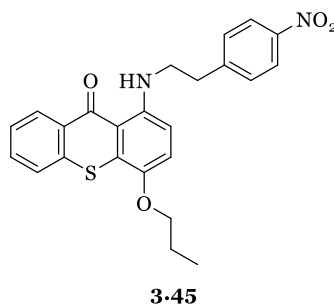


Figure 3.7 Structure of compound **3.45**.

New docking studies were performed to include the newly described tetracyclic thioxanthenes and the previously synthesized thioxanthenes.

3.4 Docking studies

The library comprised of thirteen thioxanthenes and four tetracyclic thioxanthenes was subject to another round of docking studies. This time, the studies included all three portions of the AcrAB-TolC efflux system, as well as the NorA EP, as it is described as a relevant MFS pump. However, NorA does not have a crystal structure deposited in the Protein Data Bank, and a homology model needed to be built. Reference compounds PA β N (**3.14**, **Figure 3.3**) and reserpine were included as AcrAB-TolC and NorA positive controls, respectively.

The docking studies were then performed on the available crystal structures of AcrB (PDB: 4DX5), AcrA (PDB: 2F1M), and TolC (PDB: 1EK9). The sites studied in AcrB were the hydrophobic trap and the substrate binding site, as was previously done. For AcrA, the sites chosen were the helical hairpin, an important region in AcrA linked to the transition between the ligand bound and unbound states, critical for the opening of TolC to form the

active pump, and the lipoyl domain, which interfaces with AcrB (48, 54, 55). Regarding TolC, the region chosen was the one comprising the lysine residues that interacted with the dithiobis(sulfosuccinimidyl propionate), a bifunctional crosslinker (55).

Considering the NorA efflux pump homology model, the cavity predicted to be the binding core region and the cavity turned to the cytoplasmic side were chosen as the sites to perform these studies (56). The docking scores for the compounds and positive controls are present in

Table 3.3.

Table 3.3 Docking scores for the tioxanthenes in relevant bacterial EPs.

Compounds	Docking scores (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
3.5	-6.1	-1.0	-5.4	-5.4	-6.3	-5.6	-4.9
3.6	-7.5	4.2	-6.2	-6.3	-7.5	-7.8	-6.2
3.7	-6.9	-5.0	-5.6	-4.7	-6.8	-3.9	-5.7
3.8	-6.5	-5.1	-5.4	-5.2	-6.2	-5.3	-5.3
3.11	-7.6	4.8	-5.7	-4.9	-7.7	-2.3	-5.5
3.12	-6.3	17.5	-5.4	-4.2	-6.2	-5.2	-4.9
3.22	-8.1	-2.5	-6.0	-5.2	-7.3	-6.2	-5.0
3.33	-8.2	-5.7	-6.9	-6.4	-7.8	-5.5	-5.7
3.34	-7.8	-4.8	-6.5	-6.0	-7.1	-6.2	-5.1
3.36	-7.5	1.6	-6.1	-5.7	-7.2	-6.1	-5.7
3.37	-7.3	-3.6	-5.8	-4.8	-6.9	-4.0	-5.8
3.39	-6.3	-4.7	-5.4	-5.3	-6.4	-4.7	-5.7
3.40	-6.5	-5.6	-5.7	-5.7	-6.6	-5.8	-5.4
3.41	-7.4	-5.7	-5.8	-6.1	-7.0	-5.5	-5.6
3.42	-7.9	-6.2	-6.9	-5.6	-9.0	-5.2	-6.5
3.43	-8.5	32.9	-6.8	-5.7	-8.4	4.0	-7.4
3.44	-7.2	-5.7	-5.8	-5.3	-6.7	-6.1	-5.7
3.45	-7.4	-3.4	-6.5	-6.6	-6.9	-7.4	-5.3
PAβN	-7.1	-4.7	-5.8	-4.9	-7.1	-	-
Reserpine	-	-	-	-	-	1.0	-4.6

SBS: Substrate binding site; **HT:** Hydrophobic trap; **HH:** Helical hairpin; **LD:** Lipoyl domain; **BCR:** Binding core region; **CS:** Cytoplasmic side; **PAβN:** Phenyl-arginine β-naphthylamide.

The results predicted the synthesized derivatives to be at least as effective as the EPis used, considering they present comparable docking scores, and are generally more effective than starting materials (**3.12** and **3.39**).

It could also be predicted that the compounds were more active towards the AcrB portion, specifically the substrate binding site, and would present the least affinity towards

the hydrophobic trap of the same portion. Generally, the AcrA portion was the one with the least affinity overall.

In terms of the NorA homology model, the predicted affinities seem to be equally distributed among the two tested sites. The results obtained herein lead to the assumption that the derivatives could potentially be bacterial EPIs, as they present docking scores similar to those of previously reported EPIs. As such, all the compounds were selected for assays in resistant bacterial models.

3.5 Biological activity

3.5.1 Antibacterial activity and potentiation of antibiotics

The synthesized library of 11 thioxanthone derivatives was tested for its antimicrobial activity against six bacterial strains: the Gram-negative *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *Salmonella enterica* serovar Typhimurium SL1344, a strain with the *acrA* gene deleted, and the Gram-positive *S. aureus* 25923, *E. faecalis* ATCC 29212, and the methicillin and ofloxacin-resistant *S. aureus* 272123. Although the aim of this project was not to find new antibacterial compounds, this assay served as a guide to establish the concentration of the compounds that could be used in other assays without harm to the bacterial strains.

The compounds were also studied for their capacity of potentiating antibiotics in resistant strains. Two clinically relevant bacterial strains were used: the ESBL-producing *E. coli* SA/2 and the VRE *E. faecalis* B3/101 (57). The antibiotics used in this scope were the third-generation cephalosporin cefotaxime (CTX) and the glycopeptide vancomycin (VAN), respectively.

From the analysis of the results in **Table 3.4**, it can be inferred that only two derivatives displayed antimicrobial activity against the tested strains. Compounds **3.5** and **3.33** were active against *S. aureus* ATCC 25923 and *E. faecalis* ATCC 21212, with MICs of 83 μ M and 34 μ M, respectively, with compound **3.33** also displaying a MIC of 50 μ M for *S. Typhimurium* SL1344. These results suggest that this compound was more active than thioxanthenes that had been previously studied for antimicrobial activity (24).

Since sulfonamides are known antibacterials, 4-sulfamoylbenzoic acid, the precursor of compound **3.33**, was tested against the strains for which it was active and did not display an observable MIC at the highest concentration tested (100 μ M). Sulfonamides are *p*-aminobenzoic acid antimetabolites, and the *para*-amino group, which is not present in **3.33**, is essential for the antibacterial activity. Consequently, it can be hypothesized that **3.33** presents a mechanism of action distinct from that of sulfonamides.

Table 3.4 Minimum inhibitory concentration of the thioxanthone derivatives tested against bacteria, and potentiation of antibiotics.

Compound	Antibacterial activity						Potentiation of antibiotics ¹	
	Minimum inhibitory concentration (MIC) (μM)						MIC reduction	
	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 27853	<i>E. faecalis</i> ATCC 29212	<i>S. aureus</i> ATCC 25923	<i>S. aureus</i> 272123	<i>S. Typhimurium</i> SL1344	<i>E. coli</i> SA/2	<i>E. faecalis</i> B3/101
							CTX MIC = 1124 μM	VAN MIC = 707 μM
3.5	>100	>100	83	83	>100	>100	-	-
3.6	-	-	-	-	>100	>100	-	-
3.7	-	-	>100	>100	>100	>100	-	-
3.8	-	-	-	-	>100	>100	-	-
3.11	-	-	-	-	>100	>100	-	-
3.22	>100	>100	>100	>100	>100	>100	4-fold	No effect
3.33	>100	>100	34	34	>100	50	16-fold	No effect
3.34	>100	>100	>100	>100	>100	>100	No effect	No effect
3.36	>100	>100	>100	>100	>100	>100	No effect	No effect
3.37	>100	>100	>100	>100	>100	>100	No effect	16-fold
3.39	>100	>100	>100	>100	>100	>100	No effect	No effect
3.40	>100	>100	>100	>100	>100	>100	No effect	No effect
3.41	>100	>100	>100	>100	>100	>100	No effect	No effect
3.42	>100	>100	>100	>100	>100	>100	No effect	No effect
3.43	>100	>100	>100	>100	>100	>100	2-fold	No effect
3.44	>100	>100	>100	>100	>100	>100	2-fold	No effect
3.45	-	-	-	-	>100	>100	-	-

¹ The compounds were tested at the maximum concentration that did not cause effects in the bacteria; - compounds with no data had previously been tested for these strains (24); **CTX**: cefotaxime; **VAN**: vancomycin.

Considering the potential of increasing the activity of antibiotics in resistant strains, it was observed that thioxanthenes interact preferentially towards the Gram-negative strain, as the four compounds **3.22**, **3.33**, **3.43**, and **3.44**, were able to decrease the MIC of CTX in *E. coli* SA/2. Notably, compound **3.33** was able to improve the potency of CTX by 16-fold, showing that this compound is promising on its own and in combination with antibiotics. Compound **3.9** caused the same 16-fold decrease in the MIC of VAN against *E. faecalis* B3/101. Since *E. coli* SA/2 is an ESBL producer and *E. faecalis* B3/101 had the capacity to modify the peptidoglycan synthesis pathway, it can be theorized that the compounds could interact at this level.

3.5.2 Bacterial efflux pump inhibition assay

In order to deepen the knowledge about the potential of thioxanthenes as antimicrobial adjuvants, studies regarding the inhibition of efflux were performed. The assay used was

the real-time EtBr accumulation, based on the fact that ethidium bromide is both an EP substrate and a DNA intercalant agent capable of emitting fluorescence (58, 59). Thus, if efflux is impaired, EtBr will bind to the bacterial DNA and emit fluorescence, which can then be detected and measured. **Figure 3.8** illustrates the usefulness of EtBr in this context.

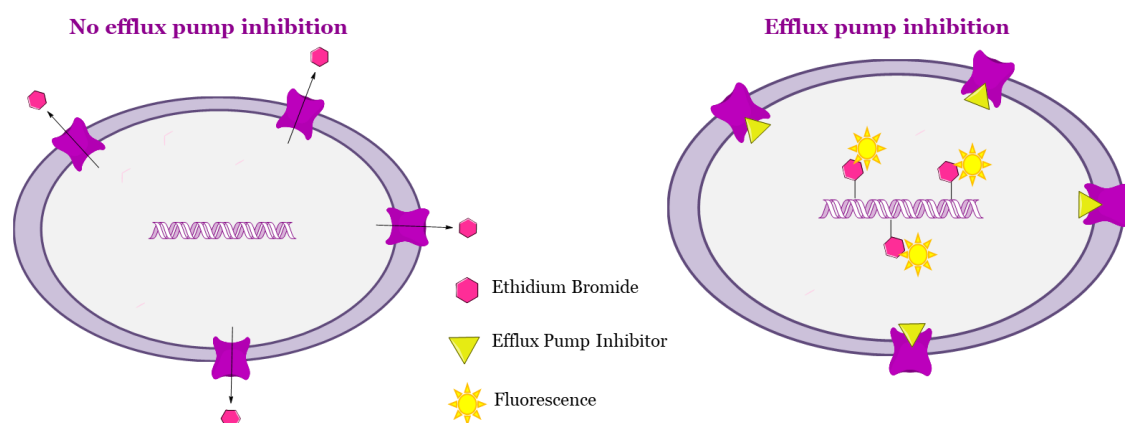


Figure 3.8 Graphical explanation of the real-time EtBr accumulation assay.

Compounds **3.5 – 3.8**, **3.11**, **3.22**, **3.33**, **3.34**, **3.36**, **3.37**, and **3.39 – 3.45** were submitted to a preliminary screening, to assess their potential as bacterial EPIs by the modulation of EtBr accumulation. Two strains were used for this assay: *S. Typhimurium* SL1344 and *S. aureus* 272123. *S. aureus* 272123 is a Gram-positive clinical strain, whose expression of the *norA* gene were not changing comparing to reference levels (60). However, the *norA* gene is a core gene of this strain, which implies that the NorA pump exists across the *S. aureus* species, and this strain had previously been used to study the effect of natural products against EtBr efflux (60-62).

On the other hand, the *Salmonella* strain used herein was chosen based on the docking studies, which predicted the least affinity of the compounds towards the AcrA portion of the AcrAB-TolC efflux system.

To study the effects of the compounds against the efflux of EtBr, they needed to be used in sub-MIC concentrations, in order to assure bacterial viability. As such, compound **3.33** was tested at one-third of its MIC, 17 μ M, against *S. Typhimurium* SL1344, and the 50 μ M against *S. aureus* 272123. Similarly, the other compounds were tested at 50 μ M for both strains, as their MIC was over 100 μ M for these strains.

The results were presented as the relative fluorescence index (RFI), which was calculated based on the means of the relative fluorescence units and are present in **Table 3.5**. As positive controls, reserpine and CCCP were used for *S. aureus* 272123 and *S. Typhimurium* SL1344, respectively, at the sub-MIC concentration of 25 μ M.

Table 3.5 RFI of the tested thioxanthone derivatives.

Compound	RFI $\pm$ SD	
	<i>S. aureus</i> 272123	<i>S. Typhimurium</i> SL1344
3.5	0.75 $\pm$ 0.08	-0.02 $\pm$ 0.04
3.6	-0.13 $\pm$ 0.04	-0.20 $\pm$ 0.01
3.7	1.50 $\pm$ 0.04	0.67 $\pm$ 0.01
3.8	0.11 $\pm$ 0.26	-0.10 $\pm$ 0.05
3.11	-0.10 $\pm$ 0.02	0.49 $\pm$ 0.11
3.22	0.24 $\pm$ 0.07	0.74 $\pm$ 0.08
3.33	0.29 $\pm$ 0.19	-0.21 $\pm$ 0.07
3.34	-0.18 $\pm$ 0.05	1.23 $\pm$ 0.18
3.36	0.89 $\pm$ 0.06	1.37 $\pm$ 0.54
3.37	0.14 $\pm$ 0.02	1.54 $\pm$ 0.09
3.39	1.13 $\pm$ 0.13	0.87 $\pm$ 0.11
3.40	-0.07 $\pm$ 0.50	0.54 $\pm$ 0.11
3.41	0.96 $\pm$ 0.27	7.52 $\pm$ 0.01
3.42	9.69 $\pm$ 0.95	25.51 $\pm$ 2.93
3.43	0.20 $\pm$ 0.03	0.20 $\pm$ 0.02
3.44	4.71 $\pm$ 0.78	6.00 $\pm$ 0.80
3.45	0.09 $\pm$ 0.11	-0.20 $\pm$ 0.01
Reserpine	0.50 $\pm$ 0.04	-
CCCP	-	0.23 $\pm$ 0.04

RFI: Relative fluorescence index; **SD**: Standard deviation; **CCCP**: carbonyl cyanide 3-chlorophenylhydrazone.

The results show that compound **3.5** was able to decrease the efflux of EtBr in *S. aureus* 272123, compounds **3.11**, **3.22**, **3.34**, **3.37**, and **3.40**, were effective at inhibiting efflux in *S. Typhimurium* SL1344, and compounds **3.7**, **3.36**, **3.39**, **3.41**, **3.42**, and **3.44** were effective against both strains. It can be seen in **Table 3.5** that some compounds display negative RFI values. This is due to the fact that these compounds display a lower extent of EtBr inhibition than the control, and for these purposes were considered ineffective.

Another factor that should be taken into account when analyzing these results is the origin of the fluorescence, that is, if it is caused by an increase in EtBr accumulation or if the compound is fluorescent by itself. Compounds **3.7**, **3.36**, **3.39**, **3.41**, **3.42**, and **3.44** are the ones that present higher RFI values in both strains and were then further analyzed in a similar way as the previous assay over the same time, and the fluorescence curves were analyzed, as can be seen in **Figure 3.9**.

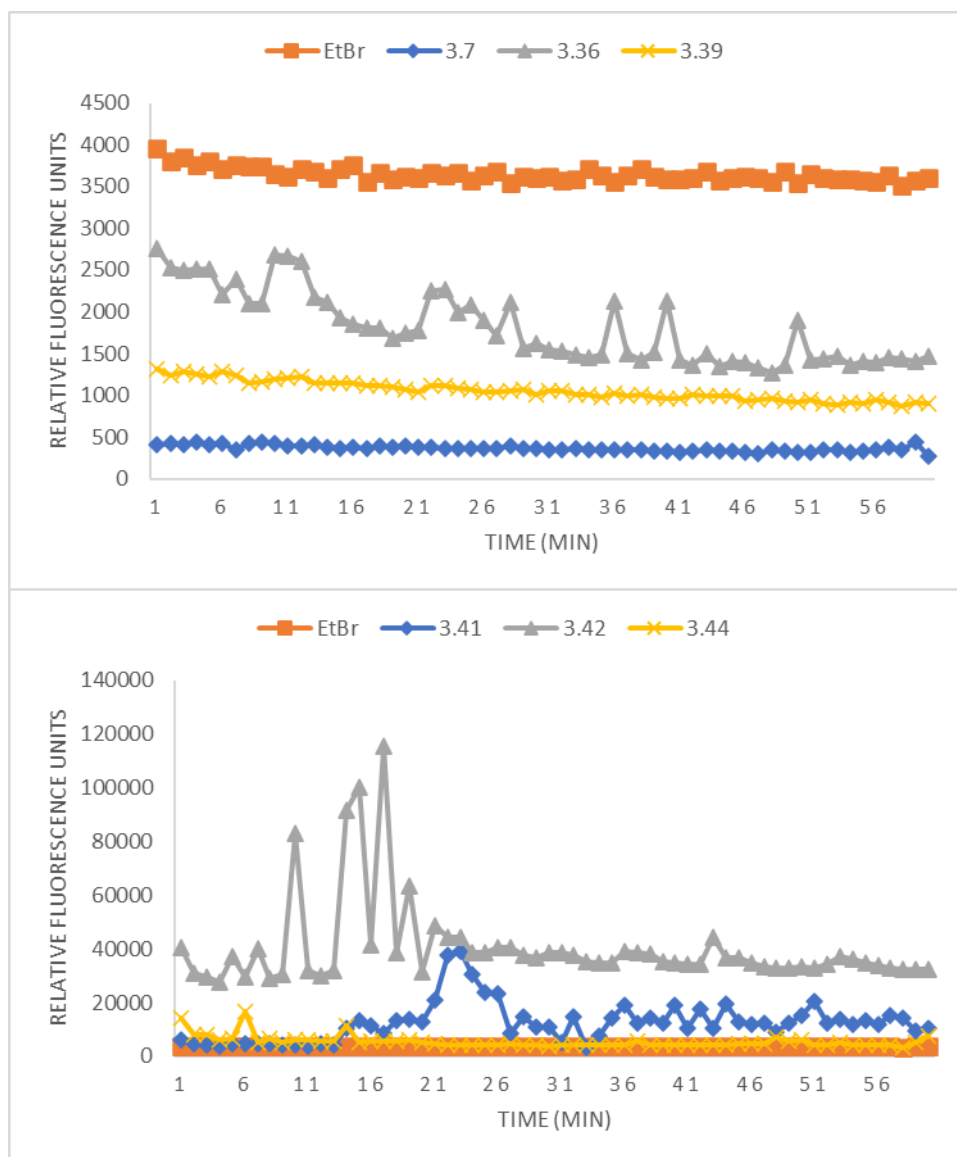


Figure 3.9 Fluorescence curves of EtBr and compounds **3.7**, **3.36**, and **3.39** (top), and **3.41**, **3.42**, and **3.44** (bottom).

Compound **3.7** was shown to display a low, constant fluorescence pattern, oppositely to the other compounds. With this knowledge, compounds **3.36**, **3.39**, **3.41**, **3.42**, and **3.44** were dismissed as EPIs, as their fluorescence patterns do not allow for any conclusions to be drawn regarding the modulation of EtBr efflux. The fact that none of the compounds present antibacterial activity against the strains they inhibit EPs is an advantage, as it makes them less prone to the appearance of AMR (63).

The thioxanthone derivatives appear to interfere with EPs preferentially in Gram-negative bacteria, with six active compounds (**3.7**, **3.11**, **3.22**, **3.34**, **3.37**, and **3.40**), against two (**3.5** and **3.7**) active for Gram-positive. This emphasizes the potential of a tricyclic scaffold with a sulfur heteroatom as a Gram-negative EPI, as was previously described for the antipsychotic chlorpromazine (**3.10**, **Figure 3.3**) (37). However, bacteria

present multiple efflux pumps of multiple families on their membranes, and in the absence of deeper studies it is not possible to point out which EP is being inhibited.

A visual inspection on the possible predicted interaction of the active compounds with their putative targets was established using PyMOL (64). According to the results obtained *in vitro*, compounds **3.5** and **3.7** were inspected in the homology model of the NorA pump, specifically in the binding core region, since this was predicted to be the site they would bind with the most affinity. At a first glance, the two compounds are predicted to bind in opposite positions in relation to each other (**Figure 3.10, A**). Compound **3.5** appears to establish a hydrogen interaction between the amine in C1 and Glu-222, as well as a π - π stacking between one of the aromatic rings and Phe-47 of the homology model (**Figure 3.10, B**). On the other hand, compound **3.7** appears to interact with Tyr-292 through the oxygen in the propoxy group (**Figure 3.10, C**).

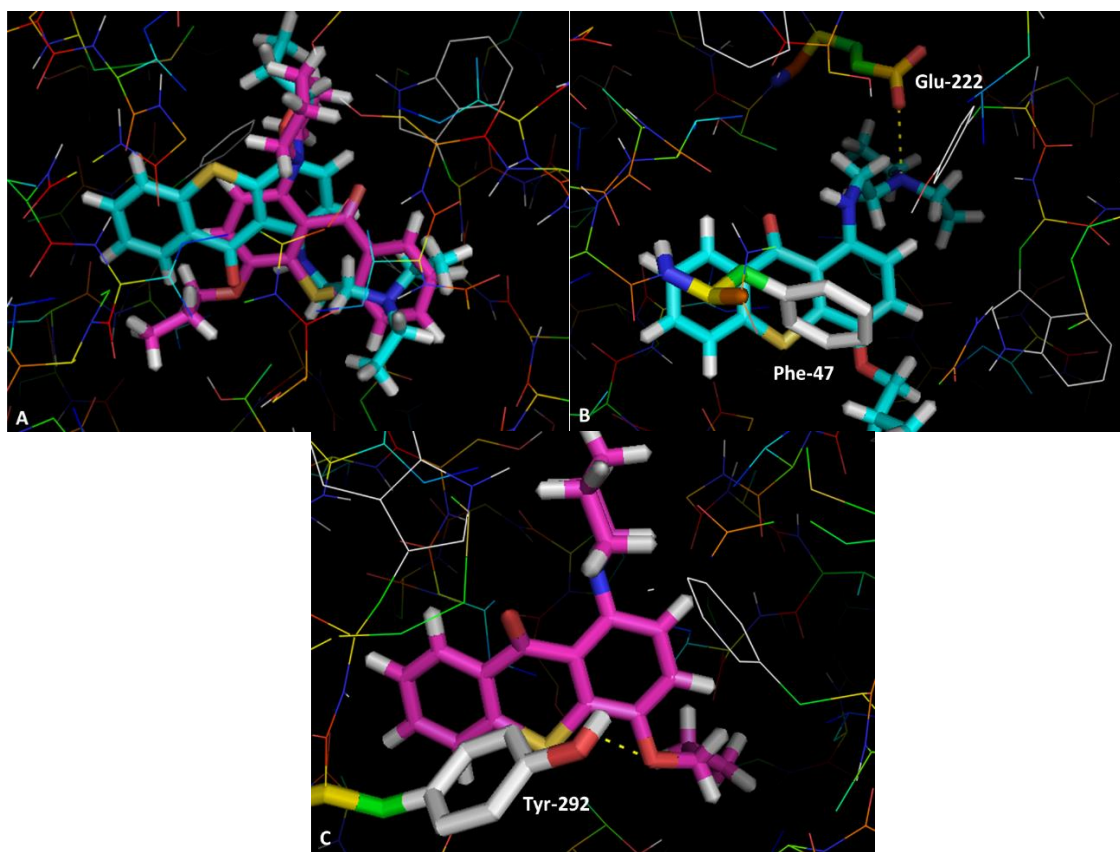


Figure 3.10 Molecular visualization of thioxanthenes in the binding core region of the NorA homology model
(A) General view of compounds **3.5** (blue) and **3.7** (pink); (B) Interaction between compound **3.5** and NorA;
(C) Interaction between compound **3.7** and NorA.

The same study was performed for AcrB and compounds **3.7**, **3.11**, **3.22**, **3.34**, **3.37**, and **3.40**. A general view shows that all compounds are predicted to bind approximately in the same site, but it seems they are likely to interact with different residues (**Figure 3.11, A**).

Compound **3.7** has the simplest and most flexible C1 substituent out of all the derivatives tested and is the only compound out of this library to be predicted to interact with Arg-620 through a hydrogen interaction with the oxygen in the propoxy chain (**Figure 3.11, B**).

Regarding compound **3.11**, the oxygen in the acetylpiperazine moiety can establish a hydrogen interaction with Gln-89, as can the oxygen in the propoxy chain with Asn-274, and the C9 carbonyl with Gln-176 (**Figure 3.11, C**).

Through the visualization of compound **3.22** in AcrB, dipole interactions can be seen between the nitrogen of the pyrimidine moiety and Gln-89 and Gln-176, as well as a T-shaped π - π interaction between the aromatic ring and Phe-615. Additionally, the C9 carbonyl can establish a hydrogen interaction with Gln-89 (**Figure 3.11, D**).

For compound **3.34**, a few hydrogen interactions are predicted to happen. The oxygens directly bound to the sulfur atom in the sulfonamide moiety seem to interact with Thr-87 and Arg-620, whereas the aromatic amine interacts with Ser-46. The C9 carbonyl is also likely to play a role in this context, as it forms a hydrogen interaction with a water molecule, which interacts with Gln-176 and Leu-177. Lastly, the oxygen in the propoxy chain seems to establish a hydrogen interaction with Gln-89 (**Figure 3.11, E**).

Considering compound **3.37**, the nitrogen in the piperazin-1-one moiety forms a hydrogen interaction with Gln-125, and the propoxy group displays the same hydrogen interaction with Gln-89 that has been previously described (**Figure 3.11, F**).

Finally, compound **3.40** is predicted to establish a hydrogen interaction between a nitrogen and Gly-126 (**Figure 3.11, G**).

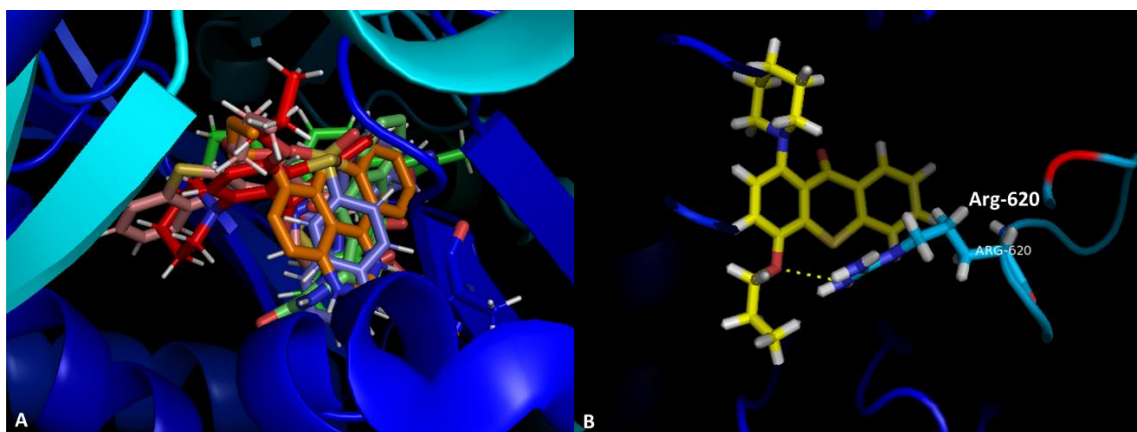


Figure 3.11 Molecular visualization of thioxanthenes in the substrate binding site of AcrB. (A) General view of compounds **3.7** (red), **3.11** (pink), **3.22** (light green), **3.34** (blue), **3.37** (dark green), and **3.40** (orange); (B) Interactions between compound **3.7** and AcrB; (C) Interactions between compound **3.11** and AcrB; (D) Interactions between compound **3.22** and AcrB; (E) Interactions between compound **3.34** and AcrB; (F) Interactions between compound **3.37** and AcrB; (G) Interactions between compound **3.40** and AcrB.

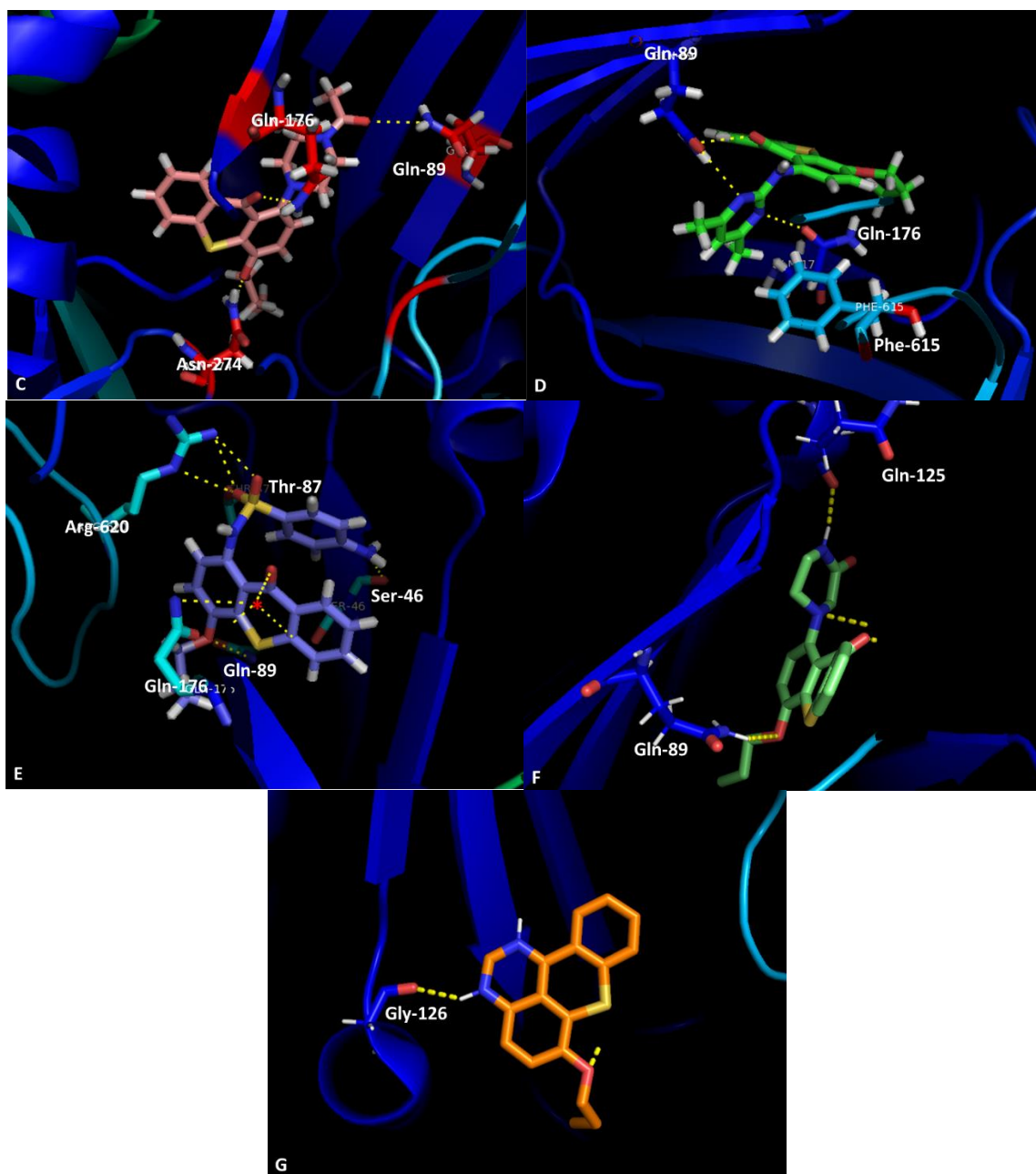


Figure 3.11 Molecular visualization of thioxanthenes in the substrate binding site of AcrB. (A) General view of compounds **3.7** (red), **3.11** (pink), **3.22** (light green), **3.34** (blue), **3.37** (dark green), and **3.40** (orange); (B) Interactions between compound **3.7** and AcrB; (C) Interactions between compound **3.11** and AcrB; (D) Interactions between compound **3.22** and AcrB; (E) Interactions between compound **3.34** and AcrB; (F) Interactions between compound **3.37** and AcrB; (G) Interactions between compound **3.40** and AcrB (cont.).

Even though this molecular visualization consists of computational predictions, it can constitute a useful tool in guiding future works concerning these efflux pumps, as the compounds have displayed EPI activity and, in the case of AcrB, there seems to be a couple of common residues they are likely to interact with. These are Gln-89 and Gln-176, which are polar amino acids, and were predicted to be involved in the interactions with compounds **3.11**, **3.22**, **3.34**, and **3.37**.

As thioxanthone derivatives showed promising results as bacterial EPs, they were tested for other resistance mechanisms related to EPs.

3.5.3 Inhibition of biofilm formation

Biofilm is a hydrated matrix of proteins and sugars which forms a slimy layer in which bacteria embed themselves in. This makes them able to adhere to surfaces, such as medical devices, and cause persistent infections, and subsequently evade host defenses and develop AMR. However, bacteria in biofilms are not necessarily resistant to antibacterial drugs, but rather tolerant, due to either being in a non-metabolizing, dormant state, which makes antibiotics that act on bacteria that are actively performing metabolic activities fail, or due to biofilm phenotypic factors (65, 66).

Despite this, AMR also develops in biofilm communities. Antibiotics penetrate in a slower rate through biofilms, or in an incomplete manner, the microenvironment is altered within the biofilm, with zones rich in waste accumulation and depleted of nutrients that could lead to antagonistic effects, and the formation of a subpopulation resembling spores, which are uniquely and highly protected (66, 67).

Bacterial EPs are closely related to the formation of biofilm, as they are responsible for the transport of extracellular polymeric substances, the regulation of the expression of genes that control biofilm formation, and can influence the way bacterial cells aggregate and adhere to surfaces (68).

Considering that EPs and biofilm are related phenomena, the thioxanthones derivatives were tested for their potential of inhibiting biofilm formation in two Gram-positive strains: *S. aureus* 25923 and the multi-resistant *S. aureus* 272123. The biofilm inhibition was presented in percentage (%) and was calculated based on the mean of absorbance units. The compounds were applied at 100 μ M or at half its MIC, as was the case of compounds **3.5** and **3.33** for *S. aureus* ATCC 25923 (41 and 12 μ M, respectively). Reserpine was used as a positive control in both strains, due to its potential as an EPI and an inhibitor of biofilm formation (69, 70). The results obtained in this assay are present in **Table 3.6**.

Table 3.6 Inhibition of biofilm formation by thioxanthone derivatives.

Compound	Inhibition of biofilm formation (%) $\pm$ SD	
	<i>S. aureus</i> ATCC 25923	<i>S. aureus</i> 272123
3.5	0.53 $\pm$ 1.05	81.1 $\pm$ 1.18
3.6	0.37 $\pm$ 0.47	0
3.7	93.4 $\pm$ 0.86	93.5 $\pm$ 0.33
3.8	0.17 $\pm$ 0.83	47.9 $\pm$ 1.08
3.11	0	47.1 $\pm$ 7.54
3.22	0	0

Compound	Inhibition of biofilm formation (%) $\pm$ SD	
	<i>S. aureus</i> ATCC 25923	<i>S. aureus</i> 272123
3.33	53.3 $\pm$ 7.81	96.5 $\pm$ 0.67
3.34	0.42 $\pm$ 1.36	72.0 $\pm$ 6.66
3.36	0	20.2 $\pm$ 4.00
3.37	5.18 $\pm$ 1.02	31.5 $\pm$ 8.99
3.39	0	2.46 $\pm$ 0.72
3.40	0	76.0 $\pm$ 2.06
3.41	0	73.6 $\pm$ 1.80
3.42	0	6.32 $\pm$ 4.32
3.43	19.7 $\pm$ 7.46	1.62 $\pm$ 2.43
3.44	0	55.2 $\pm$ 4.20
3.45	0	0
Reserpine	30.37 $\pm$ 7.32	72.10 $\pm$ 1.54

SD: Standard deviation.

The analysis of **Table 3.6** shows that compounds **3.5**, **3.7**, **3.33**, **3.34**, **3.40**, and **3.41** show a biofilm inhibition over 70% for *S. aureus* 272123. This reflects the potential of the compounds to act against this mechanism, as the compounds show biofilm formation inhibition comparable to that of reserpine. Furthermore, it is interesting to notice that compound **3.7** can inhibit biofilm formation in equal extent in both strains tested, which suggests that its mechanism remains unaltered in both reference and resistant strains. Lastly, this compound also displayed EP inhibition against *S. aureus* 272123, which could be related to these results.

3.5.4 Inhibition of quorum-sensing

QS is a communication mechanism used by bacteria, which comprises the production of autoinducers, extracellular signaling molecules, the detection by receptors in the membrane or cytoplasm, and the response to these molecules, which activates gene expression and boosts autoinducer production. It is an indicator of bacterial population density, used by bacteria to trail modifications in their cell counts and collectively change gene expression, particularly those which control activities that are advantageous when bacteria are acting in synchrony, such as bioluminescence, biofilm formation and secretion of virulence factors, among others (71).

There are differences between QS in Gram-positive and Gram-negative bacteria. The former uses autoinducing peptides as signaling molecules, which bind to a membrane-bound histidine kinase receptor, which, when phosphorylated, activates the transcription of genes, or are transported back to the cytoplasm where they can interact with transcription factors and modulate changes in gene expression (71, 72). The latter use mostly acyl-

homoserine lactones (AHL), which diffuse freely across the bacterial membranes, and bind to transcription factors and kinase receptors similar to those described for Gram-positive bacteria (73).

Even though some QS molecules can travel across membranes through diffusion, others need to be transported either by vesicles or EPs. As such, impairment in bacterial EPs can also have an impact in QS, and EPIs can also act to reverse the virulence of some bacteria (74).

Considering this, a QS assay was performed, using three different Gram-negative models: *C. violaceum* 026 (CV026), a sensor strain, and *Sphingomonas paucimobilis* Ezf 10-17 (EZF), an AHL-producing strain, which were inoculated as parallel lines; *Serratia marcescens* AS-1, which produces and responds to AHL; and *C. violaceum* wild-type 85 (wt85), which also produces AHL and responds to it. The inhibition of QS by the thioxanthone derivatives was measured by the reduction of pigment production, in millimeters (mm), using promethazine (PMZ) as a positive control (75, 76). The results are present in **Table 3.7**.

Table 3.7 Quorum-sensing inhibition by thioxanthone derivatives.

Compound	Quorum-sensing inhibition (mm) $\pm$ SD		
	<i>S. marcescens</i> AS-1	wt85	EZF + CV026
3.5	0	0	0
3.6	0	0	0
3.7	0	0	31 $\pm$ 0.8
3.8	0	0	0
3.11	0	0	30 $\pm$ 0.5
3.22	0	0	32 $\pm$ 0.1
3.33	0	0	0
3.34	0	0	51 $\pm$ 0.1
3.36	31 $\pm$ 0.8	0	0
3.37	0	0	0
3.39	0	0	0
3.40	32 $\pm$ 0.5	0	50 $\pm$ 0.1
3.41	0	0	29 $\pm$ 0.8
3.42	0	0	49 $\pm$ 0.5
3.43	0	0	0
3.44	0	0	0
3.45	0	0	0
PMZ	18 $\pm$ 0.8	40 $\pm$ 0.1	41 $\pm$ 0.5

wt85: *C. violaceum* wild-type 85; **EZF:** *S. paucimobilis* Ezf 10-17; **CV026:** *C. violaceum* 026; **SD:** Standard deviation; **PMZ:** Promethazine.

Due to the structural similarity between thioxanthenes and PMZ, used herein as a positive control, it was expected that the derivatives would display QS inhibition. After an analysis of the results, it could be seen that pigment production was affected mostly in the system EZF + CV026, with seven compounds active (**3.7**, **3.11**, **3.22**, **3.34**, and **3.40** – **3.42**). Compounds **3.36** and **3.40** were effective in *S. marcescens* AS-1. It is interesting to notice that compounds **3.7**, **3.11**, **3.22**, **3.34**, and **3.40** were also effective as Gram-negative EPIs, and **3.36**, **3.41** and **3.42** displayed inconclusive results due to fluorescence. As it has been reported that bacteria of the *Chromobacterium* genus have RND efflux systems, a relationship could be theorized for these results, since EPs have influence in QS (77).

3.5.5 Antifungal activity

Fungal diseases also constitute a large global burden, especially considering that individuals with compromised immune function are increasing. As antifungals are becoming inadequate, the search for new antifungal agents acquires a great impact (78).

As the need arises for new antifungal drugs with new targets, high safety and bioavailability, the thioxanthone derivatives herein described were tested against relevant strains: the yeast *Candida albicans* ATCC 10231, the filamentous fungus *Aspergillus fumigatus* ATCC 204305, and the dermatophyte *Trichophyton rubrum* FF5. The results regarding these assays are present in **Table 3.8**.

Table 3.8 Minimum inhibitory concentration of the thioxanthone derivatives tested against fungi.

Compound	Minimum inhibitory concentration (µg/mL)		
	<i>C. albicans</i> ATCC 10231	<i>A. fumigatus</i> ATCC 204305	<i>T. rubrum</i> FF5
3.5	64-128	16-32	16
3.6	>128	>128	>128
3.7	>128	>128	>128
3.8	>128	>128	>128
3.11	>128	>128	>128
3.22	>128	>128	>128
3.33	>128	>128	>128
3.34	>128	>128	>128
3.36	>128	>128	>128
3.37	>128	>128	>128
3.39	>128	>128	>128
3.40	>128	>128	>128
3.41	>128	>128	>128
3.42	>128	>128	>128

Compound	Minimum inhibitory concentration (µg/mL)		
	<i>C. albicans</i> ATCC 10231	<i>A. fumigatus</i> ATCC 204305	<i>T. rubrum</i> FF5
3.43	>128	>128	>128
3.44	>128	>128	>128
3.45	>128	>128	>128

Thioxanthone derivative **3.5** was the only compound active against the tested strains, having a mild activity on *C. albicans* ATCC 10231, with a MIC of 64 – 128 µg/mL, while for *A. fumigatus* ATCC 204305 and *T. rubrum* FF5 it displayed a lower MIC, of 16 – 32 and 16 µg/mL, respectively. Noteworthy is also the fact that this compound is fungicidal for *T. rubrum* at 16 µg/mL.

3.5.6 *In vitro* studies in mammalian cells

The thioxanthone derivatives were also tested in mammalian cells. Their safety was evaluated through the determination of their cytotoxicity in mouse embryonic fibroblasts (NIH/3T3 cell line) and in human colorectal adenocarcinoma cells (Caco-2 cell line). Lastly, their effects on P-gp expression and activity were evaluated.

3.5.6.1 Cytotoxicity in mouse embryonic fibroblasts

The cytotoxicity of the most promising compounds, **3.5**, **3.22**, **3.33**, **3.34**, and **3.40**, which presented the best and broadest overall results, was evaluated using mouse embryonic fibroblast cell line (NIH/3T3). The half maximal inhibitory effect (IC₅₀) is present in **Table 3.9**. Doxorubicin was used as a positive control.

Table 3.9 Cytotoxicity of the selected compounds in NIH/3T3 cell line.

Compound	IC ₅₀ (µM)
3.5	>100
3.22	>100
3.33	64.5 ± 4.54
3.34	>100
3.40	>100
Doxorubicin	12.1 ± 0.81

IC₅₀: half maximal inhibitory effect.

In general, the compounds were shown to be safe at high concentrations for the tested cell line. Only compound **3.33** showed measurable cytotoxicity although above bacterial inhibitory concentrations.

3.5.6.2 Cytotoxicity in human cells

The newly synthesized thioxanthenes **3.33**, **3.34**, **3.36**, **3.37**, **3.39** – **3.43** were investigated for their potential as P-gp inhibitors. As such, the cytotoxicity of these compounds (0 – 20 μ M) was initially evaluated, in Caco-2 cells, 24 hours after exposure, using two different cytotoxicity assays, the neutral red (NR) uptake and the sulforhodamine B (SRB) binding assays, which are based in different principles to measure cellular dysfunction as previously studied for compounds **3.5** – **3.8**, **3.11**, and **3.45** (12, 23, 79). In these studies, the tested derivatives showed efficacy in the modulation of P-gp expression and/or activity at the non-cytotoxic concentration of 20 μ M and after 24 hours of exposure, which justifies the incubation time and concentration range used (79).

As observed in

Figure 3.12, no significant differences in NR uptake were observed 24 hours after exposure to compounds **3.33**, **3.36**, **3.37**, **3.39**, and **3.43**, at any tested concentration (10 and 20 μ M), and when compared to control cells (0 μ M), demonstrating the lack of lysosomal impairment. For compounds **3.40** – **3.42**, a slight but significant decrease in the NR uptake was observed for the highest tested concentration (20 μ M), and when compared to control cells (NR uptake significantly decreased to 92.2, 94.1 and 95.5%, 24 hours after exposure to compounds **3.40**, **3.41**, and **3.42**). For compound **3.34**, no significant effect on NR uptake was detected 24 hours after exposure to the highest tested concentration (20 μ M), while a slight but significant decrease in the NR uptake was observed for the 10 μ M concentration (NR uptake significantly decreased to 95.5% over control cells, 24 hours after exposure to 10 μ M compound **3.34**).

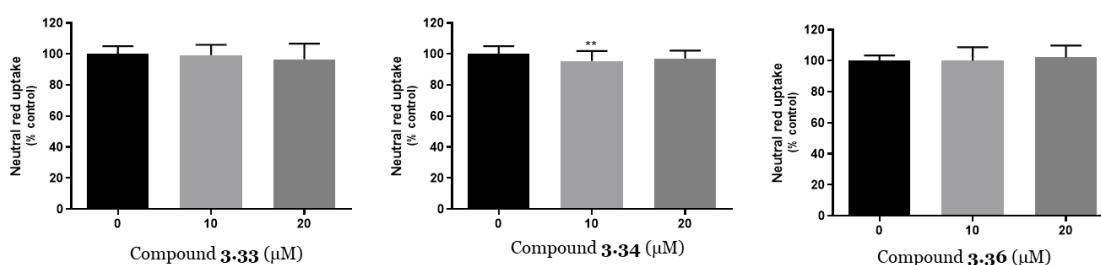


Figure 3.12 Cytotoxicity of the thioxanthone derivatives **3.33**, **3.34**, **3.36**, **3.37**, **3.39** – **3.43** (0 – 20 μ M), in Caco-2 cells, by the NR uptake assay, 24 hours after exposure. The results are presented as mean $\pm$ SD from four independent experiments, carried out in triplicate. Statistical comparisons were performed using the One-way ANOVA parametric method, followed by Dunnett's multiple comparisons test [$** p < 0.01$ vs. control (0 μ M)].

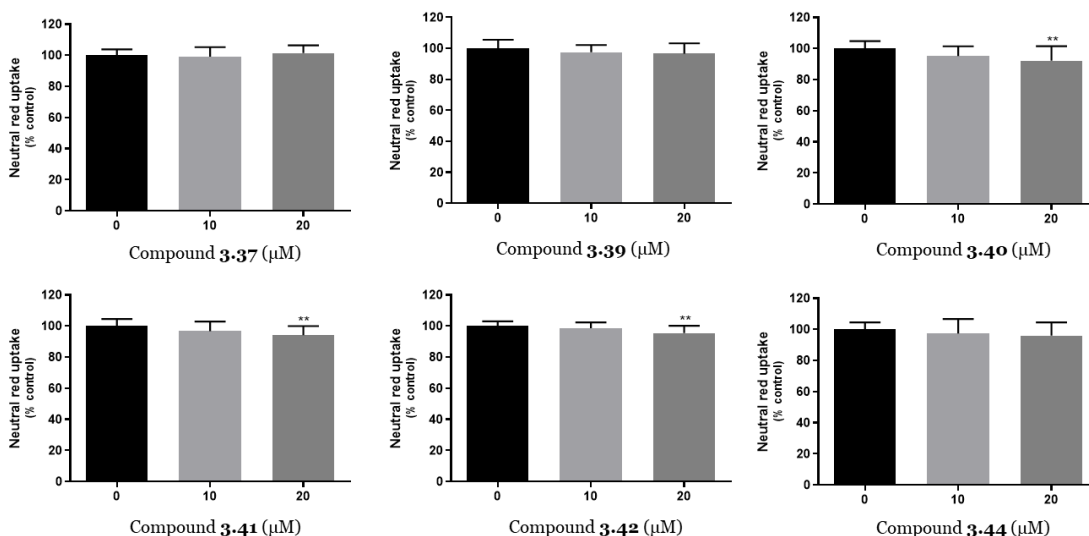


Figure 3.12 Cytotoxicity of the thioxanthone derivatives **3.33**, **3.34**, **3.36**, **3.37**, **3.39** – **3.43** (0 – 20 μM), in Caco-2 cells, by the NR uptake assay, 24 hours after exposure. The results are presented as mean ± SD from four independent experiments, carried out in triplicate. Statistical comparisons were performed using the One-way ANOVA parametric method, followed by Dunnett's multiple comparisons test [****** $p < 0.01$ vs. control (0 μM)] (cont.).

In the SRB binding assay, and as observed in **Figure 3.13**, no significant differences were detected in the ability of the dye to bind to the basic amino acids of cellular proteins, 24 hours after exposure to compounds **3.33**, **3.34**, **3.36**, **3.37**, **3.39**, **3.40** – **3.42**, at any tested concentration (10 and 20 μM), demonstrating the lack of significant differences in the total protein mass and, consequently, in the number of cells in culture. Concerning compound **3.43**, no significant differences in SRB binding were detected 24 hours after exposure to 10 μM concentration, but a slight but significant increase in SRB binding was observed for the highest tested concentration (20 μM), and when compared to control cells (SRB binding significantly increased to 105.8 % 24 hours after exposure to 20 μM compound **3.43**).

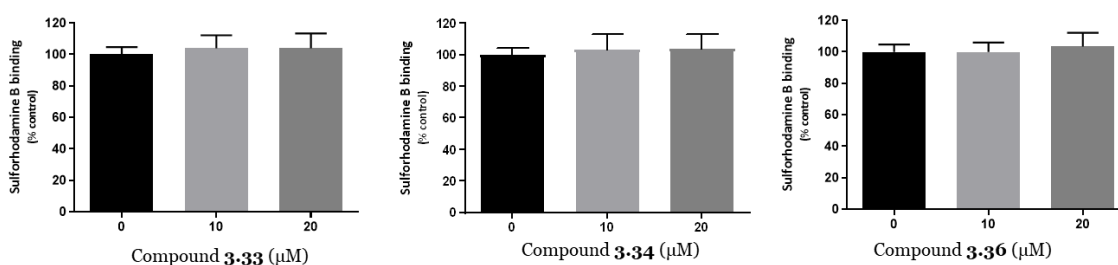


Figure 3.13 Cytotoxicity of the thioxanthone derivatives **3.33**, **3.34**, **3.36**, **3.37**, **3.39** – **3.43** (0 – 20 μM), in Caco-2 cells, by the SRB binding assay, 24 hours after exposure. The results are presented as mean ± SD from four independent experiments, carried out in triplicate. Statistical comparisons were performed using the One-way ANOVA parametric method, followed by Dunnett's multiple comparisons test [****** $p < 0.01$ vs. control (0 μM)].

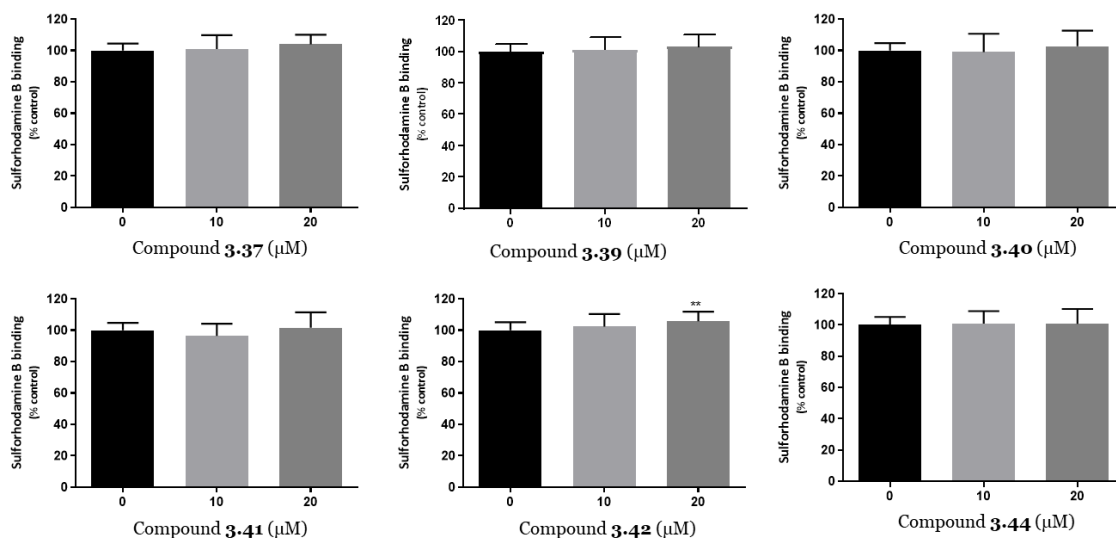


Figure 3.13 Cytotoxicity of the thioxanthone derivatives **3.33**, **3.34**, **3.36**, **3.37**, **3.39** – **3.43** (0 – 20 μM), in Caco-2 cells, by the SRB binding assay, 24 hours after exposure. The results are presented as mean $\pm$ SD from four independent experiments, carried out in triplicate. Statistical comparisons were performed using the One-way ANOVA parametric method, followed by Dunnett's multiple comparisons test [$** p < 0.01$ vs. control (0 μM)] (cont.).

Overall, the obtained results demonstrate the lack of relevant cytotoxicity of the thioxanthone derivatives towards Caco-2 cells, for concentrations up to 20 μM , and 24 hours after exposure. Furthermore, the high agreement between the results obtained in the NR uptake and SRB binding assays should be highlighted, which provides a high confidence in the obtained data. Based on these results, the non-cytotoxic 20 μM concentration was selected for the evaluation of the potential effects of compounds in P-gp expression and activity. In fact, the ability of similar thioxanthone derivatives **3.5** – **3.8**, **3.11**, and **3.45** for P-gp induction/activation has already been reported, at the same concentration and using the same *in vitro* model, the Caco-2 cells (79).

3.5.6.3 Effects on P-glycoprotein expression and activity

The effect of compounds on P-gp protein expression was evaluated, by flow cytometry, 24 hours after exposure, at 20 μM using the monoclonal UIC2-PE antibody, as was previously studied for the other compounds **3.5** – **3.8**, **3.11**, and **3.45** (20). However, the ability of compounds **3.33**, **3.34**, **3.36**, **3.37**, **3.39** – **3.43** to interfere with the fluorescence of the UIC2-PE antibody was firstly assessed.

For this purpose, as Caco-2 cells were incubated for 24 hours, in the presence or absence of thioxanthone derivatives at 20 μM , and subsequently analysed, by flow cytometry, as described for the P-gp expression assay. A remarkable increase in the intracellular fluorescence of cells exposed to compounds **3.33**, **3.34**, **3.36**, **3.40** – **3.42**, and **3.44** was

observed, making it impossible to assess the effect of these thioxanthone derivatives on P-gp expression. Only compounds **3.37** and **3.39** were tested further for their potential to modulate P-gp expression.

As observed in **Figure 3.14**, no significant differences in P-gp expression in cells pre-exposed to compound **3.37** for 24 hours were detected, when compared to control cells (0 μ M). In contrast, compound **3.39** caused a significant reduction in P-gp expression levels (to 83%), when compared to control cells (0 μ M, 100%).

The effects of the compounds (20 μ M) on P-gp activity were further assessed, by fluorescence spectroscopy, using rhodamine 123 as a P-gp fluorescent substrate and zosuquidar as a specific third generation P-gp inhibitor. For that purpose, two experimental designs were performed.

In the first experimental approach, the accumulation of the P-gp fluorescent substrate was assessed in Caco-2 cells pre-exposed to compounds (20 μ M) for 24 hours, aiming to elucidate possible changes in P-gp activity resulting from changes in P-gp expression, given the long period of incubation with the thioxanthone derivatives. The combination of the evaluation of both P-gp expression and activity is extremely important as an increased P-gp protein expression may not be necessarily accompanied by an increased activity of the pump (79, 80).

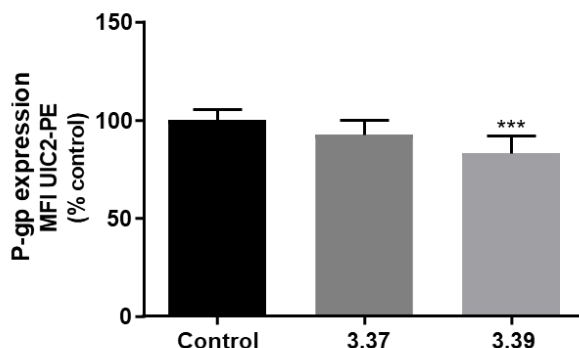


Figure 3.14 Evaluation of the expression levels of P-glycoprotein (P-gp), by flow cytometry, in Caco-2 cells exposed to compounds **3.37** and **3.39** (20 μ M) for 24 hours. The results are presented as mean $\pm$ SD of three independent experiments (performed in triplicate). Statistical comparisons were made using the One-way ANOVA parametric method, followed by Dunnett's multiple comparisons test [*** $p < 0.001$ vs. control (0 μ M)].

Preliminary studies to assess the potential interference of compounds autofluorescence in the analysis of P-gp activity were also performed. Accordingly, compounds **3.36**, **3.40**, **3.41**, and **3.42** were not tested for their ability to modulate P-gp activity, given their remarkable fluorescence in the experimental conditions used to evaluate rhodamine 123 intracellular fluorescence. As observed in **Figure 3.15**, none of the tested compounds significantly changed the P-gp activity 24 hours after exposure.

Concerning the second experimental approach used to evaluate the effects of the compounds in P-gp activity, Caco-2 cells were exposed to thioxanthone derivatives (20 μ M) only during the 90 minutes of incubation with the fluorescent substrate (rhodamine 123, 10 μ M). This experimental approach aims to evaluate potential immediate effects of thioxanthone derivatives on P-gp activity due to direct inhibition or activation of the protein, *i.e.*, not to change the protein expression.

According to the obtained results, and as observed in **Figure 3.15**, no significant differences in P-gp activity were detected in Caco-2 cells exposed to compounds **3.37** and **3.44**, when compared to control cells (0 μ M). In contrast, compounds **3.33** and **3.34** caused a significant and immediate decrease in P-gp activity (to 82 and 85%, respectively), when compared to control cells (0 μ M). On the other hand, the incubation of Caco-2 cells with compound **3.39** resulted in a slight, but significant, immediate increase in P-gp activity (116%), when compared to control cells (100%).

Therefore, compounds **3.33** and **3.34** demonstrated potential as P-gp inhibitors, while compound **3.39** demonstrated to be an activator of this important efflux protein, overall suggesting a potential new source of new P-gp modulators. Furthermore, although compound **3.39** significantly reduced P-gp expression in Caco-2 cells 24 hours after exposure (**Figure 3.14**), this decrease did not translate into a concomitant decrease in the protein activity (**Figure 3.15**).

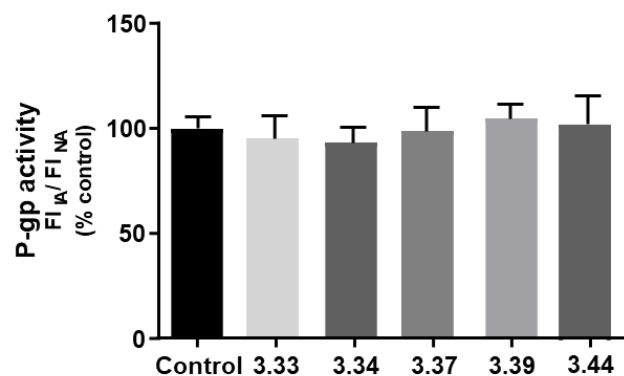


Figure 3.15 P-glycoprotein (P-gp) activity assessed by fluorescence spectroscopy in Caco-2 cells pre-exposed to the thioxanthone derivatives **3.33**, **3.34**, **3.37**, **3.39**, and **3.44** (20 μ M) for 24 hours. The results are presented as mean $\pm$ SD of five independent experiments, performed in triplicate. Statistical comparisons were made using the One-way ANOVA parametric method, followed by Dunnett's multiple comparisons test.

A possible explanation for this result could be a possible contribution of the protein activation mediated by the compound **3.39**, that could have remained intracellularly (after the 24 hours pre-exposure period), in agreement with its P-gp activation potential, observed in the experimental approach of evaluation of P-gp activity in Caco-2 cells incubated with

the compound **3.39** only during the accumulation with the P-gp fluorescent substrate (Figure 3.16).

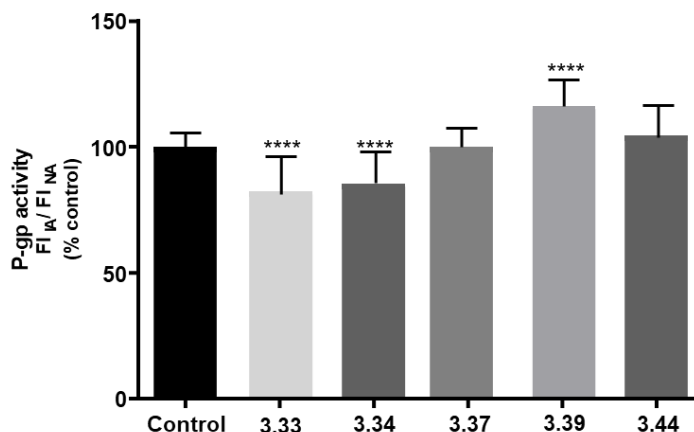


Figure 3.16 P-gp activity assessed by fluorescence spectroscopy in Caco-2 cells pre-exposed to thioxanthone derivatives **3.33**, **3.34**, **3.37**, **3.39**, and **3.44** (20 μ M) only during the 90 minutes of the incubation period with the fluorescent substrate (rhodamine 123, 10 μ M). The results are presented as mean $\pm$ SD of four independent experiments, performed in triplicate. Statistical comparisons were made using the One-way ANOVA parametric method, followed by Dunnett's multiple comparisons test [**** $p < 0.0001$ vs. control (0 μ M)].

3.6 Concluding remarks

In this chapter, the aim was to synthesize a library of thioxanthone derivatives with EPI activity. Based on the structures of previously described EPIs, a virtual library was designed and filtered according to their affinity to AcrB, a relevant target and to their drug-likeness. The docking studies employed showed that 20 out of the 1000 compounds were predicted to be similarly active towards AcrB as P-gp. The virtual screening employed was useful, as it allowed the rational choice of compounds to be synthesized.

The synthetic protocol followed yielded only five out of the 20 virtual hit compounds, **3.22**, **3.33**, **3.34**, **3.36**, **3.37**, and **3.44**. This happened because four of the compounds, **3.40** – **3.43**, experienced a dehydrative cyclization and formed tetracyclic thioxanthenes, which was unforeseen when designing the virtual library. Additionally, a halogen-substitution reaction was performed, yielding the 1-bromo derivative **3.39**, which was predicted to lead to better yields. The synthesis of other thioxanthone derivatives was attempted, but the yields were either very low, or the reaction did not happen. Overall, eleven new thioxanthone derivatives were synthesized and purified successfully.

These derivatives were then tested for their biological activity, and two compounds, **3.5** and **3.33**, were effective against Gram-positive strains, with **3.33** also displaying antibacterial activity against the Gram-negative *S. Typhimurium* SL1344, showing the

broad spectrum of activity of this last compound. Additionally, these compounds could also decrease the MIC of CTX by 16-fold in the ESBL-producing strain *E. coli* SA/2. For the same strain, compounds **3.22**, **3.43**, and **3.44** could also decrease the MIC of CTX by 4-, 2-, and 2-fold, respectively. Another impressive potentiation result was the one obtained with **3.37** and VAN, in *E. faecalis* B3/101, where a 16-fold MIC reduction was observed, while not displaying any antibacterial activity. Compound **3.5** was also the only compound to display antifungal activity, albeit broad spectrum, as it was active against the three tested strains.

Since the results obtained in the previous assay were unspecific, an assay directly related to the inhibition bacterial EPs was performed, and compounds **3.5** and **3.7** were found active against *S. aureus* 272123, while **3.7**, **3.11**, **3.22**, **3.34**, **3.37**, and **3.40** were active against *S. Typhimurium* SL1344. No specific structural features stand out as essential for activity, which was expected, since bacterial EPs are known to recognize a multiplicity of substrates with very diverse scaffolds. Out of the five virtual hits, three of them were EPIs, which is a positive result in this regard.

Other mechanisms related to EPs were also evaluated, such as biofilm formation and QS, which allowed to analyze the compounds in a wider scope. Compound **3.7** was notable in this regard, as it was able to drastically inhibit biofilm formation in the two *S. aureus* strains tested but could also inhibit QS in the model of EZF+CV026, which could be related to the EP inhibition displayed in both strains tested. The fact that compound **3.7** also did not show cytotoxicity makes it a promising compound, as it showed broad activity for the resistance mechanisms tested, and its use as an antibacterial adjuvant should be further investigated. Derivative **3.22** was also able to decrease EP activity in the *Salmonella* strain used, as well as QS in EZF+CV026. The fact that it shows efficacy in restoring the MIC of the antibiotic CTX in *E. coli* suggests that it can permeate the membrane of Gram-negative organisms, which makes it a good candidate for further studies.

The sulfonamide derivatives **3.33** and **3.34** have shown different profiles. Compound **3.33** was the only of the new derivatives to show antibacterial activity, against the reference strains of *S. aureus* and *E. faecalis* used, as well as against *S. Typhimurium*. It could also decrease the formation of biofilm in *S. aureus* 272123. However, this compound displayed cytotoxicity and inhibition of P-gp, which warrants further modifications to improve its safety and selectivity. Compound **3.34**, on the other hand, displayed effectivity at inhibiting efflux in *S. Typhimurium* SL1344 and biofilm formation in *S. aureus* 272123, but also inhibited P-gp. It is not clear which efflux pump is being inhibited in the EPI assay performed herein, and the fact that the sulfonamide derivatives inhibit both bacterial and mammalian EPs could mean they are acting on ABC efflux pumps, although further studies are needed to confirm this. In fact, and considering previous studies with aminated thioxanthenes, compounds **3.5**, **3.7**, and **3.11**, and in the light of the results described

throughout the previous chapters, these compounds also display broad spectrum activity, inhibiting mammalian and bacterial EPs, or are inhibiting pumps of the ABC family. This can pose a limitation in their use, as P-gp is involved in detoxification pathways, which could be impaired if these compounds were used. Compound **3.39**, however, showed activation of P-gp, which can make it useful in other situations unrelated to the scope of this work.

The tetracyclic thioxanthenes that were obtained unexpectedly also displayed high fluorescence, and their potential as bacterial EPIs could not be studied due to this characteristic, except for **3.40**, which proved to be useful against Gram-negative EPs. In order to study the real potential of this new class of compounds, an assay that does not rely on fluorescence could be used, such as genetic assays aiming to analyze the expression of genes coding for EPs. However, these assays are laborious and time-consuming, and it was not possible to perform them in this instance. Outside of the scope of this thesis, the fluorescence displayed by the tetracyclic thioxanthene derivatives was used as an advantage, since the compounds showed antitumor potential. By allying the cytotoxic properties demonstrated by these compounds with their fluorescence, they can be proposed as useful theranostic tools (81).

Overall, thioxanthenes show an interesting profile to be used against the circumvention of antibacterial resistance mechanisms, even though no structure-activity relationships (SAR) could be established with these results. Synergy studies with these strains could be suggested for further studies, particularly with antibiotics that are prone to efflux by EPs.

3.7 Experimental section

3.7.1 Chemistry

3.7.1.1 General considerations

All reagents and solvents used for the synthesis were purchased from Sigma Aldrich (Sigma-Aldrich Co. Ltd., Gillingham, UK) and no further purification process was implemented. Solvents were evaporated using a rotary evaporator under reduced pressure, Buchi Waterchath B-480. All reactions were monitored by thin-layer chromatography (TLC) carried out on precoated plates with 0.2 mm of thickness using Merck silica gel 60 (GF₂₅₄) with appropriate mobile phases. Compounds were easily detectable at 254 nm or 365 nm.

Flash column chromatography using silica gel 60 (0.040–0.063 mm, Merck, Darmstadt, Germany) was used in the purification of the synthesized compounds. Melting points (mp) were measured in a Köfler microscope (Wagner and Munz, Munich, Germany) and are uncorrected. ¹H- and ¹³C-nuclear magnetic resonance (NMR) spectra were recorded at the

University of Aveiro, Department of Chemistry in CDCl₃ or dimethyl sulfoxide (DMSO)-*d*₆ (Deutero GmbH, Ely, UK) at room temperature on a Bruker Avance 300 spectrometer (300.13 MHz for ¹H and 75.47 MHz for ¹³C, Bruker Biosciences Corporation, Billerica, MA, USA). Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference. Coupling constants are reported in hertz (Hz). ¹³C-NMR assignments were made by bidimensional heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) NMR experiments (long-range C, H coupling constants were optimized to 7 Hz) or by comparison with the assignments of similar molecules. High-resolution mass spectroscopy (HRMS) spectra were measured on a Bruker FTMS APEX III mass spectrometer (Bruker Corporation, Billerica, MA, USA) and recorded as electrospray ionization (ESI) mode in Centro de Apoio Científico e Tecnológico à Investigação (CACTI, University of Vigo, Pontevedra, Spain) or on a LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) at CEMUP, University of Porto, Portugal.

Quantitative and qualitative gas chromatography-mass spectrometry (GC-MS) analyses were performed on a Trace GC 2000 Series ThermoQuest gas chromatograph equipped with ion-trap GCQ Plus ThermoQuest Finnigan mass detector (Austin, Texas, USA). Chromatographic separation was achieved using a capillary column (30 m 0.25 mm 0.25 μm, cross-linked 5% diphenyl and 95% dimethyl polysiloxane) from Restek® (Pennsylvania, USA) and high-purity helium C60 as carrier gas. An initial temperature of 80 °C was maintained for 1 minute, increased to 300 °C at 10 °C/minute rate, and held for 5 minutes, giving a total run time of 45 minutes. The flow of the carrier gas was maintained at 1.5 mL/min. The injector port was set at 280 °C.

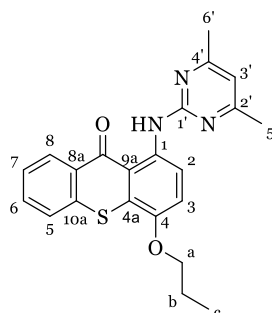
The peak purity of the new compounds was determined by high performance liquid chromatography (HPLC). The HPLC system consisted of Thermo Scientific SpectraSystem P4000 pump, equipped with a degasser, a Thermo Scientific SpectraSystem AS3000 autosampler fitted with a maximum volume 100 μL loop, and a Thermo Scientific SpectraSystem UV8000 DAD detector (USA). Data acquisition was performed using ChromQuest 5.0 software, version 3.2.1. The column used in this study was a reverse phase C18 HPLC column (ACE® 5 C18, 150 × 4.6 mm I.D., particle size 5 μm) manufactured by Advanced Chromatography Technologies Ltd (Aberdeen, Scotland, UK). The mobile phase composition was water and acetonitrile (30:70 *v/v*) or water, acetonitrile and diethylamine (30:70:0.1 *v/v/v*) (for **3.22** and **3.42**), all of which were HPLC grade solvents obtained from Merck Life Science S.L.U. (Darmstadt, Germany). The flow rate was 1.0 mL/minute and the UV detection wavelength was set at 261 nm. Analyses were performed at room temperature in an isocratic mode in a 30 minute run. Peak purity index was determined by total peak UV-Vis spectra between 210-650nm with a step of 4 nm.

Compounds **3.34** and **3.39** – **3.41** were also elucidated by X-ray crystallography. Diffraction data were collected at 291 K with a Gemini PX Ultra (Rigaku/Oxford, Neu-Isenburg, Germany) equipped with CuK α radiation (λ = 1.54184 Å). The structures were solved by direct methods using SHELXS-97 (82) and refined with SHELXL-97 (82). Carbon, oxygen, nitrogen, and sulfur atoms were refined anisotropically. Hydrogen atoms were either placed at their idealized positions using appropriate HFIX instructions in SHELXL, and included in subsequent refinement cycles, or were directly found from difference Fourier maps and were refined freely with isotropic displacement parameters. Full details of the data collection and refinement and tables of atomic coordinates, bond lengths and angles, and torsion angles have been deposited with the Cambridge Crystallographic Data Centre (CCDC).

3.7.1.2 General procedure for the synthesis of aminated thioxanthenes and tetracyclic thioxanthenes

The syntheses of compounds **3.5** – **3.8**, **3.11**, and **3.45** have been previously reported (12). Compounds **3.22**, **3.33**, **3.34**, **3.36**, **3.37**, and **3.40** – **3.44** through a copper-catalyzed Ullmann-type C – N coupling, starting from compound **3.12** or **3.39** (**Scheme 3.1**). Compounds **3.22**, **3.33**, **3.34**, **3.36**, **3.37**, and **3.40** – **3.44** were obtained by adding the appropriate amine or sulfamide to a suspension of 1-chloro-4-propoxy-9*H*-thioxanthen-9-one (**3.12**, 0.250 mg, 0.8 mmol) (**Scheme 3.1**, procedure **a**) or 1-bromo-4-propoxy-9*H*-thioxanthen-9-one (**3.39**, 0.250 mg, 0.7 mmol) (**Scheme 3.1**, procedure **c**) in methanol (25 mL), CuI (2 mg) and K₂CO₃ (0.1 mmol). The suspension was heated at 100 °C for 48 hours, in a sealed flask.

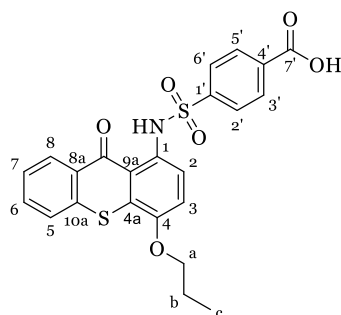
1-[(4,6-Dimethylpyrimidin-2-yl)amino]-4-propoxy-9*H*-thioxanthen-9-one (**3.22**)



3% as red dust (procedure **a**). mp 179.7 – 181.7 °C (methanol). IR (KBr) ν_{max} : 3445, 2966, 2919, 2878, 2851, 1594, 1575, 1505, 1475, 1465, 1443, 1401, 1384, 1335, 1291, 1267, 1240, 1226, 1166, 1116, 1086, 1063, 973, 890, 822, 789, 769, 629. ¹H NMR (CDCl₃, 300.13 MHz) δ (ppm): 12.8 (1H, s, NH), 9.01 (1H, d, J = 9.21, H2), 8.62 (1H, dd, J = 8.13 and 1.94 Hz, H8), 7.58 (2H, m, H5 and H6), 7.45 (1H, ddd, J = 8.19, 5.10 and 3.14 Hz, H7), 7.22 (1H, d, J =

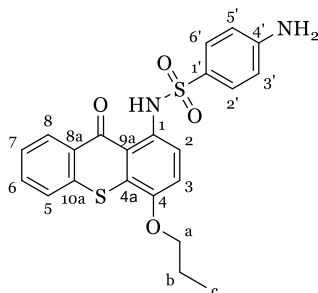
9.24 Hz, H3), 6.53 (1H, s, H3'), 4.12 (2H, t, $J = 6.44$ Hz, H_a), 2.42 (6H, s, H5' and H6'), 1.93 (2H, st, $J = 7.22$ Hz, H_b), 1.14 (3H, t, $J = 7.41$ Hz, H_c). ¹³C NMR (CDCl₃, 75.48 MHz) δ (ppm): 184.0 (C9), 167.5 (C2' and C4'), 159.9 (C1'), 146.7 (C4), 139.9 (C1), 136.9 (C10a), 132.1 (C6), 130.2 (C8a), 129.9 (C8), 129.0 (C4a), 126.3 (C7), 126.0 (C5), 116.6 (C3), 116.4 (C9a), 115.6 (C2), 112.2 (C3'), 71.8 (Ca), 24.2 (C5' and C6'), 22.8 (Cb), 10.9 (Cc) (**Figure S3.1**). HRMS (ESI+): m/z [C₂₂H₂₁N₃O₂S + H]⁺ calcd. for [C₂₂H₂₂N₃O₂S]: 392.1432; found 392.1437 (**Figure S3.2**). Peak purity [HPLC – C18; water, acetonitrile and diethylamine (30:70:0.1 v/v/v)]: 97.2% (**Figure S3.3**).

4-[N-(9-Oxo-4-propoxy-9H-thioxanthen-1-yl)sulfamoyl]benzoic acid (**3.33**)



3% (procedure **a**) and 48% as orange powder (procedure **b**). mp 324.6 – 326.4 °C (chloroform). IR (KBr) $\nu_{\max}$: 3440, 2967, 2879, 1595, 1554, 1460, 1437, 1383, 1357, 1292, 1266, 1205, 1165, 1133, 1087, 1061, 992, 832, 787, 751, 729, 699, 628, 604, 557. ¹H NMR (DMSO, 300.13 MHz) δ (ppm): 12.7 (1H, s, OH), 8.44 (1H, dd, $J = 8.28$ and 1.44, H8), 7.88 (3H, d, $J = 8.07$ Hz, H5, H3' and H5'), 7.77 (1H, ddd, $J = 8.22$, 6.92 and 1.38 Hz, H6), 7.71 (2H, d, $J = 8.01$, H2' and H6'), 7.59 (1H, ddd, $J = 8.25$, 6.95 and 1.38 Hz, H7), 7.51 (1H, d, $J = 8.94$ Hz, H2), 7.43 (1H, d, $J = 8.94$ Hz, H3), 4.10 (2H, t, $J = 6.30$ Hz, H_a), 1.78 (2H, st, $J = 7.04$ Hz, H_b), 1.02 (3H, t, $J = 7.38$ Hz, H_c). ¹³C NMR (DMSO, 75.48 MHz) δ (ppm): 185.2 (C9), 168.1 (C7'), 149.2 (C4), 145.2 (C4'), 138.0 (C1'), 136.1 (C10a), 134.2 (C1), 133.5 (C6), 129.6 (C3' and C5'), 129.1 (C8), 128.8 (C4a), 128.0 (C9a), 127.2 (C7 and C8a), 126.8 (C5), 126.2 (C2' and C6'), 115.9 (C2 and C3), 72.3 (Ca), 22.7 (Cb), 10.5 (Cc) (**Figure S3.4**). HRMS (ESI+): m/z [C₂₃H₁₉NO₆S₂ + H]⁺ calcd. for [C₂₃H₂₀NO₆S₂]: 470.0732; found 470.0726 (**Figure S3.5**). Peak purity [HPLC – C18; water and acetonitrile (30:70 v/v)]: 99.9% (**Figure S3.6**).

4-Amino-*N*-(9-oxo-4-propoxy-9*H*-thioxanthen-1-yl)benzenesulfonamide (**3.34**)



28% as orange crystals (procedure **c**). mp 211.9 – 213.5 °C [dichloromethane (9):acetone (1)]. IR (KBr) ν_{max} : 3479, 3377, 2968, 2879, 1630, 1591, 1569, 1500, 1460, 1435, 1362, 1316, 1287, 1264, 1211, 1185, 1176, 1147, 1089, 1061, 1008, 984, 939, 829, 745, 722, 673, 659, 635, 563, 547, 527, 474, 459. ^1H NMR (CDCl_3 , 300.13 MHz) δ (ppm): 12.4 (1H, s, NH), 8.51 (1H, dd, $J = 8.18$ and 1.97 Hz, H8), 7.66 (1H, d, $J = 8.94$ Hz, H3), 7.60 (4H, m, H5, H6, H2' and H6'), 7.47 (1H, ddd, $J = 8.22$, 6.05 and 2.21 Hz, H7), 7.06 (1H, d, $J = 9.00$ Hz, H2), 6.51 (2H, dt, $J = 9.24$ and 2.36 Hz, H3' and H5'), 4.07 (2H, t, $J = 6.36$ Hz, Ha), 1.90 (2H, st, $J = 7.37$ Hz, Hb), 1.12 (3H, t, $J = 7.41$ Hz, Hc). ^{13}C NMR (CDCl_3 , 125.77 MHz) δ (ppm): 184.2 (C9), 150.2 (C1), 149.2 (C4), 137.3 (C4'), 137.2 (C10a), 136.2 (C8a and C9a), 132.7 (C6), 129.8 (C8), 129.6 (C2' and C6'), 129.3 (C1'), 126.6 (C7), 126.4 (C4), 117.9 (C4a), 116.6 (C3), 115.0 (C2), 114.2 (C3' and C5'), 71.5 (Ca), 22.7 (Cb), 10.8 (Cc) (**Figure S3.7**). HRMS (ESI+): m/z [$\text{C}_{22}\text{H}_{20}\text{N}_2\text{O}_4\text{S}_2 + \text{Na}$] $^+$ calcd. for [$\text{C}_{23}\text{H}_{19}\text{NO}_6\text{S}_2\text{Na}$]: 462.0683; found 462.2658 (**Figure S3.8**). Peak purity [HPLC – C18; water and acetonitrile (30:70 v/v)]: 99.9% (**Figure S3.9**).

Crystal was monoclinic, space group $\text{P}2_1/\text{n}$, cell volume 2018.0(11) Å³ and unit cell dimensions $a = 11.016$ (3) Å, $b = 8.542$ (4) Å and $c = 21.450$ (4) Å, and $\beta = 91.252$ (17)° (uncertainties in parentheses). The refinement converged to R (all data) = 7.92% and wR_2 (all data) = 17.94%. CCDC 2081536 (**Figure 3.17**).

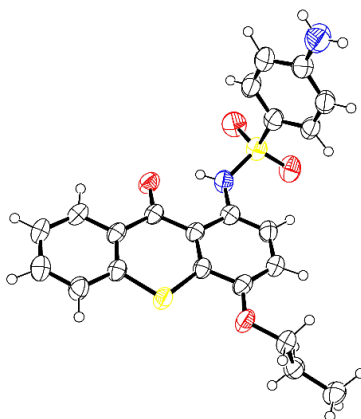
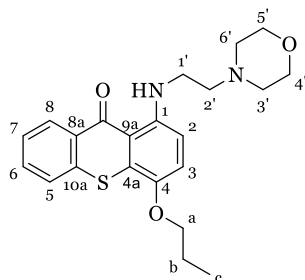


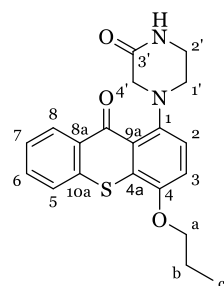
Figure 3.17 Oak Ridge thermal ellipsoid plot (ORTEP) view of compound **3.33**.

1-[(2-Morpholinoethyl)amino]-4-propoxy-9*H*-thioxanthen-9-one (**3.36**)



45% as orange needles (procedure **a**). mp 126.4 – 127.7 °C (methanol). IR (KBr) ν_{max} : 3448, 3287, 2963, 2924, 2853, 2818, 2363, 1739, 1615, 1574, 1497, 1466, 1443, 1435, 1392, 1294, 1270, 1245, 1226, 1171, 1147, 1115, 1072, 1037, 794, 746, 722. ^1H NMR (CDCl_3 , 500.13 MHz) δ (ppm): 9.90 (1H, t, $J = 4.50$ Hz, NH), 8.52 (1H, dd, $J = 8.01$ and 1.86 Hz, H8), 7.52 (2H, m, H5 and H6), 7.40 (1H, ddd, $J = 8.16$, 4.79 and 3.44 Hz, H7), 7.13 (1H, d, $J = 9.06$ Hz, H3), 6.52 (1H, d, $J = 9.06$ Hz, H2), 4.01 (2H, t, $J = 6.50$ Hz, Ha), 3.79 (4H, t, $J = 4.65$ Hz, H3' and H4'), 3.36 (2H, q, $J = 6.51$ Hz, H1'), 2.76 (2H, t, $J = 6.59$ Hz, H2'), 2.57 (4H, t, $J = 4.55$ Hz, H3' and H6'), 1.88 (2H, st, $J = 7.32$ Hz, Hb), 1.11 (3H, t, $J = 7.40$ Hz, Hc). ^{13}C NMR (CDCl_3 , 75.48 MHz) δ (ppm): 183.4 (C9), 149.2 (C1), 142.6 (C4), 136.8 (C10a), 131.7 (C6), 130.4 (C8a), 129.8 (C4a), 129.3 (C8), 126.0 (C5), 125.9 (C7), 120.3 (C3), 113.2 (C10a), 106.5 (C2), 72.8 (Ca), 67.2 (C4' and C5'), 57.2 (C2'), 53.8 (C3' and C6'), 40.6 (C1'), 23.1 (Cb), 10.7 (Cc) (**Figure S3.10**). HRMS (ESI+): m/z [$\text{C}_{22}\text{H}_{26}\text{N}_2\text{O}_3\text{S} + \text{H}$] $^+$ calcd. for [$\text{C}_{22}\text{H}_{27}\text{N}_2\text{O}_3\text{S}$]: 399.1742; found 399.1749 (**Figure S3.11**). Peak purity [HPLC – C18; water and acetonitrile (30:70 v/v)]: 99.9% (**Figure S3.12**).

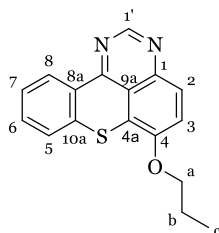
4-(9-Oxo-4-propoxy-9*H*-thioxanthen-1-yl)piperazin-2-one (**3.37**)



12% as red dust (procedure **a**). mp >350 °C (methanol). IR (KBr) ν_{max} : 3435, 2963, 2922, 2852, 2360, 1604, 1508, 1457, 1430, 1395, 1348, 1320, 1291, 1261, 1232, 1170, 1106, 1061, 998, 967, 919, 809, 744, 732, 621, 575, 529, 472, 418. ^1H NMR (DMSO, 500.13 MHz) δ (ppm): 8.55 (1H, s, NH), 7.90 (1H, dd, $J = 7.70$ and 1.38 Hz, H8), 7.51 (1H, dd, $J = 7.80$ and 1.09 Hz, H5), 7.40 (1H, td, $J = 7.98$ and 1.57 Hz, H6), 7.35 (1H, td, $J = 7.45$ and 1.27 Hz, H7), 7.00 (1H, d, $J = 9.00$ Hz, H3), 6.91 (1H, d, $J = 8.90$ Hz, H2), 3.98 (2H, t, $J = 6.38$ Hz, Ha), 3.86 (2H, t, $J = 5.30$ Hz, H1'), 3.73 (2H, t, $J = 5.53$ Hz, H2'), 3.29 (2H, s, H4'), 1.75 (2H, st, $J = 7.32$ Hz, Hb), 1.03 (3H, t, $J = 7.40$ Hz, Hc). ^{13}C NMR (DMSO, 75.48 MHz) δ (ppm): 171.8

(C3'), 163.1 (C9), 146.6 (C4), 144.9 (C1), 134.9 (C8a), 131.7 (C10a), 128.8 (C6), 126.8 (C8), 126.3 (C7), 125.2 (C5), 122.5 (C4a), 121.0 (C9a), 117.3 (C2), 114.2 (C3), 70.3 (Ca), 60.7 (C1'), 50.2 (C2'), 57.7 (C4'), 22.3 (Cb), 10.6 (Cc) (**Figure S3.13**). HRMS (ESI⁺): *m/z* [C₂₀H₂₀N₂O₃S + H]⁺ calcd. for [C₂₀H₂₁N₂O₃S]: 369.1273; found 369.1263 (**Figure S3.14**). Peak purity [HPLC – C18; water and acetonitrile (30:70 *v/v*)]: 99.8% (**Figure S3.15**).

6-propoxythiochromeno[4,3,2-*de*]quinazoline (**3.40**)



11% as yellow needles (procedure **a**). mp 89 – 91 °C [chloroform (9):acetone (1)]. IR (KBr) ν_{max} : 3443, 2922, 2852, 2360, 2342, 1570, 1560, 1530, 1502, 1442, 1369, 1344, 1281, 1262, 1210, 1101, 1069, 1028, 988, 831, 803, 750, 724, 669. ¹H NMR (CDCl₃, 300.13 MHz) δ (ppm): 9.02 (1H, s, H1'), 8.85 (1H, dd, *J* = 7.98 and 1.04 Hz, H8), 7.74 (1H, d, *J* = 9.06 Hz, H2), 7.53 (1H, d, *J* = 9.09 Hz, H3), 7.48 (1H, ddd, *J* = 8.04, 6.69 and 1.39 Hz, H6), 7.43 (1H, m, H5), 7.38 (1H, ddd, *J* = 8.07, 6.69 and 1.43 Hz, H7), 4.19 (2H, t, *J* = 6.41 Hz, Ha), 1.93 (2H, st, *J* = 7.26 Hz, Hb), 1.14 (3H, t, *J* = 7.41 Hz, Hc). ¹³C NMR (CDCl₃, 75.48 MHz) δ (ppm): 158.2 (C9), 154.4 (C1'), 149.3 (C4), 146.9 (C1), 136.0 (C1a), 131.9 (C6), 128.0 (C8), 127.8 (C8a), 126.8 (C7), 126.7 (C5), 123.2 (C2), 120.6 (C9a), 119.0 (C3), 118.6 (C4a), 71.4 (Ca), 22.9 (Cb), 10.8 (Cc) (**Figure S3.19**). HRMS (ESI⁺): *m/z* [C₁₇H₁₄N₂OS + H]⁺ calcd. for [C₁₇H₁₅N₂OS]: 295.0900; found 295.0893 (**Figure S3.20**). Peak purity [HPLC – C18; water and acetonitrile (30:70 *v/v*)]: 99.9% (**Figure S3.21**).

Crystals were monoclinic, space group P2₁/c, cell volume was 2990.30 (10) Å³ and unit cell dimensions were *a* = 12.1985 (2) Å, *b* = 13.4082 (3) Å and *c* = 18.8486 (3) Å and β = 104.0758 (18)°. There were two molecules in the asymmetric unit. The refinement converged to *R* (all data) = 7.26% and *wR*₂ (all data) = 15.53%. CCDC 2065174 (**Figure 3.18**).

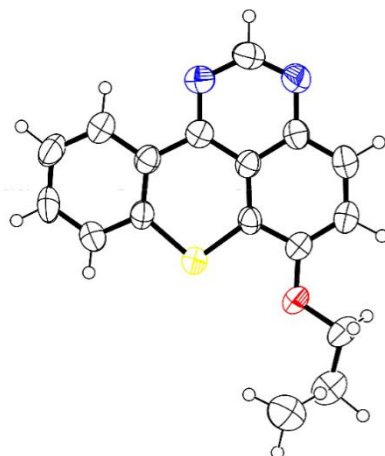
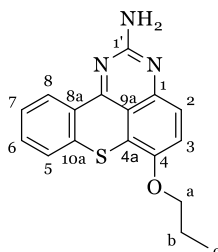


Figure 3.18 ORTEP view of compound **3.40**.

6-propoxythiochromeno[4,3,2-*de*]quinazolin-2-amine (**3.41**)



25% as red needles (procedure **a**). mp 180 – 182 °C [chloroform (8):acetone (2)]. IR (KBr) ν_{max} : 3462, 3297, 3157, 2963, 1638, 1608, 1569, 1558, 1545, 1502, 1453, 1436, 1405, 1391, 1368, 1318, 1261, 1232, 1152, 1123, 1100, 1064, 980, 906, 823, 800, 765, 726, 643, 607, 572, 539, 475. ^1H NMR (CDCl_3 , 500.13 MHz) δ (ppm): 8.79 (1H, dd, J = 8.10 and 1.58 Hz, H8), 7.46 (1H, ddd, J = 8.05, 6.75 and 1.35 Hz, H6), 7.43 (1H, dd, J = 8.00 and 1.58 Hz, H5), 7.39 (1H, d, J = 9.00 Hz, H3), 7.36 (1H, ddd, J = 8.15, 6.70 and 1.53 Hz, H7), 7.32 (1H, d, J = 9.00 Hz, H2), 5.05 (2H, s, NH₂), 4.12 (2H, t, J = 6.45 Hz, Ha), 1.90 (2H, st, J = 7.35 Hz, Hb), 1.12 (3H, t, J = 7.43 Hz, Hc). ^{13}C NMR (CDCl_3 , 75.48 MHz) δ (ppm): 159.8 (C9), 159.6 (C1'), 149.2 (C1), 146.6 (C4), 136.4 (C10a), 131.6 (C6), 127.9 (C8), 127.7 (C8a), 126.7 (C4a), 126.4 (C7), 120.2 (C3), 120.1 (C2), 119.6 (C4a), 115.4 (C9a), 71.7 (Ca), 23.0 (Cb), 10.8 (Cc) (**Figure S3.22**). HRMS (ESI⁺): m/z [$\text{C}_{17}\text{H}_{15}\text{N}_3\text{OS} + \text{H}$]⁺ calcd. for [$\text{C}_{17}\text{H}_{16}\text{N}_3\text{OS}$]: 310.1009; found 310.0910 (**Figure S3.23**). Peak purity [HPLC – C18; water and acetonitrile (30:70 v/v)]: 99.9% (**Figure S3.24**).

Crystals were triclinic, space group P-1, cell volume was 966.33 (18) Å³ and unit cell dimensions were a = 7.4849 (5) Å, b = 11.7086 (16) Å and c = 12.4900 (13) Å and α = 63.635 (12)°, β = 80.550 (7)°, γ = 88.220 (8)°. One molecule of **3.41** and one molecule of toluene (solvent) were found in the asymmetric unit. The refinement converged to R (all data) = 9.89% and wR_2 (all data) = 13.55%. CCDC 2069933 (**Figure 3.19**).

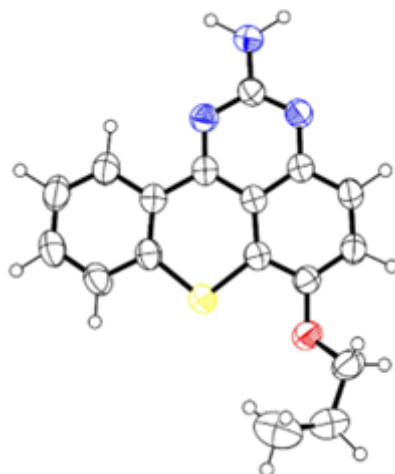
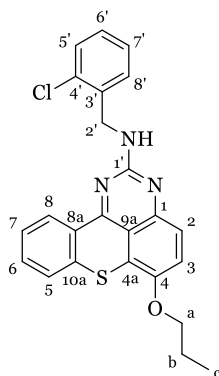


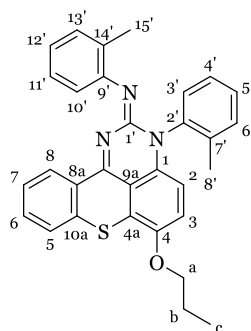
Figure 3.19 ORTEP view of compound **3.41**.

N-(2-chlorobenzyl)-6-propoxythiochromeno[4,3,2-*de*]quinazolin-2-amine (**3.42**)



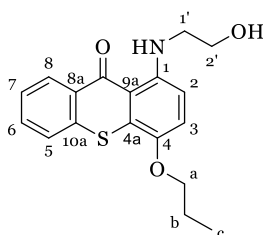
45% as red needles (procedure **c**). mp 166.9 – 168.0 °C (methanol). IR (KBr) ν_{max} : 3245, 2962, 2936, 2880, 1611, 1553, 1533, 1473, 1439, 1401, 1351, 1335, 1316, 1260, 1251, 1229, 1075, 1049, 1039, 990, 819, 794, 761, 742, 721. ^1H NMR (CDCl_3 , 300.13 MHz) δ (ppm): 8.77 (1H, dd, $J = 7.89$ and 1.16 Hz, H8), 7.38 (1H, d, $J = 9.18$ Hz, H3), 7.38 (7H, m, H5, H6, H7, H5', H6', H7' and H8'), 7.20 (1H, d, $J = 9.27$ Hz, H2), 5.55 (1H, t, $J = 5.49$ Hz, NH), 4.90 (2H, d, $J = 5.97$ Hz, H2'), 4.11 (2H, t, $J = 6.45$ Hz, Ha), 1.89 (2H, st, $J = 7.28$ Hz, Hb), 1.12 (3H, t, $J = 7.40$ Hz, Hc). ^{13}C NMR (CDCl_3 , 75.48 MHz) δ (ppm): 159.2 (C9), 159.0 (C1'), 149.2 (C1), 146.3 (C4), 137.32 (C4'), 136.3 (C10a), 133.7 (C3'), 131.4 (C6), 129.8 (C8'), 127.8 (C8), 127.0 (C8a), 119.5 (C4a), 71.8 (Ca), 43.5 (C3'), 23.0 (Cb), 10.8 (Cc), Car (C2, C3, C5, C7, C9a, C5', C6' and C7 (**Figure S3.25**)). HRMS (ESI⁺): m/z [$\text{C}_{24}\text{H}_{20}\text{ClN}_3\text{OS} + \text{H}$]⁺ calcd. for [$\text{C}_{24}\text{H}_{21}\text{ClN}_3\text{OS}$]: 434.1094; found 434.1099 (**Figure S3.26**). Peak purity [HPLC – C18; water and acetonitrile (30:70 v/v)]: 97.2% (**Figure S3.27**).

(*E*)-6-propoxy-*N*,3-di-*o*-tolylthiochromeno[4,3,2-*de*]quinazolin-2(3*H*)-imine (**3.43**)



13% as red dust (procedure **a**). mp 194.6 – 196.0 °C (chloroform:*n*-hexane). IR (KBr) ν_{max} : 3423, 2964, 2935, 2876, 1601, 1578, 1542, 1467, 1421, 1384, 1337, 1317, 1263, 1124, 1080, 1070, 1043, 1023, 1004, 938, 806, 791, 774, 761, 749, 722, 671, 654, 584. ^1H NMR (CDCl_3 , 300.13 MHz) δ (ppm): 8.44 (1H, dd, $J = 8.19$ and 0.98 Hz, H8), 7.51 (1H, m, H10'), 7.49 (1H, m, H6), 7.43 (3H, m, H4', H11' and H13'), 7.32 (2H, m, H3' and H5'), 7.12 (3H, m, H5, H6' and H12'), 6.99 (1H, d, $J = 9.03$ Hz, H3), 6.91 (1H, td, $J = 6.48$ and 2.05 Hz, H7), 6.13 (1H, d, $J = 8.94$ Hz, H2), 4.03 (2H, t, $J = 6.39$ Hz, Ha), 2.25 (3H, s, H15'), 2.08 (3H, s, H8'), 1.89 (2H, st, $J = 7.24$ Hz, Hb), 1.11 (3H, t, $J = 7.40$ Hz, Hc). ^{13}C NMR (CDCl_3 , 75.48 MHz) δ (ppm): 157.5 (C9), 147.9 (C2'), 147.0 (C4), 140.0 (C1), 137.9 (C1'), 136.8 (C9'), 135.8 (C10a), 132.0 (C13'), 131.8 (C6'), 131.8 (C6), 131.2 (C8a), 129.8 (C6'), 129.2 (C4'), 129.1 (C8), 129.0 (C3'), 128.4 (C11'), 127.7 (C14'), 127.0 (C5'), 126.4 (C10'), 125.6 (C12'), 123.5 (C4a), 123.1 (C5), 121.9 (C7), 117.2 (C3), 111.7 (C9a), 109.6 (C2), 71.6 (Ca), 22.8 (Cb), 18.6 (C8'), 17.8 (C15'), 10.8 (Cc) (**Figure S3.28**). HRMS (ESI^+): m/z [$\text{C}_{31}\text{H}_{28}\text{N}_3\text{OS} + \text{H}$] $^+$ calcd. for [$\text{C}_{31}\text{H}_{29}\text{N}_3\text{OS}$]: 490.1953; found 490.1948 (**Figure S3.29**). Peak purity [HPLC – C18; water and acetonitrile (30:70 v/v)]: 96.8% (**Figure S3.30**).

1-[(2-Hydroxyethyl)amino]-4-propoxy-9*H*-thioxanthen-9-one (**3.44**)



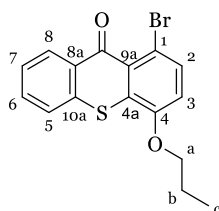
7% as red needles (procedure **a**). mp 173.1 – 173.9 °C (methanol). IR (KBr) ν_{max} : 3447, 2360, 2342, 1636, 669, 649, 494, 458. ^1H NMR (CDCl_3 , 300.13 MHz) δ (ppm): 10.1 (1H, s, OH), 8.49 (1H, td, $J = 8.1$ and 1.1 Hz, H8), 7.53 (2H, m, H5 and H7), 7.40 (1H, ddd, $J = 8.2$, 4.6 and 3.7 Hz, H6), 7.12 (1H, d, $J = 9.0$ Hz, H3), 6.61 (1H, d, $J = 9.1$ Hz, H2), 4.01 (2H, t, $J = 6.5$ Hz, Ha), 3.96 (2H, t, $J = 5.4$ Hz, H2'), 3.46 (2H, t, $J = 5.4$ Hz, H1'), 1.88 (2H, st, $J = 7.4$ Hz, Hb), 1.12 (3H, t, $J = 7.4$ Hz, Hc). ^{13}C NMR (CDCl_3 , 125.77 MHz) δ (ppm): 183.6 (C9), 148.5 (C1), 143.1 (C4), 136.9 (C10a), 132.6 (C5), 130.2 (C9a), 129.8 (C8a), 129.3 (C8), 126.0

(C6 and C7), 119.3 (C3), 112.8 (C4a), 107.8 (C2), 72.7 (Ca), 61.3 (C2'), 45.9 (C1'), 23.0 (Cb), 10.9 (Cc) (**Figure S3.31**). HRMS (ESI⁺): *m/z* calcd. for [C₁₈H₁₉NO₃S]: 329.1086; found 329.1066 (**Figure S3.32**). Peak purity [HPLC – C18; water and acetonitrile (30:70 *v/v*)]: 99.9% (**Figure S3.33**).

3.7.1.3 General procedure for the synthesis of 1-bromo-4-propoxy-9*H*-thioxanthen-9-one (**3.39**)

From a suspension of 1-chloro-4-propoxy-9*H*-thioxanthen-9-one (**3.12**) (400 mg, 1 mmol) in nitrobenzene (10 mL), KBr (5 mmol), CuI (0.01 mmol) and H₃PO₄ (8 mmol) (**Scheme 3.1**, procedure **b**), compound **3.39**, a bromine derivative, was obtained. The reaction mixture stirred at 200 °C (reflux) for 48 hours.

1-Bromo-4-propoxy-9*H*-thioxanthen-9-one (**3.39**)



54% as brown needles (procedure **b**); mp 88 – 90 °C (methanol). IR (KBr) ν_{max} : 3442, 2961, 2874, 2360, 1637, 1592, 1580, 1560, 1460, 1427, 1375, 1307, 1254, 1173, 1114, 1053, 961, 911, 865, 819, 807, 787, 753, 745, 677, 642, 566, 478. ¹H NMR (CDCl₃, 300.13 MHz) δ (ppm): 8.45 (1H, dd, *J* = 8.01 and 0.83 Hz, H8), 7.66 (1H, d, *J* = 8.61 Hz, H3), 7.58 (2H, m, H5 and H6), 7.44 (1H, ddd, *J* = 8.13, 5.40 and 2.80 Hz, H7), 6.89 (1H, d, *J* = 8.61 Hz, H2), 4.11 (2H, t, *J* = 6.39 Hz, Ha), 1.94 (2H, st, *J* = 7.35 Hz, Hb), 1.14 (3H, t, *J* = 7.41 Hz, Hc). ¹³C NMR (CDCl₃, 75.48 MHz) δ (ppm): 180.5 (C9), 153.3 (C4), 135.7 (C8a), 133.2 (C3), 132.1 (C6), 130.6 (C1), 130.5 (C9a), 129.8 (C8), 127.4 (C10a), 126.7 (C7), 126.1 (C5), 113.7 (C4a), 113.2 (C2), 71.4 (Ca), 22.6 (Cb), 10.8 (Cc) (**Figure S3.16**). MS (EI) *m/z* (%): 349.9 (9) [M + 2]⁺, 347.9 (10) [M]⁺, 307.9 (32), 305.9 (32), 279.9 (20), 277.9 (20), 227.0 (65), 199.0 (57), 171.0 (100) (**Figure S3.17**). Peak purity [HPLC – C18; water and acetonitrile (30:70 *v/v*)]: 99.9% (**Figure S3.18**).

Crystal was triclinic, space group P-1, cell volume 2018.0 (11) Å³ and unit cell dimensions *a* = 11.3197 (8) Å, *b* = 11.9631 (8) Å and *c* = 12.7932 (10) Å, and cell angles α = 108.678 (6)°, β = 111.986 (7)° and γ = 99.290 (6)°. The refinement converged to *R* (all data) = 11.96% and *wR*₂ (all data) = 26.62%. CCDC 2081537 (**Figure 3.20**).

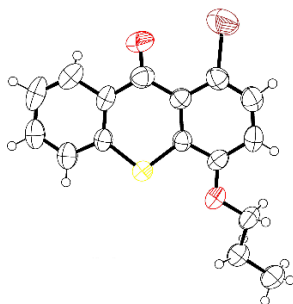


Figure 3.20 ORTEP view of compound **3.39**.

3.7.2 Docking studies

The crystal structure of the AcrB (PDB: 4DX5) (83), AcrA (PDB: 2F1M) (84), and TolC (PDB: 1EK9) (85) portions of the AcrAB-TolC bacterial efflux system, downloaded from the protein databank (PDB) (28), were used for this study. The known EPIs PA β N, reserpine and verapamil, along with the tested compounds were drawn with ChemDraw (PerkinElmer Informatics) and minimized using ArgusLab. Docking was carried out using AutoDock Vina (Scripps, CA, USA) (51), in the sites described in (45, 55). The NorA efflux pump does not have an available crystal structure, and a homology model was prepared. The model was generated using the Swiss Model server (86) and the sequence deposited in Uniprot (Q5HHX4) (87), using the EmrD pump from *E. coli* (PDB: 2GFP) as the homolog, as described in (56).

Regarding P-gp, the 3D structure of the ATP-bound P-gp in the outward-facing conformation, which has a low affinity for substrates and modulators, was established by cryo-electron microscopy (88). Although the human P-gp structure in the inward-facing conformation, which has higher affinity for substrates and modulators, is still not described, there are several mice P-gp structures obtained by X-ray structures (89) and by electron density maps already published (90, 91).

Therefore, a human P-gp homology model was built using as template the mice 3D P-gp with the highest % identity and the availability of 3D coordinates with the best resolution, which was 4Q9H mice P-gp, with a % identity of 89.0%, and a resolution of 3.4 Å (91). After single sequence alignment, model creation, incorporation of missing loops to the model, insertion of side chains, verification of overall geometry, addition of hydrogen atoms, adding of charges, and final staged minimization performed using Sybyl (Tripos Certara, MO, USA), the human P-gp model was obtained and validated by single-point energy calculation and Ramachandran plot analysis (98.7%, i.e., 1179/1194 of all residues were in the allowed regions). The top nine poses were collected for each molecule and the lowest docking score value was associated with the most favorable binding conformation.

PyMOL (Schrödinger, NY, USA) (64) was used for visual inspection of results and graphical representations.

3.7.3 Biological activity

3.7.3.1 Culture media and chemicals

The culture media used in the experiments were the following: cation-adjusted Mueller-Hinton broth (MHB II; Sigma-Aldrich, St- Louis, MO, USA and Biokar Diagnostics, Allone, Beauvais, France), Luria-Bertani broth (LB-B; Sigma, St. Louis, MO, USA), Tryptic Soy broth (TSB; Scharlau Chemie S. A., Barcelona), and Tryptic-Soy agar (TSA; Biokar Diagnostics, Allone, Beauvais, France), were purchased. Sabouraud Dextrose Agar (SDA) was purchased from bio-Mérieux, Marcy L'Etoile, France, RPMI-1640 broth medium from Biochrom AG, Berlin, Germany, which was buffered with 3-(*N*-morpholino) propanesulfonic acid (MOPS), purchased from Sigma-Aldrich, St. Louis, MO, USA, to pH 7.0. The modified Luria-Bertani agar (LB*-A), used for the QS inhibition assays, was prepared in-house, according to the formula: 1.0 g yeast extract (Merck, Darmstadt, Germany), 10.0 g tryptone (Biolab, Budapest, Hungary), 10.0 g NaCl (Molar Chemicals, Halásztelek, Hungary), 1.0 g K₂HPO₄ (Biolab, Budapest, Hungary), 0.3 g MgSO₄ x 7H₂O (Reanal, Budapest, Hungary), 5 ml Fe- ethylenediaminetetraacetic acid (EDTA) stock solution and 20.0 g of bacteriological agar (Molar Chemicals, Halásztelek, Hungary) per 1 L of media.

Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, NR solution 0.33%, SRB, rhodamine 123, zosuquidar, DMSO and Trizma® base were obtained from Sigma (St. Louis, Missouri, USA). Nonessential amino acids, heat-inactivated fetal bovine serum, antibiotic (10 000 U/mL penicillin, 10 000 µg/mL streptomycin), Hank's Balanced Salt Solution with or without calcium and magnesium [HBSS (+/+) and HBSS (-/-), respectively] and 0.25% trypsin/1 mM EDTA were purchased from Gibco Laboratories (Lenexa, Kansas, USA). Triton™ X100 was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dulbecco's Phosphate-Buffered Saline (PBS) solution without calcium, magnesium and phenol red [PBS (-/-)] was purchased from Grisp (Porto, Portugal). P-gp monoclonal antibody (clone UIC2) conjugated with phycoerythrin, was purchased from Abcam (Cambridge, United Kingdom). Reagents used in flow cytometry, namely, the cleaning solution, decontamination solution and flow cell extended clean solution were purchased from BD Biosciences (San Jose, CA, USA).

DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), PBS (pH 7.4), EtBr, reserpine, CCCP, PMZ, and crystal violet (CV) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Doxorubicin 2

mg/mL was purchased from Teva Pharmaceuticals, Budapest, Hungary. The antibiotic CTX was purchased from Duchefa Biochemie (Haarlem, The Netherlands) and VAN from Oxoid (Basingstoke, England). Bacteria were purchased from ATCC, the cell line NIH/3T3 was purchased from Sigma (Steinheim, Germany) and Caco-2 cells were obtained from the European Collection of Cell Culture (ECACC, UK).

3.7.3.2 Microorganisms

As Gram-positive strains, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, methicillin and ofloxacin-resistant *S. aureus* 272123 clinical isolate, and environmental isolate VRE *E. faecalis* B3/101 (57) were used. As Gram-negative strains, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, the *acrA* gene inactivated mutant *S. enterica* serovar Typhimurium SL1344, and clinical isolates of the ESBL *E. coli* SA/2 were investigated in this study.

For the QS tests, all the strains used were Gram-negative. The bacteria used were *C. violaceum* wild type 85 characterized by the AHL signal molecule-mediated production of the purple violacein pigment, capable of endogenous QS-signal molecule-production (*N*-hexanoyl-L-HSL), *C. violaceum* CV026 (CV026), a Tn5 transposase-mutant, AHL-signal molecule indicator strain (produces purple violacein pigment in the presence of AHL), which is incapable of endogenous QS-signal molecule-production, but useful in the detection of external stimuli, *S. paucimobilis* Ezf 10-17 (EZF), AHL-producing-strain (used with *C. violaceum* CV026), and *S. marcescens* AS-1, characterized by the production AHL signal molecule-mediated production of the orange-red pigment prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin), capable of endogenous QS-signal molecule-production (*N*-hexanoyl- L-HSL) (76).

As yeast *C. albicans* ATCC 10231, and as filamentous fungus *A. fumigatus* ATCC 204305 and a dermatophyte (*T. rubrum* FF5, clinical isolate) were used. Cultures were obtained in SDA.

3.7.3.3 Antibacterial assay and potentiation of antibiotics

The MIC for each compound was determined by broth microdilution method in a 96-well plate according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (92). The media used was MHB II. The concentrations tested ranged from 100 µM to 0.195 µM and were prepared from a stock solution of 10 mM in DMSO, for *S. aureus* 272123 and *S. Typhimurium* SL1344; for the other strains, the concentrations tested ranged from 64 µg/mL to 4 µg/mL and were prepared from a stock solution of 10 mg/mL in DMSO. DMSO

was used in subinhibitory concentrations (1 % *v/v*). The MIC was determined by visual inspection.

The combined effect of the compounds and clinically relevant antimicrobial drugs was evaluated by determining the antibiotic MIC in the presence of each compound. Briefly, the MIC of CTX and VAN, for *E. coli* SA/2 and *E. faecalis* B3/101, respectively, were determined in the presence of the highest concentration of each compound tested that did not affect bacterial growth when the compound was used alone. The antibiotic tested was serially diluted whereas the concentration of each compound was kept fixed. In the case of compound **3.33** the concentration used was 8 µg/mL. For all other compounds, the concentration used was 64 µg/mL. Antibiotic MICs were determined as described above.

3.7.3.4 Efflux pump inhibition assay

The compounds were evaluated for their ability to inhibit the efflux of EtBr, an efflux pump substrate in *S. enterica* serovar Typhimurium SL1344 and *S. aureus* 272123 strains, through real-time fluorimetry, monitoring the intracellular accumulation of EtBr, an efflux pump substrate.

This was determined by the automated method using a CLARIOstar Plus plate reader (BMG Labtech, Ortenberg, Germany). Reserpine and CCCP were applied at 25 µM as positive controls, and the solvent DMSO was applied at 1 % *v/v*. The bacterial strains were incubated in an appropriate culture media (TSB – *S. aureus* 272123; LB-B – *S. Typhimurium* SL1344) at 37 °C until they reached an optical density (OD) of 0.6 at 600 nm. The culture was centrifuged at 13,000 × *g* for 3 minutes, and the pellet washed and resuspended with phosphate buffered saline (PBS, pH 7.4). The suspension was centrifuged again in the same conditions and resuspended in PBS.

Compound **3.33** was applied at one-third MIC concentration in *S. Typhimurium* SL1344, 17 µM, and the rest was applied at 50 µM, in a solution of a non-toxic concentration of EtBr (1 µg/mL) in PBS. Then, 50 µL of this solution were transferred into a 96-well black microtiter plate (Greiner Bio-One Hungary Kft, Mosonmagyaróvár, Fertőszor, Hungary), and 50 µL of bacterial suspension (OD₆₀₀ = 0.6) were added to each well. The plates were placed into the CLARIOstar plate reader, and the fluorescence was monitored at excitation and emission wavelengths of 530 nm and 600 nm, respectively, every minute for one hour in a real-time basis. From the real-time data, the activity of the compounds, namely the RFI of the last time point (minute 60) of the EtBr accumulation assay, was calculated according to the following equation:

$$RFI = \frac{RF_{\text{treated}} - RF_{\text{untreated}}}{RF_{\text{untreated}}}$$

where RF_{treated} is the relative fluorescence (RF) at the last time point of EtBr accumulation curve in the presence of the compound, and $RF_{\text{untreated}}$ is the RF at the last time point of the EB accumulation curve of the untreated control, having only the solvent (DMSO) control (93). The samples were tested in triplicate, and the RFI presented comes from the average of these three values. The SD was calculated automatically.

3.7.3.5 Inhibition of biofilm formation

The thioxanthone derivatives were tested for their ability to decrease the formation of biofilm. The bacterial strains used were the Gram-positive *S. aureus* ATCC 25923 and *S. aureus* 272123.

The detection of the biofilm formation was carried out using the dye CV (0.1 % v/v). The initial inoculum was incubated in TSB overnight, and then diluted to an OD_{600} of 0.1. Then, the bacterial suspension was added to 96-well microtiter plates and the compounds were added at a sub-MIC concentration. This meant that compound **3.33** was added at 50 μM for *S. aureus* ATCC 25923, while the rest of the compounds were added at the concentration of 100 μM . The final volume in each well was 200 μL . Reserpine was used as positive control, as it was the same compound used in the efflux pump inhibition assay and it has shown activity in the inhibition of biofilm formation in *S. aureus* strains (69). The plates were incubated at 30 °C for 48 hours, with gentle stirring (100 rpm). After this incubation period, the TSB medium was discarded, and the plates were washed with tap water to remove unattached cells. Afterwards, 200 μL of a 0.1 % v/v CV solution were added to the wells and incubated for 15 minutes at room temperature. Then, the CV solution was removed from the wells, and the plates were washed again with tap water, and 200 μL of a 70 % ethanol solution were added to the wells. The biofilm formation was determined by measuring the OD_{600} using a Multiscan EX ELISA plate reader (Thermo Labsystems, Cheshire, WA, USA). The anti-biofilm effect of the compounds was expressed in the percentage (%) of decrease of biofilm formation.

3.7.3.6 Inhibition of quorum-sensing

The QS inhibitory effect of the compounds was examined on the EZF and the sensor CVO26 strains, on the wt85 strain, and on *S. marcescens*. The method used was the parallel inoculation method, where pair combinations of the used sensor strain CVO26 and the AHL producing strain EZF were inoculated directly onto the LB*-A agar surface in parallel, at an approximate distance of 5 mm from each other. *S. marcescens* AS-1 and wt85 were inoculated as a single line.

Filter paper disks (7 mm in diameter) were placed on the center of the inoculated line(s) and impregnated with 8 μ L of a solution of 10 mM of the compounds. PMZ was used as positive control, as previous results have demonstrated its activity as a QS inhibitor (75).

The agar plates were incubated at room temperature (20 °C) for 24 – 48 hours. The QS inhibition was accessed visually, through the inhibition of pigment production. The discolored, but intact, bacterial colonies were measured with a ruler (75).

3.7.3.7 Antifungal activity

The susceptibility tests for yeasts were performed based on the CLSI description for the broth microdilution method on the reference document M27A-3 for yeasts (94) and M38-A2 (95) for filamentous fungi, in RPMI-1640 broth culture media. The concentrations tested ranged from 128 μ g/mL to 32 μ g/mL.

The MIC was considered the lowest concentration that was able to totally inhibit the growth when comparing to control (non-treated microorganism in culture medium). A DMSO control (microorganism in RPMI with DMSO (1% *v/v*) was included.

3.7.3.8 *In vitro* studies in mammalian cells

3.7.3.8.1 Cytotoxicity in NIH/3T3 cell line

A 10.0 mM stock solution of each compound was prepared in DMSO. All stock solutions were stored at –80 °C and freshly diluted on the day of the experiment in fresh cell culture medium (ensuring that DMSO did not exceed 0.1 % DMSO concentration of the exposure media).

Mouse fibroblasts (NIH/3T3, ATCC CRL-1658TM) were cultivated in DMEM (Gibco 52100-039) and supplemented with 10 % heat-inactivated fetal bovine serum (Biowest, VWR International Kft, Debrecen, Hungary), 2 mM of L-glutamine, 1 mM Na pyruvate, 100 U/L and 10 mg/L penicillin/streptomycin mixture (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), respectively, and 0.1 % nystatin (8.3 g/L in ethylene glycol). The adherent cells were detached using a combination of 0.25 % Trypsin-Versene (EDTA) solution for 5 minutes at 37 °C. Before each cytotoxicity assay using this cell line, cells were seeded in untreated 96-well flat-bottom microtiter plates, following a 4-hour incubation period in a humidified atmosphere (5 % CO₂, 95 % air) at 37 °C (96).

The cytotoxicity of **3.5**, **3.22**, **3.33**, **3.34**, and **3.40** was assessed in NIH/3T3 cells, using MTT assay. Prior to the assay, the cells were seeded for 4 hours using 1×10^4 cells/well. The compounds were added by 2-fold serial dilutions to the cells distributed into 96-well flat bottom microtiter plates starting with 100 μ M. The plates were incubated for 24 hours, after which a solution of MTT in PBS was added to each well and incubated for another 4 hours.

After this, 100 µL of SDS (10% in a 0.01 M HCl solution) were added to each well and incubated overnight at 37 °C. Doxorubicin was used as positive control. Cell growth was determined in quadruplicate by measuring OD at λ=540 nm (reference 630 nm) in a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). The percentage of inhibition of cell growth was determined according to the equation.

$$100 - \left(\frac{OD_{\text{sample}} - OD_{\text{medium control}}}{OD_{\text{cell control}} - OD_{\text{medium control}}} \right) \times 100$$

3.7.3.8.2 Cytotoxicity in Caco-2 cell line

A 20.0 mM stock solution of each compound was prepared in DMSO. All stock solutions were stored at –80°C and freshly diluted on the day of the experiment in fresh cell culture medium (ensuring that DMSO did not exceed 0.1 % DMSO concentration of the exposure media).

Caco-2 cells were routinely cultured in 75 cm² flasks using DMEM with 4.5 g/L glucose supplemented with 10 % fetal bovine serum, 1 % non-essential amino acids and 1 % antibiotic. Cells were maintained at 37 °C, in a 5 % CO₂ – 95 % air atmosphere, and the cell culture medium was changed every 2 – 3 days. Cultures were passaged weekly by trypsinization (0.25 % trypsin/1 mM EDTA). In all experiments, the cells were seeded at a density of 60 000 cells/cm² and used three days after seeding, when confluence was reached. The cells used in all experiments were taken between the 55th and 64th passages.

The cytotoxicity of the compounds (0 - 20 µM) was evaluated by the NR uptake and SRB binding assays, 24 hours after exposure. Caco-2 cells were seeded onto 96-well plates at a density of 60 000 cells/cm² and exposed, after reaching confluence (three days after seeding), to the compounds (0 – 20 µM), in fresh cell culture medium. Triton™ X-100 (0.1%) was used as positive control.

3.7.3.8.3 Neutral red uptake assay

The NR assay is based on the ability of viable cells to incorporate and bind the supravital dye NR into the lysosomes, thus providing a quantitative estimation of the number of viable cells in a culture (97).

Twenty-four hours after exposure to the compounds, the cell culture medium was removed, followed by the addition of fresh cell culture medium containing 50 µg/mL NR, and incubation, at 37°C in a humidified 5 % CO₂ – 95% air atmosphere, for 90 minutes. After this incubation period, the cell culture medium was removed, the dye absorbed only by viable cells extracted with absolute ethyl alcohol/distilled water (1:1) with 5% acetic acid,

and the absorbance measured at 540 nm in a multi-well plate reader (PowerWaveX BioTek Instruments, Vermont, USA). The percentage of NR uptake relatively to that of the control cells (0 μ M) was used as the cytotoxicity measure. Five independent experiments were performed, in triplicate.

3.7.3.8.4 Sulforhodamine B binding assay

The SRB binding assay is based in the pH dependent binding of SRB to basic amino acids of cellular proteins under mild acidic conditions. The colorimetric evaluation of bounded SRB thus provides an estimation of the total protein mass, which is related to the number of cells in culture (98).

Twenty-four hours after exposure to the compounds, the cell culture medium was removed, the cells were washed with Hank's Balanced Salt Solution (+/+) and fixed overnight, at -20°C , with a methanolic solution of 1 % acetic acid (*v/v*). The fixation medium was then removed, and the cells incubated with a 0.05 % SRB solution (prepared in 1% acetic acid) for 60 minutes, at 37°C .

After incubation, the SRB solution was removed, and the cells washed with 1 % acetic acid (*v/v*) to remove the unbound dye. The bounded SRB was then extracted with a Tris base solution (10 mM, pH 10.5) and the absorbance measured, at 540 nm, in a multi-well plate reader (PowerWaveX BioTek Instruments, Vermont, USA). The percentage of SRB binding relatively to that of the control cells (0 μ M) was used as the cytotoxicity measure. Six independent experiments were performed, in triplicate.

3.7.3.8.5 Flow cytometry analysis of P-glycoprotein expression

The effect of the compounds on P-gp expression was evaluated by flow cytometry, 24 hours after exposure, using a P-gp monoclonal antibody (UIC2 clone conjugated with PE), as previously described (99).

Briefly, Caco-2 cells were seeded in 24-well plates at a density of 60 000 cells/cm² and exposed, three days after seeding, to a non-cytotoxic concentration of the tested compounds (20 μ M). After 24 hours of exposure, the cell culture medium was removed, the cells washed with Hank's Balanced Salt Solution (-/-) and harvested by trypsinization (0.25 % trypsin/1 mM EDTA) to obtain a cell suspension. The obtained cell suspension was transferred into 99ppendorf tubes, centrifuged (300 g for 5 minutes, at 4°C) and re-suspended in Hank's Balanced Salt Solution (+/+) buffer containing the P-gp antibody (the antibody dilution was applied according to the manufacturer's instructions for flow cytometry).

The incubation with the P-gp antibody was performed at 37°C , in the dark, and under gentle shaking, for 60 minutes. The cells were then washed twice with Hank's Balanced Salt

Solution (+/+), centrifuged (300 g for 5 minutes, at 4°C) and kept on ice until analysis. Cells were then re-suspended on ice-cold PBS (-/-) buffer immediately before the cytometer analysis, which was performed using a Becton a BD Accuri™ C6 flow cytometer (BD Biosciences, CA, USA), equipped with the FCS Express analysis software.

The fluorescence of the P-gp antibody (UIC2-PE) was measured by a 585 ± 40 nm band-pass filter (FL2). Logarithmic fluorescence was recorded and displayed as a single parameter histogram and based on the acquisition of data for at least 20,000 cells. The parameter used for comparison was the mean fluorescence intensity, calculated as percentage of control cells (0 μ M). The cells autofluorescence (unlabelled cells with or without exposure to the tested compounds) was also evaluated to eliminate its potential contribution to the analysed fluorescence signals. Given the high autofluorescence of several of the tested compounds, only compounds **3.37** and **3.39** were able to be tested for their potential effect on P-gp expression. Three independent experiments were performed, in triplicate. Mouse IgG2a-PE was used as an isotype-matched negative control to estimate nonspecific binding of the PE-labelled anti-P-gp antibody (UIC2-PE).

3.7.3.8.6 Evaluation of P-glycoprotein transport activity

The effect of the tested compounds on P-gp activity was evaluated using rhodamine 123 as a P-gp fluorescent substrate, and zosuquidar as a specific third-generation P-gp inhibitor. Indeed, several studies report the use of rhodamine 123 accumulation/efflux assays for the direct assessment of P-gp activity through the quantification of the intracellular fluorescence of the P-gp substrate (79).

Caco-2 cells were seeded in 24-well plates at a density of 60 000 cells/cm² and, three days after seeding, submitted to two different protocols of rhodamine 123 accumulation: 1) rhodamine 123 accumulation in cells pre-exposed to the compounds (20 μ M) for 24 hours; 2) rhodamine 123 accumulation in the presence of the tested compounds (20 μ M) for 90 minutes, as previously described (99).

The first protocol, given the longer pre-incubation with the tested compounds for 24 hours, aimed to assess whether potential compounds-induced increases (or decreases) in P-gp protein expression levels can be translated into potential increases (or decreases) in the pump activity.

For that purpose, 3 days after seeding, Caco-2 cells were exposed to compounds (20 μ M) in fresh cell culture medium. Twenty-four hours after exposure, the cell culture medium containing the compounds was removed, the cells washed with Hank's Balanced Salt Solution (+/+) and incubated with RHO rhodamine 123 in the absence of the tested compounds, and in the presence or absence of the specific P-gp inhibitor, zosuquidar, accordingly to the following procedures:

- Rhodamine 123 accumulation under normal conditions (NA): pre-incubation with Hank's Balanced Salt Solution (+/+), for 30 minutes at 37 °C, followed by incubation with RHO 123 (5 µM) for 90 minutes, at 37 °C, in a humidified 5 % CO₂ – 95 % O₂ air atmosphere;
- Rhodamine 123 accumulation in the presence of the P-gp inhibitor (IA): pre-incubation with zosuquidar (5 µM), prepared in Hank's Balanced Salt Solution (+/+), for 30 minutes at 37 °C, followed by incubation with rhodamine 123 (5 µM) for 90 minutes, at 37 °C, in a humidified 5 % CO₂ – 95 % O₂ air atmosphere.

After the 90 minutes incubation with rhodamine 123, the cells were washed twice with Hank's Balanced Salt Solution (-/-) and lysed with 1 % Triton™ X100, for 30 minutes, in the dark, at room temperature. RHO 123 intracellular fluorescence was then measured at excitation/emission wavelengths of 485/528 nm, in a multi-well plate reader (PowerWave-X, BioTek Instruments, Vermont, USA) and expressed as fluorescence intensity (FI). The ratio between the FI after inhibited rhodamine 123 accumulation (IA) and the FI of non-inhibited RHO 123 accumulation (NA) was the parameter used for comparison, and the results were expressed as percentage of control cells, as present in the following equation.

$$\text{Rhodamine 123 Accumulation} = \frac{\text{FI of RHO123 accumulation under P – gp inhibition (IA)}}{\text{FI of RHO123 accumulation under normal conditions (NA)}}$$

Given the high autofluorescence of several of the tested compounds at the conditions used to measure RHO 123 fluorescence, only compounds **3.33**, **3.34**, **3.37**, **3.39** and **3.44** were able to be tested for their potential effect on P-gp activity. Five independent experiments were performed, in triplicate.

On the other hand, in the second protocol, the cells were exposed to the tested compounds 30 minutes prior to addition of the P-gp fluorescent substrate, rhodamine 123, and remained in contact with the cells only during the substrate accumulation period, thus aiming to detect immediate effects of the tested compounds on P-gp activity as a result of a direct P-gp activation or inhibition. For that purpose, 3 days after seeding, the cell culture medium was removed, and the cells submitted to the following procedures:

- Rhodamine 123 accumulation under normal conditions (NA): pre-exposure to compounds (20 µM), prepared in Hank's Balanced Salt Solution (+/+), for 30 minutes at 37 °C, followed by incubation with rhodamine 123 (5 µM) for 90 minutes, at 37 °C, in a humidified 5 % CO₂ – 95 % O₂ air atmosphere. Control cells were only exposed to rhodamine 123.
- Rhodamine 123 accumulation in the presence of the P-gp inhibitor (IA): simultaneous pre-exposure to compounds (20 µM) and zosuquidar (5 µM), both

prepared in Hank's Balanced Salt Solution (+/+), for 30 minutes at 37 °C, followed by incubation with rhodamine 123 (5 µM) for 90 minutes, at 37 °C, in a humidified 5 % CO₂ – 95 % O₂ air atmosphere. Control cells were only exposed to zosuquidar and rhodamine 123.

After the 90 minutes incubation with rhodamine 123, the cells were washed twice with Hank's Balanced Salt Solution (-/-) and lysed with 1 % Triton™ X100, for 30 minutes, in the dark, at room temperature. RHO 123 intracellular fluorescence was then measured at excitation/emission wavelengths of 485/528 nm, in a multi-well plate reader (PowerWave-X, BioTek Instruments, Vermont, USA) and expressed as FI.

The ratio between the FI after inhibited rhodamine 123 accumulation (IA) and the FI of non-inhibited rhodamine 123 accumulation (NA) was the parameter used for comparison, and the results were expressed as percentage of control cells.

Given the high autofluorescence of several of the tested compounds at the conditions used to measure RHO 123 fluorescence, only compounds **3.33**, **3.34**, **3.37**, **3.39** and **3.44** were able to be tested for their potential immediate effect on P-gp activity. Four independent experiments were performed, in triplicate.

3.7.3.8.7 Statistical analysis

The results of the cytotoxicity in the NIH/3T3 cell line were expressed as the mean ± SD, and the IC₅₀ values were obtained by best fitting the dose-dependent inhibition curves in GraphPad Prism 5.03 for Windows software.

Statistical analysis of the P-gp assays was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, California, EUA). The normality of data distribution was evaluated with three different tests: KS normality test, D'Agostino & Pearson omnibus normality test and Shapiro-Wilk normality test. For data with parametric distribution One-way ANOVA was used to perform the statistical comparisons, followed by the Dunnett's multiple comparisons test. Details of the performed statistical analysis are described in each figure legend. In all cases, *p* values smaller than 0.05 were considered significant.

3.8 Supporting information

The ¹H and ¹³C NMR spectra for the newly synthesized thioxanthone derivatives **3.22**, **3.33**, **3.34**, **3.36**, **3.37**, and **3.39** – **3.44**, as well as the HRMS and GC-MS spectra, and HPLC chromatograms are displayed in **Figure S3.1** – **Figure S3.33**.

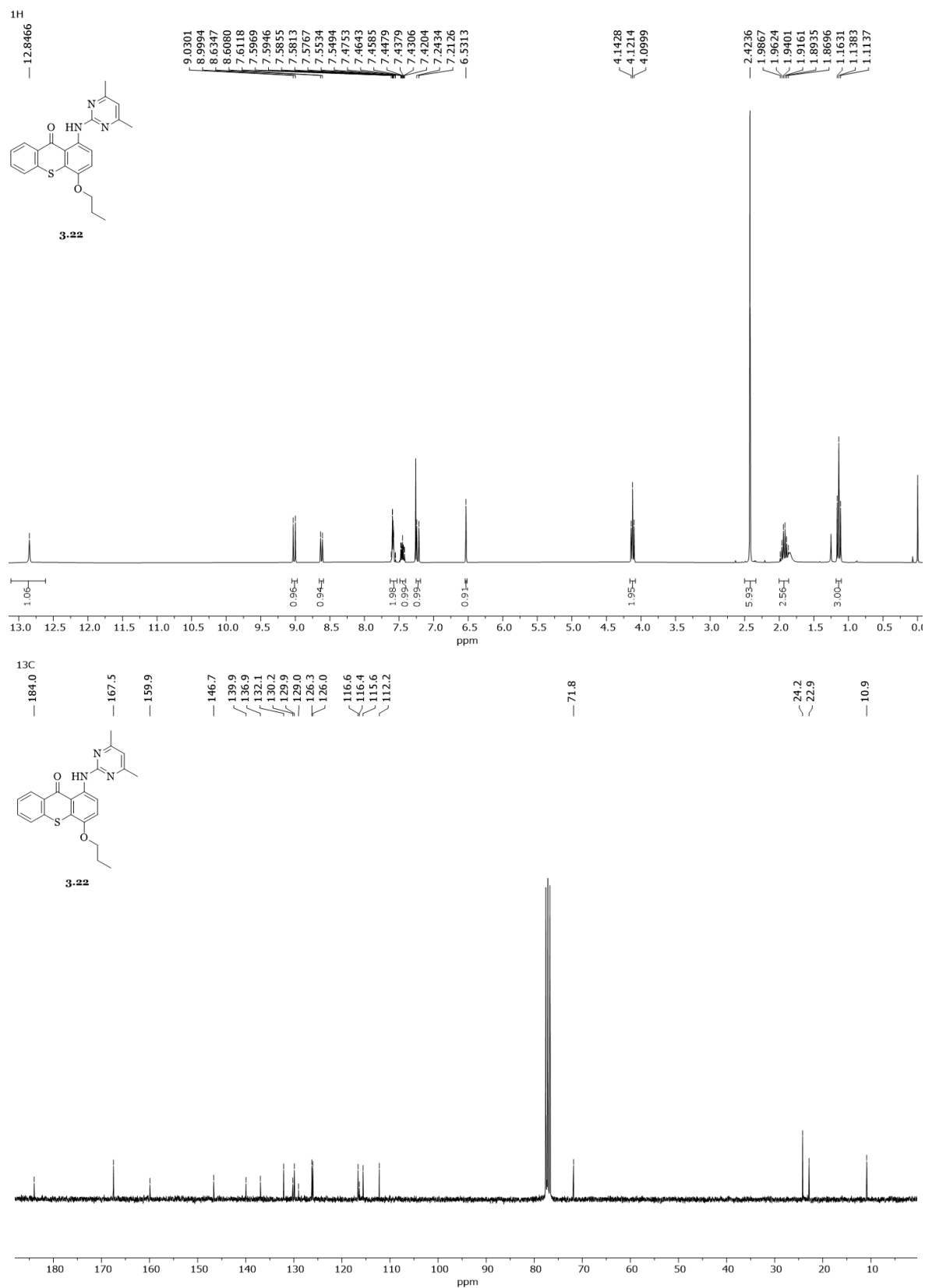
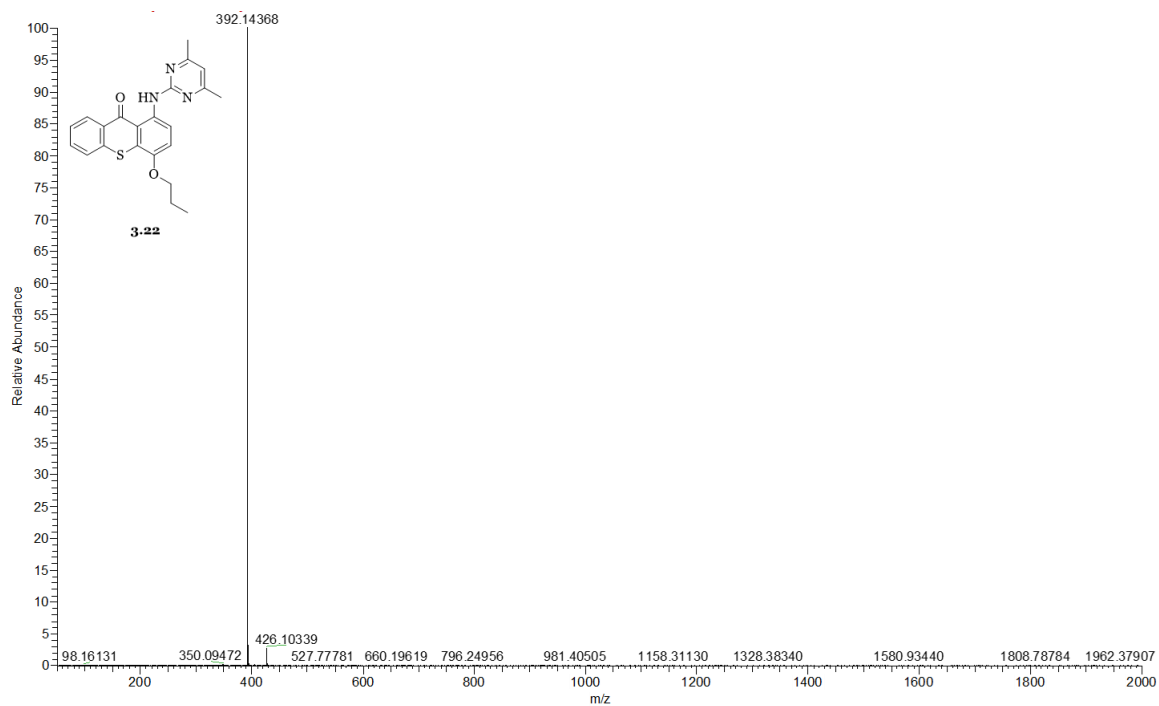


Figure S3.1 ¹H NMR (top, 300.13 MHz, CDCl₃) and ¹³C NMR (bottom, 75.48 MHz, CDCl₃) for compound **3.22**.



Meas. m/z	Formula	m/z	err [ppm]
392.1437	C ₂₂ H ₂₂ N ₃ O ₂ S	392.1432	1.28

Figure S3.2 ESI data for compound **3.22**.

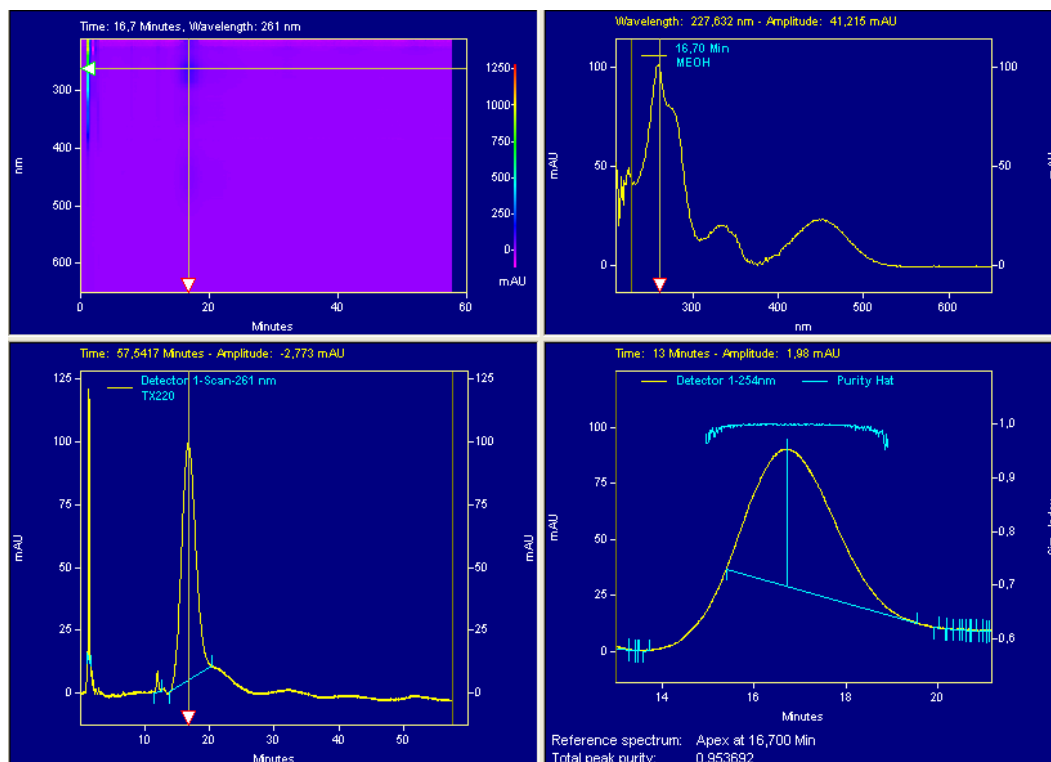


Figure S3.3 Peak purity of compound **3.22**.

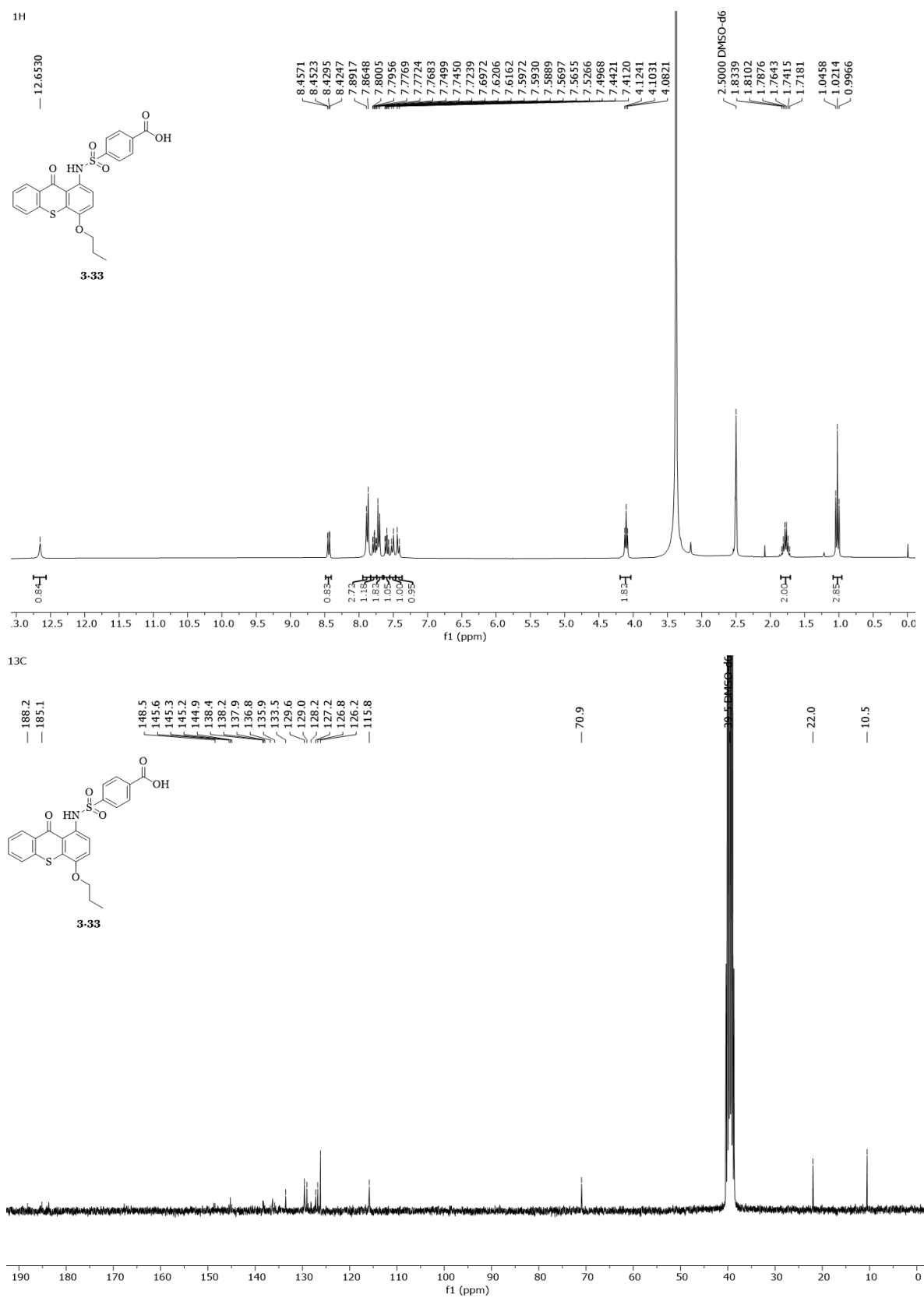


Figure S3.4 ¹H NMR (top, 300.13 MHz, DMSO) and ¹³C NMR (bottom, 75.48 MHz, DMSO) for compound **3-33**.

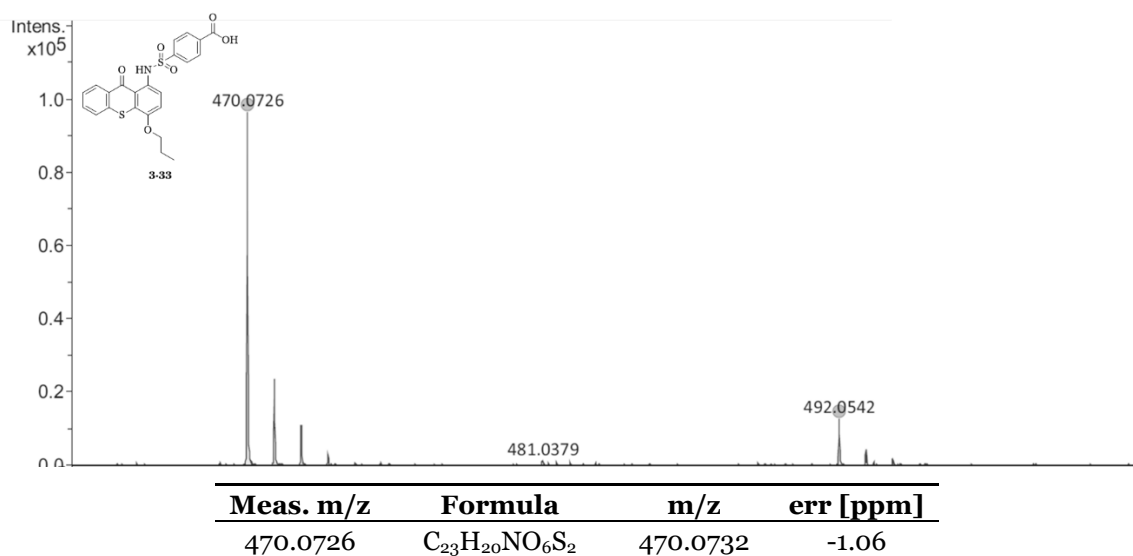


Figure S3.5 ESI data for compound **3-33**.

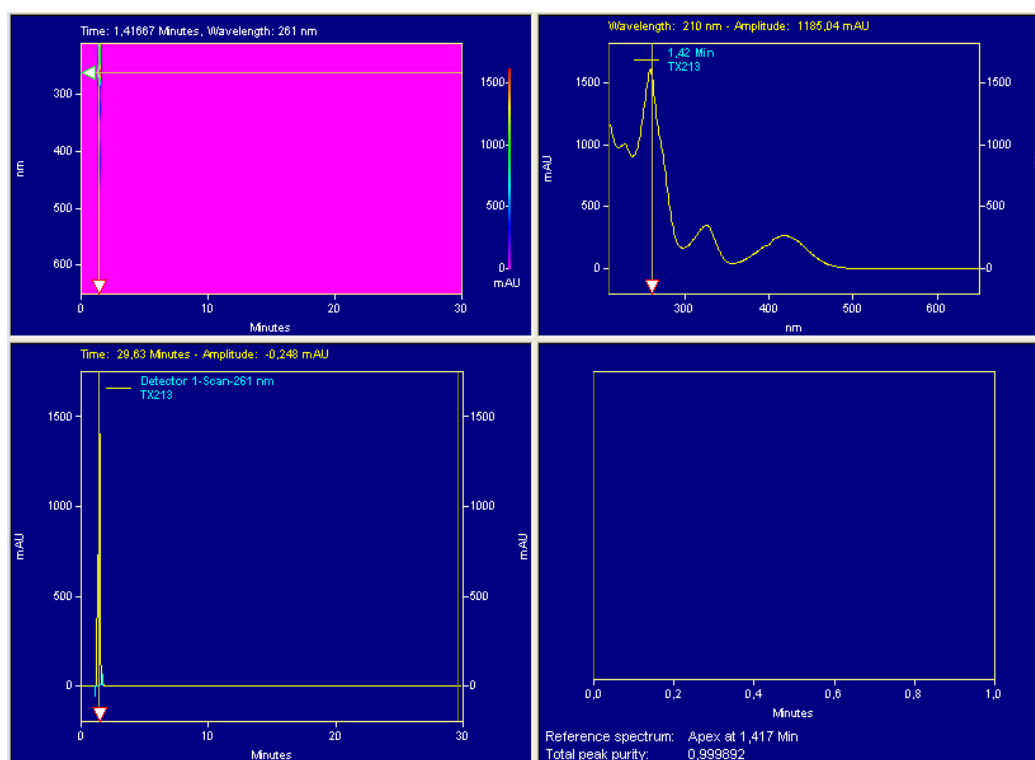


Figure S3.6 Peak purity of compound **3-33**.

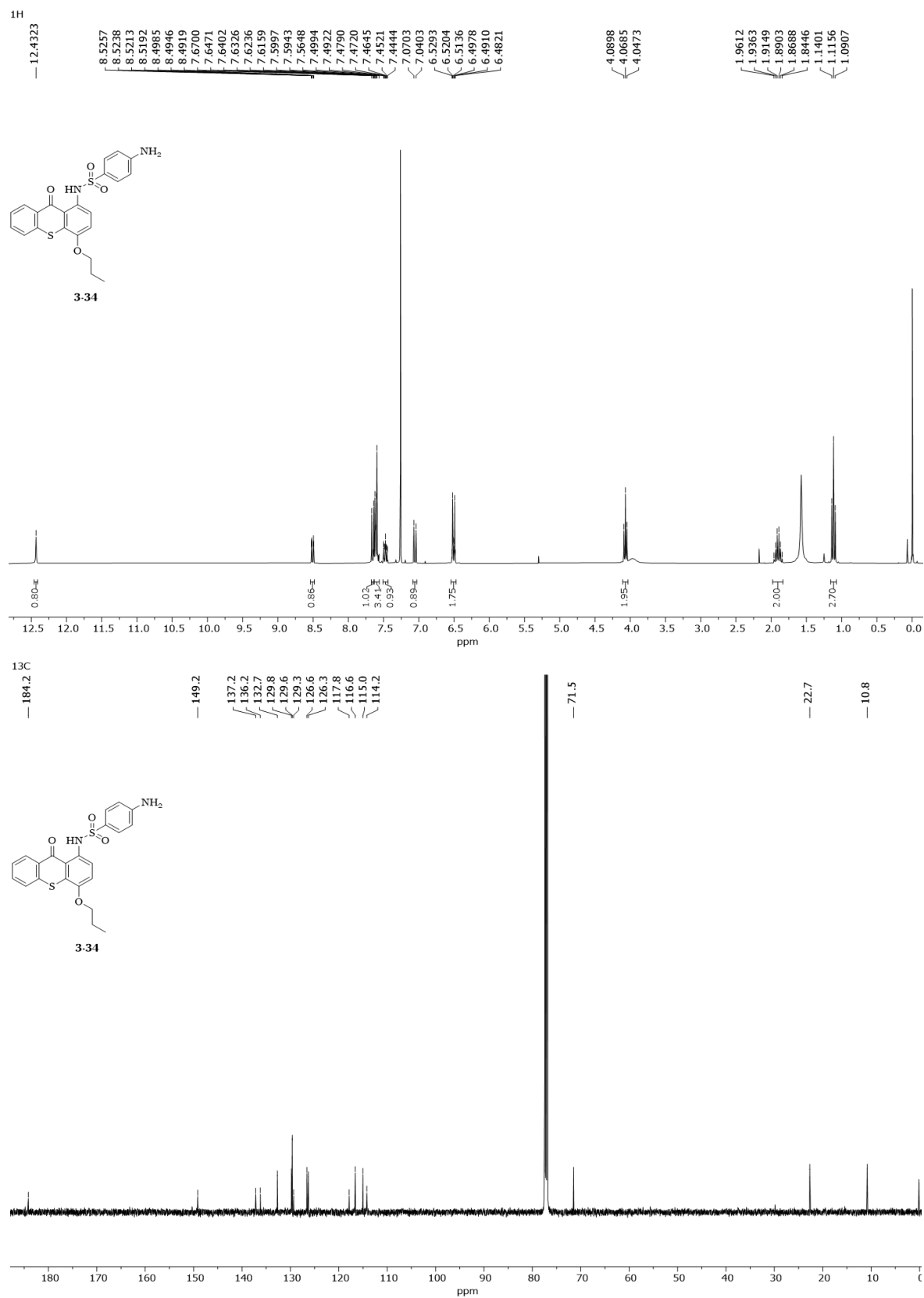
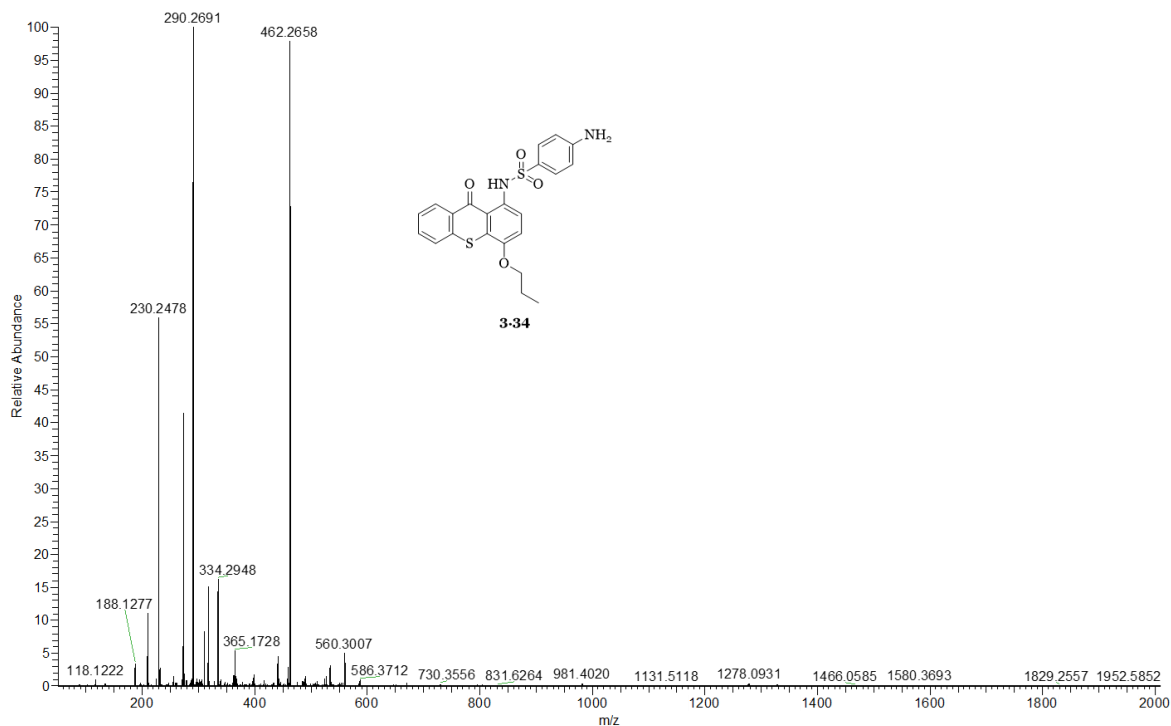


Figure S3.7 ¹H NMR (top, 300.13 MHz, CDCl₃) and ¹³C NMR (bottom, 75.48 MHz, CDCl₃) for compound **3-34**.



Meas. m/z	Formula	m/z	err [ppm]
462.2658	$C_{23}H_{19}NO_6S_2Na$	462.0683	427.43

Figure S3.8 ESI data for compound **3-34**.

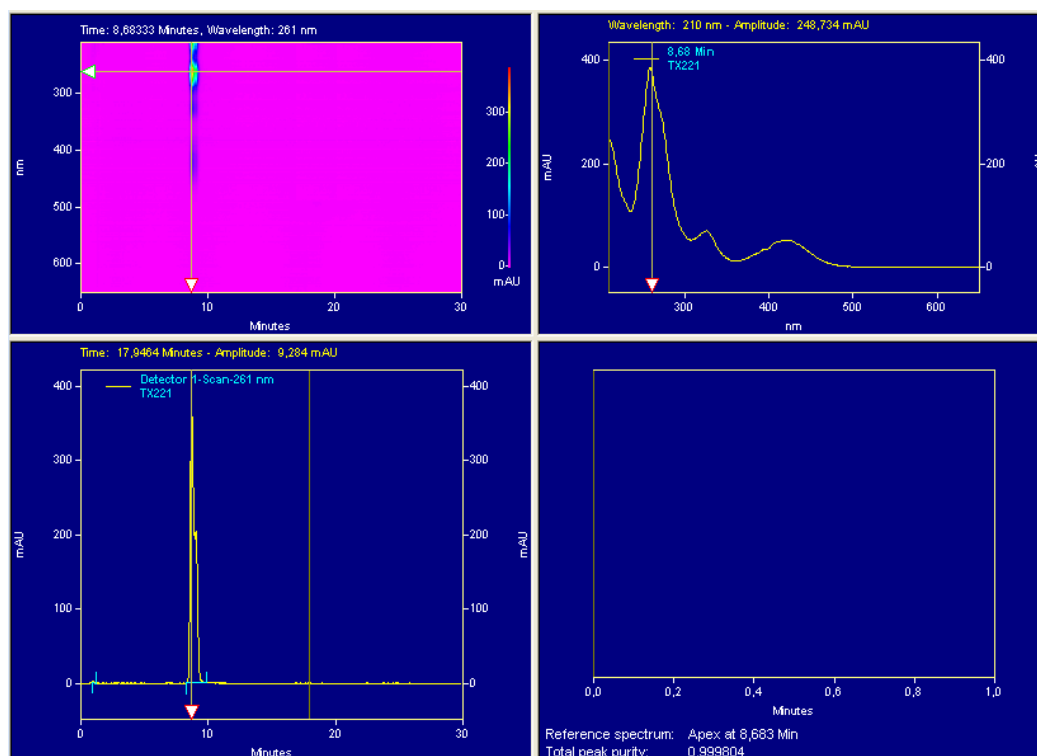


Figure S3.9 Peak purity of compound **3-34**.

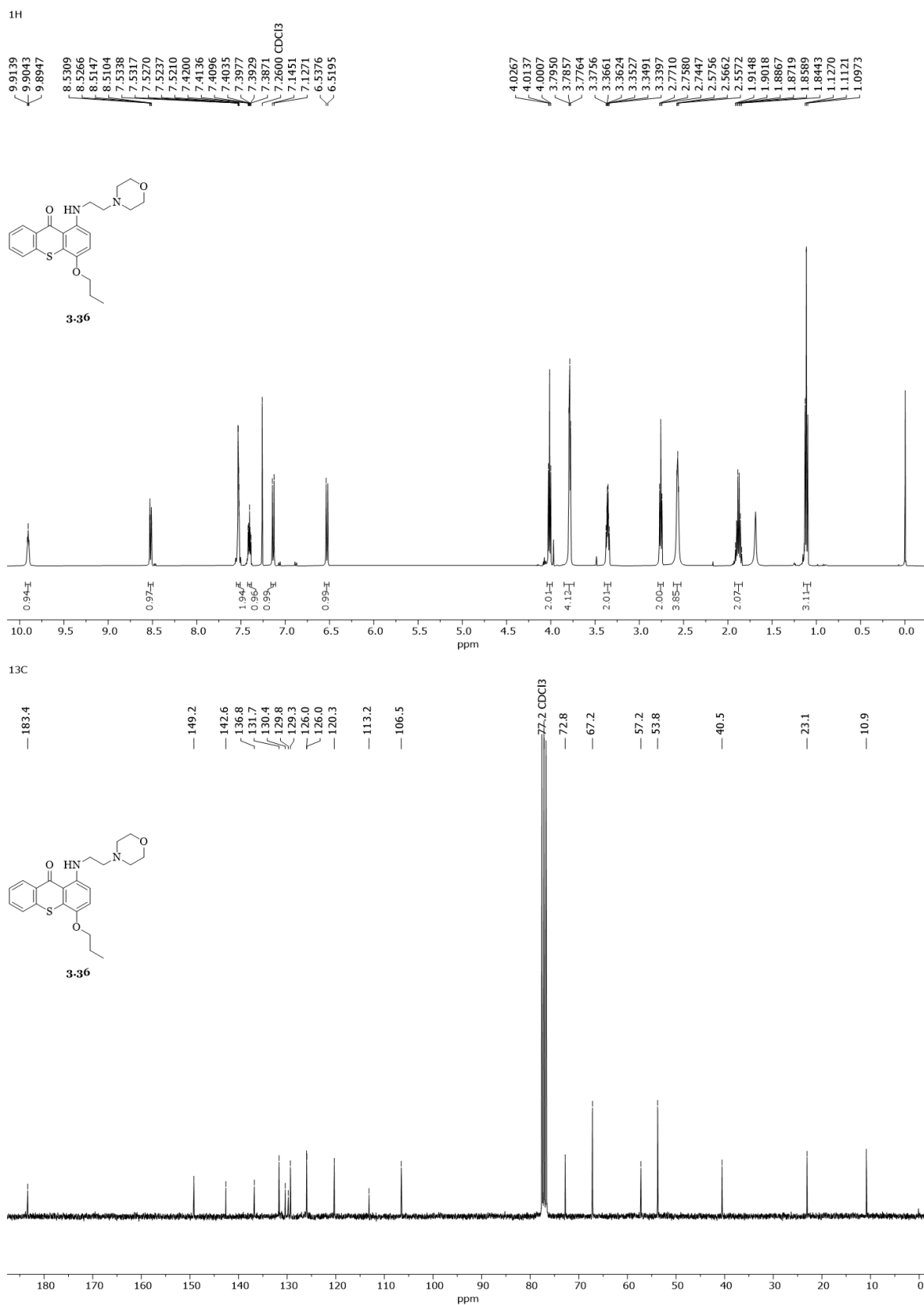
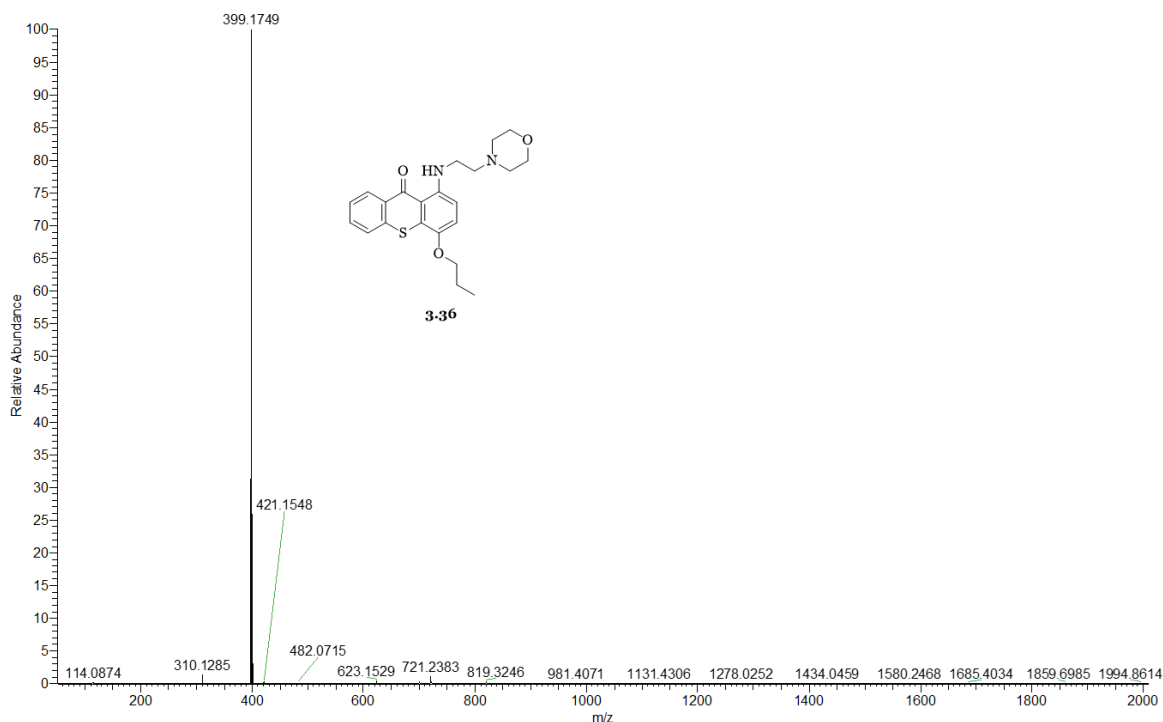


Figure S3.10 ¹H NMR (top, 500.13 MHz, CDCl₃) and ¹³C NMR (bottom, 75.48 MHz, CDCl₃) for compound **3-36**.



Meas. m/z	Formula	m/z	err [ppm]
399.1749	C ₂₂ H ₂₇ N ₂ O ₃ S	399.1742	1.75

Figure S3.11 ESI data for compound **3.36**.

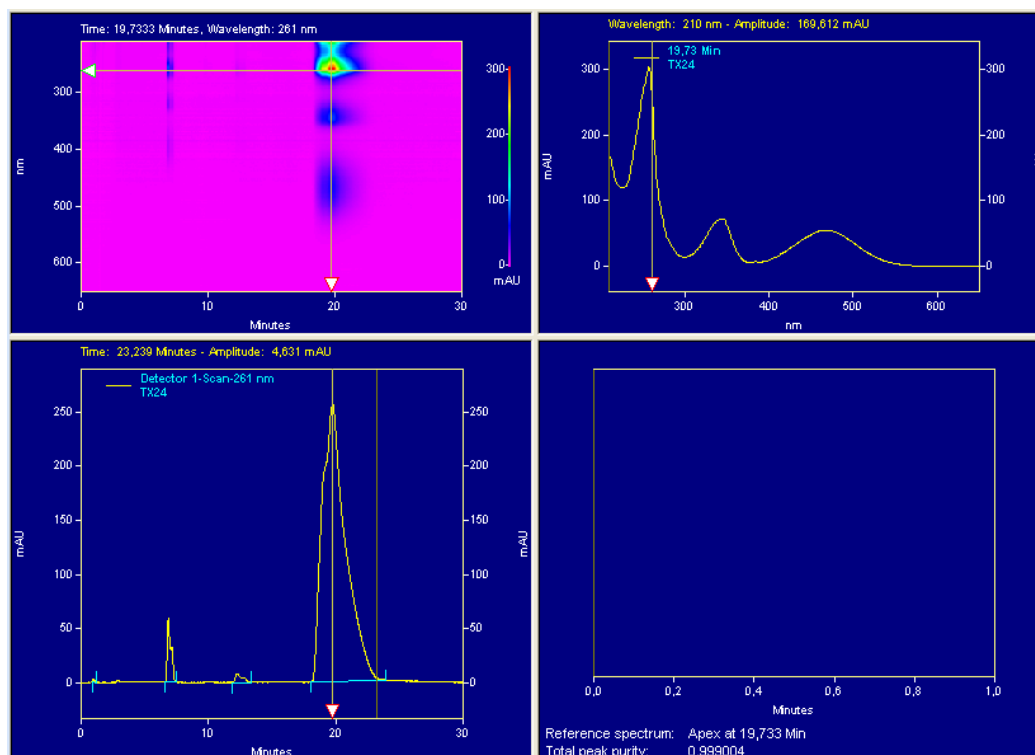


Figure S3.12 Peak purity of compound **3.36**.

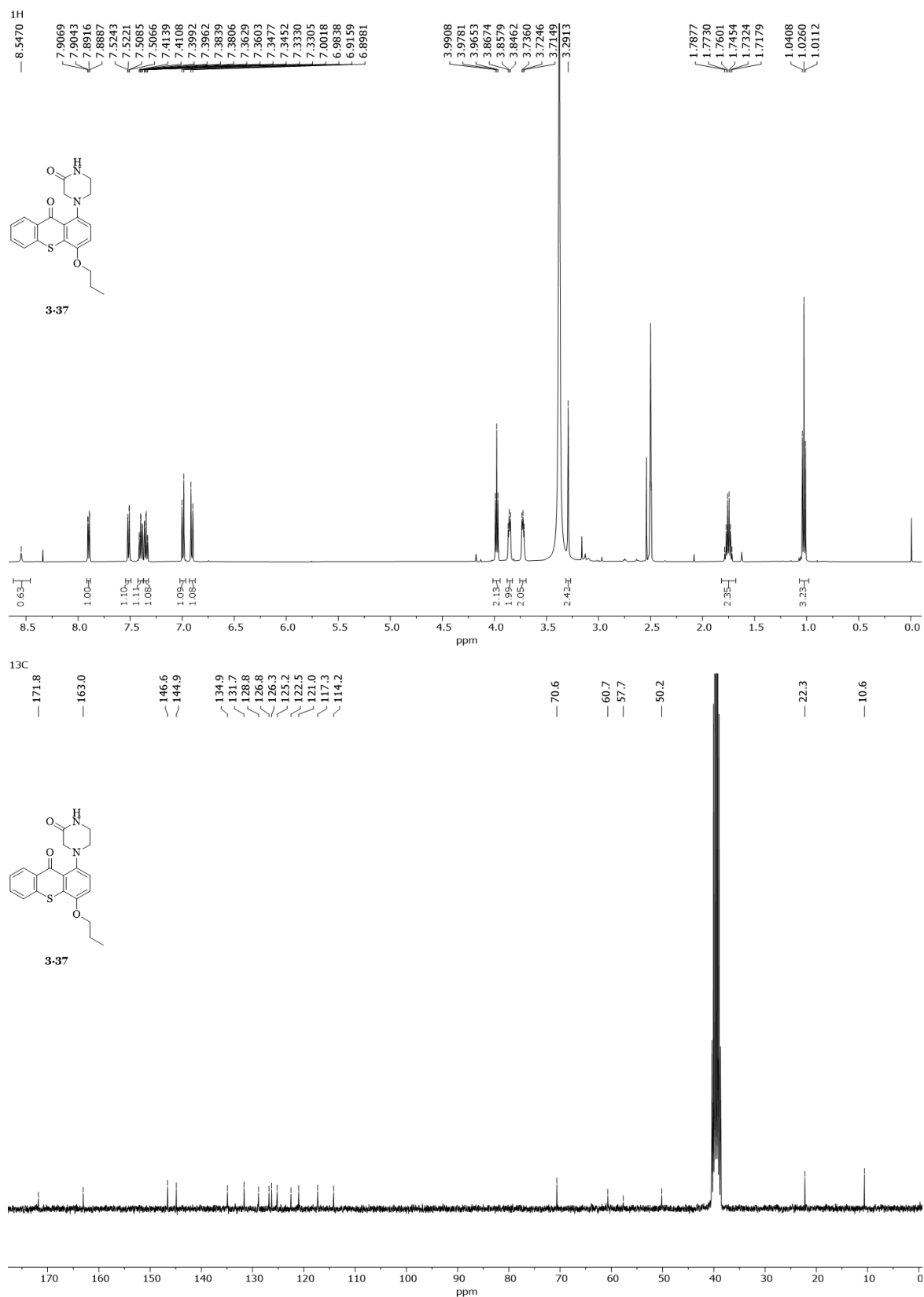
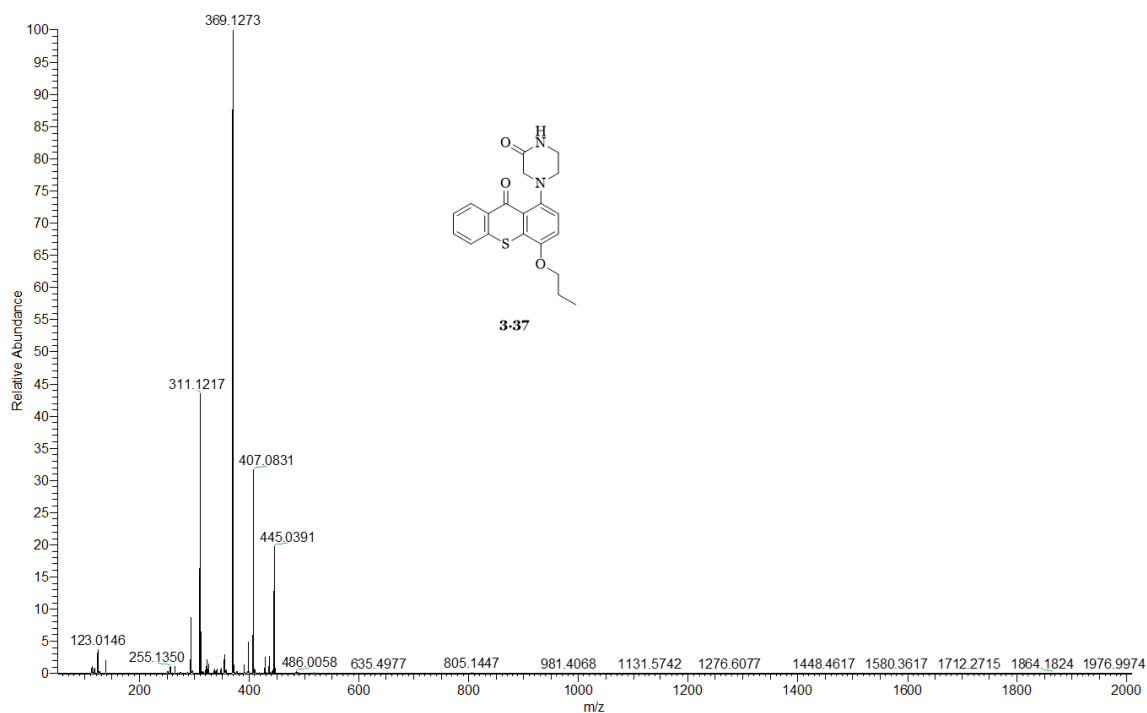


Figure S3.13 ¹H NMR (top, 500.13 MHz, DMSO) and ¹³C NMR (bottom, 75.48 MHz, DMSO) for compound **3-37**.



Meas. m/z	Formula	m/z	err [ppm]
369.1269	C ₂₀ H ₂₁ N ₂ O ₃ S	369.1273	-1.08

Figure S3.14 ESI data for compound **3.37**.

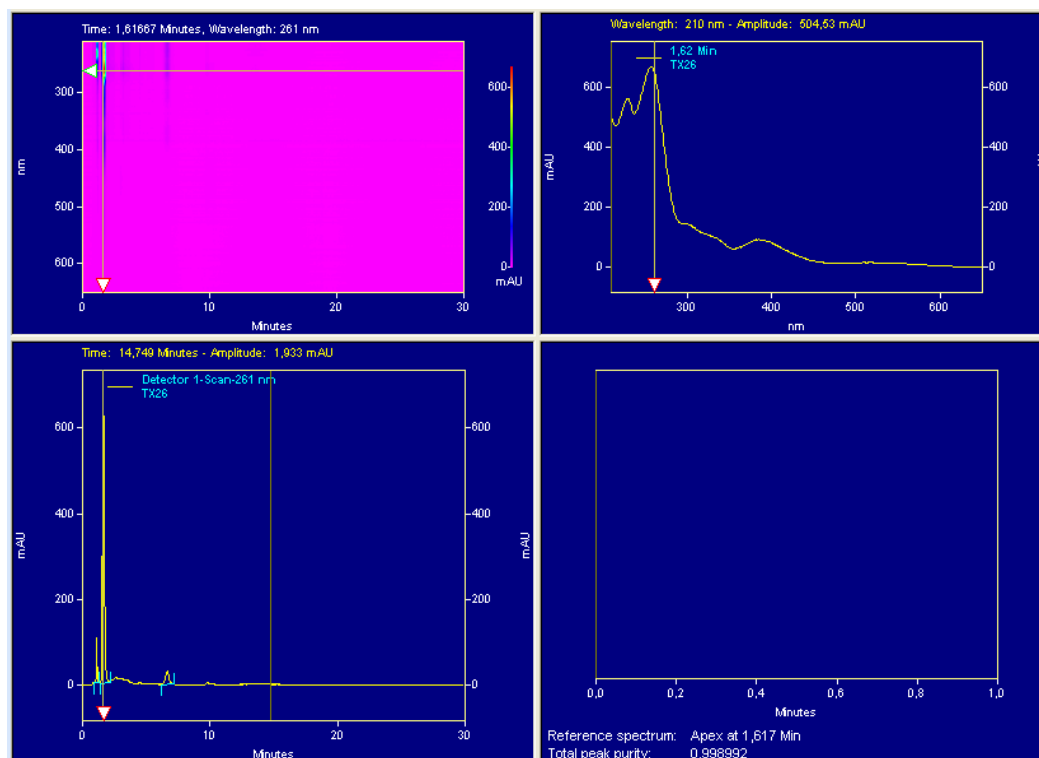


Figure S3.15 Peak purity of compound **3.37**.

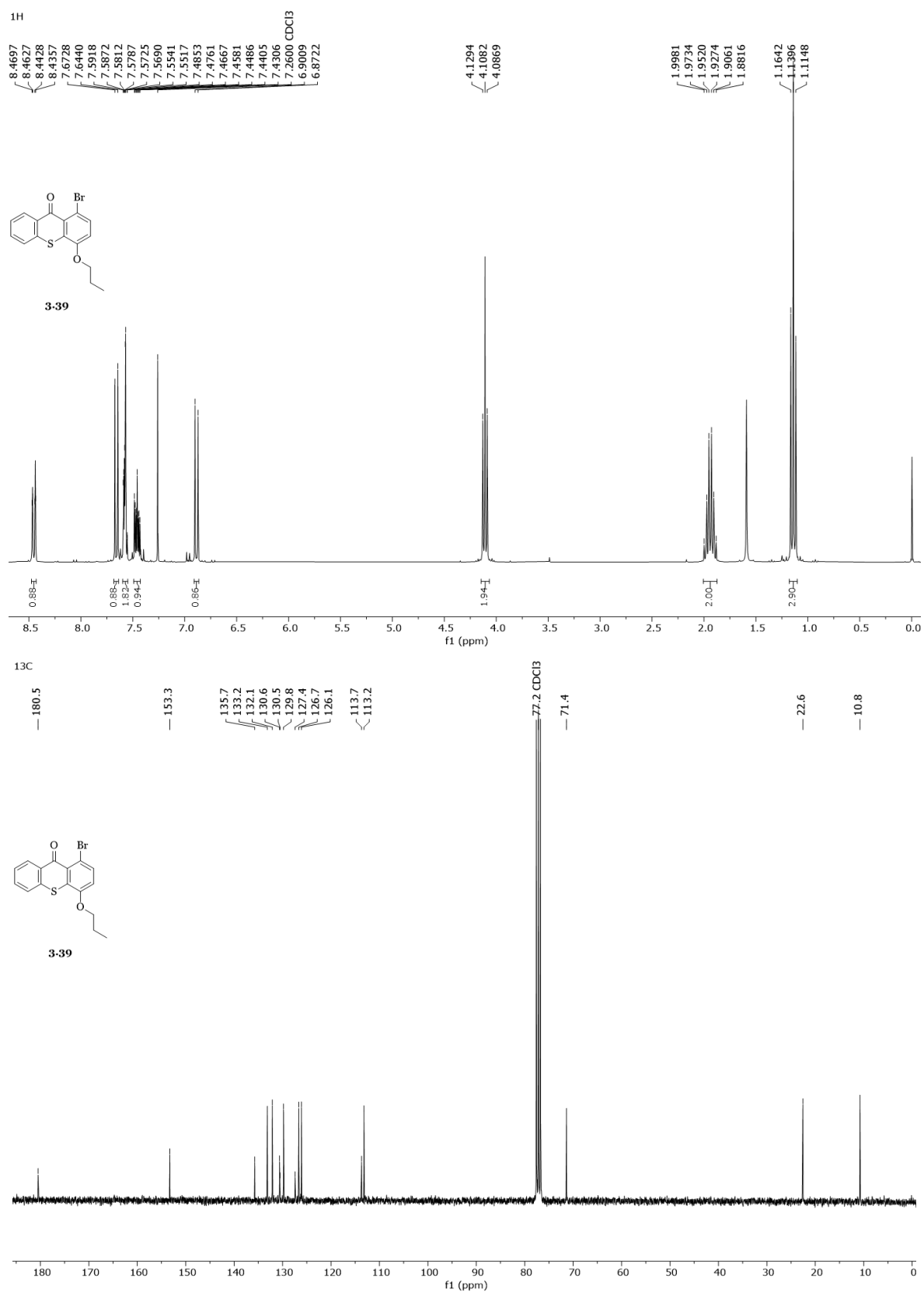


Figure S3.16 ¹H NMR (top, 300.13 MHz, CDCl₃) and ¹³C NMR (bottom, 75.48 MHz, CDCl₃) for compound **3-39**.

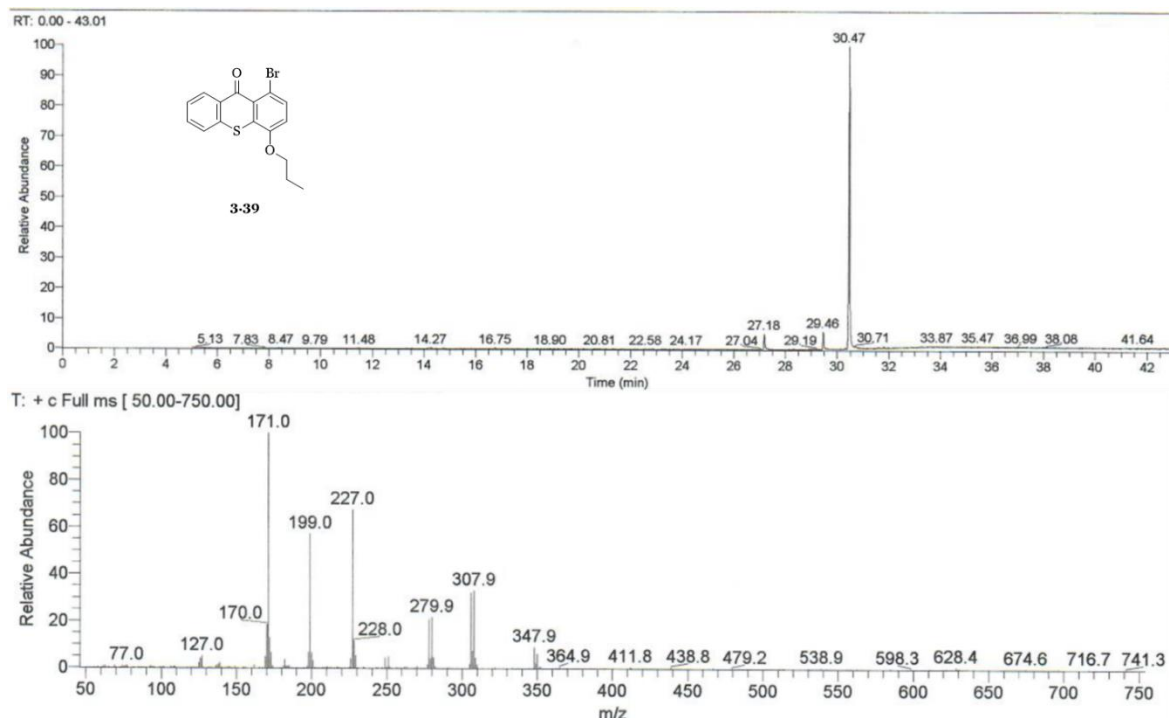


Figure S3.17 GC-MS spectra of compound 3.39.

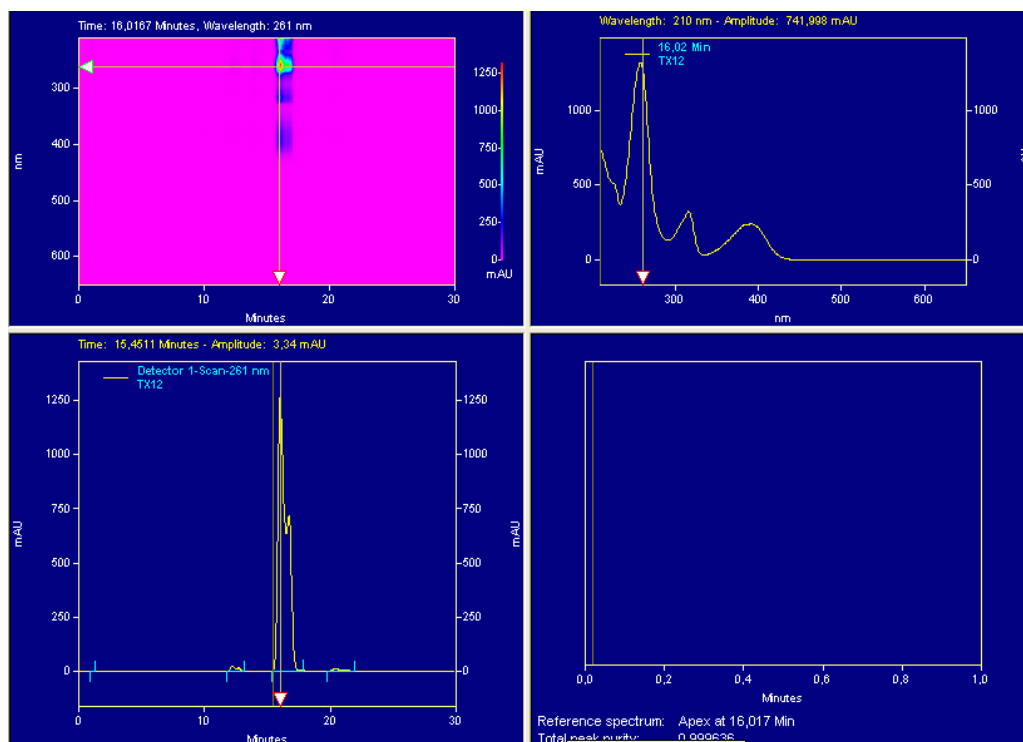


Figure S3.18 Peak purity of compound 3.39.

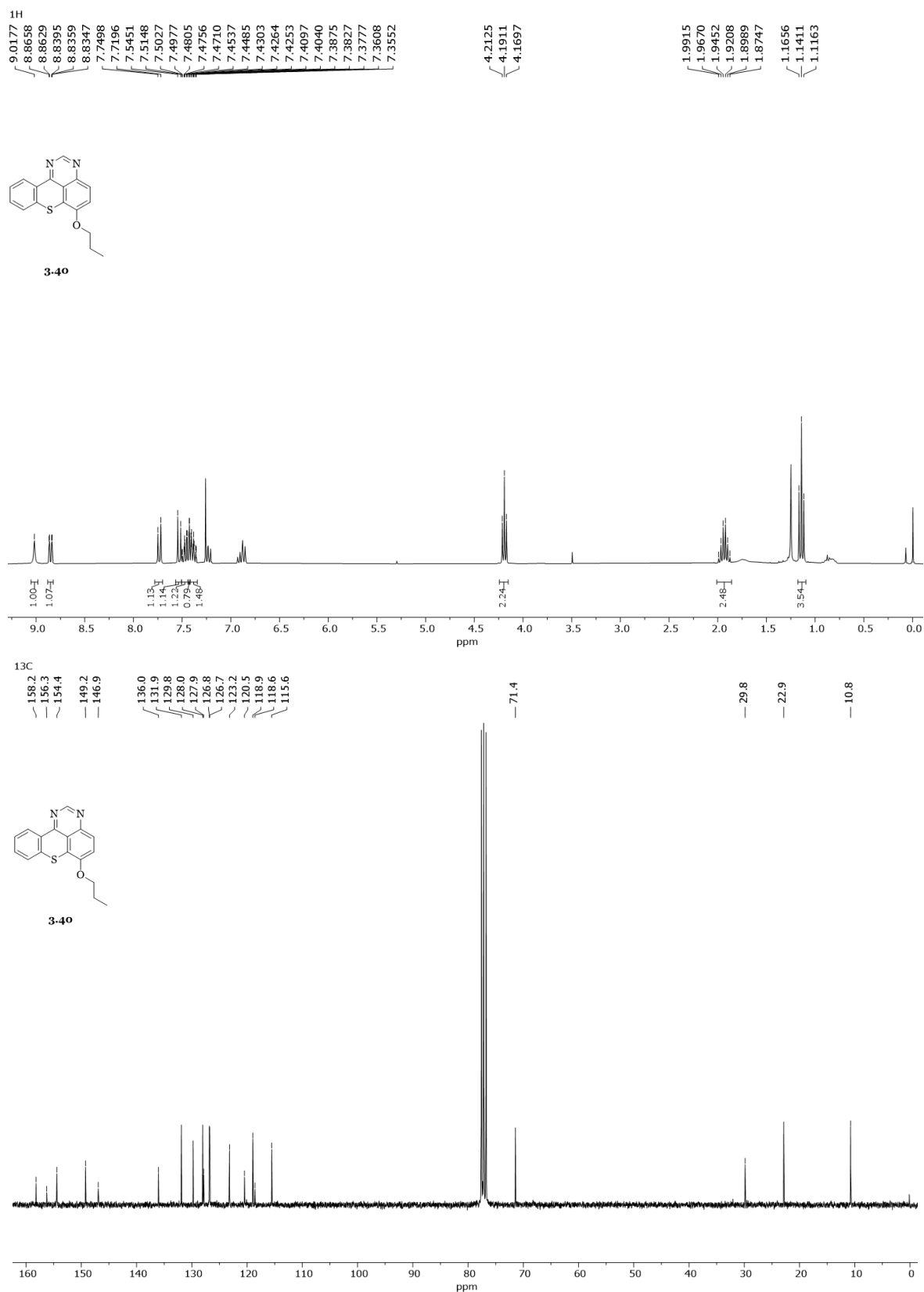


Figure S3.19 ¹H NMR (top, 300.13 MHz, CDCl₃) and ¹³C NMR (bottom, 75.48 MHz, CDCl₃) for compound **3-40**.

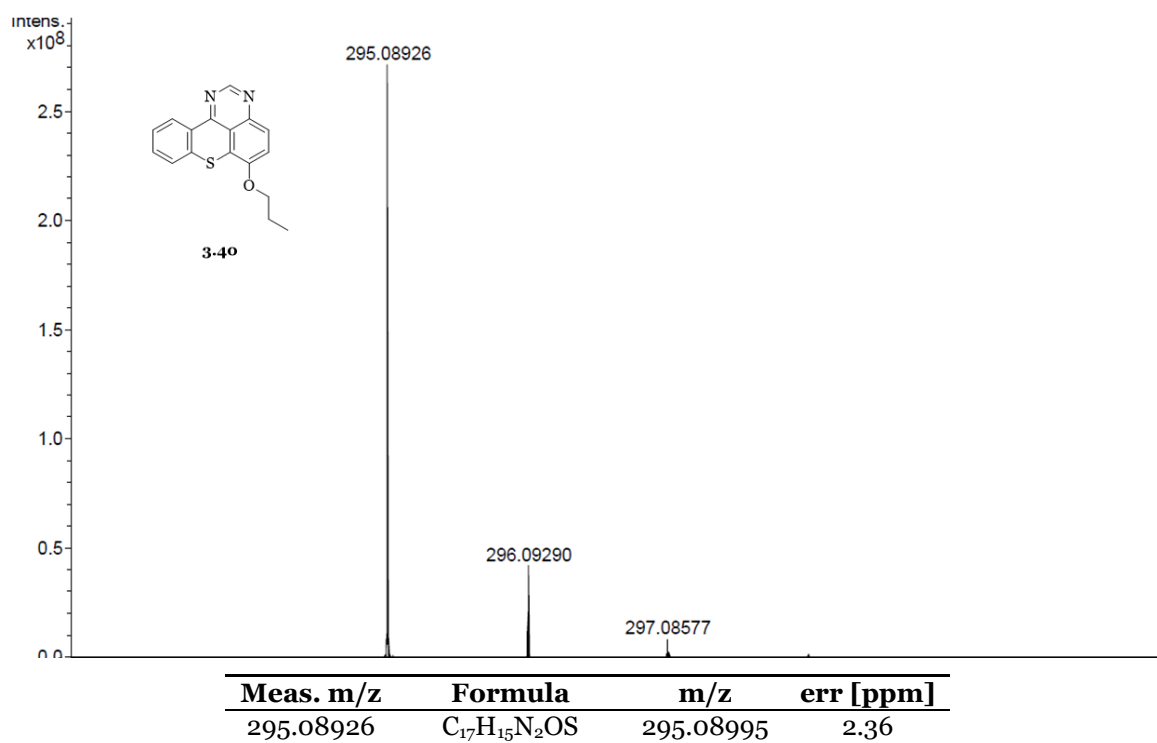


Figure S3.20 ESI data for compound **3.40**.

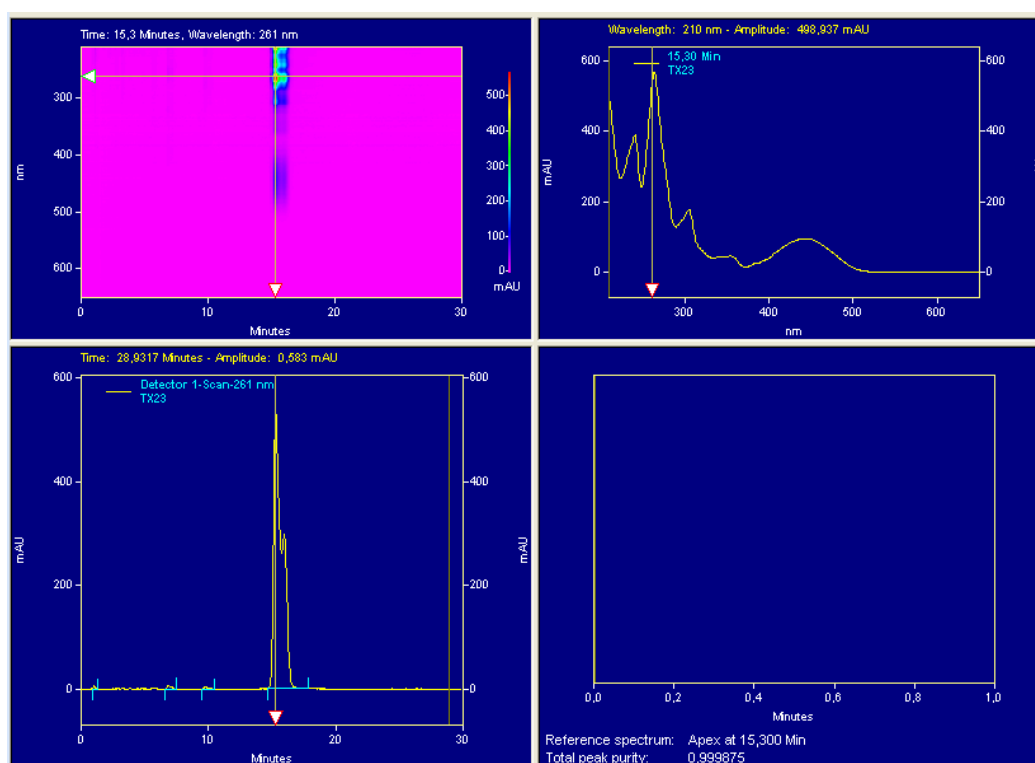


Figure S3.21 Peak purity of compound **3.40**.

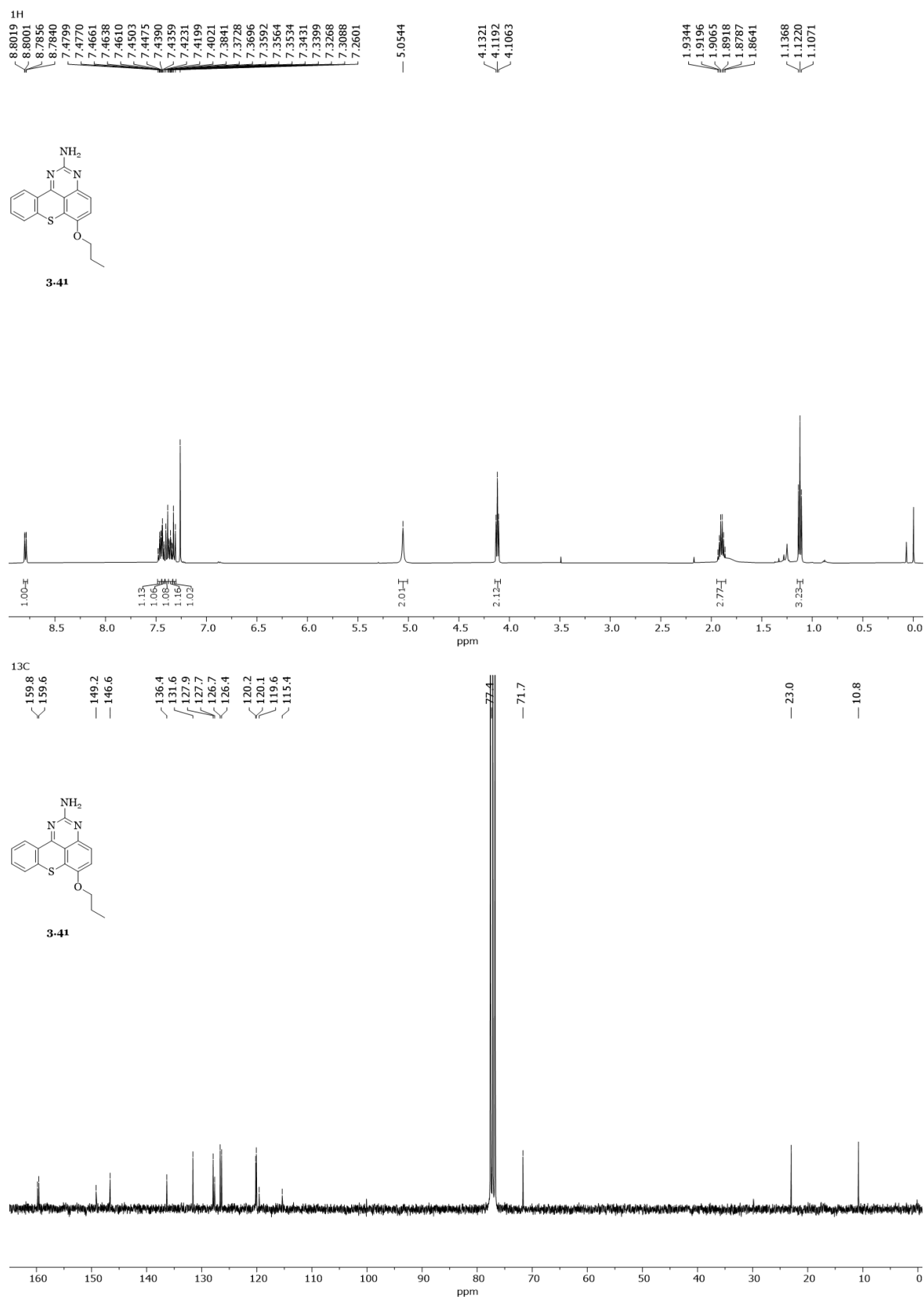


Figure S3.22 ¹H NMR (top, 500.13 MHz, CDCl₃) and ¹³C NMR (bottom, 75.48 MHz, CDCl₃) for compound **3-41**.

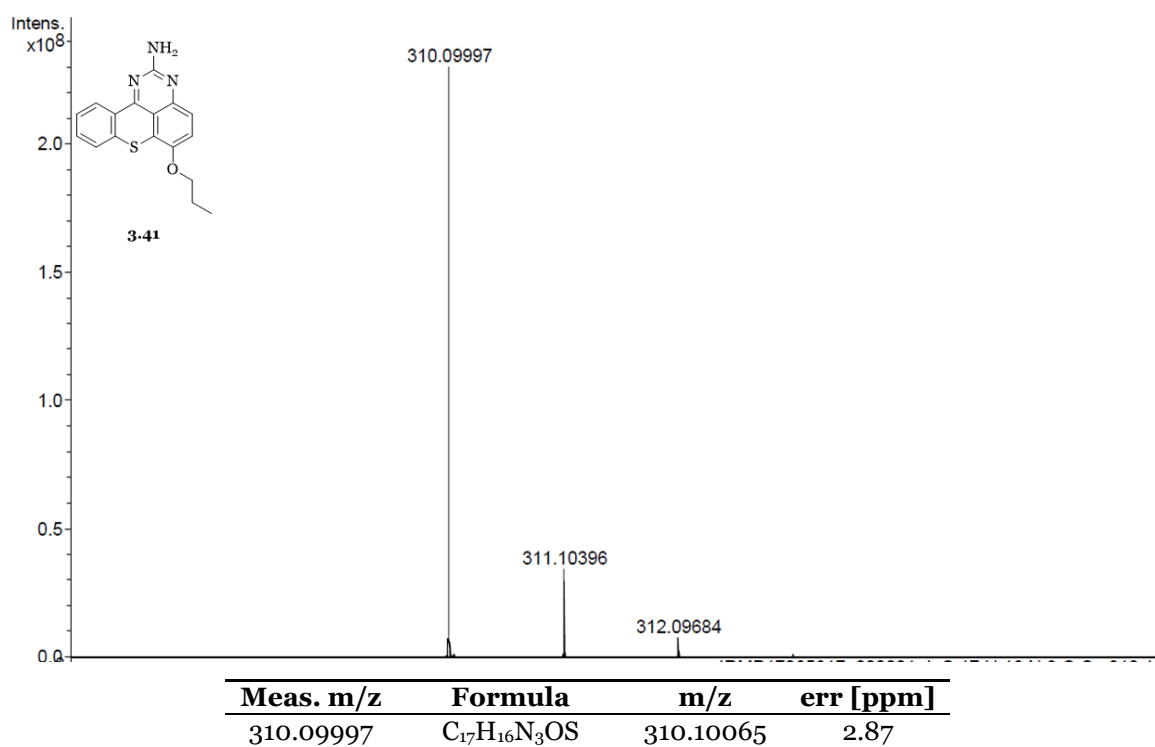


Figure S3.23 ESI data of compound **3.41**.

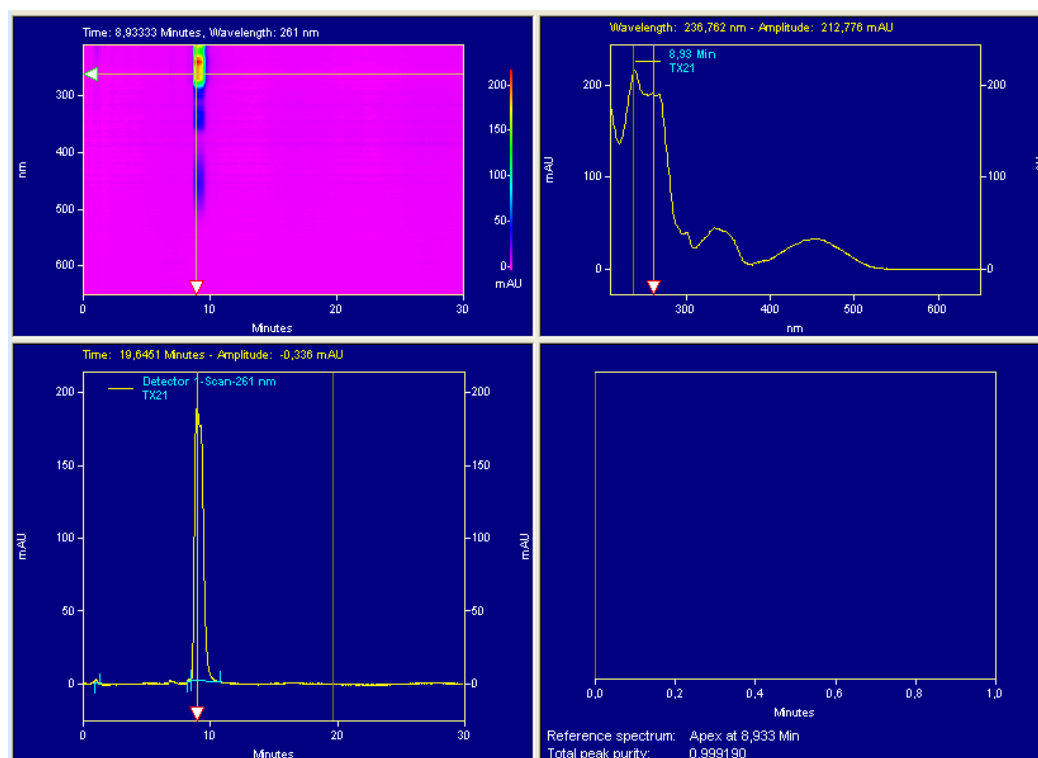


Figure S3.24 Peak purity of compound **3.41**.

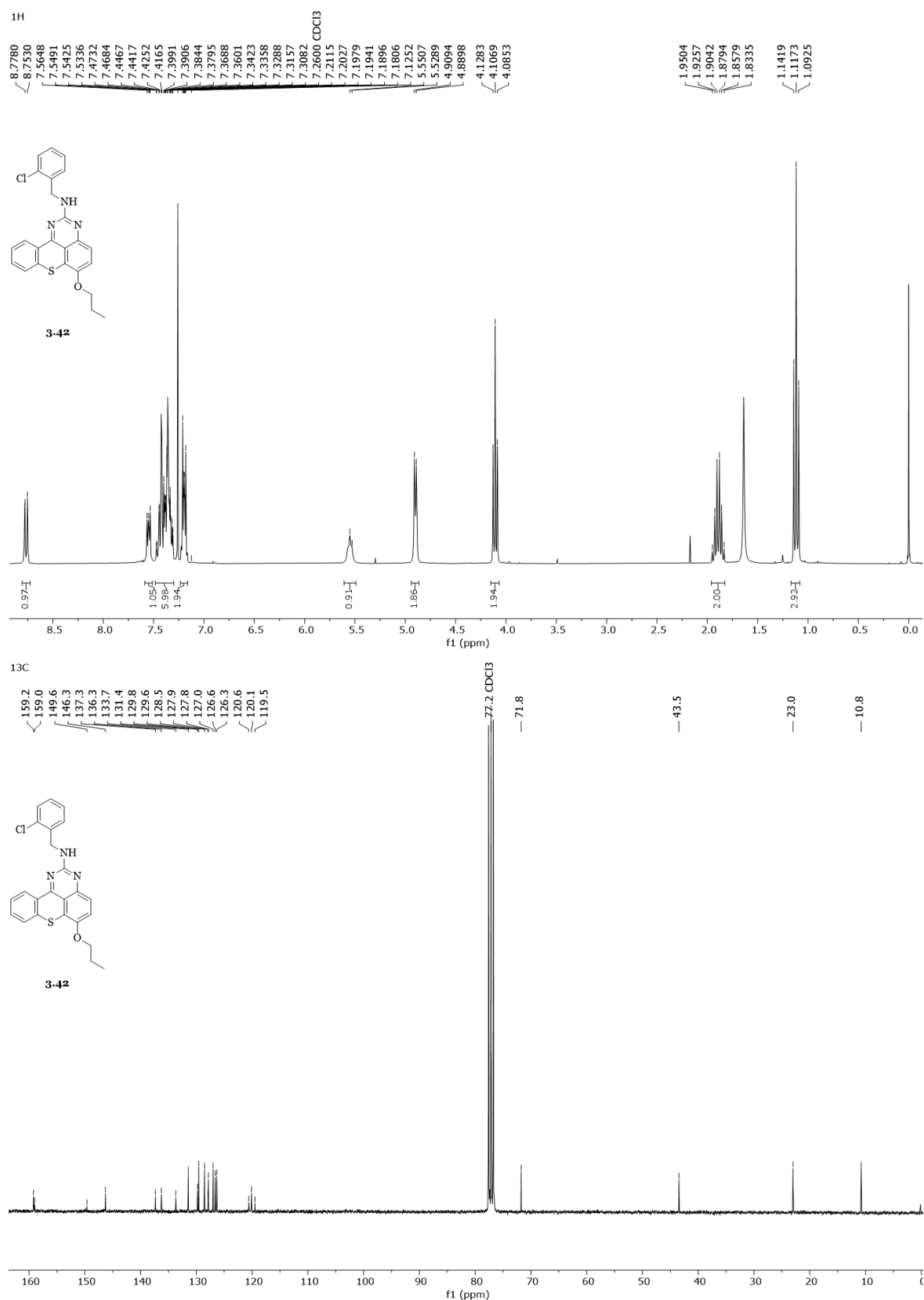
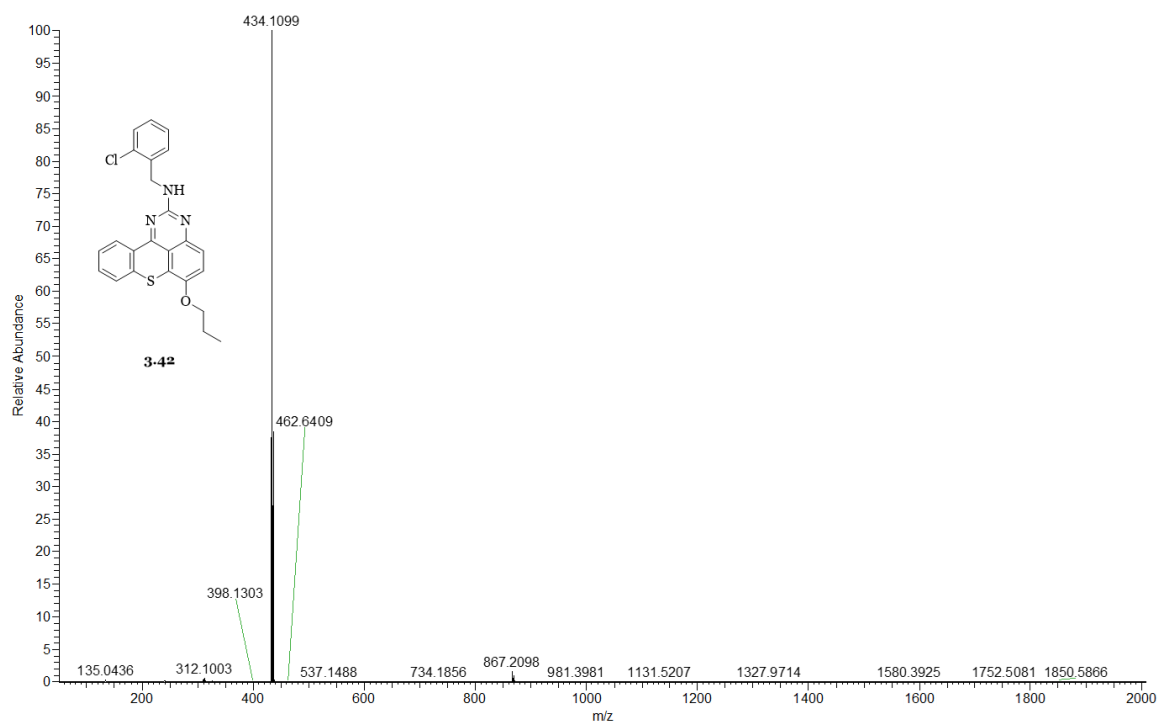


Figure S3.25 ¹H NMR (top, 300.13 MHz, CDCl₃) and ¹³C NMR (bottom, 75.48 MHz, CDCl₃) for compound **3-42**.



Meas. m/z	Formula	m/z	err [ppm]
434.1099	C ₂₄ H ₂₀ ClN ₃ OS	434.1094	1.1518

Figure S3.26 ESI data for compound **3.42**.

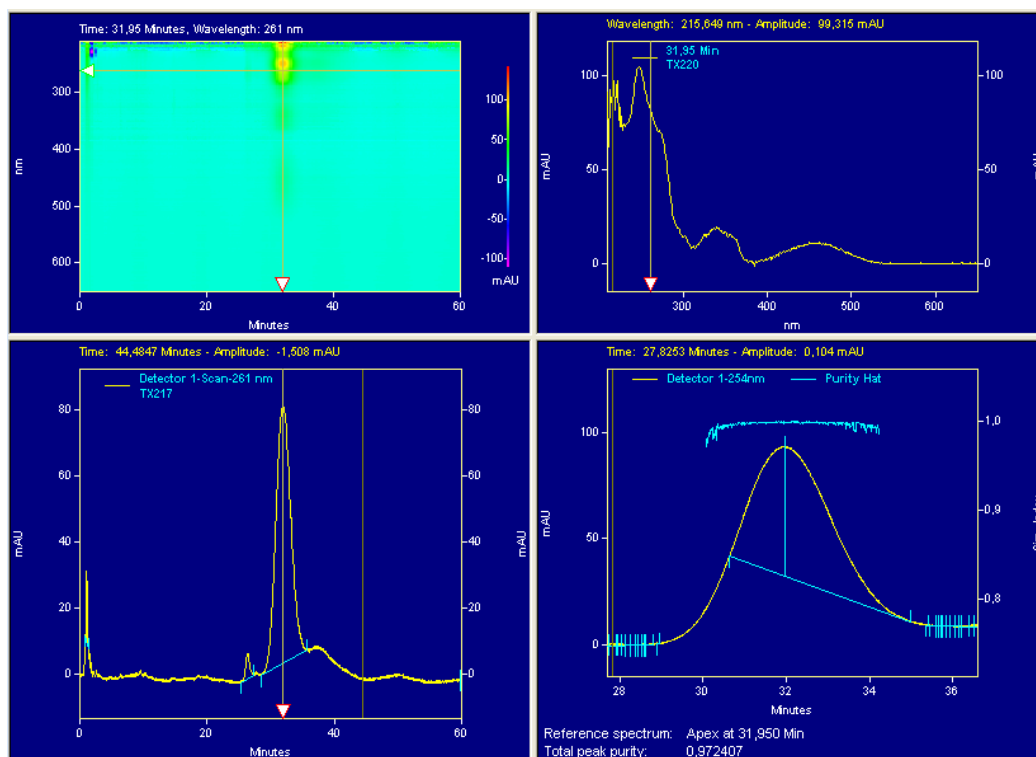


Figure S3.27 Peak purity of compound **3.42**.

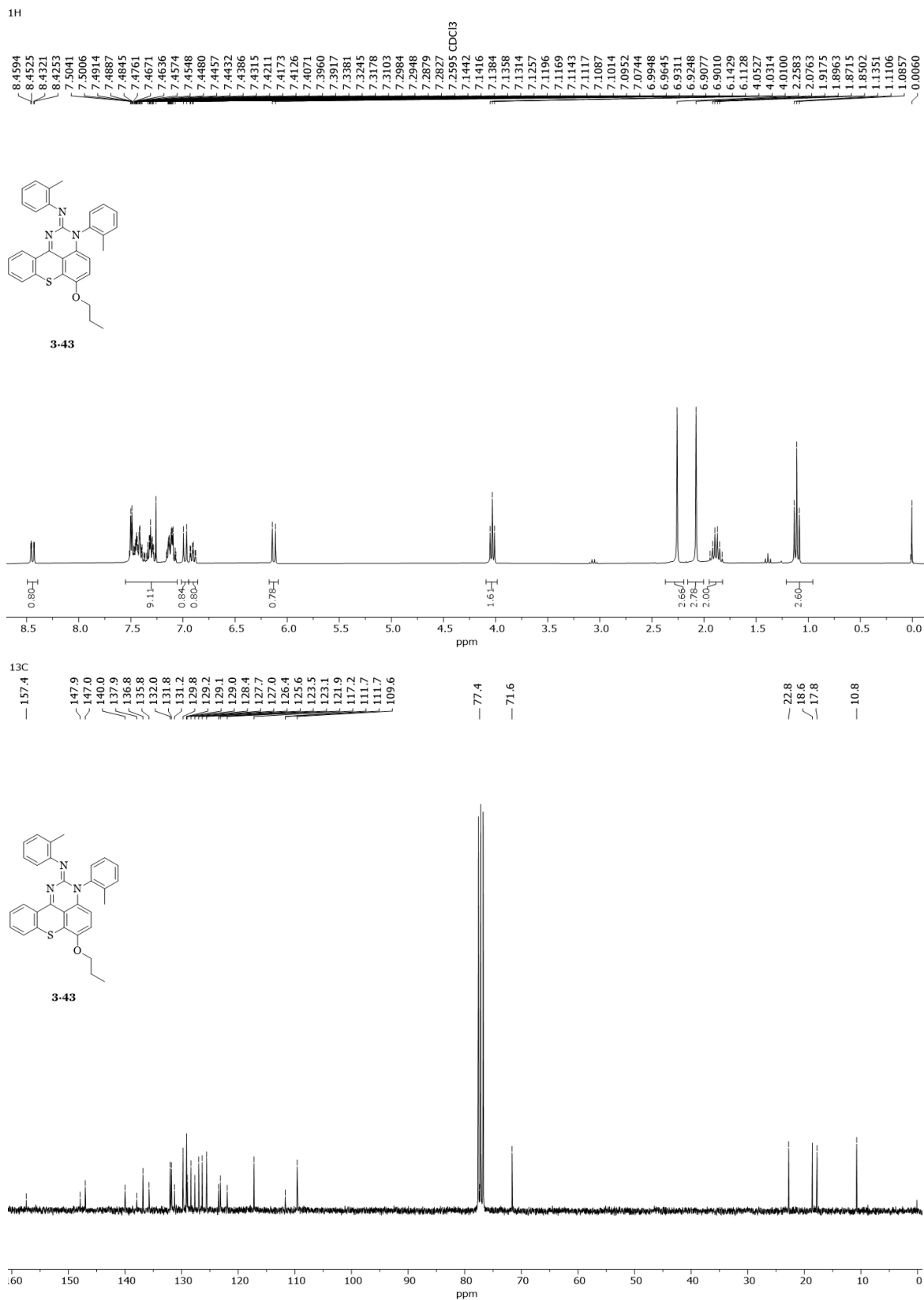


Figure S3.28 ¹H NMR (top, 300.13 MHz, CDCl₃) and ¹³C NMR (bottom, 75.48 MHz, CDCl₃) for compound **3-43**.

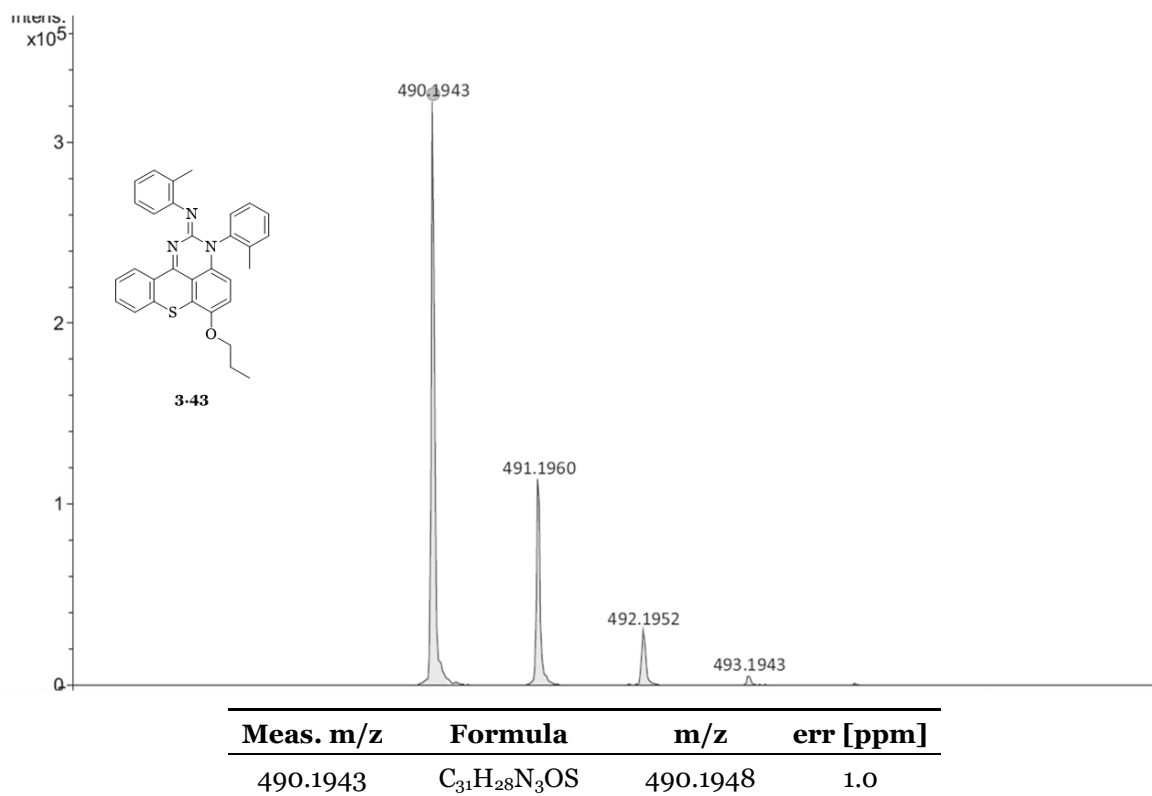


Figure S3.29 ESI data for compound **3.43**.

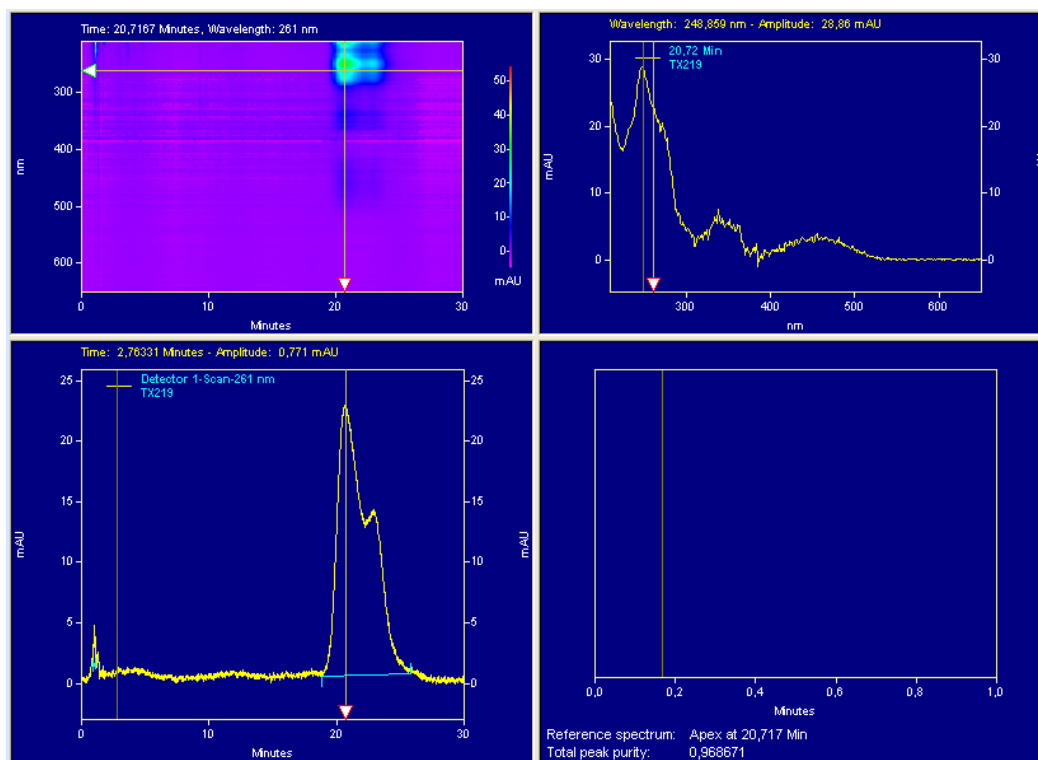


Figure S3.30 Peak purity of compound **3.43**.

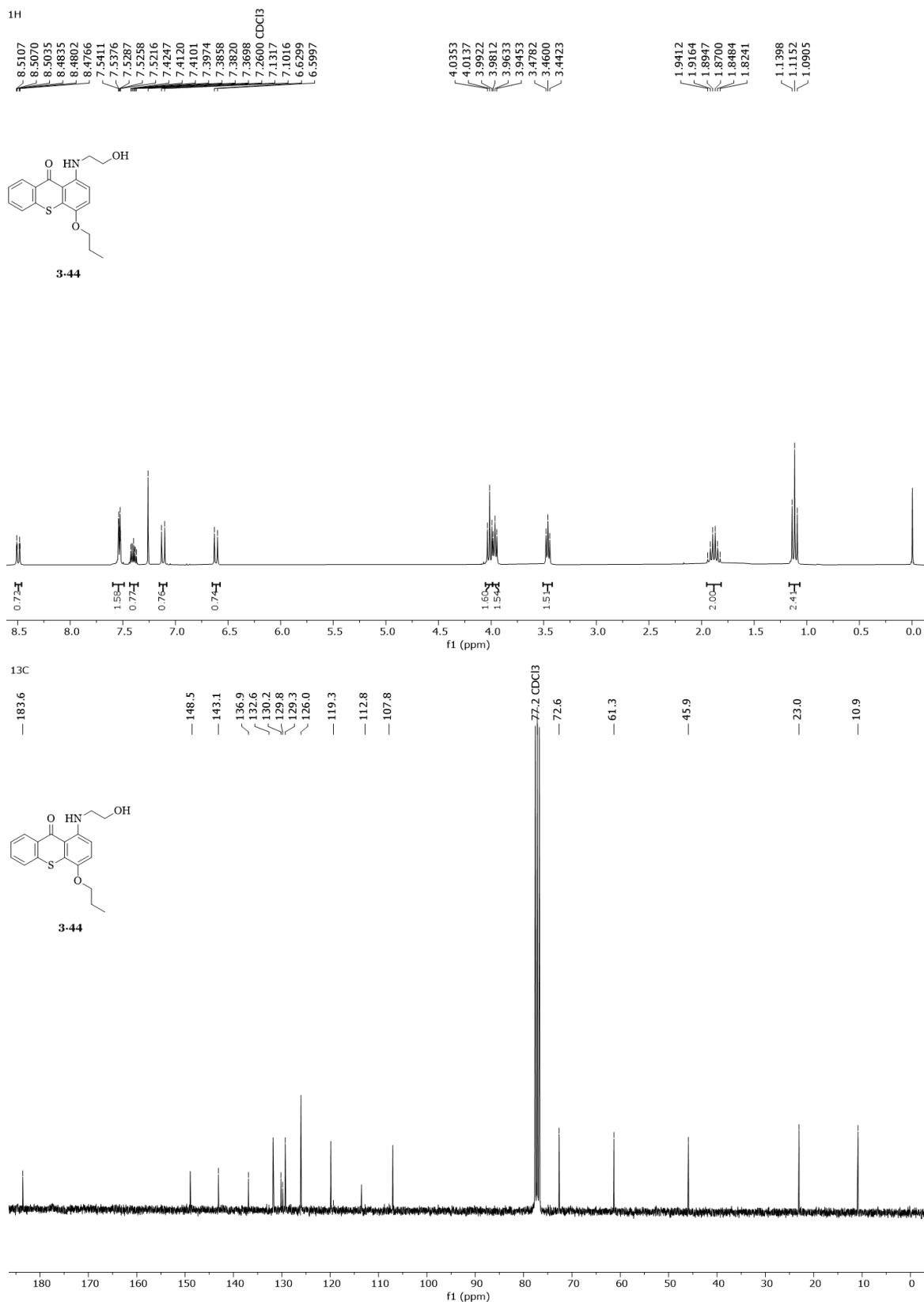


Figure S3.31 ¹H NMR (top, 300.13 MHz, CDCl₃) and ¹³C NMR (bottom, 75.48 MHz, CDCl₃) for compound **3-44**.

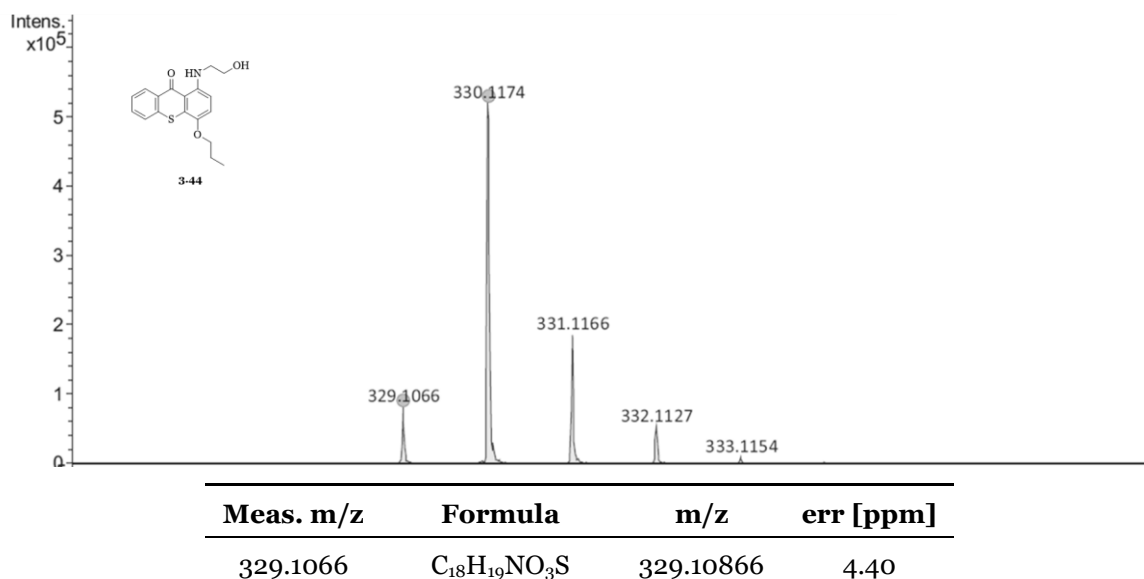


Figure S3.32 ESI data for compound **3-44**.

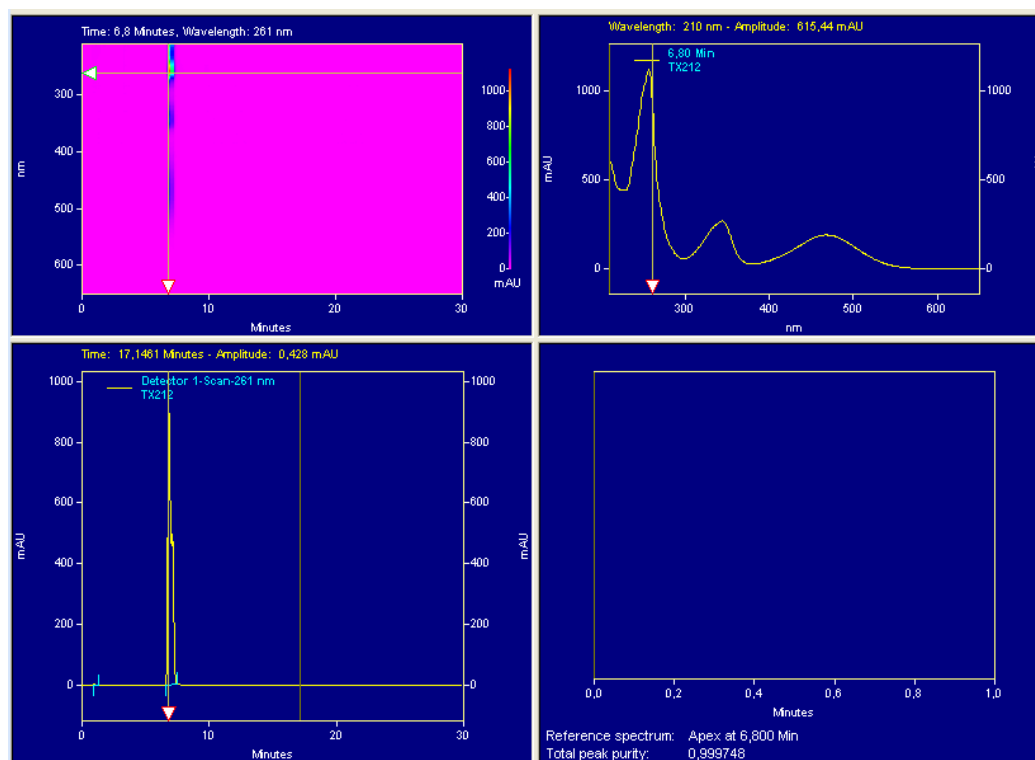


Figure S3.33 Peak purity of compound **3-44**.

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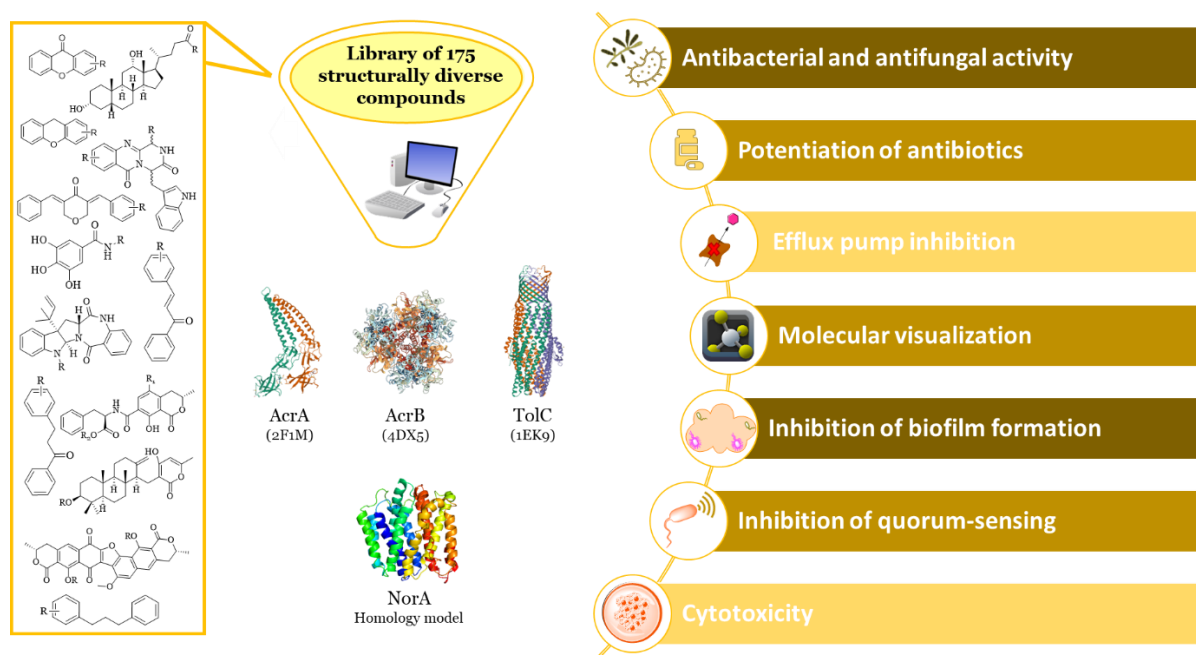
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Chapter 4 – Screening for antimicrobial activity and docking studies on structurally-diverse compounds with potential to circumvent antimicrobial resistance mechanisms ²



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Neves, A. R., Durães, F., et al. *Int J Mol Sci* 2022, 23, 14468.

Author contributions: The synthesis of the xanthone derivatives **4.14 – 4.18** and **4.33 – 4.74** were performed by Dr. Diana Resende, under the supervision of Prof. Emília Sousa and Prof. Madalena Pinto. The synthesis of chiral derivatives of xanthenes **4.19 – 4.32** was performed by Prof. Carla Fernandes. The synthesis of the xanthene derivatives **4.75 – 4.93** was performed by Miguel Maia, under the supervision of Prof. Emília Sousa. The syntheses of the gallic acid derivatives **4.94 – 4.103** and deoxycholic acid derivatives **4.104** and **4.105** were performed by Ana Rita Neves, under the supervision of Prof. Marta Correia da Silva and Prof. Emília Sousa. The synthesis of the chalcone derivatives **4.106 – 4.117** was performed by Daniela Pereira, under the supervision of Prof. Honorina Cidade and Prof. Marta Correia da Silva. The syntheses of the chalcone derivatives **4.117 – 4.123** and **4.130**, dihydrochalcone derivatives **4.131 – 4.136**, and diarylpropane derivatives **4.137 – 4.141** were performed by Ana Jesus, under the supervision of Prof. Honorina Cidade. The syntheses of chalcone derivatives **4.124 – 4.129** and diarylpentanoids **4.142 – 4.164** were performed by Joana Moreira, under the supervision of Prof. Honorina Cidade and Prof. Madalena Pinto. The synthesis of fiscalin derivatives **4.165 – 4.169** was performed by Solida Long, under the supervision of Prof. Emília Sousa and Prof. Madalena Pinto. The metabolites from marine-derived fungi **4.170 – 4.188** were isolated and characterized by Decha Kumla, under the supervision of Prof. Anake Kijjoa. The antibacterial and potentiation of antibiotics assay was performed by Fernando Durães, under the supervision of Prof. Paulo Martins da Costa. The efflux pump inhibition, inhibition of biofilm formation, inhibition of quorum sensing, and cytotoxicity assays were performed by Fernando Durães and Nikoletta Szemerédi, under the supervision of Prof. Gabriella Spengler. The antifungal assays were performed by Joana Freitas da Silva, under the supervision of Prof. Eugénia Pinto. The docking studies were performed by Fernando Durães, under the supervision of Prof. Emília Sousa and Prof. Madalena Pinto.

4.1 Introduction

4.1.1 Compound screening

The search for new antibacterial compounds has been a major topic over the past years. The number of antibacterials approved has dropped significantly in the 21st century, with no new approvals in this regard since 2019 (1, 2).

Phenotypic screening of structurally diverse compounds can offer a solution for this scarcity, as they can provide insights into chemical spaces that have been previously undiscovered, and offer a wider variety of chemical diversity that had not previously been studied or tested (3).

As the development of new antibiotics has slowed down, strategies to redevelop old antibiotics, such as what happened with colistin, have gained popularity (4). However, the appearance of resistances will lead to a loss of efficacy of these drugs. Therefore, screening of compounds should not just focus on antibacterial activity, but also in the potential to reverse AMR (5).

If the focus is switched towards ways to reverse resistance to antimicrobials, such as the inhibition of EPs, the need to find new antimicrobials decreases, as old drugs will regain their activity. In this scope, screenings of compounds of natural and synthetic sources for their potential as EPIs have been performed (6-10). Drug repurposing screenings have also been useful towards finding new structures that could be promising for this purpose, particularly drugs that act on the central nervous system, such as antipsychotics and antidepressants (11-15).

Considering the screenings that were performed for finding new EPIs, the structural diversity is noticeable, which can bring advantages and disadvantages. On the one hand, there are a lot of structural possibilities, which also leads to a better understanding of the architecture of the target and the substrates which it can accommodate. On the other hand, it makes it impossible to establish structural features that would make a compound inhibit bacterial EPs (16).

Therefore, screening structurally diverse compounds arises as an attractive strategy, as it discloses hit compounds that would show activity, which could be a starting point towards finding potent and safe EPIs through hit optimization.

Throughout this chapter, a phenotypic screening of a library of 175 in-house structurally diverse compounds was developed for antimicrobial activity and for their potential to reverse AMR mechanisms. A virtual screening was performed to check the predicted affinity of the library to relevant EPs, such as AcrAB-TolC and NorA.

4.1.2 Library of structurally diverse compounds

The library of compounds tested herein is composed by derivatives of natural products and can be divided according to the classes of compounds present in **Figure 4.1**.

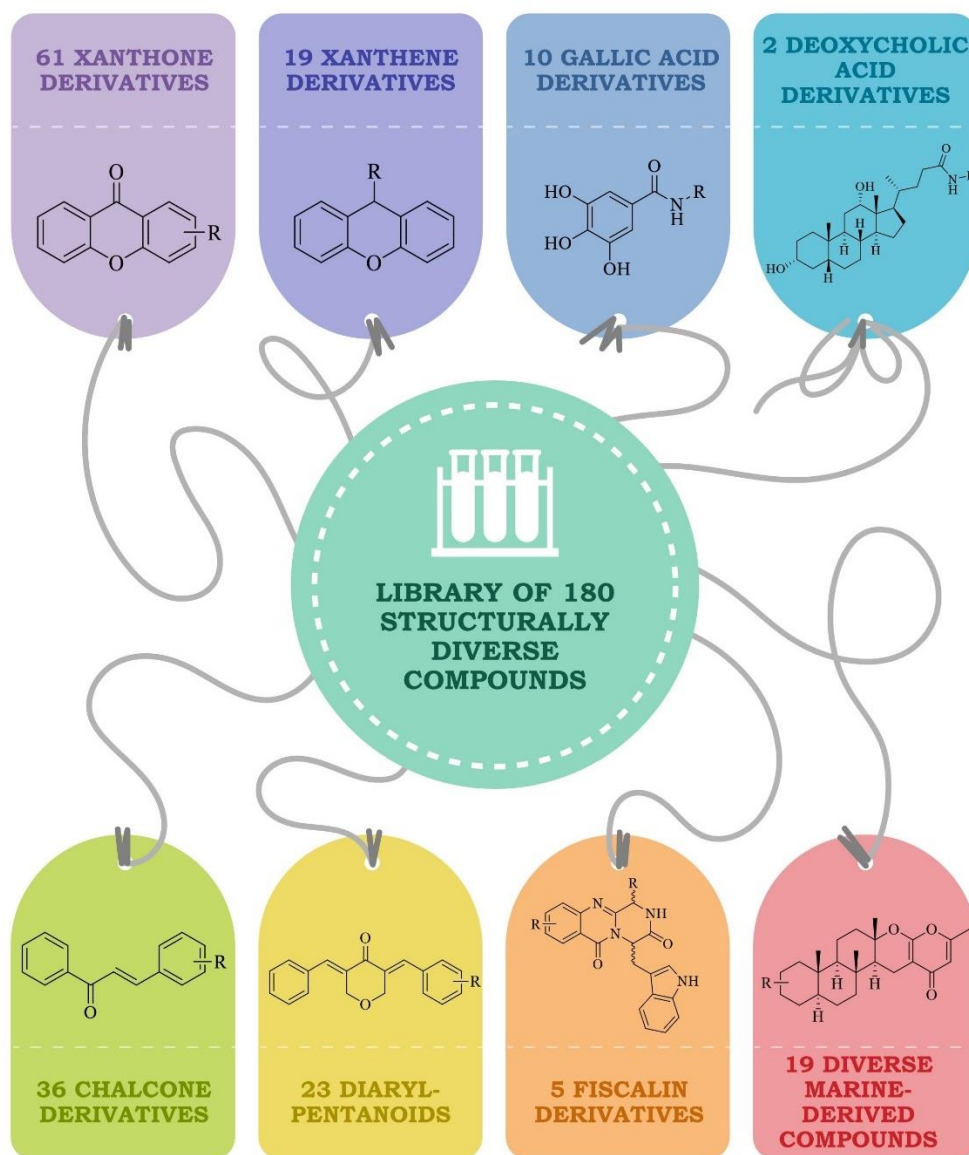


Figure 4.1 List of the library of structurally diverse compounds tested.

The tested library contained 61 xanthenes with different substitution patterns. These included mono-, di-, tri- and tetrasubstituted derivatives, with substitutions in different carbons. Xanthenes, structurally related to xanthenes, were modified in the C9 position, and 19 derivatives were synthesized and tested.

A library of ten gallic acid derivatives was also included. Within this group, amides were synthesized, and the scaffold was that of gallic acid and its trimethoxy derivative.

Deoxycholic acid, a bile acid, was also used as template for the synthesis of two amides, which were tested.

The other groups are related to the flavonoids' family: 36 chalcone derivatives, which could be further distributed into chalcones (25 derivatives), dihydrochalcones (six derivatives) and diarylpropanes (five derivatives), and 23 diarylpentanoids.

The other two groups of compounds tested are related to the marine environment. The first is a group of five synthetic fiscalins, which were inspired by compounds isolated from marine-derived fungi, and also a group comprising 19 structurally diverse metabolites, isolated from marine-derived fungi.

Some of the compounds herein belong to classes that had already been described as EPIs, while others are described herein as EPIs for the first time. A brief description of the referred classes and their biological activities will be provided over the next subchapters.

4.1.2.1 Xanthones

With a tricyclic dibenzo- γ -pyrone scaffold, containing an oxygen heterocycle, xanthones (**4.1**, **Figure 4.2**) occur as specialized metabolites in terrestrial and marine sources, such as plants, fungi, and lichens (17, 18).

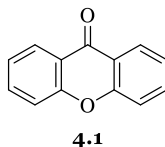


Figure 4.2 Structure of the xanthone scaffold (**4.1**).

The xanthone scaffold is considered a privileged structure, as it is prone to different substitution patterns, yielding compounds with different biological activities, which makes it a desirable class for drug development (19, 20). Among the activities described for xanthones are antioxidant (21-26), anti-inflammatory (27-31), anticoagulant (32), antitumor (25, 33-36), and anti-obesity (37).

Additionally, xanthone derivatives have also been studied for their antimicrobial activity, with derivatives proven to display antibacterial (28, 38-45), antifungal (28, 40, 46-48), and antiviral (49) activity. The number of descriptions of xanthone derivatives with antibacterial activity is encouraging and emphasizes the potential of these compounds for the planned biological assays. The fact that xanthones have displayed activity regarding the modulation of P-gp (50-52), and as isosteric derivatives, namely thioxanthones, had been described as bacterial EPIs (53), makes them interesting in this scope.

4.1.2.2 Xanthenes

Xanthenes (**4.2**, **Figure 4.3**) are structurally related to xanthenes (**4.1**, **Figure 4.2**), possessing a tricyclic dibenzo[*b,e*]pyran scaffold. It is a naturally occurring structure, with derivatives being isolated from plants and marine fungi, albeit not frequently (54).

Derivatives of this scaffold have shown potential as neuroprotective (55, 56), antitumor (57-60), anti-inflammatory and analgesic (61, 62), antidiabetic (63), antioxidant (64), antipsychotic (65), and antimicrobial agents, specifically antiparasitic (66, 67), antiviral (68), antifungal (69), and antibacterial (69-73).

Despite no xanthenes having been described for bacterial EP inhibition, the thioxanthene (*E*)-flupentixol (**4.3**, **Figure 4.3**) and the phenothiazine chlorpromazine (**4.4**, **Figure 4.3**), both C9-substituted tricyclic antipsychotics, have shown their potential in this regard (13).

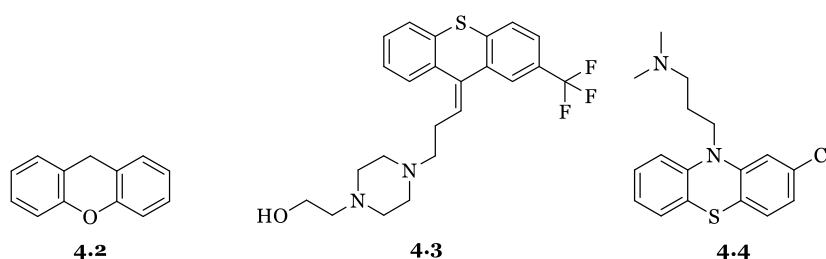


Figure 4.3 Structures of the xanthene scaffold (**4.2**) and the antipsychotics (*E*)-flupentixol (**4.3**) and chlorpromazine (**4.4**).

4.1.2.3 Gallic acid and derivatives

Gallic acid (**4.5**, **Figure 4.4**) is an antioxidant polyphenol widely produced by plants as a specialized metabolite (74). It has also showed anticancer activity (75, 76) and potentiation of anticancer drugs (77). Amide derivatives of this compound have been synthesized as an effort to improve membrane permeability, and compounds with anti-Parkinson activity were obtained (78). Gallamides have also been described as antiallergics (79), and as antibacterials (80).

Its 3,4,5-trimethoxy derivative (**4.6**, **Figure 4.4**) is present as an ester in reserpine (**4.7**, **Figure 4.4**), an antihypertensive and antipsychotic drug, which also presents potent EPI activity (81).

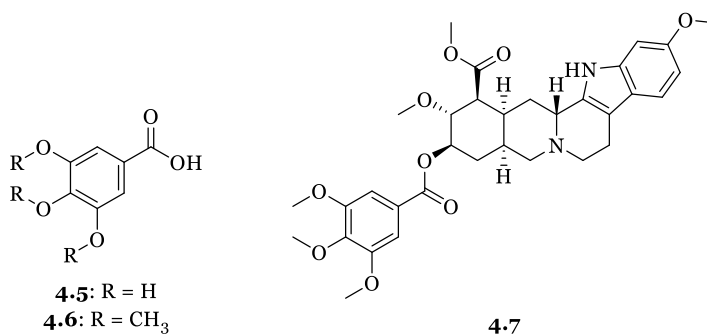


Figure 4.4 Structures of gallic acid (**4.5**), 3,4,5-trimethoxybenzoic acid (**4.6**), and reserpine (**4.7**).

4.1.2.4 Deoxycholic acid

Deoxycholic acid (**4.8**, **Figure 4.5**) is a bile acid produced by intestinal bacteria from primary bile acids. Structurally, these compounds are acidic steroids, containing 24 carbon atoms arranged in a rigid, steroidal scaffold, and an acidic side chain with varying hydroxy groups, making these compounds amphiphilic (82-84).

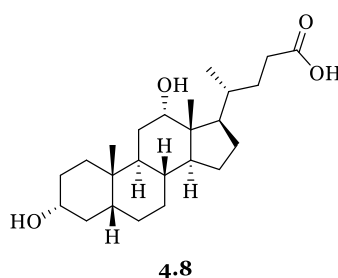


Figure 4.5 Structure of deoxycholic acid (**4.8**).

The interest on deoxycholic acid and other bile acids lies not only in the functional groups prone to chemical modifications, but also in the existence of transport systems that facilitate their cell penetration both through active and passive transport (85, 86).

Deoxycholic acid derivatives, specifically amides, have shown antibacterial (87-90), antifungal (89, 91), and antitumor activity (90, 91).

4.1.2.5 Chalcones

The chalcone scaffold (**4.9**, **Figure 4.6**) is simple and occurs naturally in plants, fruits, and vegetables. They are precursors of flavonoids, and structurally are comprised by two aromatic rings linked by a three carbon α,β -unsaturated carbonyl structure (92).

In terms of activity, these compounds have been extensively studied, and have been described as antidiabetic (93-95), anti-inflammatory (96, 97), anticancer (94, 98-100), antioxidant (101), antiparasitic (102-106), antifungal (101, 107-113), and antibacterial (112-

117). Furthermore, a few chalcone derivatives have shown potential as bacterial EPIs (7, 118).

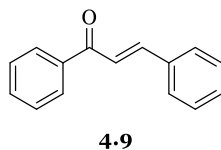


Figure 4.6 Structure of the chalcone scaffold (**4.9**).

4.1.2.6 Diarylpentanoids

Diarylpentanoids (**4.10**, **Figure 4.7**) are compounds closely related to chalcones, composed by two aromatic rings linked by a five-carbon chain, which often includes a dienone moiety. They are also known as C5-curcuminoids due to their structural relationship to curcumin (**4.11**, **Figure 4.7**).

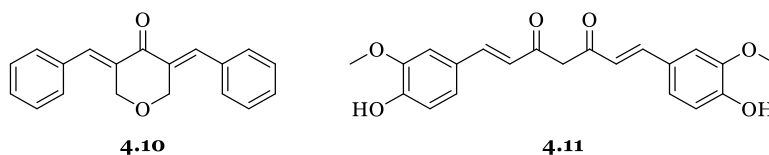


Figure 4.7 General structure of the diarylpentanoid scaffold (**4.10**) and curcumin (**4.11**).

These compounds have been described for their potential as antitumor (119-121), anti-inflammatory (122-127), antioxidant (123, 125, 128, 129), antidiabetic (130-133), neuroprotective (134), antiparasitic (135), and antibacterial agents (136).

4.1.2.7 Fiscalin and derivatives

Fiscalins (**4.12** and **4.13**, **Figure 4.8**) are valine-derived alkaloids, isolated from marine fungi, with an indolyl moiety linked to an anthranilic acid-derived moiety (137). Regarding the activities that have been associated with these compounds are neuroprotective (138, 139), anticancer (139-144), antimalarial (145), and antibacterial, with potential to synergize with antibacterial drugs (146, 147).

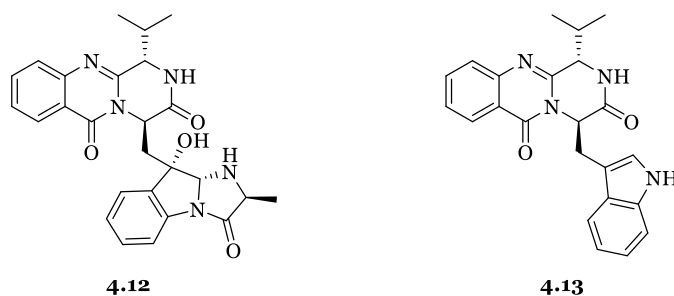


Figure 4.8 Structure of fiscalin A (**4.12**) and B (**4.13**).

4.1.2.8 Metabolites from marine-derived fungi

The marine environment has become a fruitful source of new molecules with bioactivity (148, 149). Over the past years, the focus of marine-derived compounds has been broadened past macroorganisms, with microorganisms such as bacteria and fungi being the focus of numerous studies, with several new scaffolds being produced as specialized metabolites, that have shown particular potential as anti-infective agents (150-152).

Particularly, the *Neosartorya* and *Aspergillus* genera have been prolific at providing new scaffolds for antibacterial agents, such as polyketides, alkaloids, xanthonenes, and acetophenones, among others (152-154).

4.2 Screening of a library of structurally diverse compounds

The compounds described throughout this chapter were studied for antibacterial and antifungal activity, as well as for their potential to inhibit bacterial EPs and related mechanisms, including inhibition of biofilm formation and quorum-sensing. The most promising compounds were then assessed for their safety, through a cytotoxicity assay in mouse embryonic fibroblasts (NIH/3T3 cell line). Docking studies were performed as well, both as a way to predict the affinity of the compounds towards the targets proposed (the AcrAB-TolC efflux system and the NorA pump), and as a way to visualize the predicted interactions of the compounds with the residues within the targets.

4.2.1 Xanthonenes

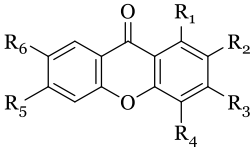
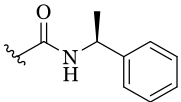
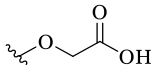
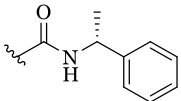
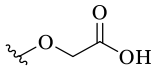
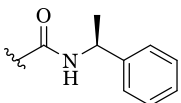
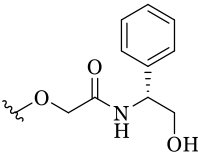
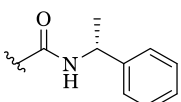
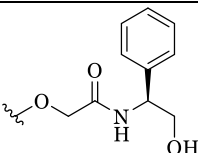
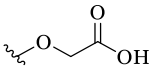
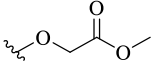
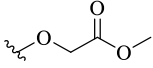
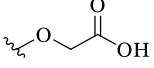
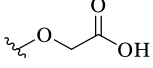
A library of 61 xanthonenes was used in this study. In terms of substitution patterns, xanthonenes are divided in 11 groups:

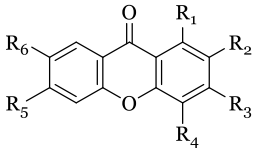
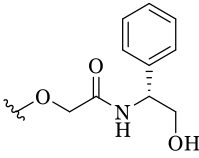
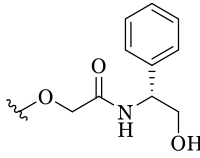
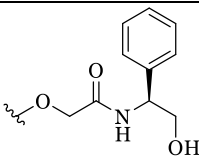
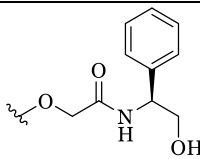
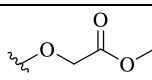
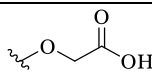
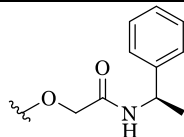
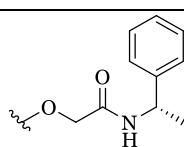
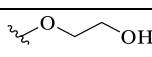
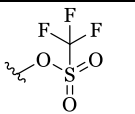
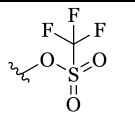
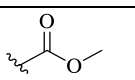
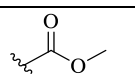
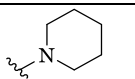
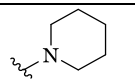
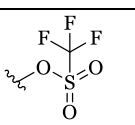
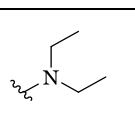
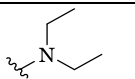
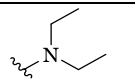
- Two monosubstituted (**4.14** and **4.15**);
- One 1,2-disubstituted (**4.16**);
- One 1,7-disubstituted (**4.17**);
- One 2,3-disubstituted (**4.18**);
- Four 2,6-disubstituted (**4.19** – **4.22**);
- Ten 3,4-disubstituted (**4.23** – **4.32**);
- Twenty-seven 3,6-disubstituted (**4.33** – **4.59**);
- One 1,2,3-trisubstituted (**4.60**);
- Eight 1,3,4-trisubstituted (**4.61** – **4.68**);
- One 1,2,3,4-tribustituted (**4.69**);
- Five 1,3,4,6-tetrasubstituted (**4.70** – **4.74**).

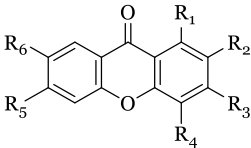
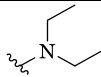
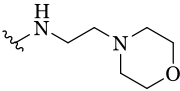
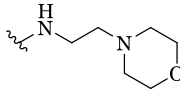
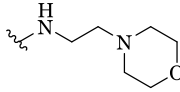
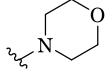
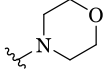
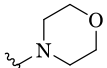
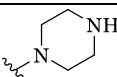
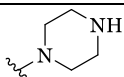
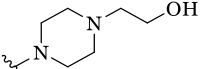
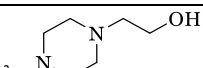
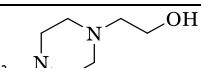
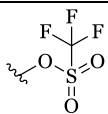
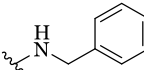
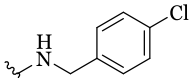
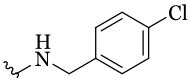
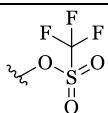
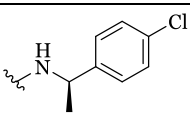
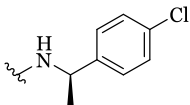
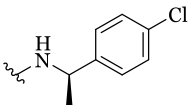
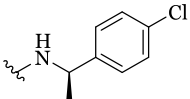
Noteworthy is the fact that the 2,6- and the 3,4-disubstituted xanthenes contained enantiomers, which were useful at providing insights into the role played by chirality in the activities tested, which had never been studied before for EP inhibition.

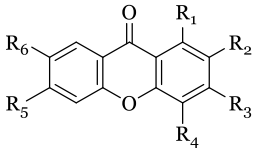
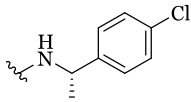
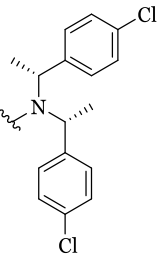
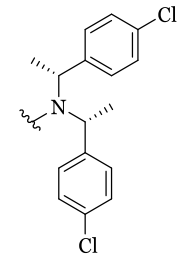
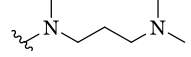
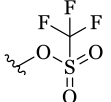
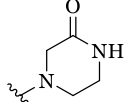
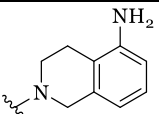
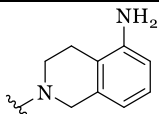
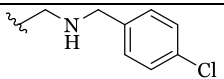
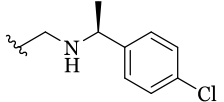
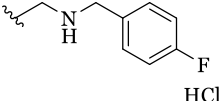
The structures of the xanthenes tested are present in **Table 4.1**.

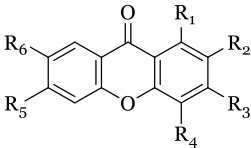
Table 4.1 Structures of the library of xanthenes tested.

 4.17 - 4.70						
Compound	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
Monosubstituted						
4.14	H	H	H	OH	H	H
4.15	OCH ₃	H	H	H	H	H
1,2-disubstituted						
4.16	OH	OH	H	H	H	H
1,7-disubstituted						
4.17	OH	H	H	H	H	OH
2,3-disubstituted						
4.18	H	OH	OH	H	H	H
2,6-disubstituted						
4.19	H		H	H		H
4.20	H		H	H		H
4.21	H		H	H		H
4.22	H		H	H		H
3,4-disubstituted						
4.23	H	H		OCH ₃	H	H
4.24	H	H			H	H
4.25	H	H			H	H

 4.17 - 4.70						
Compound	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
4.26	H	H			H	H
4.27	H	H			H	H
4.28	H	H	OCH ₃		H	H
4.29	H	H	OCH ₃		H	H
4.30	H	H	OCH ₃		H	H
4.31	H	H	OCH ₃		H	H
4.32	H	H	OCH ₃		H	H
3,6-disubstituted						
4.33	H	H	OH	H	OH	H
4.34	H	H	Cl	H	Cl	H
4.35	H	H		H		H
4.36	H	H		H		H
4.37	H	H		H		H
4.38	H	H		H		H
4.39	H	H		H		H

 4.17 - 4.70						
Compound	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
4.40	H	H	OH	H		H
4.41	H	H		H		H
4.42	H	H	OH	H		H
4.43	H	H		H		H
4.44	H	H	OH	H		H
4.45	H	H		H		H
4.46	H	H		H		H
4.47	H	H	OH	H		H
4.48	H	H	OH	H		H
4.49	H	H		H		H
4.50	H	H		H		H
4.51	H	H		H		H
4.52	H	H		H		H
4.53	H	H		H		H
4.54	H	H	OH	H		H

 4.17 - 4.70						
Compound	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
4.55	H	H	OH	H		H
4.56	H	H		H		H
4.57	H	H	OH	H		H
4.58	H	H		H		H
4.59	H	H		H		H
1,2,3-trisubstituted						
4.60	OH	CH ₃	OH	H	H	H
1,3,4-trisubstituted						
4.61	CHO	H	OH	OCH ₃	H	H
4.62	CHO	H	OCH ₃	OCH ₃	H	H
4.63	CH ₃	H	OH	OH	H	H
4.64	CH ₃	H	OCH ₃	OCH ₃	H	H
4.65		H	OCH ₃	OCH ₃	H	H
4.66		H	OCH ₃	OCH ₃	H	H
4.67		H	OCH ₃	OCH ₃	H	H
4.68		H	OCH ₃	OCH ₃	H	H
1,2,3,4-tetrasubstituted						
4.69	CH ₃	Cl	OCH ₃	OCH ₃	H	H
1,3,4,6-tetrasubstituted						

 4.17 - 4.70						
Compound	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
4.70		H	OCH ₃	OCH ₃	OCH ₃	H
4.71	CHO	H	OCH ₃	OCH ₃	OCH ₃	H
4.72		H	OCH ₃	OCH ₃	OCH ₃	H
4.73	CH ₃	H	OCH ₃	OCH ₃	OCH ₃	H
4.74	CH ₃	H	OH	OH	OH	H

4.2.1.1 Docking studies

The xanthone derivatives **4.14** – **4.74** were subject to docking studies in a similar way as the thioxanthone derivatives in the previous chapter. Briefly, the structures of the three portions of the AcrAB-TolC efflux system, AcrB (PDB: 4DX5), AcrA (PDB: 2F1M) and TolC (PDB: 1EK9), and a homology model of the NorA EP were chosen for these studies. The sites chosen for the docking studies were the hydrophobic trap and the substrate binding site for AcrB, the helical hairpin and the lipoyl domain for AcrA, and the site comprising the lysine residues that interacted with the dithiobis(sulfosuccinimidyl propionate) for TolC. For NorA, the predicted binding core region and the cavity turned to the cytoplasmic side were the sites chosen. As positive controls, PAβN and reserpine were used. The docking scores for the compounds and positive controls are present in **Table 4.2**.

Table 4.2 Docking scores for the xanthone derivatives in bacterial efflux pumps.

Compound	Docking score (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
4.14	-6.7	-5.9	-6.2	-6.2	-6.4	-6.6	-6.3
4.15	-6.8	-5.1	-6.4	-6.0	-6.7	-6.8	-6.8
4.16	-6.6	-5.5	-6.4	-6.8	-7.0	-6.5	-6.4
4.17	-6.9	-5.9	-6.4	-6.7	-6.5	-6.6	-6.3
4.18	-6.9	-6.2	-6.2	-6.7	-6.5	-6.2	-6.1
4.19	-9.2	3.2	-7.3	-6.2	-7.9	-7.0	-5.2
4.20	-8.5	0.0	-7.0	-5.5	-8.1	-8.7	-5.1
4.21	-9.6	-7.2	-7.5	-6.9	-9.0	-5.9	-6.5
4.22	-9.8	-7.1	-7.3	-5.8	-8.4	-7.1	-5.7
4.23	-7.5	-4.9	-6.1	-6.8	-6.5	-5.8	-5.3

Compound	Docking score (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
4.24	-7.3	-5.3	-5.7	-5.6	-6.5	-6.5	-6.4
4.25	-8.0	-4.4	-6.0	-5.9	-7.0	-6.1	-5.5
4.26	-7.6	-5.2	-7.7	-6.3	-7.1	-7.7	-5.8
4.27	-8.3	-5.2	-6.9	-5.8	-7.1	-5.9	-5.0
4.28	-7.3	-5.2	-5.9	-5.8	-6.5	-6.1	-5.8
4.29	-7.3	-5.1	-5.8	-6.2	-6.7	-6.2	-5.8
4.30	-7.6	1.6	-7.4	-5.4	-7.9	-8.1	-5.6
4.31	-7.3	-6.1	-6.7	-4.8	-7.8	-7.8	-5.6
4.32	-6.9	-5.6	-5.7	-5.7	-6.7	-5.4	-5.3
4.33	-6.8	-6.1	-5.6	-6.9	-6.9	-6.0	-6.0
4.34	-6.7	-5.4	-5.5	-6.2	-6.8	-6.0	-5.8
4.35	-9.9	-6.6	-7.4	-6.8	-9.2	-8.0	-7.3
4.36	-7.2	-5.5	-5.4	-5.6	-7.0	-7.3	-7.2
4.37	-8.7	-4.3	-6.3	-6.0	-8.8	-5.5	-6.4
4.38	-8.1	-4.8	-6.2	-6.4	-8.1	-7.2	-7.4
4.39	-6.7	-4.2	-5.2	-5.2	-6.9	-6.8	-6.8
4.40	-6.8	-5.4	-5.4	-5.5	-7.0	-6.6	-6.6
4.41	-8.0	-6.6	-6.3	-5.6	-7.8	-7.9	-5.8
4.42	-7.8	-7.2	-6.3	-5.9	-7.5	-7.8	-6.3
4.43	-9.0	-3.9	-6.0	-6.0	-8.3	-5.6	-5.9
4.44	-7.6	-6.2	-5.9	-6.6	-7.7	-7.2	-6.8
4.45	-8.6	-3.6	-6.0	-6.0	-8.4	-5.6	-5.8
4.46	-8.2	-5.2	-6.1	-5.8	-8.3	-6.7	-6.4
4.47	-7.5	-6.0	-6.0	-6.6	-7.6	-7.1	-6.4
4.48	-8.2	-5.7	-6.4	-5.7	-7.6	-7.7	-5.7
4.49	-8.2	-4.6	-6.4	-5.8	-7.9	-0.5	-3.1
4.50	-9.4	-7.4	-7.6	-7.2	-9.7	-9.0	-7.5
4.51	-9.1	-8.3	-7.4	-6.9	-8.5	-7.9	-6.4
4.52	-9.8	-6.7	-8.0	-7.3	-9.5	-8.9	-6.8
4.53	-9.7	-7.0	-7.6	-6.8	-9.2	-6.5	-5.5
4.54	-8.6	-7.6	-6.9	-6.3	-8.4	-7.9	-6.2
4.55	-8.3	-6.8	-6.9	-6.4	-8.0	-8.9	-7.4
4.56	-10.7	4.1	-7.4	-5.9	-8.2	0.0	-3.3
4.57	-7.1	-4.8	-5.5	-5.3	-6.7	-7.1	-6.5
4.58	-9.8	-4.9	-6.7	-6.4	-9.4	-6.3	-6.2
4.59	-10.4	11.8	-7.1	-6.5	-9.9	1.0	-8.0
4.60	-6.9	-6.2	-6.4	-6.3	-6.6	-6.2	-5.5
4.61	-7.2	-6.2	-5.7	-6.7	-6.9	-6.3	-5.3
4.62	-7.2	-5.0	-5.5	-6.1	-6.2	-4.7	-5.2
4.63	-6.9	-6.1	-6.7	-7.0	-6.7	-6.9	-6.6
4.64	-6.8	-5.9	-5.7	-5.9	-6.6	-5.1	-5.4

Compound	Docking score (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
4.65	-8.0	8.6	-7.0	-6.2	-7.9	-7.9	-5.4
4.66	-7.6	-5.5	-7.0	-6.4	-7.9	-5.6	-6.4
4.67	-7.3	-5.8	-6.3	-6.3	-6.9	-7.9	-6.0
4.68	-7.1	-5.0	-5.3	-5.5	-6.2	-3.4	-5.7
4.69	-7.0	-5.1	-5.8	-5.9	-6.4	-4.8	-5.2
4.70	-7.0	-5.0	-5.1	-5.7	-6.1	-2.9	-5.6
4.71	-7.3	-5.9	-5.3	-6.4	-6.3	-4.7	-5.1
4.72	-7.5	-5.9	-5.5	-4.9	-6.5	-4.5	-5.2
4.73	-7.1	-5.5	-5.3	-6.1	-6.6	-4.8	-5.2
4.74	-7.2	-6.4	-6.0	-7.8	-6.6	-5.9	-5.9
PAβN	-7.1	-4.7	-5.8	-4.9	-7.1	-	-
Reserpine	-	-	-	-	-	1.0	-4.6

SBS: Substrate binding site; **HT:** Hydrophobic trap; **HH:** Helical hairpin; **LD:** Lipoyl domain; **BCR:** Binding core region; **CS:** Cytoplasmic side; **PAβN:** Phenyl-arginine β-naphthylamide.

Similar to what happened with thioxanthenes, xanthone derivatives are predicted to have similar binding affinities as the positive controls towards the targets tested, presenting comparable or superior docking scores. Additionally, these derivatives also seem to present more affinity towards the AcrB portion of the AcrAB-TolC efflux system, namely the substrate binding site. On the opposite side, the hydrophobic trap was the site towards which the compounds were predicted to interact with the least affinity.

When it comes to the NorA pump, the compounds were predicted to have more affinity than reserpine as a general rule, with all the xanthone derivatives presenting more predicted affinity towards the binding core region than reserpine.

The docking studies also show that 2,6- and 3,6-substitution would be more favorable towards an interaction with the substrate binding site of AcrB, and that substitution with two bulky substituents in 3,6-disubstituted xanthenes were generally predicted to interact better with the same site than asymmetric substitutions. On the other hand, in some cases it could be seen that 3,6-asymmetrically substituted xanthenes would have better predicted affinity towards the hydrophobic trap, likely due to a decrease in steric hindrance (**4.38** and **4.40** *vs.* **4.39**, **4.41** *vs.* **4.42**, **4.43** *vs.* **4.44**, **4.46** *vs.* **4.47**, and **4.48** *vs.* **4.49**).

Due to the encouraging results obtained in the docking studies, all the derivatives were tested for the proposed biological activities.

4.2.1.2 Antibacterial activity and potentiation of antibiotics

The synthetic xanthenes **4.19** – **4.59** were tested for their antibacterial activity and for their use in potentiating the activity of antibiotics. The derivatives **4.14** – **4.18** and **4.60** – **4.74** had previously been studied for their antibacterial activity (45, 155).

In this assay, six bacterial strains were used, three Gram-negative (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. enterica* serovar Typhimurium SL1344), and three Gram-positive (*S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, and *S. aureus* 272123).

The results obtained showed that compound **4.45** was active against the Gram-negative *E. coli* ATCC 25922, showing a MIC and a minimum bactericidal concentration (MBC) of 176 μ M, being the only bactericidal compound within this series. Compound **4.31** was effective against both *S. aureus* strains tested, with a MIC of 136 μ M for *S. aureus* ATCC 25923 and 100 μ M for *S. aureus* 272123.

Similarly, compounds **4.54** and **4.55** also displayed antibacterial activity against both strains, with **4.54** showing MICs of 22 μ M and 100 μ M, and **4.55** showing MICs of 11 μ M and 100 μ M, for *S. aureus* ATCC 25923 and *S. aureus* 272123, respectively.

Compounds **4.44** and **4.56** showed a mild activity against the same strain of *S. aureus* ATCC 25923, with MICs of 142 μ M and 82 μ M, respectively. Lastly, compound **4.57** showed potential against *S. aureus* 272123, displaying a MIC of 100 μ M.

The potential of the compounds to act as antibacterial adjuvants was also evaluated through the potentiation of the activity of antibiotics to which bacteria had developed resistance towards. The ESBL-producing *E. coli* SA/2 and the VRE *E. faecalis* B3/101 were used in this scope, and the antibiotics used were CTX (MIC = 1124 μ M) and VAN (MIC = 707 μ M), respectively.

Herein, five xanthenes were able to decrease the MIC of CTX when used in combination. Compounds **4.24**, **4.30**, and **4.40** were able to decrease the MIC of CTX four times, whereas compounds **4.44** and **4.45** showed an 8-fold decrease. It should be noted that the enantiomer of compound **4.30**, compound **4.31**, did not display any changes in this assay, suggesting once again the role of enantioselectivity in this situation.

4.2.1.3 Bacterial efflux pump inhibition assay

The xanthone derivatives **4.14** – **4.74** were further investigated concerning the potential they displayed towards inhibiting bacterial EPs. The models used were the same as described before, *S. aureus* 272123 and *S. Typhimurium* SL1344, using reserpine and CCCP, respectively, at sub-inhibitory concentrations (25 μ M). The results are present in **Table 4.3**.

Considering that compounds **4.31**, **4.54**, **4.55**, and **4.57** showed a MIC of 100 μ M for *S. aureus* 272123, they were tested at 33 μ M, one-third of their MIC, in order to assure bacterial viability. The other compounds were tested at 50 μ M, for both strains.

Table 4.3 RFI of the tested xanthone derivatives.

Compound	RFI $\pm$ SD	
	<i>S. aureus</i> 272123	<i>S. Typhimurium</i> SL1344
4.14	-0.36 $\pm$ 0.03	-0.01 $\pm$ 0.05
4.15	-0.10 $\pm$ 0.06	0.28 $\pm$ 0.09
4.16	0.05 $\pm$ 0.06	0.23 $\pm$ 0.04
4.17	0.13 $\pm$ 0.08	2.90 $\pm$ 0.71
4.18	-0.69 $\pm$ 0.02	-0.57 $\pm$ 0.02
4.19	0.05 $\pm$ 0.04	0.13 $\pm$ 0.05
4.20	0.30 $\pm$ 0.02	4.64 $\pm$ 0.23
4.21	0.05 $\pm$ 0.01	0.02 $\pm$ 0.06
4.22	0.26 $\pm$ 0.07	0.96 $\pm$ 0.04
4.23	0.11 $\pm$ 0.05	0.10 $\pm$ 0.01
4.24	0.01 $\pm$ 0.04	0.05 $\pm$ 0.07
4.25	-0.14 $\pm$ 0.01	-0.06 $\pm$ 0.04
4.26	-0.21 $\pm$ 0.01	2.23 $\pm$ 0.05
4.27	3.19 $\pm$ 0.31	0.03 $\pm$ 0.06
4.28	0.03 $\pm$ 0.03	0.36 $\pm$ 0.05
4.29	-0.01 $\pm$ 0.01	-0.03 $\pm$ 0.02
4.30	-0.22 $\pm$ 0.07	0.05 $\pm$ 0.07
4.31	0.15 $\pm$ 0.12	-0.03 $\pm$ 0.07
4.32	0.14 $\pm$ 0.03	0.28 $\pm$ 0.02
4.33	-0.23 $\pm$ 0.04	-0.17 $\pm$ 0.02
4.34	-0.09 $\pm$ 0.04	0.02 $\pm$ 0.04
4.35	-0.05 $\pm$ 0.07	0.09 $\pm$ 0.02
4.36	-0.21 $\pm$ 0.04	-0.05 $\pm$ 0.03
4.37	1.12 $\pm$ 0.02	0.82 $\pm$ 0.02
4.38	1.06 $\pm$ 0.04	0.36 $\pm$ 0.01
4.39	0.32 $\pm$ 0.05	0.04 $\pm$ 0.04
4.40	0.76 $\pm$ 0.24	0.28 $\pm$ 0.14
4.41	-0.29 $\pm$ 0.04	-0.10 $\pm$ 0.05
4.42	-0.05 $\pm$ 0.08	-0.01 $\pm$ 0.04
4.43	0.12 $\pm$ 0.03	0.03 $\pm$ 0.08
4.44	-0.58 $\pm$ 0.06	-0.26 $\pm$ 0.10
4.45	0.06 $\pm$ 0.07	-0.11 $\pm$ 0.02
4.46	-0.01 $\pm$ 0.18	0.01 $\pm$ 0.06
4.47	0.88 $\pm$ 0.07	2.24 $\pm$ 0.05
4.48	-0.01 $\pm$ 0.01	0.16 $\pm$ 0.05
4.49	0.12 $\pm$ 0.02	0.03 $\pm$ 0.02

Compound	RFI $\pm$ SD	
	<i>S. aureus</i> 272123	<i>S. Typhimurium</i> SL1344
4.50	2.12 $\pm$ 0.07	3.52 $\pm$ 0.64
4.51	0.06 $\pm$ 0.05	0.17 $\pm$ 0.03
4.52	1.89 $\pm$ 0.06	1.40 $\pm$ 0.25
4.53	-0.02 $\pm$ 0.08	0.75 $\pm$ 0.05
4.54	1.46 $\pm$ 0.10	5.09 $\pm$ 0.09
4.55	1.69 $\pm$ 0.04	4.64 $\pm$ 1.64
4.56	0.29 $\pm$ 0.04	0.98 $\pm$ 0.12
4.57	0.21 $\pm$ 0.04	0.22 $\pm$ 0.09
4.58	1.09 $\pm$ 0.08	3.26 $\pm$ 0.04
4.59	-0.27 $\pm$ 0.04	0.49 $\pm$ 0.04
4.60	1.16 $\pm$ 0.18	1.74 $\pm$ 0.22
4.61	-0.28 $\pm$ 0.06	0.02 $\pm$ 0.02
4.62	0.08 $\pm$ 0.07	0.13 $\pm$ 0.09
4.63	-0.91 $\pm$ 0.01	-0.92 $\pm$ 0.01
4.64	0.15 $\pm$ 0.25	0.05 $\pm$ 0.11
4.65	-0.01 $\pm$ 0.04	0.04 $\pm$ 0.04
4.66	1.08 $\pm$ 0.12	2.09 $\pm$ 0.05
4.67	0.02 $\pm$ 0.02	2.86 $\pm$ 0.14
4.68	0.11 $\pm$ 0.30	1.75 $\pm$ 0.57
4.69	-0.04 $\pm$ 0.06	0.05 $\pm$ 0.09
4.70	0.53 $\pm$ 0.20	1.16 $\pm$ 0.64
4.71	5.49 $\pm$ 0.84	0.13 $\pm$ 0.15
4.72	-0.34 $\pm$ 0.02	-0.29 $\pm$ 0.08
4.73	0.38 $\pm$ 0.02	0.24 $\pm$ 0.05
4.74	-0.92 $\pm$ 0.01	-0.87 $\pm$ 0.02
Reserpine	0.31 $\pm$ 0.07	-
CCCP	-	0.23 $\pm$ 0.04

RFI: Relative fluorescence index; SD: Standard deviation; CCCP: carbonyl cyanide 3-chlorophenylhydrazone.

An analysis of **Table 4.3** shows that xanthone derivatives **4.27**, **4.39**, **4.71**, and **4.73** could successfully inhibit the efflux of EtBr in *S. aureus* 272123, whereas compounds **4.15**, **4.17**, **4.20**, **4.22**, **4.26**, **4.53**, **4.56**, **4.59**, **4.67**, and **4.68** were able to do the same in *S. Typhimurium* SL1344, showing a better affinity of xanthenes for efflux pumps present in Gram-negative bacteria. It was also noticed that compounds **4.37**, **4.38**, **4.40**, **4.47**, **4.50**, **4.52**, **4.54**, **4.55**, **4.58**, **4.60**, **4.66**, and **4.70**, were effective for both strains in use, and these compounds were assessed for their autofluorescence, to clarify whether the results obtained are due to the intracellular accumulation of EtBr, or to the fluorescence of the compounds. These results are in **Figure 4.9**.

The fluorescence curves in **Figure 4.9** show that only compounds **4.37**, **4.38**, **4.40**, and **4.66** did not display autofluorescence. Oppositely, compounds **4.47**, **4.50**, **4.52**, **4.54**, **4.55**, **4.58**, **4.60**, and **4.70** showed very high fluorescence and/or irregular fluorescence patterns. As such, no conclusions could be drawn regarding these derivatives with this particular assay. **Table 4.3** also shows compounds with negative RFI values, which means they presented less fluorescence than the negative control and were, therefore, considered ineffective.

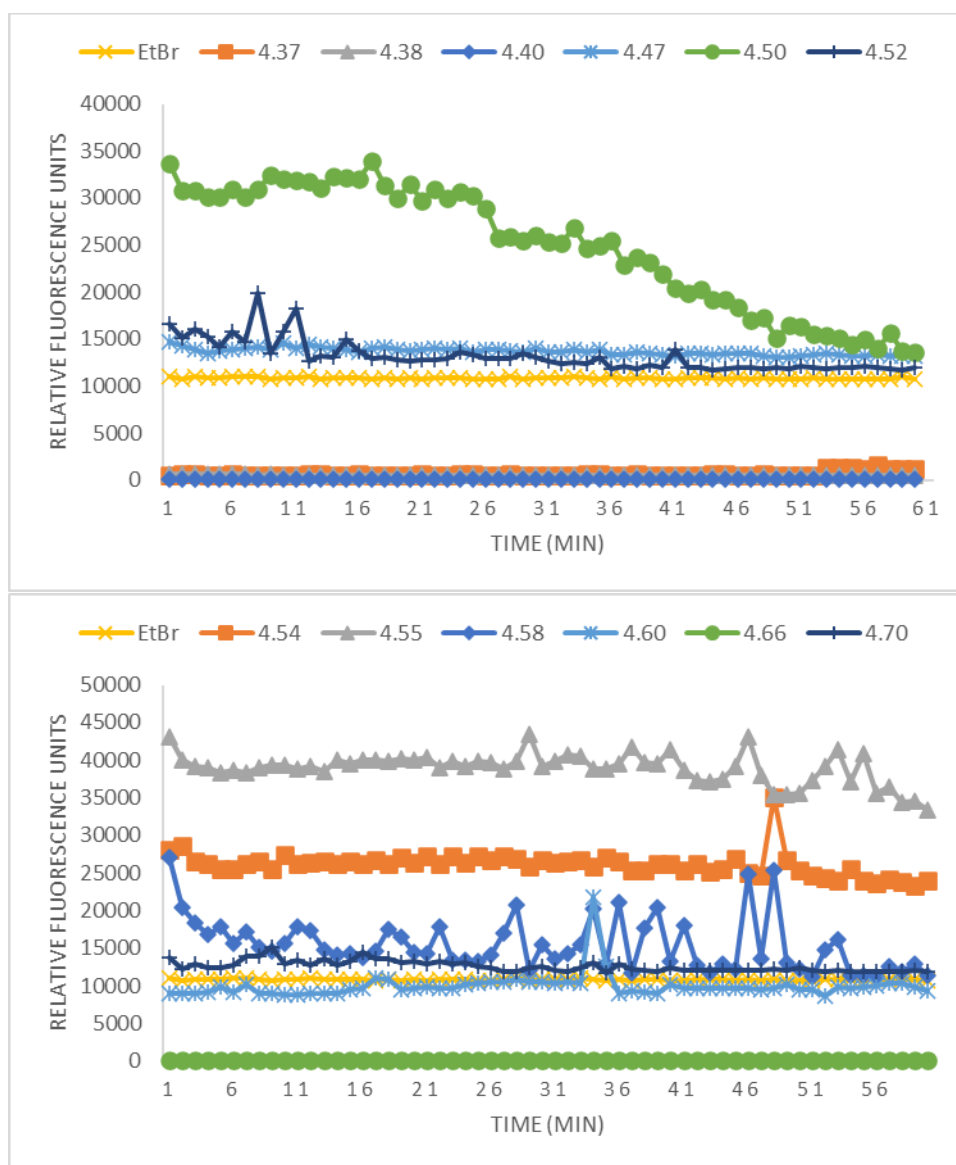


Figure 4.9 Fluorescence curves of EtBr and compounds **4.37**, **4.38**, **4.40**, **4.47**, **4.50**, and **4.52** (top), and **4.54**, **4.55**, **4.58**, **4.60**, **4.66**, and **4.70** (bottom).

Considering the data herein obtained, a few conclusions can be drawn. Firstly, in xanthenes exclusively substituted with hydroxy groups, only compound **4.17** displayed promising activity for Gram-negative EPs. This can lead to the hypothesis that a

hydroxylation in each aromatic ring is required, and possibly in the C1 and C7 positions, or at least in closer proximity to the ketone. It should be mentioned that compound **4.17** is a natural product, whose isolation from plants has been described, and had already demonstrated synergy with antibiotics in Gram-positive and Gram-negative bacteria (156, 157). However, compound **4.17** did not present synergy with a strain capable of producing ESBL, which could mean its AMR reversion happens through the inhibition of EPs (155).

Regarding the different substitution patterns, the introduction of bulky groups in the C1 position appears to be beneficial, as is the case of **4.15**, and **4.66 – 4.68**. The lack of activity regarding compounds **4.62** and **4.64** shows that the methoxy groups in C3 and C4 are not as important for EP inhibition in this case. The structurally related compounds **4.65** and **4.67** show that the presence of a fluorine atom in the phenylmethanamine moiety is more favorable than a chlorine atom.

Compounds **4.19 – 4.22**, **4.26**, **4.27**, **4.30**, **4.31**, **4.54**, and **4.55** are enantiomers, and were useful to draw information regarding the role of enantioselectivity. The fluorescence of compounds **4.54** and **4.55** did not allow for any conclusions to be drawn. However, regarding the other enantiomeric pairs, it was noted that **4.26**, an *S,S*-enantiomer, was active against *S. Typhimurium* SL1344, while the *R,R*-enantiomer **4.27** was active against *S. aureus* 272123. Compounds **4.20** and **4.22** were also active against the Gram-negative bacteria used, while their enantiomers did not show activity in this regard.

Regarding the 3,6-disubstituted derivatives, it could be seen that the diethylamine moiety is favorable in the inhibition of EPs in *S. aureus* 272123, evidenced by the activity of **4.38 – 4.40**, and by the fact that it is present in a thioxanthone that could also inhibit EPs in the same strain. On the other hand, di-substitution with this moiety appears to hinder the inhibition of EPs in *S. Typhimurium* SL1344. A piperidine moiety also seems to be a successful substituent, with compound **4.37** displaying favorable results for both strains, similar to what had happened with a thioxanthone with the same substituent. Finally, the moiety in compound **4.53**, which is the same moiety present in **4.66**, seems to also be a promising one for *S. Typhimurium* EP inhibition.

Once again, the tricyclic scaffold appears to be fruitful at yielding bacterial EPIs, due to the multiplicity of derivatives that can be obtained.

The effective compounds were visualized within the targets they are likely to interact with. Due to the docking results, the interactions of the active compounds were inspected in the binding core region of the NorA homology model (compounds **4.27**, **4.37 – 4.40**, **4.66**, **4.71**, and **4.73**) and in the substrate binding site of AcrB (**4.15**, **4.17**, **4.20**, **4.22**, **4.26**, **4.37**, **4.38**, **4.40**, **4.53**, **4.56**, **4.59**, and **4.66 – 4.68**).

Compound **4.27**, predicted to interact with NorA, shows that the substituent in C3 plays an important role, as the amide present in this moiety is likely to interact with Glu-222 by a

hydrogen interaction, like what happened with thioxanthenes. The hydroxy moiety establishes the same interaction with Phe-288, as can be seen in (**Figure 4.10, A**). Furthermore, compound **4.27** seems to fit perfectly into a pocket present in this site (**Figure 4.10, B**).

Regarding compound **4.37**, a nitrogen atom in one of the piperidine moieties was able to establish a hydrogen interaction with Asp-239, and the C9 carbonyl interacted with Ser-236 and Glu-222 (**Figure 4.10, C**). On the other hand, compound **4.38** is predicted to only establish a T-shaped π - π interaction with Tyr-292, as can be seen in **Figure 4.10, D**. Compounds **4.39** and **4.40** did not show any polar or hydrogen interactions with the NorA homology model in this instance.

Similarly, compound **4.66** did not show any relevant interactions with the residues, while compound **4.71** showed an interaction between the aldehyde and Glu-222 (**Figure 4.10, E**). Lastly, compound **4.73** showed a T-shaped π - π interaction with Phe-47 (**Figure 4.10, F**). It should however be noticed that these studies were performed in a homology model, which means the residues could vary.

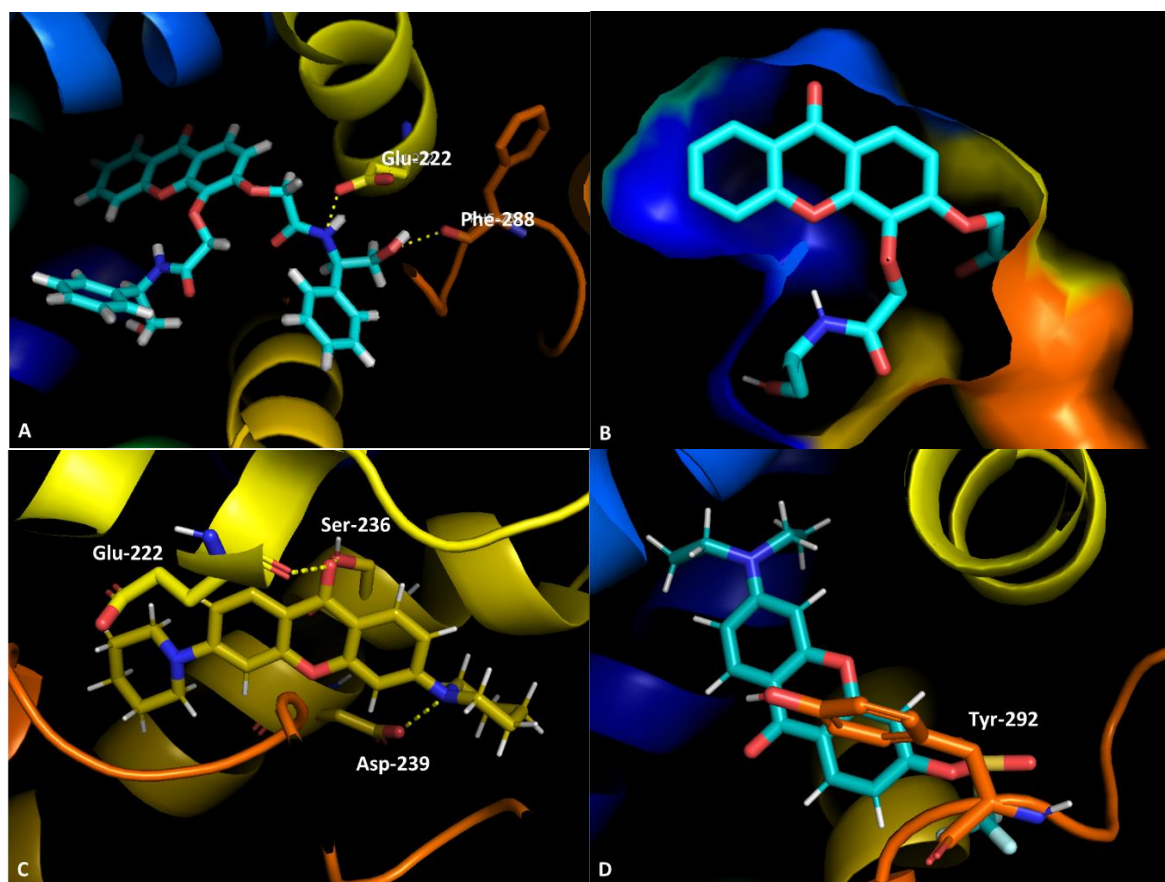


Figure 4.10 Molecular visualization of xanthenes in the binding core region of the NorA homology model (A) Interaction between compound **4.27**; (B) Surface view of compound **4.27** within the NorA homology model; (C) Interaction between compound **4.37** and NorA; (D) Interaction between compound **4.38** and NorA; (E) Interaction between compound **4.71** and NorA; (F) Interaction between compound **4.73** and NorA.

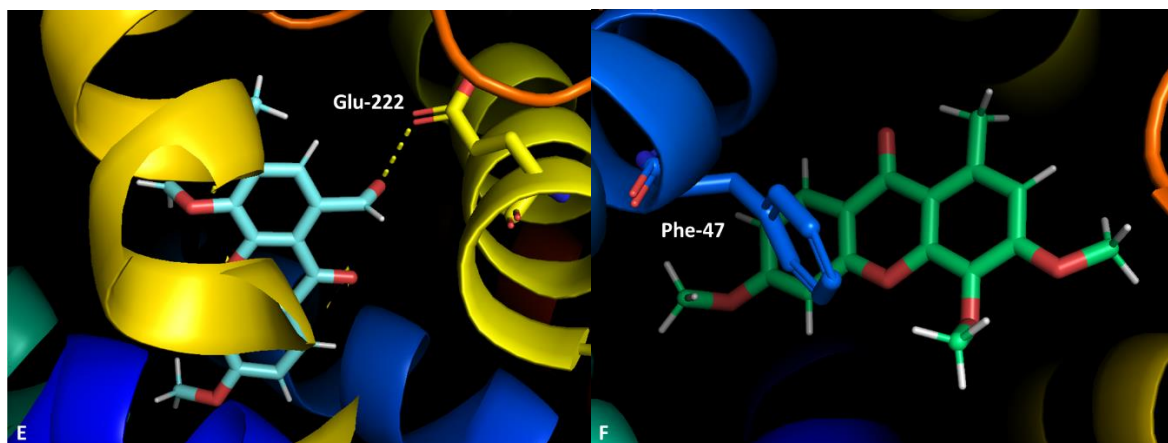


Figure 4.10 Molecular visualization of xanthenes in the binding core region of the NorA homology model (A) Interaction between compound **4.27**; (B) Surface view of compound **4.27** within the NorA homology model; (C) Interaction between compound **4.37** and NorA; (D) Interaction between compound **4.38** and NorA; (E) Interaction between compound **4.71** and NorA; (F) Interaction between compound **4.73** and NorA (cont.).

The same was done for compounds **4.15**, **4.17**, **4.20**, **4.22**, **4.26**, **4.37**, **4.38**, **4.40**, **4.53**, **4.56**, **4.59**, and **4.66** – **4.68** in the substrate binding site of the AcrB portion of the AcrAB-TolC efflux pump. Compound **4.15** is predicted to establish a hydrogen interaction with the nitrogen in Lys-292 (**Figure 4.11, A**). Compound **4.17** can establish a dipole interaction between the carbonyl at C9 and a carbonyl in Thr-87. The oxygen in the xanthone scaffold can form a hydrogen interaction with an amine in Gln-176, and the hydroxy groups at C1 and C7 interact with Arg-620 and Gly-619, respectively, as can be seen in **Figure 4.11, B**.

Compound **4.20** was predicted to interact with several residues previously described for thioxanthenes, such as Gln-89, Gln-176, and Gly-619. Additionally, this compound also interacts with Ser-180, Gln-181, and Gln-273 (**Figure 4.11, C**). Compound **4.22** is also predicted to establish hydrogen interactions mostly with residues previously described in other studies with thioxanthenes, such as Thr-87, Gln-176 and Arg-620. The hydroxy group present in the compound can establish an additional hydrogen interaction with Lys-292 (**Figure 4.11, D**). A deeper analysis of **4.26** shows interactions with Ser-46, Ser-128, Lys-163, Asn-274, and Arg-767 (**Figure 4.11, E**).

Compound **4.37** showed interactions between the carbonyl and Gly-179 and Asn-274, and between the oxygen in the xanthone moiety and Gln-125, as seen in **Figure 4.11, F**. When it comes to compound **4.38**, the sulfonate moiety is predicted to interact with Lys-163, while the carbonyl interacts with Asn-274 (**Figure 4.11, G**).

Regarding compound **4.40**, it can establish a double interaction with Arg-620, through the carbonyl and hydroxy groups present in this compound. Additionally, the carbonyl can also interact with Thr-87, and the oxygen in the xanthone core can establish a hydrogen interaction with Gln-176, as illustrated in **Figure 4.11, H**. Compound **4.53** was shown to

be predicted to also interact with Arg-620, through the carbonyl, and with Gln-176 through the oxygen in the xanthone scaffold (**Figure 4.11, I**). On the other hand, compound **4.56** is predicted to interact with Arg-620 and Thr-87, both through the carbonyl (**Figure 4.11, J**).

Compound **4.59** was predicted to establish interactions between one of the aromatic amines and Ala-159 and Asp-164. Additionally, the carbonyl interacted with Glu-130 (**Figure 4.11, K**).

Xanthones **4.66** (**Figure 4.11, L**) and **4.67** (**Figure 4.11, M**) interact with Thr-87 and Arg-320 through the carbonyl, and the oxygen in the xanthone core and the oxygen in the methoxy group in C4 can interact with Gln-176. Lastly, the carbonyl in compound **4.68** can interact Gln-176 and Leu-177, while the methoxy groups in C3 and C4 can interact with Arg-620 and Asn-274, respectively (**Figure 4.11, N**).

There are also non-polar interactions established between the compounds and both targets (not shown).

It can be seen that xanthones are predicted to bind in a few residues that have been described for thioxanthonenes, such as the polar amino acids Glu-222 and Tyr-292 in the NorA homology model, and the polar Ser-46, Thr-87, Gln-89, Gln-125, Gln-176, Asn-247, and Arg-620, and the non-polar Leu-177 for AcrB.

The xanthone derivatives that presented potential for EP inhibition were tested for their potential of inhibiting resistance mechanisms related to this phenomenon. As such, compounds **4.15**, **4.17**, **4.20**, **4.22**, **4.26**, **4.27**, **4.37 – 4.40**, **4.53**, **4.56**, **4.59**, **4.66**, **4.67**, **4.68**, **4.71**, and **4.73** were tested for their potential of inhibiting biofilm formation and QS.

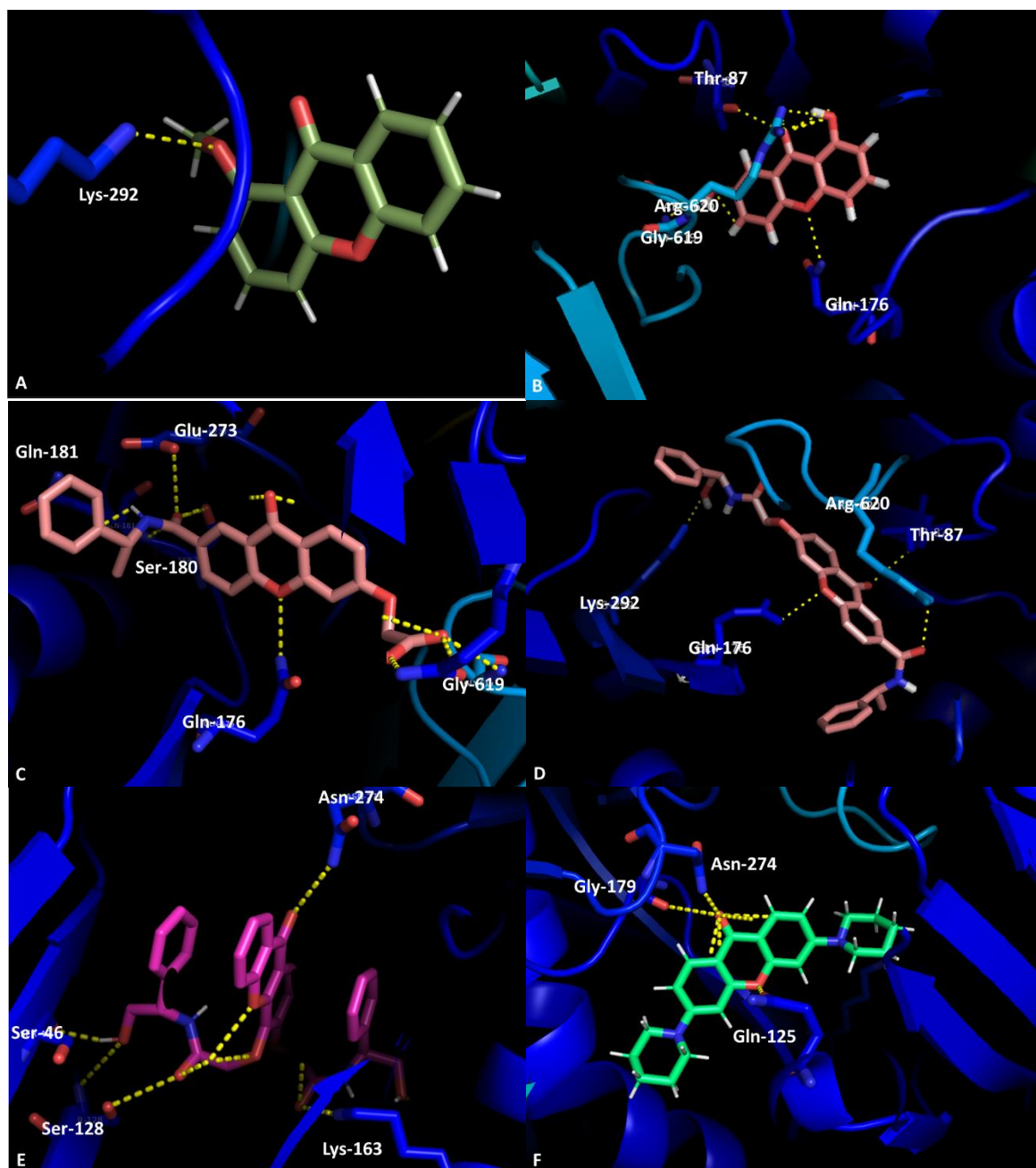


Figure 4.11 Molecular visualization of xanthenes in the substrate binding site of AcrB. (A) Interactions between compound **4.15** and AcrB; (B) Interactions between compound **4.17** and AcrB; (C) Interactions between compound **4.20** and AcrB; (D) Interactions between compound **4.22** and AcrB; (E) Interactions between compound **4.26** and AcrB; (F) Interactions between compound **4.37** and AcrB; (G) Interactions between compound **4.38** and AcrB; (H) Interactions between compound **4.40** and AcrB; (I) Interactions between compound **4.53** and AcrB; (J) Interactions between compound **4.56** and AcrB; (K) Interactions between compound **4.59** and AcrB; (L) Interactions between compound **4.66** and AcrB; (M) Interactions between compound **4.67** and AcrB; (N) Interactions between compound **4.68** and AcrB.

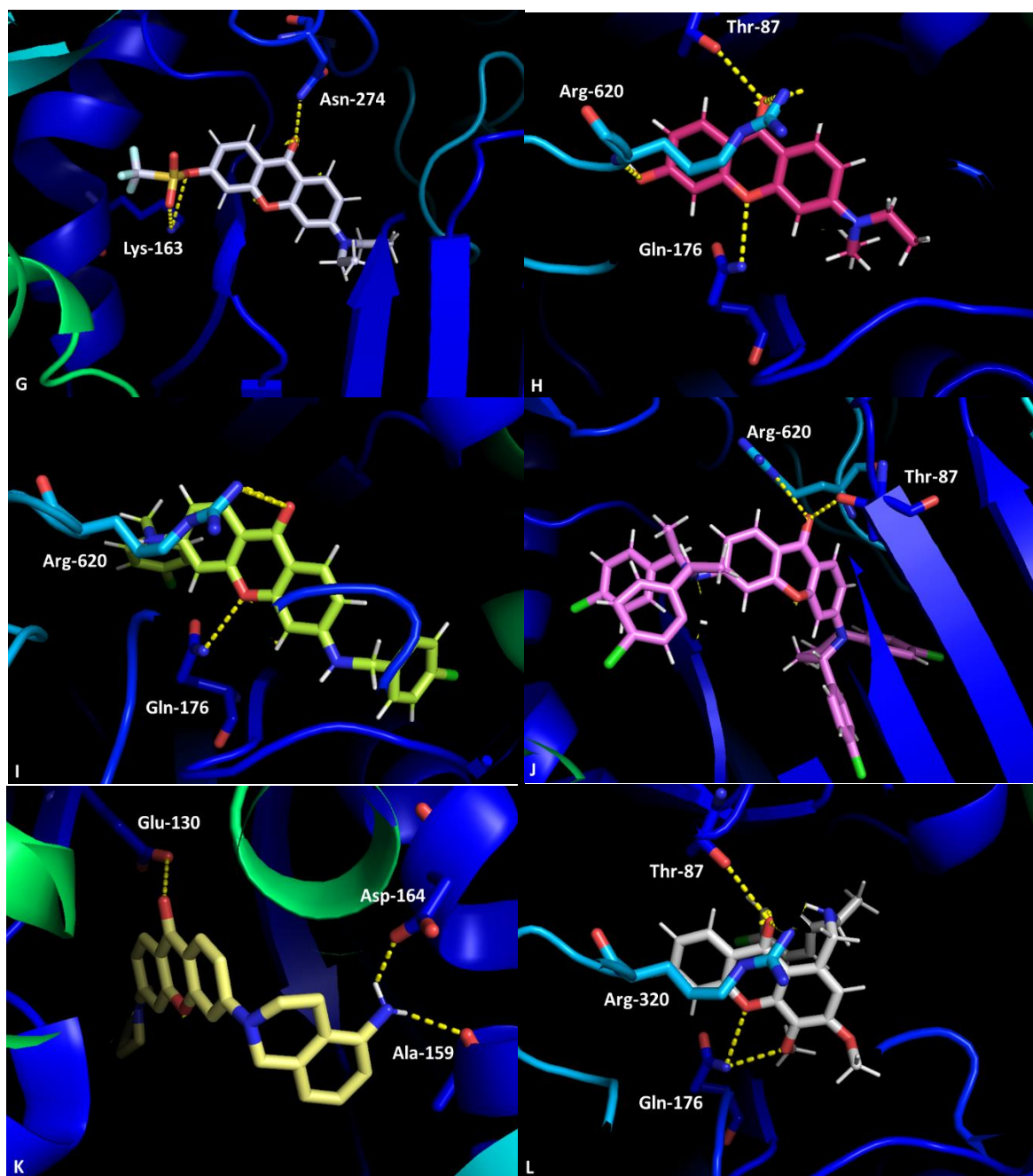


Figure 4.11 Molecular visualization of xanthenes in the substrate binding site of AcrB. (A) Interactions between compound **4.15** and AcrB; (B) Interactions between compound **4.17** and AcrB; (C) Interactions between compound **4.20** and AcrB; (D) Interactions between compound **4.22** and AcrB; (E) Interactions between compound **4.26** and AcrB; (F) Interactions between compound **4.37** and AcrB; (G) Interactions between compound **4.38** and AcrB; (H) Interactions between compound **4.40** and AcrB; (I) Interactions between compound **4.53** and AcrB; (J) Interactions between compound **4.56** and AcrB; (K) Interactions between compound **4.59** and AcrB; (L) Interactions between compound **4.66** and AcrB; (M) Interactions between compound **4.67** and AcrB; (N) Interactions between compound **4.68** and AcrB (cont.).

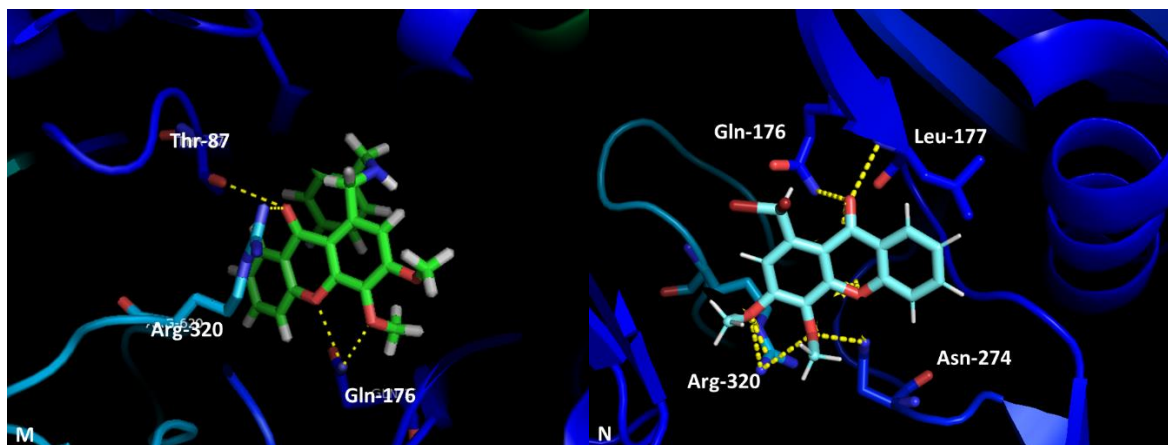


Figure 4.11 Molecular visualization of xanthenes in the substrate binding site of AcrB. (A) Interactions between compound **4.15** and AcrB; (B) Interactions between compound **4.17** and AcrB; (C) Interactions between compound **4.20** and AcrB; (D) Interactions between compound **4.22** and AcrB; (E) Interactions between compound **4.26** and AcrB; (F) Interactions between compound **4.37** and AcrB; (G) Interactions between compound **4.38** and AcrB; (H) Interactions between compound **4.40** and AcrB; (I) Interactions between compound **4.53** and AcrB; (J) Interactions between compound **4.56** and AcrB; (K) Interactions between compound **4.59** and AcrB; (L) Interactions between compound **4.66** and AcrB; (M) Interactions between compound **4.67** and AcrB; (N) Interactions between compound **4.68** and AcrB (cont.).

4.2.1.4 Inhibition of biofilm formation

The capacity of compounds **4.15**, **4.17**, **4.20**, **4.22**, **4.26**, **4.27**, **4.37** – **4.40**, **4.53**, **4.56**, **4.59**, **4.66**, **4.67**, **4.68**, **4.71**, and **4.73** to inhibit the formation of biofilm by *S. aureus* ATCC 25923 and *S. aureus* 272123 was evaluated. Even though compounds **4.15**, **4.17**, **4.20**, **4.22**, **4.26**, **4.53**, **4.56**, **4.59**, **4.67**, and **4.68** only inhibited EPs in the Gram-negative strain tested, they were also included in this assay, as the inhibition of biofilm could happen through a mechanism independent from EPs. Reserpine was used at 25 μ M as a positive control, and the compounds were tested at 100 μ M, or at half their MIC for these strains. This means that compounds **4.31**, **4.54**, **4.55**, and **4.57** were tested at 50 μ M for *S. aureus* 272123, and compounds **4.54**, **4.55**, and **4.56** were tested at 11 μ M, 7 μ M, and 40 μ M, respectively, for *S. aureus* ATCC 25923. The results are expressed in % and can be seen on **Table 4.4**.

Table 4.4 Inhibition of biofilm formation by xanthone derivatives.

Compound	Inhibition of biofilm formation (%) $\pm$ SD	
	<i>S. aureus</i> ATCC 25923	<i>S. aureus</i> 272123
4.15	0	40.0 $\pm$ 7.66
4.17	0	94.2 $\pm$ 1.31
4.20	0	46.2 $\pm$ 1.19
4.22	0	21.0 $\pm$ 1.47
4.26	0	7.85 $\pm$ 1.95

Compound	Inhibition of biofilm formation (%) $\pm$ SD	
	<i>S. aureus</i> ATCC 25923	<i>S. aureus</i> 272123
4.27	0	55.6 $\pm$ 3.26
4.37	0	13.1 $\pm$ 2.65
4.38	0.52 $\pm$ 0.56	72.2 $\pm$ 0.63
4.39	67.7 $\pm$ 0.56	66.5 $\pm$ 3.64
4.40	0	8.16 $\pm$ 0.93
4.53	5.71 $\pm$ 0.03	0
4.56	27.4 $\pm$ 0.04	91.0 $\pm$ 0.02
4.59	56.1 $\pm$ 0.05	93.6 $\pm$ 0.03
4.66	90.8 $\pm$ 1.62	77.2 $\pm$ 4.45
4.67	97.5 $\pm$ 0.62	65.0 $\pm$ 9.86
4.68	0	6.27 $\pm$ 1.41
4.71	0	59.0 $\pm$ 12.0
4.73	4.61 $\pm$ 0.01	1.88 $\pm$ 0.01
Reserpine	22.3 $\pm$ 5.10	63.1 $\pm$ 2.33

SD: Standard deviation.

The results show that the compounds are more effective towards the inhibition of biofilm in the resistant *S. aureus* 272123 strain. The most effective compounds were **4.17**, **4.38**, **4.56**, **4.59**, and **4.66**, which could inhibit the formation of biofilm above 70%. Among these, only compound **4.38** was able to inhibit the efflux of EtBr in this model. The structurally related **4.66** and **4.67** were able to decrease biofilm formation by more than 90% in the reference strain. Compound **4.39** displayed the same reduction in biofilm formation in both tested strains, which could mean it affects the same mechanism in both strains.

The results obtained show that most compounds inhibit the formation of biofilm through a mechanism different than the inhibition of EPs, at least in *S. aureus* 272123, since most of the compounds that inhibited biofilm formation for this strain did not show potential as EPIs in the previous assay.

4.2.1.5 Inhibition of quorum-sensing

The inhibition of QS triggered by xanthone derivatives **4.15**, **4.17**, **4.20**, **4.22**, **4.26**, **4.27**, **4.37** – **4.40**, **4.53**, **4.56**, **4.59**, **4.66**, **4.67**, **4.68**, **4.71**, and **4.73**, was evaluated. The models used were wt85, *S. marcescens* AS-1, and the pair EZF and CV026, and the results were the measure, in mm, of colonies that are devoid of pigment. PMZ was used as a positive control.

Although compounds **4.27**, **4.37** – **4.40**, **4.66**, **4.71**, and **4.73** only showed potential at inhibiting Gram-positive efflux, they were included in this assay for the same reasons

mentioned in the inhibition of biofilm formation assay. The results regarding the QS inhibition are in **Table 4.5**.

Table 4.5 Quorum-sensing inhibition by xanthone derivatives.

Compound	Quorum-sensing inhibition (mm) $\pm$ SD		
	<i>S. marcescens</i> AS-1	wt85	EZF + CV026
4.15	0	0	0
4.17	0	0	0
4.20	0	0	0
4.22	0	0	0
4.26	0	0	47 $\pm$ 0.1
4.27	0	0	31 $\pm$ 0.8
4.37	0	0	21 $\pm$ 0.5
4.38	0	0	53 $\pm$ 0.1
4.39	0	0	0
4.40	0	0	32 $\pm$ 0.1
4.53	0	0	59 $\pm$ 0.5
4.56	0	0	61 $\pm$ 0.8
4.59	0	0	29 $\pm$ 0.5
4.66	0	0	0
4.67	0	0	40 $\pm$ 0.1
4.68	0	0	30 $\pm$ 0.5
4.71	0	0	42 $\pm$ 0.8
4.73	0	0	38 $\pm$ 0.5
PMZ	18 $\pm$ 0.8	40 $\pm$ 0.1	41 $\pm$ 0.5

wt85: *C. violaceum* wild-type 85; **EZF**: *S. paucimobilis* Ezf 10-17; **CV026**: *C. violaceum* 026; **SD**: Standard deviation; **PMZ**: Promethazine.

An analysis of **Table 4.5** shows that the derivatives tested herein were only effective at inhibiting QS in the model that used EZF and CV026, with compounds **4.26**, **4.27**, **4.37**, **4.38**, **4.40**, **4.53**, **4.56**, **4.59**, **4.68**, **4.71**, and **4.73** showing inhibition of pigment production by the bacterial colonies. Among these, **4.26**, **4.37**, **4.38**, **4.40**, **4.53**, **4.56**, **4.59**, and **4.68** were also capable of inhibiting the efflux of EtBr in the Gram-negative model used, which could be related to these results. Interestingly, the enantiomeric pair **4.26** and **4.27** displayed inhibition of QS, showing that in this instance there is no enantioselectivity.

4.2.1.6 Antifungal activity

The xanthone derivatives **4.19** – **4.59** were assayed for their antifungal activity. Compounds **4.14** – **4.18** and **4.60** – **4.74** had previously been screened for antifungal activity, and compounds **4.63**, **4.66**, and **4.74** showed antifungal activity against the

dermatophyte *T. rubrum* FF5, with MICs of 16 μ M, 32 μ M and 128 μ M, respectively (46, 155).

The new derivatives were tested against the yeast *C. albicans* ATCC 10231, the filamentous fungus *A. fumigatus* ATCC 204305, and the dermatophyte *T. rubrum* FF5. The results obtained showed that only compound **4.45** displayed weak antifungal activity, with a MIC of 256 μ M for *T. rubrum* FF5. This compound has also shown antibacterial activity against *E. coli* ATCC 25922, emphasizing the potential of this compound as an anti-infective agent.

4.2.1.7 Cytotoxicity

The safety of the compounds was evaluated through the performance of a cytotoxicity assay, using mouse embryonic fibroblasts (NIH/3T3) as cell line. Herein, only the compounds that displayed promising activity in both EP inhibition assays and/or were able to decrease biofilm formation and inhibit QS were tested (**4.37**, **4.38**, **4.40**, **4.56**, **4.59**, **4.66**, and **4.67**). The IC₅₀ results are present in **Table 4.6**. Doxorubicin was used as positive control.

Table 4.6 Cytotoxicity of the selected compounds in NIH/3T3 cell line.

Compound	IC ₅₀ (μ M)
4.37	82.0 $\pm$ 0.27
4.38	>100
4.40	>100
4.56	18.5 $\pm$ 0.01
4.59	64.22 $\pm$ 1.09
4.66	35.1 $\pm$ 4.86
4.67	>100
Doxorubicin	12.1 $\pm$ 0.81

IC₅₀: half maximal inhibitory effect.

Compounds **4.38**, **4.40**, and **4.67** did not show observable cytotoxicity for the cell line tested, which suggests they could be used as EPI, and inhibitors of biofilm formation and QS, in a safe manner. The same is true for compounds **4.37** and **4.59**, which, even though they display cytotoxicity, their IC₅₀ is above the concentration used for them to display EPI activity and QS inhibition. The inhibition of biofilm could, however, be compromised, as the assay was performed with a concentration of 100 μ M. Lastly, compounds **4.56** and **4.66** displayed low IC₅₀ values, which compromise their safe use against the activities described herein.

To summarize, xanthenes appear to be promising regarding the circumvention of resistance mechanisms. From the results obtained, it can be noted that xanthenes with

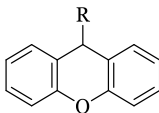
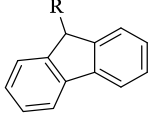
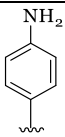
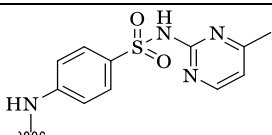
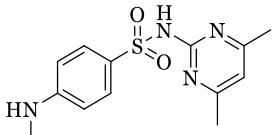
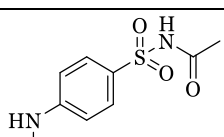
bulky substituents are the ones with the most potential, as well as a few compounds with more than one substituent. Compounds **4.37** and **4.67** were particularly interesting in this scope. Compound **4.37**, carrying a piperidine moiety which had previously shown potential in thioxanthenes, showed EP inhibition in both strains tested, as well as QS inhibition, which could be related. Compound **4.67** inhibited EPs in the Gram-negative strain tested, as well as the related resistance mechanisms studied, biofilm formation and QS. As such, these compounds could be regarded as hits in this scenario.

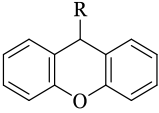
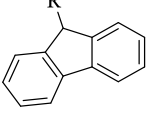
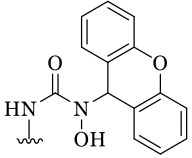
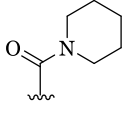
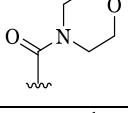
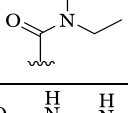
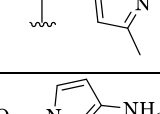
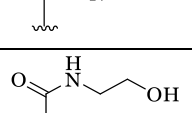
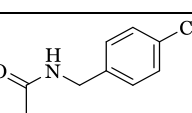
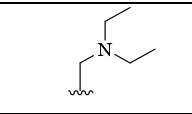
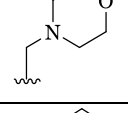
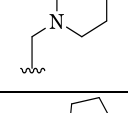
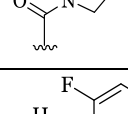
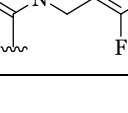
Among the xanthenes tested, 17 showed potential as EPIs. Even though this is higher than the number of thioxanthenes that displayed the same effect, it must be considered that the library of xanthenes had significantly more compounds. Nevertheless, a phenotypic screening appears to be a more effective approach, as only two thioxanthenes obtained by structure-based design showed EPI activity.

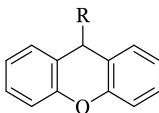
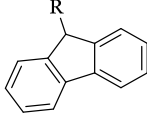
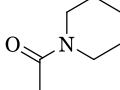
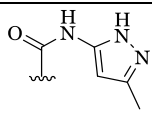
4.2.2 Xanthenes

A library of seventeen C9-substituted xanthenes and two structurally related C9-substituted fluorenes was used for the assays previously described. The structures of compounds **4.75** – **4.93** can be seen in **Table 4.7**.

Table 4.7 Structures of the library of xanthenes tested.

 4.75 - 4.91  4.92 - 4.93	
Compound	R
4.75	
4.76	
4.77	
4.78	

 4.75 - 4.91  4.92 - 4.93	
Compound	R
4.79	
4.80	
4.81	
4.82	
4.83	
4.84	
4.85	
4.86	
4.87	
4.88	
4.89	
4.90	
4.91	

	
4.75 - 4.91	4.92 - 4.93
Compound	R
4.92	
4.93	

4.2.2.1 Docking studies

The xanthene and fluorene derivatives **4.75 – 4.93** underwent docking studies similarly to what has been described previously. The docking scores are in **Table 4.8**.

Table 4.8 Docking scores for the xanthene derivatives in bacterial efflux pumps.

Compound	Docking score (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
4.75	-7.1	-6.2	-6.8	-6.5	-6.9	-7.6	-6.6
4.76	-9.5	-4.6	-8.3	-7.7	-9.0	-8.2	-6.9
4.77	-9.5	-0.8	-8.1	-7.7	-9.2	-7.9	-6.5
4.78	-8.2	3.2	-7.0	-6.8	-8.7	-6.5	-6.7
4.79	-9.6	2.2	-7.1	-6.9	-8.4	3.6	-7.1
4.80	-7.3	-5.1	-6.3	-6.3	-7.2	-6.4	-6.1
4.81	-7.3	-5.2	-6.1	-6.3	-6.8	-6.2	-6.1
4.82	-6.6	-5.0	-5.7	-5.7	-6.3	-5.3	-5.4
4.83	-8.3	-6.0	-7.3	-6.3	-7.9	-7.4	-7.1
4.84	-7.9	-6.1	-6.5	-7.1	-7.7	-6.6	-6.3
4.85	-6.6	-5.4	-6.3	-6.0	-6.7	-5.9	-6.3
4.86	-7.7	1.2	-7.0	-6.8	-8.0	-7.3	-6.7
4.87	-5.6	-4.7	-5.3	-5.6	-6.0	-6.7	-5.1
4.88	-6.9	-5.4	-6.0	-6.0	-6.8	-5.5	-5.8
4.89	-6.7	-5.6	-5.8	-5.7	-6.9	-6.6	-6.0
4.90	-7.3	-5.7	-6.3	-6.4	-6.8	-5.9	-5.9
4.91	-8.1	-5.8	-6.7	-6.7	-8.6	-7.5	-7.0
4.92	-6.8	-6.0	-6.3	-6.0	-7.4	-6.5	-6.6
4.93	-8.1	-5.9	-7.3	-7.2	-8.0	-8.7	-6.4
PAβN	-7.1	-4.7	-5.8	-4.9	-7.1	-	-
Reserpine	-	-	-	-	-	1.0	-4.6

SBS: Substrate binding site; **HT:** Hydrophobic trap; **HH:** Helical hairpin; **LD:** Lipoyl domain; **BCR:** Binding core region; **CS:** Cytoplasmic side; **PAβN:** Phenyl-arginine β-naphthylamide.

The xanthene derivatives, similar to the structurally related tricyclic xanthenes and thioxanthenes, are predicted to present more affinity towards the substrate binding site of the AcrB portion, while presenting the least favorable scores for the hydrophobic trap. Generally, the compounds were predicted to have more affinity towards the AcrAB-TolC efflux system than PA β N.

Concerning NorA, the derivatives seemed to have more affinity than reserpine to both sites. The majority of the compounds are predicted to have more affinity towards the binding core region.

In general, there was no noticeable difference between the docking scores of xanthene (**4.75** – **4.91**) and fluorene derivatives (**4.92** and **4.93**). However, it could be noticed that the compounds with the best docking scores for the substrate binding site of AcrB and TolC are that with the bulkier substituents (**4.76** – **4.79**). Since all the compounds presented favorable results, they were tested for the relevant biological activities.

4.2.2.2 Antibacterial activity and potentiation of antibiotics

Derivatives **4.75** – **4.93** were tested for their antimicrobial activity against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. enterica* serovar Typhimurium SL1344, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, and *S. aureus* 272123.

No compounds presented antimicrobial activity for these strains. Derivatives **4.76** – **4.78** present a sulfonamide moiety, which is a known bacteriostatic agent. However, SAR studies have demonstrated that a free aromatic amine in the *para* position of the sulfonamide is essential for the activity of sulfonamides. In this case, this amine is linked to a xanthenyl moiety, which can be responsible for the absence of antibacterial activity (158, 159).

Concerning the potentiation of antibacterial drugs, neither the xanthene nor the fluorene derivatives demonstrated potential at potentiating CTX against *E. coli* SA/2 or VAN against *E. faecalis* B3/101. However, none of these strains are known to overexpress EPs, and since the compounds displayed docking scores similar or better than the positive controls, they were tested for their potential as bacterial EPs.

4.2.2.3 Bacterial efflux pump inhibition assay

Xanthene derivatives **4.75** – **4.93** were assayed for their potential to act as EPs in *S. aureus* 272123 and *S. Typhimurium* SL1344, using reserpine and CCCP, respectively, at sub-inhibitory concentrations (25 μ M). The compounds were all tested at the concentration of 50 μ M, as none of them displayed antibacterial activity against these strains. The results are present in **Table 4.9**.

Table 4.9 RFI of the tested xanthene derivatives.

Compound	RFI $\pm$ SD	
	<i>S. aureus</i> 272123	<i>S. Typhimurium</i> SL1344
4.75	0.14 $\pm$ 0.05	1.66 $\pm$ 0.01
4.76	0.63 $\pm$ 0.09	2.75 $\pm$ 0.11
4.77	0.13 $\pm$ 0.04	2.18 $\pm$ 0.04
4.78	0.14 $\pm$ 0.10	2.94 $\pm$ 0.23
4.79	-0.25 $\pm$ 0.05	0.06 $\pm$ 0.21
4.80	0.27 $\pm$ 0.01	0.35 $\pm$ 0.06
4.81	0.04 $\pm$ 0.01	0.25 $\pm$ 0.07
4.82	0.07 $\pm$ 0.04	0.44 $\pm$ 0.01
4.83	0.41 $\pm$ 0.01	0.22 $\pm$ 0.05
4.84	0.29 $\pm$ 0.04	0.05 $\pm$ 0.04
4.85	0.01 $\pm$ 0.02	0.11 $\pm$ 0.10
4.86	-0.08 $\pm$ 0.08	0.14 $\pm$ 0.05
4.87	0.88 $\pm$ 0.01	0.39 $\pm$ 0.03
4.88	0.16 $\pm$ 0.02	0.25 $\pm$ 0.03
4.89	0.37 $\pm$ 0.06	0.16 $\pm$ 0.01
4.90	0.25 $\pm$ 0.04	0.22 $\pm$ 0.04
4.91	0.23 $\pm$ 0.04	0.75 $\pm$ 0.06
4.92	0.32 $\pm$ 0.04	0.27 $\pm$ 0.06
4.93	0.18 $\pm$ 0.01	0.14 $\pm$ 0.05
Reserpine	0.56 $\pm$ 0.04	-
CCCP	-	0.34 $\pm$ 0.04

RFI: Relative fluorescence index; **SD:** Standard deviation; **CCCP:** carbonyl cyanide 3-chlorophenylhydrazone.

Six xanthene derivatives, **4.75**, **4.77**, **4.78**, **4.80**, **4.82**, and **4.91**, were able to decrease the efflux of EtBr in a higher extent than CCCP, in *S. Typhimurium* SL1344. Furthermore, compounds **4.76** and **4.87** were able to inhibit EPs in both strains tested, which led to studies into their fluorescence. This was also inspected in compounds **4.80** and **4.91**, since these compounds also displayed a somewhat high fluorescence, even though they were less effective than reserpine in *S. aureus* 272123. **Figure 4.12** shows the result of this study.

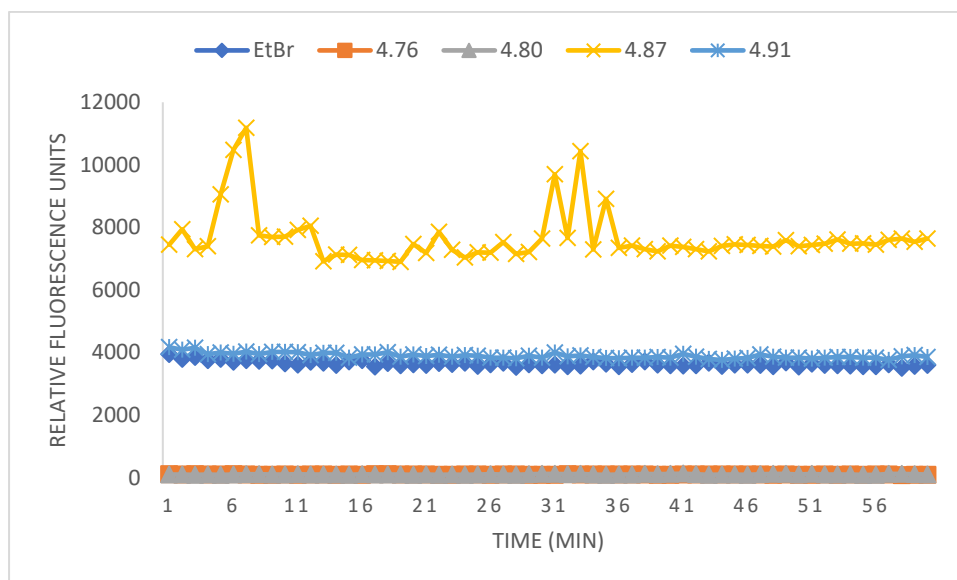


Figure 4.12 Fluorescence curves of EtBr and compounds **4.76**, **4.80**, **4.87**, and **4.91**.

Compounds **4.87** and **4.91** display an erratic and/or high fluorescence pattern, which means their results in this assay must be considered inconclusive. On the other hand, compounds **4.76** and **4.80** showed a low and steady fluorescence along the time of the assay, and they were considered inhibitors of EtBr efflux.

At a first glance, it can be noted that sulfonamide-substituted xanthenes yield effective EPIs, since derivatives **4.76** – **4.78** could all inhibit Gram-negative EPs, with **4.76** showing the ability to also inhibit Gram-positive EPs, while not displaying antibacterial activity. The moiety in compound **4.82** is also present in xanthenes **4.38** and **4.40**, which showed inhibition of efflux in *S. Typhimurium* SL1344. The use of the xanthene moiety allows substitutions in the C9 position, leading to different substitution patterns in the tricyclic scaffold that could not be explored with (thio)xanthenes.

Compound **4.76** was visualized in the binding core region of NorA, while compounds **4.75** – **4.78**, **4.80**, and **4.82** were visualized in the substrate binding site of AcrB.

The analysis of **4.76** in the binding core region of NorA shows that both oxygens in the sulfonamide moiety and the nitrogen atoms in the pyrimidine moiety can interact with Glu-222 and Ala-289 (**Figure 4.13**). The residue Glu-222 has also shown important in the interaction with other tricyclic compounds.

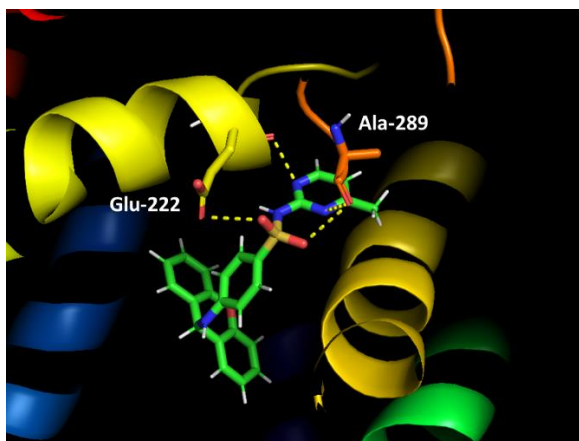


Figure 4.13 Interactions between compound **4.76** and the binding core region of NorA.

The interactions between AcrB and the xanthenes **4.75** – **4.78**, **4.80**, and **4.82** were inspected. Compound **4.75** appears to interact with Leu-177 and Gly-179, residues that had previously been described as residues with which xanthenes interact (**Figure 4.14, A**).

Compounds **4.76** (**Figure 4.14, B**) and **4.77** (**Figure 4.14, C**) have similar interactions, being able to establish hydrogen interactions with Gln-176, Ser-180, Gln-181, Arg-620, and Asn-274. Interestingly, in this case only the residues Gln-176 and Asn-274 have previously been associated with the interaction between tricyclic compounds and AcrB. The structurally related **4.78** shows interactions with Ser-48, Glu-273, Arg-620, and Tyr-772, with Arg-620 being previously associated with the binding site of xanthenes within this target (**Figure 4.14, D**).

Compound **4.80** appears to interact with Ser-48, Asn-274, and Arg-620 (**Figure 4.14, E**), and compound **4.82** is predicted to interact with Gln-176, Asn-274, and Arg-620 (**Figure 4.14, F**), residues previously described for both xanthenes and (thio)xanthenes.

The compounds that were able to inhibit EPs in the models studied were then assayed for their potential at inhibiting other resistance and virulence mechanisms, such as the formation of biofilm and the inhibition of QS, respectively.

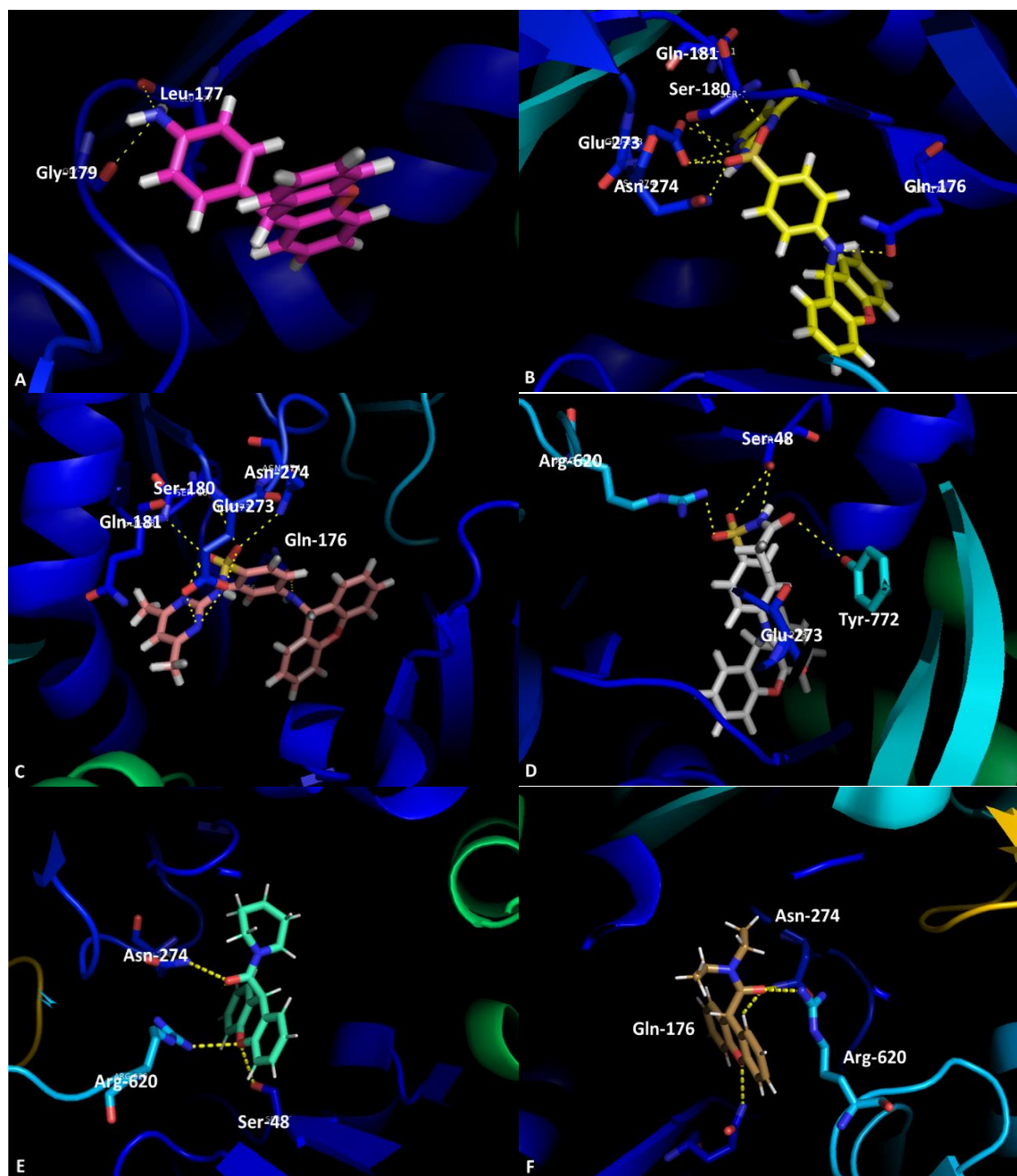


Figure 4.14 Molecular visualization of xanthenes in the substrate binding site of AcrB. (A) Interactions between compound **4.75** and AcrB; (B) Interactions between compound **4.76** and AcrB; (C) Interactions between compound **4.77** and AcrB; (D) Interactions between compound **4.78** and AcrB; (E) Interactions between compound **4.80** and AcrB; (F) Interactions between compound **4.82** and AcrB.

4.2.2.4 Inhibition of biofilm formation

The xanthene derivatives **4.75** – **4.78**, **4.80**, and **4.82** were evaluated for their capability of inhibiting the formation of biofilm in *S. aureus* ATCC 25923 and *S. aureus* 272123. Since none of the compounds showed antibacterial activity for these strains, they were tested at 100 μ M. The results are in **Table 4.10**.

Table 4.10 Inhibition of biofilm formation by xanthene derivatives.

Compound	Inhibition of biofilm formation (%) $\pm$ SD	
	<i>S. aureus</i> ATCC 25923	<i>S. aureus</i> 272123
4.75	1.80 $\pm$ 2.42	16.1 $\pm$ 0.72
4.76	4.49 $\pm$ 0.27	18.2 $\pm$ 0.27
4.77	1.91 $\pm$ 1.74	78.3 $\pm$ 9.27
4.78	0.06 $\pm$ 0.99	79.2 $\pm$ 1.12
4.80	7.77 $\pm$ 0.77	75.4 $\pm$ 2.03
4.82	0	86.8 $\pm$ 0.66
Reserpine	22.3 $\pm$ 4.21	77.6 $\pm$ 1.44

SD: Standard deviation.

Compounds **4.77**, **4.78**, **4.80**, and **4.82** showed biofilm formation inhibition above 75%, similar or above reserpine, used herein as a positive control at 25 μ M. It is interesting to notice that the only compound able to inhibit Gram-positive efflux pumps, **4.76**, did not show efficacy in the inhibition of biofilm formation. This could lead to the hypothesis that the EPs being inhibited by **4.76** are not important for the efflux of extracellular polymeric substances, used in the production of biofilm.

4.2.2.5 Inhibition of quorum-sensing

The same set of compounds that was evaluated for the inhibition of biofilm formation was investigated for their potential in inhibiting quorum-sensing. The models used were wt85, *S. marcescens* AS-1, and the pair EZF and CVO26, and the results were the measure, in mm, of colonies that are devoid of pigment. PMZ was used as a positive control (inhibition of 41 $\pm$ 0.5 mm). The results obtained showed that only two compounds were effective in this assay, **4.76** and **4.77**, which showed an inhibition of pigment production in EZF + CVO26 of 48 $\pm$ 0.1 mm and 41 $\pm$ 0.8 mm, respectively.

4.2.2.6 Antifungal activity

The xanthene derivatives **4.75** – **4.93** were tested against the yeast *C. albicans* ATCC 10231, the filamentous fungus *A. fumigatus* ATCC 204305, and the dermatophyte *T. rubrum* FF5. Xanthenes **4.77** and **4.81** both showed a MIC of 128 μ g/mL against *T. rubrum* FF5.

The xanthene derivatives mentioned herein were not evaluated for their cytotoxicity in this instance.

In comparison to the previous series of xanthenes and thioxanthenes, what is more notable is the fact that xanthenes are not as effective at inhibiting QS, which may lead to the conclusion that the lack of a carbonyl at C9 results in a reduction of effectiveness regarding

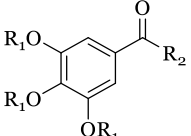
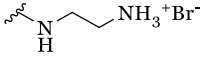
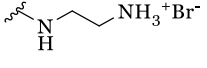
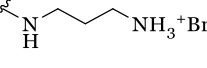
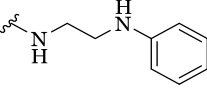
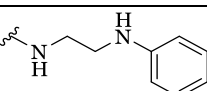
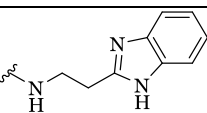
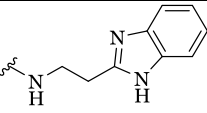
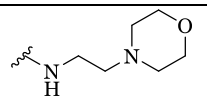
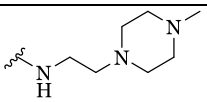
this mechanism. Considering the results obtained in the EP inhibition, it cannot be inferred that C9-substitution has a positive effect.

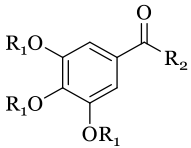
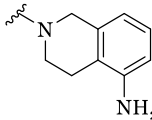
4.2.3 Gallic acid derivatives

A library of ten gallic acid derivatives was synthesized and tested for their potential as antimicrobial agents, and at reversing AMR. Trimethoxybenzoic acid, a derivative of gallic acid, occurs in the ester form in the EPI reserpine (**4.7**, **Figure 4.4**). As such, in an attempt to simplify the complex structure of this alkaloid, the simple scaffold of gallic and trimethoxybenzoic acids was combined with an amine, as this is an important motif for target interaction.

The compounds obtained and tested, **4.94** – **4.102**, can be seen in **Table 4.11**.

Table 4.11 Structures of the library of gallamides tested.

 94 - 103		
Compound	R ₁	R ₂
4.94	H	
4.95	CH ₃	
4.96	H	
4.97	H	
4.98	CH ₃	
4.99	H	
4.100	CH ₃	
4.101	CH ₃	
4.102	CH ₃	

 94 - 103		
Compound	R ₁	R ₂
4.103	CH ₃	

4.2.3.1 Docking studies

The ten gallic amides were subject to docking studies in the portions of the AcrAB-TolC efflux system and in the NorA pump. The docking scores regarding derivatives **4.94 – 4.102** are in **Table 4.12**.

Table 4.12 Docking scores for the gallic acid derivatives in bacterial efflux pumps.

Compound	Docking score (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
4.94	-5.8	-4.8	-5.0	-6.0	-5.7	-6.0	-5.7
4.95	-5.5	-4.5	-4.7	-5.3	-5.7	-4.8	-4.6
4.96	-6.0	-4.9	-5.0	-5.8	-5.9	-6.1	-6.0
4.97	-7.5	-6.3	-5.8	-6.7	-6.5	-8.2	-7.8
4.98	-7.0	-5.5	-5.7	-4.7	-6.4	-7.8	-6.4
4.99	-7.6	-7.1	-6.5	-7.1	-7.3	-8.4	-7.0
4.100	-7.1	-6.4	-6.4	-5.4	-6.7	-7.5	-5.9
4.101	-6.6	-4.6	-5.0	-5.0	-5.9	-6.3	-6.3
4.102	-6.4	-3.2	-5.0	-5.6	-5.9	-6.2	-4.4
4.103	-7.1	-4.9	-6.1	-5.5	-6.4	-5.6	-5.3
PAβN	-7.1	-4.7	-5.8	-4.9	-7.1	-	-
Reserpine	-	-	-	-	-	1.0	-4.6

SBS: Substrate binding site; **HT:** Hydrophobic trap; **HH:** Helical hairpin; **LD:** Lipoyl domain; **BCR:** Binding core region; **CS:** Cytoplasmic side; **PAβN:** Phenyl-arginine β-naphthylamide.

Firstly, it can be noticed that compounds **4.94 – 4.96** were predicted to be the least effective against the substrate binding site of AcrB. These compounds share a structural feature, which is the presence of an ammonium bromide moiety, which could imply that compounds with an ionic moiety may not be as effective in this case. The AcrA portion is predicted to be the portion with the general lowest docking scores.

When it comes to the NorA pump, all the compounds were predicted to have more affinity towards the binding core region.

Given that the compounds present docking scores approximate to those of the positive control of AcrAB-TolC, and higher than the positive control for NorA, they were all tested for biological activity.

4.2.3.2 Antibacterial activity and potentiation of antibiotics

Compounds **4.94** – **4.103** were tested against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. enterica* serovar Typhimurium SL1344, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, and *S. aureus* 272123.

Compound **4.99** showed antibacterial activity against *S. aureus* 272123, with a MIC of 100 μ M, and *S. Typhimurium* SL1344, with a MIC of 50 μ M. The MIC for the other compounds was higher than 100 μ M, for all the strains used.

When it comes to the potentiation of antibiotics, compound **4.94** was able to decrease the MIC of CTX against *E. coli* SA/2 by 2-fold. Considering *E. faecalis* B3/101, compounds **4.102** and **4.103** could decrease the MIC of VAN by 2-fold.

4.2.3.3 Bacterial efflux pump inhibition assay

The potential of compounds **4.94** – **4.103** to act as bacterial EPIs was evaluated in *S. aureus* 272123 and *S. Typhimurium* SL1344. The concentration used was 50 μ M for all the compounds except compound **4.99**, as it showed antibacterial activity towards both strains used for this assay. As such, it was used at 17 μ M for *S. aureus* 272123 and at 33 μ M for *S. Typhimurium* SL1344, one-third of its MIC for each strain. As positive controls, reserpine and CCCP were used, for the Gram-positive and Gram-negative strain, respectively, at the concentration of 25 μ M. The results are present in **Table 4.13**.

Table 4.13 RFI of the tested gallic acid derivatives.

Compound	RFI $\pm$ SD	
	<i>S. aureus</i> 272123	<i>S. Typhimurium</i> SL1344
4.94	-0.02 $\pm$ 0.01	-0.07 $\pm$ 0.03
4.95	-0.21 $\pm$ 0.03	-0.36 $\pm$ 0.01
4.96	-0.07 $\pm$ 0.03	-0.07 $\pm$ 0.05
4.97	-0.35 $\pm$ 0.01	-0.41 $\pm$ 0.01
4.98	0.08 $\pm$ 0.07	0.22 $\pm$ 0.02
4.99	-0.21 $\pm$ 0.03	-0.36 $\pm$ 0.01
4.100	0.30 $\pm$ 0.05	0.22 $\pm$ 0.11
4.101	0.17 $\pm$ 0.03	-0.01 $\pm$ 0.01
4.102	0.14 $\pm$ 0.02	0.06 $\pm$ 0.01
4.103	0.14 $\pm$ 0.02	0.05 $\pm$ 0.02
Reserpine	0.30 $\pm$ 0.12	-
CCCP	-	0.16 $\pm$ 0.06

RFI: Relative fluorescence index; SD: Standard deviation; CCCP: carbonyl cyanide 3-chlorophenylhydrazone.

From the results obtained, it could be noted that only compound **4.100** inhibits the efflux of EtBr in *S. aureus* 272123, which could also inhibit the efflux in *S. Typhimurium* SL1344. Additionally, compound **4.98** can also act as an efflux inhibitor in the *Salmonella* strain used. The rest of the compounds presented mostly negative RFI values, which means the fluorescence is lower than that of the negative control. Since compound **4.100** displayed inhibition for both strains, its autofluorescence was evaluated, and can be seen in **Figure 4.15**.

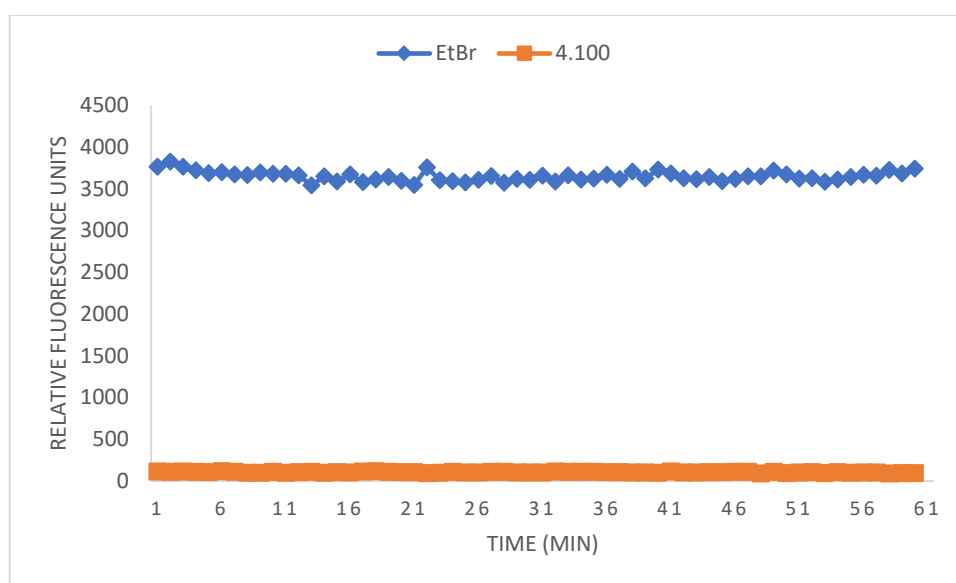


Figure 4.15 Fluorescence curves of EtBr and compound **4.100**.

The fluorescence appears to not be an issue, as it remains steady throughout the duration of the assay. As such, the results obtained for this compound were considered as positive results. An analysis of the common structural features between compounds **4.98** and **4.100** may lead to the conclusion that an aromatic amine linked to a trimethoxybenzamide moiety by a two-carbon chain could be the ideal substitution in order for these compounds to be bacterial EPIs and might be a guide in the synthesis of compounds with this aim.

The effective compounds **4.98** and **4.100** were visualized in the substrate binding site of AcrB, and compound **4.100** was visualized in the binding core region of the NorA homology model.

Considering compound **4.100** and the NorA homology model, two interactions seem to occur. One of them is between the carbonyl and Asp-352, and the other is a T-shaped π - π interaction between Phe-221 and the trimethoxybenzoic moiety. These interactions are present in **Figure 4.16**.

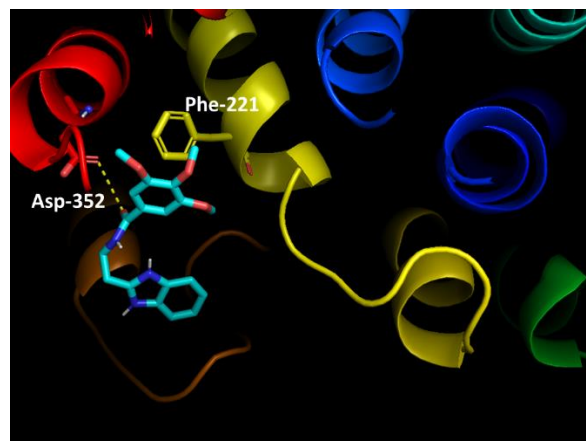


Figure 4.16 Interactions between compound **4.100** and NorA.

Regarding the AcrB portion, compounds **4.98** and **4.100** are predicted to bind in approximately the same site. While for **4.98**, the only interaction detected was with Phe-617 (**Figure 4.17, A**), for compound **4.100** an additional hydrogen interaction between the tertiary amine and Ser-134 was also found (**Figure 4.17, B**).

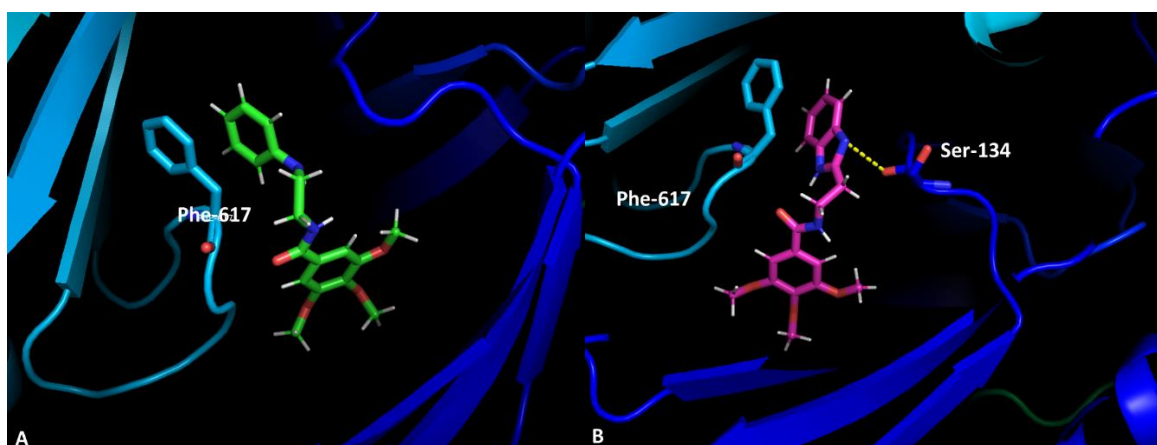


Figure 4.17 Molecular visualization of gallic amides in the substrate binding site of AcrB. (A) Interactions between compound **4.98** and AcrB; (B) Interactions between compound **4.100** and AcrB.

Since compounds **4.98** and **4.100** displayed EPI activity, they were assayed for their potential as inhibitors of biofilm formation and QS.

4.2.3.4 Inhibition of biofilm formation

As compounds **4.98** and **4.100** proved to be effective at inhibiting the efflux of EtBr, they were tested for their potential at inhibiting the formation of biofilm, a process intrinsically related to EPs, in *S. aureus* ATCC 25923 and *S. aureus* 272123. Neither compound showed antibacterial activity for these strains, hence they were tested at 100 μ M.

It was shown that compound **4.98** did not show any modification in the production of biofilm compared to the control, in both strains tested. Compound **4.100** showed a very low percentage of biofilm formation inhibition in *S. aureus* 272123, $21.3 \pm 4.91\%$, compared with $72.1 \pm 4.24\%$ inhibition displayed by reserpine at 25 μM . This compound did not display any inhibition in *S. aureus* ATCC 25923.

4.2.3.5 Inhibition of quorum-sensing

The two compounds, **4.98** and **4.100**, were tested regarding their ability to inhibit quorum-sensing in three models: wt85, *S. marcescens* AS-1, and the pair EZF and CV026. The antipsychotic and QS inhibitor PMZ was used as a positive control, and showed an inhibition of 41 ± 0.5 mm. Neither compound showed any sign of QS inhibition concerning these models.

4.2.3.6 Antifungal activity

The gallamides **4.94** – **4.103** were tested against the yeast *C. albicans* ATCC 10231, the filamentous fungus *A. fumigatus* ATCC 204305, and the dermatophyte *T. rubrum* FF5. No compounds showed activity to any of the fungal strains used in this assay.

None of the compounds were tested for their cytotoxic activity in the scope of this study.

The results obtained within this class are not as promising as the ones previously described for tricyclic compounds, especially xanthenes and thioxanthenes. Allied to the fact that the only two derivatives with activity comprise the bulkiest substituents, a rigid aromatic moiety, and a trimethoxylated scaffold (versus trihydroxylated), it can be concluded that the molecular size is an important factor in this matter.

4.2.4 Deoxycholic acid derivatives

Methyl deoxycholate (**4.104**) and one deoxycholic amide (**4.105**), synthesized from the bile acid deoxycholic acid, were studied for the aforementioned biological activities. The structures of derivatives **4.104** and **4.105** are present in **Figure 4.18**.

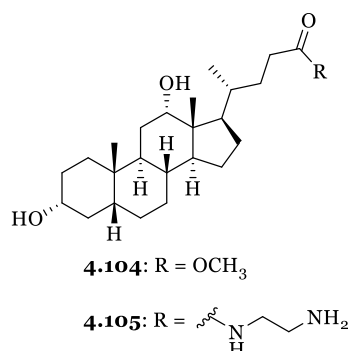


Figure 4.18 Structures of the deoxycholic acid derivatives tested.

4.2.4.1 Docking studies

The two derivatives were studied for their predicted affinity towards AcrAB-TolC and NorA, through docking studies. The docking scores of derivatives **4.104** and **4.105** can be seen in **Table 4.14**.

Table 4.14 Docking scores for the deoxycholic acid derivatives in bacterial efflux pumps.

Compound	Docking score (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
4.104	-7.6	5.6	-5.9	-5.6	-7.8	0.2	-5.8
4.105	-7.8	3.0	-5.9	-5.4	-7.4	-2.4	-5.5
PAβN	-7.1	-4.7	-5.8	-4.9	-7.1	-	-
Reserpine	-	-	-	-	-	1.0	-4.6

SBS: Substrate binding site; **HT**: Hydrophobic trap; **HH**: Helical hairpin; **LD**: Lipoyl domain; **BCR**: Binding core region; **CS**: Cytoplasmic side; **PAβN**: Phenyl-arginine β-naphthylamide.

Compounds **4.104** and **4.105** are predicted to have very unfavorable affinity towards the hydrophobic trap of AcrB. On the other hand, they showed very good affinity towards the substrate binding site of AcrB and the site studied in TolC. Both sites studied in AcrA showed that the predicted affinity was similar to that of PAβN, the positive control, but were still the lowest comparing to the other portions.

When it comes to NorA, these compounds showed more predicted affinity towards the cavity present in the cytoplasmic side than the binding core region.

Both compounds were studied for their antimicrobial potential, as well as for mechanisms related to AMR.

4.2.4.2 Antibacterial activity and potentiation of antibiotics

Compounds **4.104** and **4.105** were evaluated for their antibacterial potential in the six strains previously described: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. enterica* serovar Typhimurium SL1344, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, and *S. aureus* 272123. The ester derivative **4.104** only showed antibacterial activity towards *S. aureus* ATCC 25923, with a MIC of 25 μM.

The amide derivative **4.105**, on the other hand, showed antibacterial activity for all the strains tested. This prompted an evaluation of the MBC as well, in order to assess if the compound was also bactericidal, as these compounds possess a steroidal scaffold, which can enter cells through specific transporters, but can also interfere with the membrane fluidity, leading to cell death (83).

The results regarding the antibacterial activity of compound **4.105** are present in **Table 4.15**.

Table 4.15 Minimum inhibitory concentration of compound **4.105** against the tested bacteria.

MIC of compound 4.105 (μM)											
<i>E. coli</i> ATCC 25922		<i>P. aeruginosa</i> ATCC 27853		<i>E. faecalis</i> ATCC 29212		<i>S. aureus</i> ATCC 25923		<i>S. aureus</i> 272123		<i>S. Typhimurium</i> SL1344	
MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
73	147	147	147	37	73	37	37	12.5	ND	12.5	ND

MIC: Minimum inhibitory concentration; **MBC**: Minimum bactericidal concentration; **ND**: Not determined.

Compound **4.105** presents a broad spectrum of activity, with observable MIC for every strain tested. It also showed bactericidal activity for the reference strains of *E. coli*, *P. aeruginosa*, *E. faecalis*, and *S. aureus*.

The derivatives were also tested for their capacity of potentiating the activity of the antibiotics CTX and VAN in *E. coli* SA/2 and *E. faecalis* B3/101, respectively. In this case, the compounds showed no alteration in the MIC of the antibiotics.

4.2.4.3 Bacterial efflux pump inhibition assay

The deoxycholic acid derivatives **4.104** and **4.105** were evaluated for their capability of inhibiting the efflux of EtBr in *S. aureus* 272123 and *S. Typhimurium* SL1344, in comparison to reserpine and CCCP, respectively, at 25 μM . Compound **4.104** was used at 50 μM , while compound **4.105** was used at 4 μM for *S. aureus* 272123 and *S. Typhimurium* SL1344. The RFI values obtained can be seen in **Table 4.16**.

Table 4.16 RFI of the tested deoxycholic acid derivatives.

Compound	RFI $\pm$ SD	
	<i>S. aureus</i> 272123	<i>S. Typhimurium</i> SL1344
4.104	1.06 $\pm$ 0.12	0.32 $\pm$ 0.02
4.105	0.15 $\pm$ 0.04	1.07 $\pm$ 0.08
Reserpine	0.35 $\pm$ 0.04	-
CCCP	-	0.16 $\pm$ 0.08

RFI: Relative fluorescence index; **SD**: Standard deviation; **CCCP**: carbonyl cyanide 3-chlorophenylhydrazine.

As it can be observed, compound **4.104** is effective in both strains tested, whereas **4.105** is effective for *S. Typhimurium* SL1344, at a low concentration of 4 μM . As is customary in situations where a compound displays a favorable RFI for both strains, compound **4.104** was screened for its relative fluorescence alone, which can be seen in **Figure 4.19**.

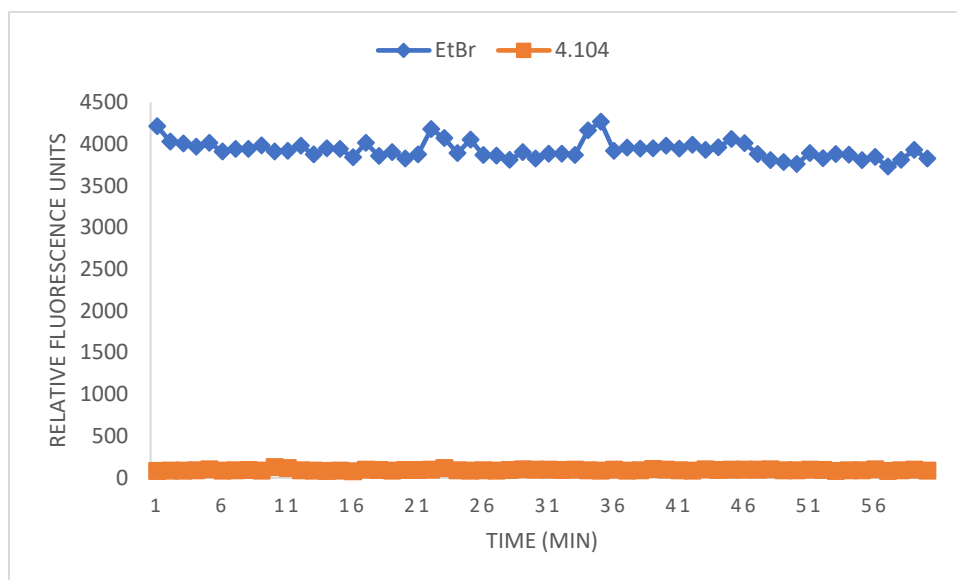


Figure 4.19 Fluorescence curves of EtBr and compound **4.104**.

The fluorescence of compound **4.104** remained constant and low over the duration of the study, which means it does not interfere with the results obtained. As such, deoxycholic acid derivatives appear to be promising as EPIs, particularly in Gram-negative bacteria.

Both compounds were visualized for the main pumps present in the strains they were effective towards. Both **4.104** and **4.105** were visualized in the substrate binding site of AcrB, and compound **4.104** was also visualized in the cavity present in the cytoplasmic side of The NorA pump, as this was the site predicted towards which **4.104** would have more affinity towards.

The visualization of **4.104** in the cavity on the cytoplasmic side of NorA shows that a nitrogen in Lys-31 and both oxygens in the ester moiety are able to establish hydrogen interactions. This interaction can be seen in **Figure 4.20**.

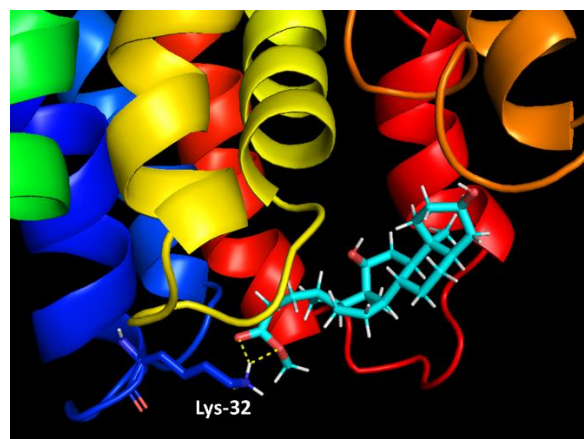


Figure 4.20 Interactions between compound **4.104** and NorA.

In the case of AcrB, compound **4.104** is predicted to establish interactions between the carbonyl and Ser-48 and Arg-620, which has also been described in the interaction with other scaffolds. The hydroxy groups in the steroid scaffold can also interact with Arg-620, as well as Gly-619. **Figure 4.21, A** shows this.

Although it is structurally related, compound **4.105** shows interactions with different residues than **4.104**. In fact, **4.105** is predicted to interact with Ser-46 and Gln-89 through the carbonyl, and the primary and secondary amines interact with Asp-174 and Gln-176, respectively. The hydroxy groups show interactions with Lys-163 and Leu-177, which are present in **Figure 4.21, B**.

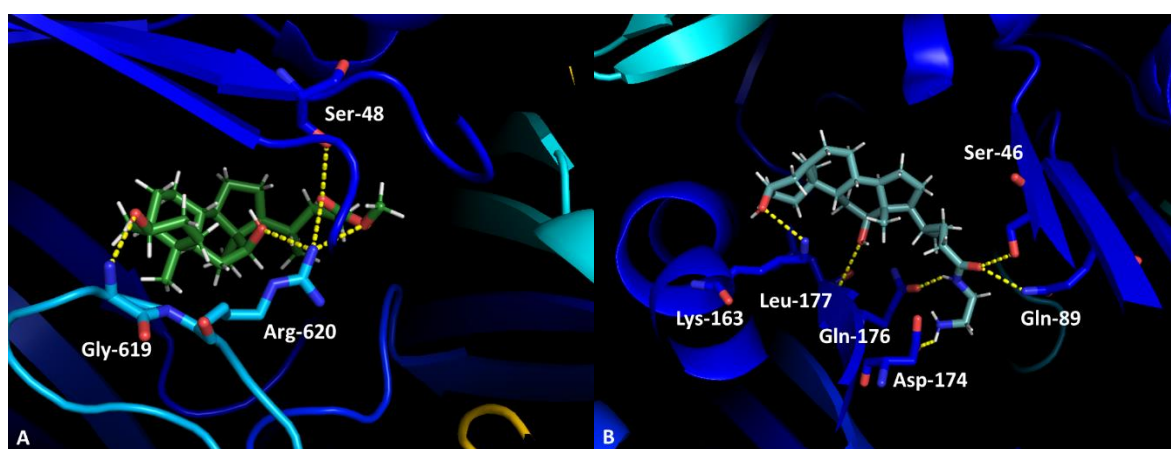


Figure 4.21 Molecular visualization of deoxycholic acid derivatives in the substrate binding site of AcrB. (A) Interactions between compound **4.104** and AcrB; (B) Interactions between compound **4.105** and AcrB.

4.2.4.4 Inhibition of biofilm formation

The deoxycholic acid derivatives **4.104** and **4.105** were evaluated for their ability to decrease the formation of biofilm in *S. aureus* ATCC 25923 and *S. aureus* 272123. Herein, the compounds were used at half their MIC, which means that compound **4.104** was used at 12.5 μ M against *S. aureus* ATCC 25923 and at 100 μ M in *S. aureus* 272123, while **4.105** was applied at 19 μ M for *S. aureus* ATCC 25923 and at 6.25 μ M for *S. aureus* 272123. Reserpine was used as a positive control at the concentration of 25 μ M.

This assay showed that compound **4.104** showed an inhibition of biofilm formation of $93.9 \pm 0.61\%$ for *S. aureus* 272123, compared to 72.1 ± 4.24 displayed by reserpine. This compound showed no activity against *S. aureus* ATCC 25923. The opposite was observed for compound **4.105**, with a reduction of $91.4 \pm 2.18\%$ in biofilm formation in *S. aureus* ATCC 25923, with reserpine showing a $22.3 \pm 4.21\%$ inhibition, and no effect in *S. aureus* 272123.

4.2.4.5 Inhibition of quorum-sensing

The two compounds **4.104** and **4.105** were tested for QS inhibition in wt85, *S. marcescens* AS-1, and the pair EZF and CV026. Compound **4.105** showed an inhibition of 20 ± 0.1 mm in EZF + CV026, while **4.104** did not show inhibition for the three models tested.

4.2.4.6 Antifungal activity

The deoxycholic acid derivatives **4.104** and **4.105** were tested for antifungal activity, in three fungi: *C. albicans* ATCC 10231, *A. fumigatus* ATCC 204305, and *T. rubrum* FF5. Compound **4.104** did not show activity against the strains tested. Compound **4.105**, on the other hand, was active against the three fungi used.

For *C. albicans* ATCC 10231, **4.105** showed a MIC of 64 $\mu\text{g/mL}$, with fungicidal activity, for *A. fumigatus* ATCC 204305 a concentration of 128 $\mu\text{g/mL}$ was fungistatic, and for *T. rubrum* FF5 it showed a MIC of 32 $\mu\text{g/mL}$, but its minimum fungicidal concentration was 64 $\mu\text{g/mL}$.

4.2.4.7 Cytotoxicity

As the two compounds showed a broad diversity of activities in the scope tested, which led to an increased interest in this scaffold, they were both tested for their cytotoxicity in the NIH/3T3 cell line (mouse embryonic fibroblasts). Doxorubicin was used as positive control and the IC_{50} results of compounds **4.104** and **4.105** can be seen in **Table 4.17**.

Table 4.17 Cytotoxicity of the deoxycholic acid derivatives against NIH/3T3 cell line.

Compound	IC_{50} (μM)
4.104	>100
4.105	56.8 ± 1.87
Doxorubicin	26.9 ± 1.02

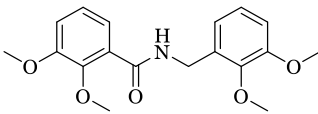
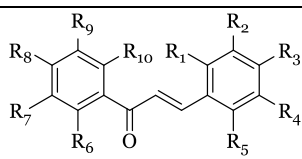
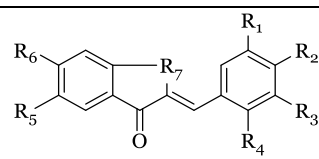
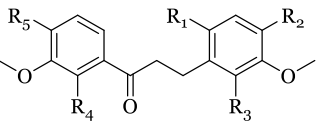
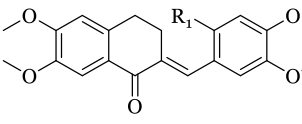
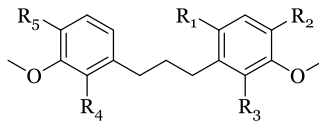
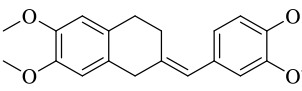
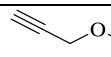
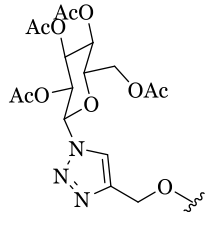
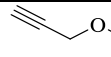
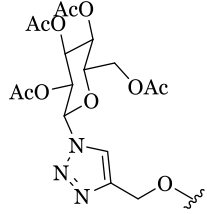
IC_{50} : half maximal inhibitory effect.

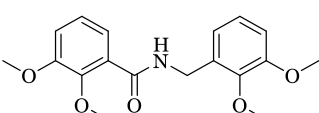
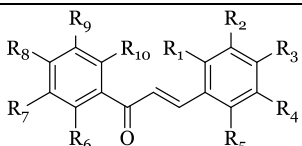
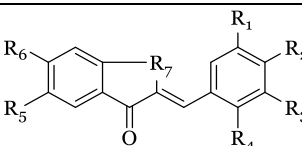
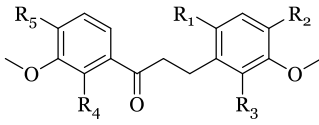
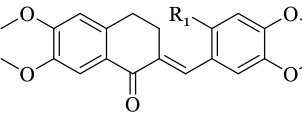
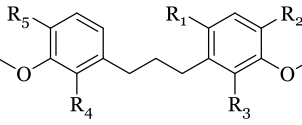
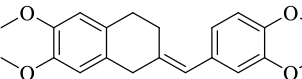
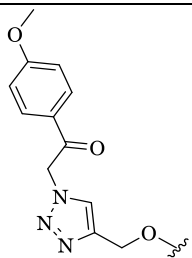
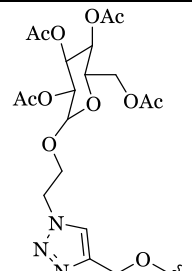
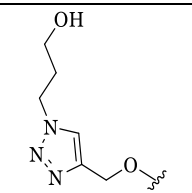
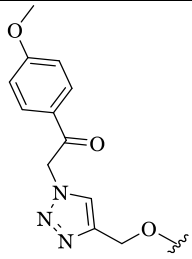
Compound **4.104** proved to be safe in the cell line tested, with an IC_{50} above the maximum concentration tested (100 μM). Compound **4.105** showed a lower IC_{50} , of approximately 57 μM . However, this value only compromises the possible use of **4.105** as a Gram-negative antibacterial, since it was tested at concentrations below 57 μM for all the other activities studied.

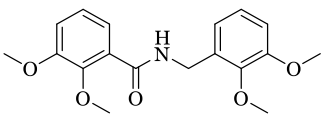
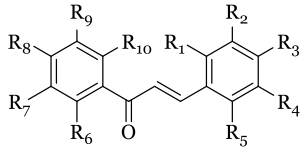
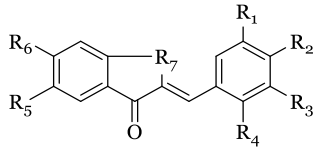
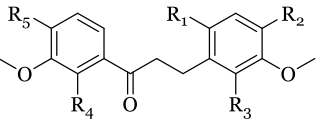
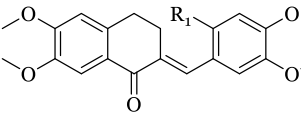
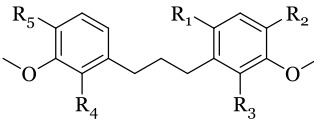
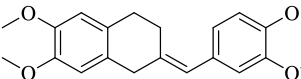
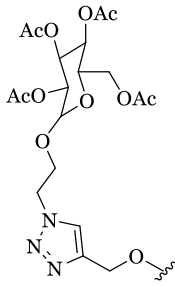
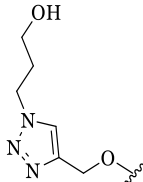
4.2.5 Chalcones

A library of 36 chalcone derivatives was studied for antimicrobial activity and related AMR mechanisms. The library could be further divided into 25 chalcones, six dihydrochalcones, and five diarylpropanes. The structures of these compounds can be seen in **Table 4.18**.

Table 4.18 Structures of the chalcone derivatives tested.

 4.106  4.107 - 4.123  4.124 - 4.130  4.131 - 4.134  4.135 - 4.136  4.137 - 4.140  4.141										
Compound	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	R ₉	R ₁₀
Chalcones										
4.107	H	H	OCH ₃	OCH ₃	H	OH	H		H	H
4.108	H	H	OCH ₃	OCH ₃	H	OH	H		H	H
4.109	H	OCH ₃	OCH ₃	OCH ₃	H	OH	H		H	H
4.110	H	OCH ₃	OCH ₃	OCH ₃	H	OH	H		H	H

 4.106  4.107 - 4.123  4.124 - 4.130  4.131 - 4.134  4.135 - 4.136  4.137 - 4.140  4.141										
Compound	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	R ₉	R ₁₀
4.111	H	H	OCH ₃	OCH ₃	H	OH	H		H	H
4.112	H	H	OCH ₃	OCH ₃	H	OH	H		H	H
4.113	H	H	OCH ₃	OCH ₃	H	OH	H		H	H
4.114	H	OCH ₃	OCH ₃	OCH ₃	H	OH	H		H	H

 4.106  4.107 - 4.123  4.124 - 4.130  4.131 - 4.134  4.135 - 4.136  4.137 - 4.140  4.141										
Compound	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	R ₉	R ₁₀
4.115	H	OCH ₃	OCH ₃	OCH ₃	H	OH	H		H	H
4.116	H	OCH ₃	OCH ₃	OCH ₃	H	OH	H		H	H
4.117	H	H	H	OCH ₃	OCH ₃	OCH ₃	OCH ₃	H	H	H
4.118	H	H	OCH ₃	OCH ₃	H	H	OCH ₃	OCH ₃	H	H
4.119	H	Br	OCH ₃	Br	H	H	Br	OCH ₃	Br	H
4.120	H	Br	H	OCH ₃	OCH ₃	OCH ₃	OCH ₃	H	Br	Br
4.121	H	H	H	OCH ₃	OCH ₃	OCH ₃	OCH ₃	H	Br	Br
4.122	Br	H	OCH ₃	OCH ₃	H	H	OCH ₃	OCH ₃	H	Br
4.123	H	H	OCH ₃	OCH ₃	H	H	OCH ₃	OCH ₃	H	Br
4.124	H	H	H	OCH ₃	H	H	CH ₂	-	-	-
4.125	OCH ₃	OCH ₃	H	OCH ₃	H	H	CH ₂	-	-	-
4.126	OCH ₃	OCH ₃	H	OCH ₃	H	H	CH ₂ S	-	-	-
4.127	H	Cl	H	H	H	H	CH ₂ S	-	-	-
4.128	H	Cl	H	H	H	H	CH ₂ CH ₂	-	-	-
4.129	H	H	H	OCH ₃	H	H	CH ₂ CH ₂	-	-	-
4.130	H	OCH ₃	OCH ₃	H	OCH ₃	OCH ₃	CH ₂ CH ₂	-	-	-
Dihydrochalcones										
4.131	H	H	OCH ₃	OCH ₃	H	-	-	-	-	-
4.132	Br	H	OCH ₃	OCH ₃	H	-	-	-	-	-
4.133	H	OCH ₃	H	H	OCH ₃	-	-	-	-	-

 4.106 4.107 - 4.123 4.124 - 4.130 4.131 - 4.134 4.135 - 4.136 4.137 - 4.140 4.141										
Compound	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	R ₉	R ₁₀
4.134	Br	OCH ₃	H	H	OCH ₃	-	-	-	-	-
4.135	Br	-	-	-	-	-	-	-	-	-
4.136	H	-	-	-	-	-	-	-	-	-
Diarylpropanes										
4.137	H	H	OCH ₃	OCH ₃	H	-	-	-	-	-
4.138	Br	H	OCH ₃	OCH ₃	Br	-	-	-	-	-
4.139	H	OCH ₃	H	OCH ₃	H	-	-	-	-	-
4.140	Br	OCH ₃	H	OCH ₃	Br	-	-	-	-	-

4.2.5.1 Docking studies

Docking studies were performed, regarding this library of 36 chalcones, using the AcrAB-TolC and NorA EPs. The docking scores of compounds **4.106 – 4.141** are present in **Table 4.19**.

Table 4.19 Docking scores for the chalcone derivatives in bacterial efflux pumps.

Compound	Docking score (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
4.106	-7.0	-3.5	-5.6	-5.4	-6.3	-6.7	-5.2
4.107	-6.8	-4.7	-6.0	-5.7	-6.6	-8.4	-7.2
4.108	-8.2	1.4	-5.5	-4.8	-6.7	-3.5	-5.1
4.109	-7.1	-3.1	-6.0	-6.2	-6.5	-7.3	-5.3
4.110	-9.2	1.2	-5.7	-5.1	-6.6	-4.3	-3.8
4.111	-8.6	-3.9	-6.9	-6.5	-7.7	-9.1	-5.8
4.112	-8.6	4.7	-5.7	-5.0	-6.0	-2.3	-4.4
4.113	-7.5	-6.7	-6.5	-6.3	-6.9	-8.5	-7.6
4.114	-8.4	-6.9	-7.0	-6.1	-7.4	-7.6	-5.2
4.115	-7.5	6.1	-6.6	-6.4	-6.9	-7.4	-5.0
4.116	-7.9	-4.5	-6.1	-6.0	-7.0	-7.3	-4.2

Compound	Docking score (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
4.117	-6.8	-4.8	-5.3	-5.8	-6.6	-6.3	-5.2
4.118	-7.0	-5.3	-5.7	-5.4	-6.5	-7.7	-7.3
4.119	-6.9	-1.4	-5.6	-5.3	-6.7	-5.8	-5.3
4.120	-6.7	-3.4	-5.5	-5.1	-6.6	-4.5	-4.8
4.121	-7.1	-3.0	-5.8	-5.2	-6.6	-5.3	-5.3
4.122	-6.7	-2.3	-5.6	-5.4	-5.8	-5.5	-5.2
4.123	-6.7	-0.9	-5.4	-5.4	-6.3	-7.5	-5.6
4.124	-6.7	-5.5	-5.8	-6.1	-7.0	-7.9	-7.8
4.125	-6.7	-4.5	-5.5	-5.8	-6.7	-7.0	-7.1
4.126	-6.9	-4.0	-5.6	-6.0	-6.6	-6.0	-5.2
4.127	-6.5	-5.8	-5.7	-5.6	-6.9	-6.8	-6.5
4.128	-6.5	-5.8	-6.1	-6.2	-7.1	-7.6	-7.7
4.129	-7.3	-5.4	-5.7	-5.8	-7.1	-8.1	-7.8
4.130	-7.6	1.2	-6.0	-5.5	-6.7	-5.7	-5.6
4.131	-6.8	-4.5	-5.7	-5.7	-6.6	-6.9	-5.1
4.132	-6.6	-4.4	-5.5	-5.9	-6.2	-5.0	-4.9
4.133	-7.0	-4.5	-5.4	-5.5	-6.2	-7.6	-7.5
4.134	-6.5	-3.6	-5.6	-5.4	-5.8	-6.8	-6.8
4.135	-7.3	-5.4	-6.0	-5.7	-6.7	-6.8	-5.6
4.136	-7.5	-3.5	-5.8	-5.9	-6.9	-5.4	-5.6
4.137	-6.4	-4.7	-5.5	-5.2	-6.4	-6.8	-5.1
4.138	-6.1	-4.1	-5.3	-4.6	-5.8	-4.5	-4.8
4.139	-6.9	-5.0	-5.5	-4.8	-6.4	-7.5	-7.4
4.140	-6.1	-2.2	-5.1	-5.0	-6.3	-6.2	-6.1
4.141	-6.8	-5.1	-5.7	-5.2	-7.2	-7.4	-7.2
PAβN	-7.1	-4.7	-5.8	-4.9	-7.1	-	-
Reserpine	-	-	-	-	-	1.0	-4.6

SBS: Substrate binding site; **HT**: Hydrophobic trap; **HH**: Helical hairpin; **LD**: Lipoyl domain; **BCR**: Binding core region; **CS**: Cytoplasmic side; **PAβN**: Phenyl-arginine β-naphthylamide.

The results obtained show the general best predicted affinity for AcrB, specifically the substrate binding site, and the least for the hydrophobic trap in the same portion, as has been described for the other derivatives tested so far. Noteworthy are derivatives **4.108**, **4.110**, **4.112**, and **4.115**, which present very high docking scores for the hydrophobic trap. These molecules contain the bulkier groups, which can explain these unfavorable values.

For the NorA homology model, most of the compounds are predicted to show better affinity towards the binding core region. In both portions, the compounds showed comparable or better docking scores than the positive controls, thus they were all tested for relevant biological activities.

4.2.5.2 Antibacterial activity and potentiation of antimicrobials

Chalcones **4.106** – **4.141** were tested against six strains of bacteria: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. enterica* serovar Typhimurium SL1344, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, and *S. aureus* 272123. In this case, only compound **4.111** displayed antibacterial activity, with a MIC of 60 μ M against *E. faecalis* ATCC 29212.

The case is very different when the potentiation of antibiotics is considered. In fact, when used in combination with CTX against *E. coli* SA/2, or with VAN against *E. faecalis* B3/101, these compounds display favorable results, as can be seen in **Table 4.20**.

Table 4.20 Potentiation of antibiotics by chalcone derivatives **4.106** – **4.141**.

Compound	MIC reduction	
	<i>E. coli</i> SA/2	<i>E. faecalis</i> B3/101
	CTX MIC = 1124 – 562 μ M	VAN MIC = 707 μ M
4.106	No effect	2-fold
4.107	No effect	2-fold
4.108	No effect	No effect
4.109	No effect	2-fold
4.110	2-fold	No effect
4.111	No effect	8-fold
4.112	2-fold	2-fold
4.113	No effect	4-fold
4.114	No effect	No effect
4.115	No effect	No effect
4.116	No effect	No effect
4.117	2-fold	4-fold
4.118	2-fold	2-fold
4.119	2-fold	4-fold
4.120	No effect	2-fold
4.121	2-fold	2-fold
4.122	2-fold	4-fold
4.123	No effect	2-fold
4.124	No effect	2-fold
4.125	2-fold	2-fold
4.126	No effect	2-fold
4.127	No effect	No effect
4.128	2-fold	No effect
4.129	2-fold	2-fold
4.130	2-fold	2-fold
4.131	2-fold	2-fold
4.132	2-fold	2-fold
4.133	2-fold	2-fold

Compound	MIC reduction	
	<i>E. coli</i> SA/2	<i>E. faecalis</i> B3/101
	CTX MIC = 1124 – 562 μ M	VAN MIC = 707 μ M
4.134	2-fold	2-fold
4.135	2-fold	2-fold
4.136	No effect	4-fold
4.137	2-fold	2-fold
4.138	No effect	2-fold
4.139	2-fold	2-fold
4.140	2-fold	2-fold
4.141	2-fold	2-fold

MIC: Minimum inhibitory concentration; CTX: Cefotaxime; VAN: Vancomycin.

An analysis of the results of the potentiation of antibiotics shows that the chalcone scaffold is promising in regard to the decrease of the MIC of antibiotics in the resistant bacterial strains tested. In fact, only five compounds, **4.108**, **4.114 – 4.116**, and **4.127**, did not display any kind of potentiation.

The patterns of methoxylation in derivatives **4.107 – 4.116** appear to play a part in the potentiation of VAN in *E. faecalis* B3/101, particularly when it is accompanied by substitution with bulky groups. In this case, if there is a bulky substituent, only dimethoxylated chalcones present effectivity (**4.111 – 4.113**). However, compound **4.108**, with a similar substitution pattern does not show any potentiation in this instance, which can be related to the existence of a sugar moiety linked directly to the triazole, as compound **4.112**, with a linker between these moieties, is able to display potentiation.

The use of chlorine as a substituent also appears to be a hindrance for this activity, as chalcones **4.127** and **4.128** did not show any effect against *E. faecalis* B3/101.

Concerning *E. coli* SA/2, it could be noted that a thiopyranone moiety leads to lack of activity (**4.126** and **4.127**). Most of the dihydrochalcones and diarylpropanes present a 2-fold reduction in the MIC of CTX, even though no SAR could be established.

4.2.5.3 Bacterial efflux pump inhibition assay

Derivatives **4.106 – 4.141** were evaluated as bacterial EPIs according to their potential of modulating the accumulation of EtBr in *S. aureus* 272123 and *S. Typhimurium* SL1344, in comparison to reserpine and CCCP, respectively. All the compounds were tested at 50 μ M, as they presented no antibacterial activity against the strains used, and the controls were applied at 25 μ M. The results are in **Table 4.21**.

Table 4.21 RFI of the tested chalcone derivatives.

Compound	RFI $\pm$ SD	
	<i>S. aureus</i> 272123	<i>S. Typhimurium</i> SL1344
4.106	0.14 $\pm$ 0.09	0.29 $\pm$ 0.07
4.107	-0.06 $\pm$ 0.11	0.01 $\pm$ 0.02
4.108	0.06 $\pm$ 0.05	0.45 $\pm$ 0.02
4.109	-0.04 $\pm$ 0.20	0.02 $\pm$ 0.03
4.110	-0.09 $\pm$ 0.05	0.15 $\pm$ 0.05
4.111	0.06 $\pm$ 0.11	0.85 $\pm$ 0.02
4.112	0.20 $\pm$ 0.08	0.40 $\pm$ 0.06
4.113	0.10 $\pm$ 0.09	-0.01 $\pm$ 0.01
4.114	0.05 $\pm$ 0.02	0.59 $\pm$ 0.12
4.115	0.33 $\pm$ 0.04	0.52 $\pm$ 0.07
4.116	0.17 $\pm$ 0.06	-0.06 $\pm$ 0.03
4.117	0.81 $\pm$ 0.06	0.23 $\pm$ 0.09
4.118	0.32 $\pm$ 0.04	0.12 $\pm$ 0.05
4.119	-0.15 $\pm$ 0.06	0.26 $\pm$ 0.10
4.120	0.37 $\pm$ 0.08	0.29 $\pm$ 0.06
4.121	0.66 $\pm$ 0.09	-0.02 $\pm$ 0.08
4.122	0.31 $\pm$ 0.03	0.13 $\pm$ 0.03
4.123	0.29 $\pm$ 0.09	0.06 $\pm$ 0.01
4.124	0.05 $\pm$ 0.03	0.25 $\pm$ 0.05
4.125	-0.34 $\pm$ 0.01	0.11 $\pm$ 0.01
4.126	0.36 $\pm$ 0.01	-0.02 $\pm$ 0.04
4.127	0.28 $\pm$ 0.03	0.32 $\pm$ 0.08
4.128	0.60 $\pm$ 0.09	0.30 $\pm$ 0.12
4.129	0.88 $\pm$ 0.05	1.32 $\pm$ 0.06
4.130	0.37 $\pm$ 0.03	0.29 $\pm$ 0.01
4.131	0.75 $\pm$ 0.04	0.12 $\pm$ 0.04
4.132	1.20 $\pm$ 0.01	0.05 $\pm$ 0.02
4.133	0.23 $\pm$ 0.01	0.05 $\pm$ 0.05
4.134	0.88 $\pm$ 0.05	0.30 $\pm$ 0.08
4.135	0.43 $\pm$ 0.02	0.03 $\pm$ 0.06
4.136	0.67 $\pm$ 0.04	0.29 $\pm$ 0.09
4.137	1.30 $\pm$ 0.08	0.04 $\pm$ 0.03
4.138	0.22 $\pm$ 0.05	0.21 $\pm$ 0.06
4.139	0.53 $\pm$ 0.04	-0.02 $\pm$ 0.07
4.140	0.18 $\pm$ 0.02	0.15 $\pm$ 0.05
4.141	0.70 $\pm$ 0.07	0.17 $\pm$ 0.08
Reserpine	0.41 $\pm$ 0.01	-
CCCP	-	0.34 $\pm$ 0.03

RFI: Relative fluorescence index; **SD:** Standard deviation; **CCCP:** carbonyl cyanide 3-chlorophenylhydrazone.

A few interesting remarks can be made from these results. Firstly, the triazole substituted chalcones were only active against efflux inhibition in the Gram-negative strain. Specifically, **4.108**, **4.111**, **4.112**, **4.114**, and **4.115** displayed better results than CCCP in *S. Typhimurium* SL1344. Chalcone **4.129** was also effective in this case.

Oppositely, the triazole substituted chalcones did not show any activity against *S. aureus* 272123. In this instance, chalcones **4.117**, **4.121**, **4.128**, and **4.129**, dihydrochalcones **4.131**, **4.132**, **4.134**, and **4.135**, and diarylpropanes **4.136**, **4.137**, **4.139**, and **4.141** showed favorable results at inhibiting EPs in the Gram-positive strain.

As compound **4.129** showed inhibition of both strains tested, its fluorescence was evaluated. This study can be seen in **Figure 4.22**.

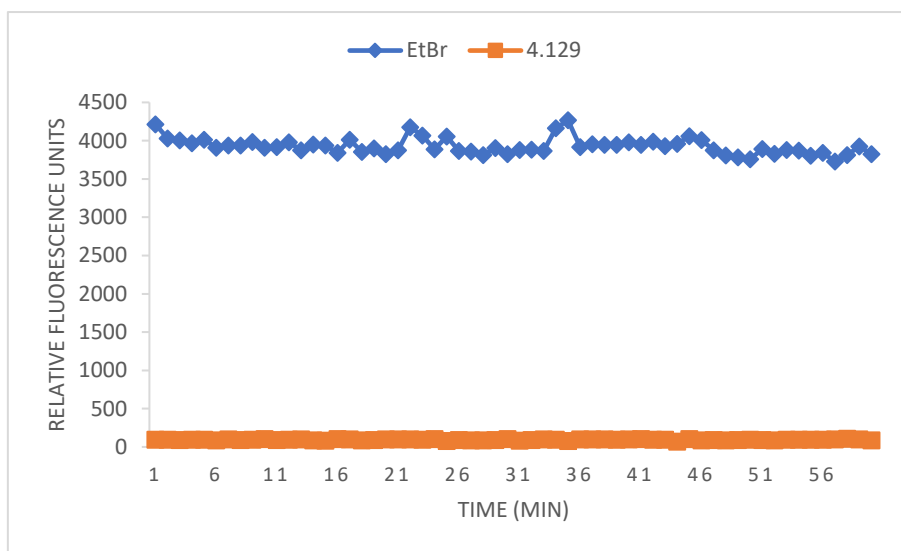


Figure 4.22 Fluorescence curves of EtBr and compound **4.129**.

As the fluorescence was kept low and stable over the duration of the assay, the results of the assay were attributed to the accumulation of EtBr. Thus, the chalcone scaffold can be considered promising in the search for bacterial EPIs.

Compounds **4.108**, **4.111**, **4.112**, **4.114**, **4.115**, and **4.129** were visualized in the substrate binding site of AcrB, and compounds **4.117**, **4.121**, **4.128**, **4.129**, **4.131**, **4.132**, **4.134** – **4.137**, **4.139**, and **4.141** were visualized in the binding core region of the NorA homology model.

A closer inspection of the interaction between compounds **4.117**, **4.135**, and **4.136** and NorA shows predicted interactions with Lys-31, through a hydrogen interaction between a methoxy and the amine in the residue (**Figure 4.23, A**). Compound **4.121** is predicted to interact with NorA through a π - π stacking with Tyr-225 (**Figure 4.23, B**).

Chalcone **4.129** can establish a polar interaction with Glu-222, as seen in **Figure 4.23, C**. The structurally related dihydrochalcone **4.131** and diarylpropane **4.137** can establish

the same hydrogen interaction between one of the methoxy groups and the amide in Ser-226 (**Figure 4.23, D**). Compound **4.132** can interact with Phe-288 via a π - π stacking (**Figure 4.23, E**). Considering compounds **4.134**, **4.139**, and **4.141**, which were predicted to bind in a different site as the previous ones, it could be noted that only **4.141** had a relevant interaction with a residue of the homology model, as it was predicted that one of the oxygens in the methoxy group interacted with the amide group of Asp-291 (**Figure 4.23, F**). Compound **4.128** did not show any relevant interactions as well, but weaker interactions with the target, such as van der Waals, still happen (not shown).

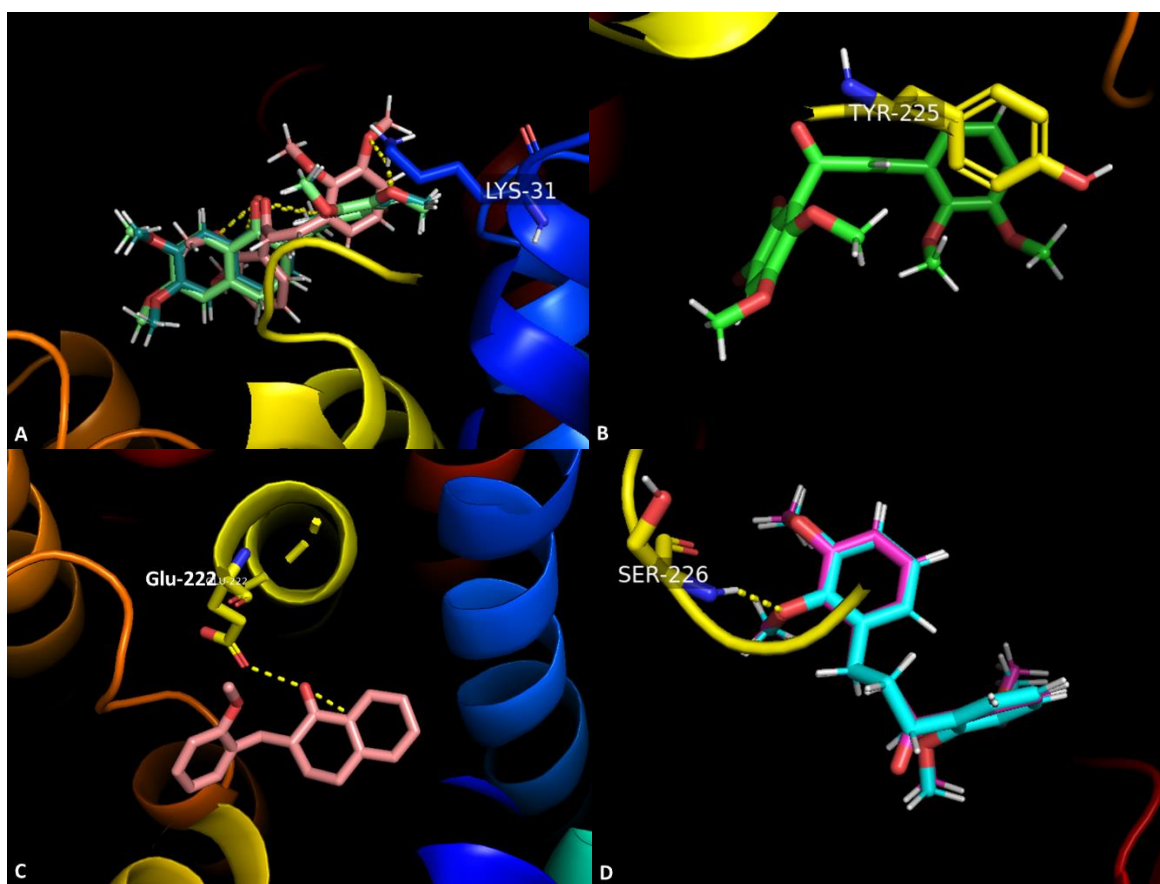


Figure 4.23 Molecular visualization of chalcone derivatives in the binding core region of the NorA homology model. (A) Interactions between compounds **4.117** (magenta), **4.135** (green), and **4.136** (teal) and NorA; (B) Interactions between compound **4.121** and NorA; (C) Interactions between compound **4.129** and NorA; (D) Interactions between compounds **4.131** (pink) and **4.137** (blue), and NorA; (E) Interactions between compound **4.132** and NorA; (F) Interactions between compounds **4.134** (blue), **4.139** (gray), **4.141** (orange) and NorA.

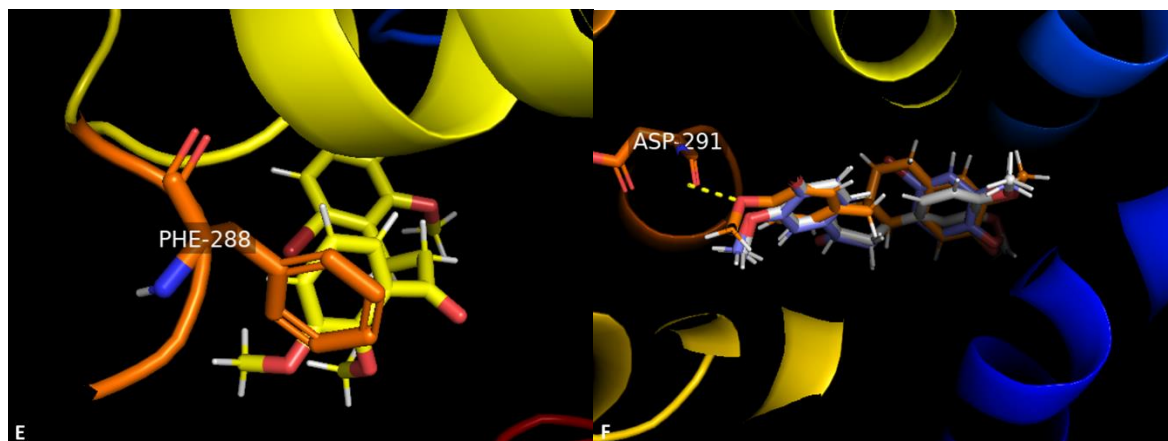


Figure 4.23 Molecular visualization of chalcone derivatives in the binding core region of the NorA homology model. (A) Interactions between compounds **4.117** (magenta), **4.135** (green), and **4.136** (teal) and NorA; (B) Interactions between compound **4.121** and NorA; (C) Interactions between compound **4.129** and NorA; (D) Interactions between compounds **4.131** (pink) and **4.137** (blue), and NorA; (E) Interactions between compound **4.132** and NorA; (F) Interactions between compounds **4.134** (blue), **4.139** (gray), **4.141** (orange) and NorA (cont.).

Concerning AcrB, compound **4.108** showed predicted interactions between Ser-46, Thr-87, Gln-176, and Asn-274, through polar interactions between carbonyls and hydroxy groups present in both the compound and the efflux pump. Other polar interactions observed were with Gln-125, Ser-128, Asp-174, and Glu-237, while hydrogen interactions were observed between compound **4.108** and Gly-126, Glu-130, Gln-181, and Lys-770, as can be seen in **Figure 4.24, A**.

Compound **4.111** is predicted to interact with Gly-126 through a hydrogen interaction established via the triazole group. The carbonyl at position C1 can establish hydrogen interactions with Gln-181, Lys-770 and Arg-767, while the oxygen at the methoxy group in ring B can establish a hydrogen interaction with Asn-274. In terms of polar interaction, one can be found between the methoxy in the substituent ring and Arg-620 (**Figure 4.24, B**).

Chalcone derivative **4.112** presented hydrogen interactions with Ser-128, Glu-130, Gln-176, Glu-181, and Gln-273 (**Figure 4.24, C**). The triazole group in compound **4.114** is predicted to establish a hydrogen interaction with Ser-46, Gln-125 and Gly-126. The hydroxy group in ring A can also form a hydrogen interaction with Val-175, and the carbonyl at C1 with Gln-176 and Leu-177. The two last hydrogen interactions predicted are between the methoxy groups in the B ring and Asn-274 and Arg-620 (**Figure 4.24, D**).

Concerning compound **4.115**, it was predicted to interact with Asp-164, through a hydrogen interaction between a hydroxy group in ring A and the carbonyl group of the residue, Asp-174 and Val-175, whose carbonyls establish hydrogen interactions with the nitrogen atoms in the triazole ring, Gln-176, Leu-177 and Gln-181, with a polar interaction being established between the carbonyl and an oxygen in the compound, and Asn-274 can

establish a hydrogen interaction between the nitrogen in the residue and an oxygen in the methoxy groups present in ring B (**Figure 4.24, E**).

Lastly, compound **4.129** showed a polar interaction between the methoxy group and Thr-87, and a hydrogen interaction between the carbonyl and Asn-274 (**Figure 4.24, F**).

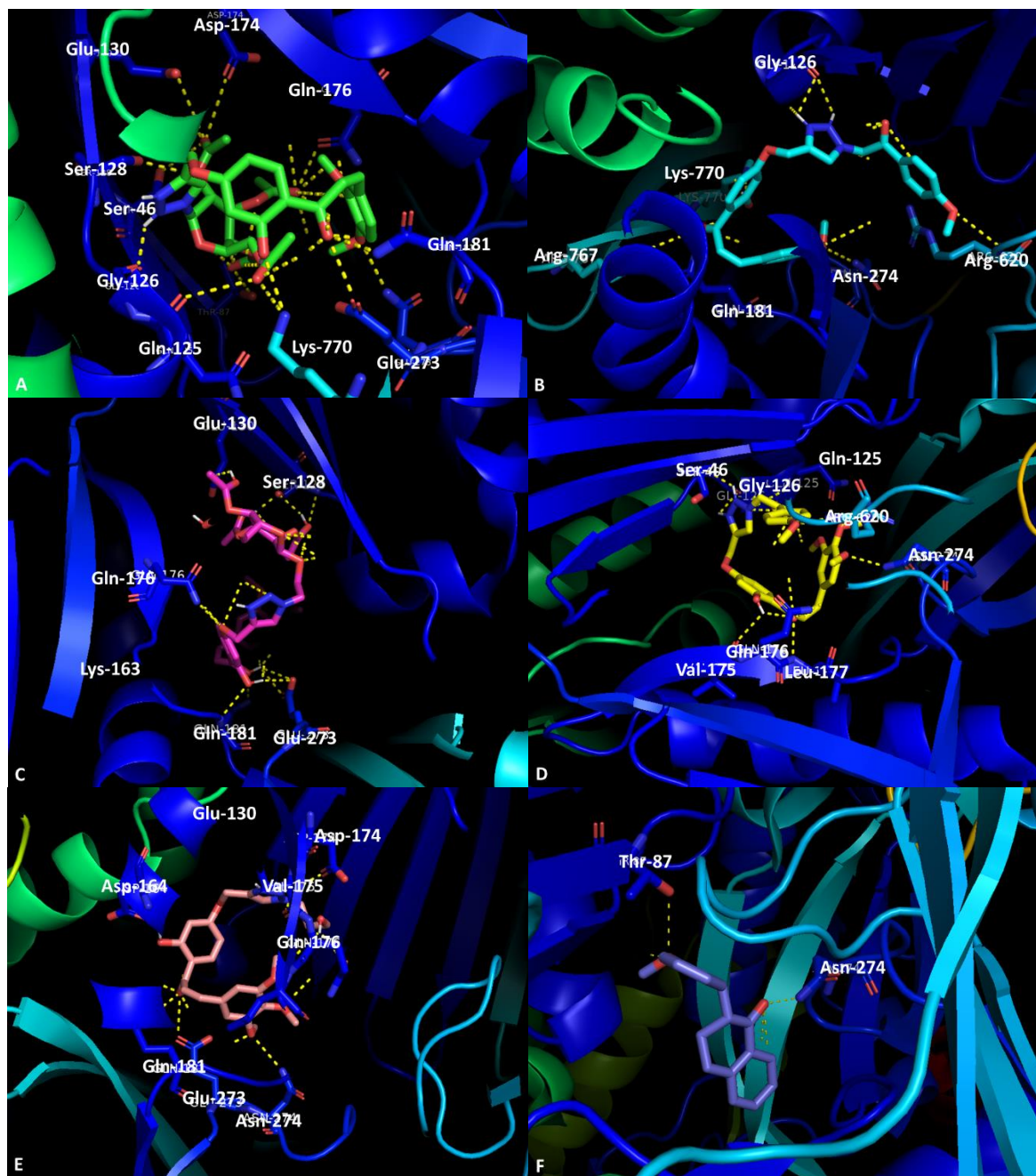


Figure 4.24 Molecular visualization of chalcone derivatives in the substrate binding site of AcrB. (A) Interactions between compound **4.108** and AcrB; (B) Interactions between compound **4.111** and AcrB; (C) Interactions between compound **4.112** and AcrB; (D) Interactions between compound **4.114** and AcrB; (E) Interactions between compound **4.115** and AcrB; (F) Interactions between compound **4.129** and AcrB.

The compounds that showed potential as EPIs were analyzed in further studies.

4.2.5.4 Inhibition of biofilm formation

Compounds **4.108**, **4.111**, **4.112**, **4.114**, **4.115**, **4.117**, **4.121**, **4.128**, **4.129**, **4.131**, **4.132**, **4.134** – **4.137**, **4.139**, and **4.141** were assayed for their potential against the formation of biofilm by *S. aureus* ATCC 25923 and *S. aureus* 272123. Since none of the compounds showed antibacterial activity for either strain, they were all tested at 100 μ M, while reserpine, used as a positive control, was tested at 25 μ M. The results obtained are compiled in **Table 4.22**.

Table 4.22 Inhibition of biofilm formation by chalcone derivatives.

Compound	Inhibition of biofilm formation (%) $\pm$ SD	
	<i>S. aureus</i> ATCC 25923	<i>S. aureus</i> 272123
4.108	0	85.8 $\pm$ 2.61
4.111	0.55 $\pm$ 2.00	89.4 $\pm$ 0.56
4.112	0	36.9 $\pm$ 1.37
4.114	0	72.3 $\pm$ 0.89
4.115	1.29 $\pm$ 0.42	59.6 $\pm$ 2.51
4.117	0	87.3 $\pm$ 3.84
4.121	3.05 $\pm$ 1.41	7.76 $\pm$ 2.14
4.128	0	0
4.129	0	83.3 $\pm$ 0.01
4.131	0	6.89 $\pm$ 2.41
4.132	0	7.08 $\pm$ 2.72
4.134	13.3 $\pm$ 6.67	7.95 $\pm$ 0.65
4.135	0	8.15 $\pm$ 0.64
4.136	0	10.7 $\pm$ 0.76
4.137	0	23.8 $\pm$ 0.13
4.139	0	49.6 $\pm$ 0.39
4.141	0	71.3 $\pm$ 1.09
Reserpine	22.3 $\pm$ 4.21	81.11 $\pm$ 1.18

SD: Standard deviation.

The chalcone derivatives tested did not show potential to inhibit the formation of biofilm in *S. aureus* ATCC 25923. In *S. aureus* 272123, compounds **4.108**, **4.111**, **4.114**, **4.117**, **4.129**, and **4.141** were able to show a decrease higher than 70% in biofilm formation. It is noteworthy that compounds **4.117**, **4.129**, and **4.141** can also inhibit EPs in *S. aureus* 272123, which can be related to this decrease in biofilm formation.

There does not seem to be a common structural feature responsible for the inhibition of biofilm formation.

4.2.5.5 Inhibition of quorum-sensing

The chalcone derivatives tested against the formation of biofilm were also evaluated for their potential of inhibiting QS, in wt85, *S. marcescens* AS-1, and EZF and CVO26, used herein as a pair. PMZ was used as a positive control, and displayed an inhibition of pigment production of 41 ± 0.5 mm.

The results obtained showed that three compounds presented potential to inhibit QS only in the pair EZF + CVO26: compound **4.121** showed 29 ± 0.5 mm, compound **4.129** displayed inhibition of 48 ± 0.8 mm, and compound **4.136** inhibited pigment production by 41 ± 0.1 mm.

Compound **4.129** has proven potential as a potentiator of antibiotics, inhibitor of EPs, biofilm, and QS, making this chalcone an interesting starting point for the synthesis of derivatives.

4.2.5.6 Antifungal activity

The chalcone derivatives **4.106** – **4.141** were tested for their potential to act as antifungal in three distinct fungi: the yeast *C. albicans* ATCC 10231, the filamentous fungus *A. fumigatus* ATCC 204305, and the dermatophyte *T. rubrum* FF5.

Out of the three strains tested, the chalcone derivatives were only active against *T. rubrum* FF5, with compound **4.106** demonstrating a MIC of 256 $\mu\text{g/mL}$, **4.117** showing a MIC of 16 – 32 $\mu\text{g/mL}$, and **4.118** showing inhibition of fungal growth at 128 $\mu\text{g/mL}$. None of the compounds displayed fungicidal activity.

4.2.5.7 Cytotoxicity

The most promising compounds, **4.108**, **4.117**, **4.118**, **4.121**, **4.129**, **4.131**, **4.132**, **4.134**, **4.136**, **4.137**, **4.139**, and **4.141**, were evaluated for their cytotoxicity against the mouse embryonic fibroblast cell line (NIH/3T3). The results can be seen in **Table 4.23**.

Table 4.23 Cytotoxicity of the chalcone derivatives against NIH/3T3 cell line.

Compound	IC ₅₀ (μM)
4.108	$80,07 \pm 3,59$
4.117	$30,16 \pm 1,04$
4.118	$45,36 \pm 3,09$
4.121	$28,31 \pm 0,25$
4.129	$88,82 \pm 1,34$
4.131	> 100
4.132	> 100
4.134	> 100

Compound	IC ₅₀ (μM)
4.136	> 100
4.137	> 100
4.139	> 100
4.141	> 100
Doxorubicin	12.1 ± 0.81

IC₅₀: half maximal inhibitory effect.

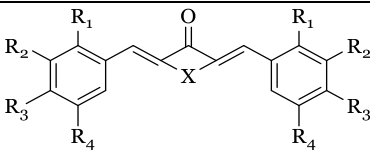
From the analysis of **Table 4.23**, it is noticeable that the dihydrochalcone (**4.131**, **4.132**, **4.134**, and **4.136**) and diarylpropane derivatives (**4.137**, **4.139**, and **4.141**) do not present cytotoxicity for this cell line, unlike chalcones **4.108**, **4.117**, **4.118**, **4.121**, and **4.129**. This is possibly due to the presence of an α, β-unsaturated ketone, which can act as a Michael acceptor, leading to covalent binding to biomolecules (160).

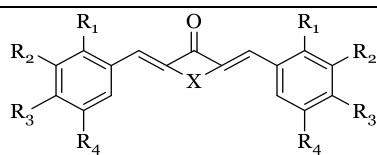
As such, and considering the lack of antibacterial activity and cytotoxicity of compounds **4.131**, **4.132**, **4.134**, **4.136**, **4.137**, **4.139**, and **4.141**, they can be considered hit compounds for the inhibition of bacterial EPs.

4.2.6 Diarylpentanoids

Diarylpentanoids are also known as C₅ curcuminoids and are structurally related to chalcones. Given the promising results obtained with chalcones, a library of 23 diarylpentanoids was tested for the same purposes. The structures of compounds **4.142** – **4.164** are in **Table 4.24**.

Table 4.24 Structures of the diarylpentanoids tested.

 142 - 164					
Compound	R ₁	R ₂	R ₃	R ₄	X
4.143	Cl	H	H	H	CH ₂ OCH ₂
4.143	Cl	H	Cl	H	CH ₂ OCH ₂
4.144	F	H	H	H	CH ₂ OCH ₂
4.145	H	F	H	H	CH ₂ OCH ₂
4.146	H	H	F	H	CH ₂ OCH ₂
4.147	H	H	CF ₃	H	CH ₂ OCH ₂
4.148	Br	H	H	H	CH ₂ OCH ₂
4.149	H	H	Br	H	CH ₂ OCH ₂
4.150	OCH ₃	H	H	H	CH ₂ OCH ₂
4.151	H	OCH ₃	H	H	CH ₂ OCH ₂
4.152	OCH ₃	OCH ₃	H	H	CH ₂ OCH ₂



142 - 164

Compound	R ₁	R ₂	R ₃	R ₄	X
4.153	OCH ₃	H	OCH ₃	H	CH ₂ OCH ₂
4.154	H	OCH ₃	OCH ₃	H	CH ₂ OCH ₂
4.155	H	OCH ₃	H	OCH ₃	CH ₂ OCH ₂
4.156	H	OCH ₃	OCH ₃	OCH ₃	CH ₂ OCH ₂
4.157	CH ₃	H	H	H	CH ₂ OCH ₂
4.158	H	CH ₃	H	H	CH ₂ OCH ₂
4.159	H	H	CH ₃	H	CH ₂ OCH ₂
4.160	OCH ₃	H	H	H	CH ₂ CH ₂ CH ₂
4.161	H	H	Cl	H	CH ₂ SCH ₂
4.162	OCH ₃	H	H	H	CH ₂ SCH ₂
4.163	H	H	Cl	H	CH ₂ CH ₂
4.164	OCH ₃	H	H	H	CH ₂ CH ₂

4.2.6.1 Docking studies

The library of diarylpentanoids presented underwent docking studies in AcrAB-TolC and NorA, the most relevant EPs in Gram-negative and Gram-positive bacteria, respectively. The docking scores obtained are compiled in **Table 4.25**.

Table 4.25 Docking scores for the diarylpentanoids in bacterial efflux pumps.

Compound	Docking score (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
4.142	-6.7	-4.5	-6.1	-6.3	-7.8	-7.9	-7.9
4.143	-7.4	-4.7	-6.5	-6.3	-7.3	-7.5	-6.1
4.144	-7.7	-5.2	-6.3	-6.3	-8.0	-8.6	-8.6
4.145	-7.4	-5.5	-6.3	-6.3	-7.5	-9.1	-9.0
4.146	-7.4	-5.1	-6.3	-6.5	-7.4	-8.2	-8.0
4.147	-8.4	-4.9	-6.9	-6.4	-8.3	-8.3	-6.7
4.148	-6.7	-4.8	-5.9	-5.9	-7.8	-6.6	-6.6
4.149	-7.1	-4.6	-5.9	-5.6	-7.5	-7.7	-5.8
4.150	-7.1	-5.1	-5.7	-5.9	-7.1	-7.2	-7.2
4.151	-7.4	-4.8	-6.1	-5.7	-7.6	-9.1	-8.3
4.152	-7.0	-2.6	-5.9	-5.3	-7.2	-6.0	-5.9
4.153	-7.4	-2.0	-6.0	-5.5	-6.8	-7.1	-5.6
4.154	-7.7	-4.3	-6.2	-5.7	-7.3	-6.6	-6.1
4.155	-7.7	-2.7	-5.8	-5.7	-6.8	-8.3	-7.5

Compound	Docking score (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
4.156	-7.2	-0.1	-6.0	-4.9	-6.9	-5.2	-5.5
4.157	-7.5	-5.3	-6.0	-6.4	-7.5	-7.6	-7.6
4.158	-7.6	-5.5	-6.2	-6.3	-7.8	-9.5	-9.5
4.159	-7.7	-4.9	-6.3	-6.5	-7.6	-7.3	-7.6
4.160	-7.1	-5.3	-5.9	-5.5	-7.4	-9.0	-8.9
4.161	-7.3	-3.9	-6.3	-5.8	-7.2	-7.8	-7.0
4.162	-7.1	-3.6	-6.0	-5.7	-7.0	-8.1	-8.0
4.163	-7.6	-4.8	-5.9	-5.9	-7.8	-8.0	-6.4
4.164	-6.9	-3.7	-5.7	-5.6	-7.0	-9.0	-8.9
PAβN	-7.1	-4.7	-5.8	-4.9	-7.1	-	-
Reserpine	-	-	-	-	-	1.0	-4.6

SBS: Substrate binding site; **HT:** Hydrophobic trap; **HH:** Helical hairpin; **LD:** Lipoyl domain; **BCR:** Binding core region; **CS:** Cytoplasmic side; **PAβN:** Phenyl-arginine β-naphthylamide.

The conclusions drawn are similar to what has been described for other compounds, with the substrate binding site having the best predicted affinity by most compounds. On the other hand, the hydrophobic trap is predicted to be the site towards which the diarylpentanoids have the least predicted affinity. In general, AcrA has lower predicted affinities than AcrB and TolC, even though the docking scores of derivatives **4.142 – 4.164** are similar or higher to that of PAβN.

Concerning the NorA pump, the compounds were predicted to have more affinity towards the binding core region, even though the difference between both sites is not very significant. All the compounds present better docking scores than reserpine, used as a positive control.

Considering this, all the compounds were tested for their potential as antimicrobials and were tested for their capability of reversing AMR mechanisms.

4.2.6.2 Antibacterial activity and potentiation of antimicrobials

The group of compounds **4.142 – 4.164** were tested in order to determine their antibacterial potential. The strains used were the Gram-negative *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. Typhimurium* SL1344, and the Gram-positive *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, and *S. aureus* 272123. Out of the 23 derivatives tested, only **4.161** displayed antibacterial activity, with a MIC of 89 μM against *S. aureus* ATCC 25923.

Differently, these derivatives showed promising results concerning the potentiation of antibiotics, like the structurally related chalcones. The results of compounds **4.142 – 4.164**

in the potentiation of CTX and VAN against *E. coli* SA/2 and *E. faecalis* B3/101, respectively, can be seen in **Table 4.26**.

Table 4.26 Potentiation of antibiotics by diarylpentanoids **4.142** – **4.164**.

Compound	MIC reduction	
	<i>E. coli</i> SA/2	<i>E. faecalis</i> B3/101
	CTX MIC = 1124 – 562 μ M	VAN MIC = 707 – 350 μ M
4.142	2-fold	No effect
4.143	2-fold	No effect
4.144	2-fold	No effect
4.145	No effect	2-fold
4.146	8-fold	No effect
4.147	4-fold	No effect
4.148	No effect	2-fold
4.149	8-fold	No effect
4.150	2-fold	No effect
4.151	No effect	4-fold
4.152	16-fold	32-fold
4.153	8-fold	No effect
4.154	2-fold	No effect
4.155	No effect	4-fold
4.156	2-fold	No effect
4.157	No effect	2-fold
4.158	2-fold	2-fold
4.159	2-fold	2-fold
4.160	No effect	2-fold
4.161	No effect	2-fold
4.162	No effect	4-fold
4.163	2-fold	2-fold
4.164	No effect	2-fold

MIC: Minimum inhibitory concentration; CTX: Cefotaxime; VAN: Vancomycin.

All the compounds showed some extent of potentiation of antibiotics, with 10 compounds showing potentiation of CTX, nine compounds showing the same with VAN, and four compounds showing activity for both. Remarkably, compounds **4.146**, **4.149**, and **4.153** reduced the MIC of CTX against *E. coli* SA/2 eight times, while compound **4.152** showed a 16-fold reduction. Additionally, compound **4.152** caused a 32-fold decrease in the MIC of VAN for *E. faecalis* B3/101, showing a broad spectrum of activity.

Considering the results obtained, SAR could be proposed for the potentiation of CTX. In the case of *E. coli* SA/2, *para*-substitution of the phenyl rings in the diarylpentanoid scaffold with an electron withdrawing moiety, such as halogens (**4.146**, **4.147**, and **4.149**), showed better potentiation than *ortho*-substitution (**4.142**, **4.144**, and **4.148**).

It was also shown that *ortho*- or *para*-substitution is essential in di- or trisubstituted derivatives (**4.143**, **4.152** – **4.154**, and **4.156**), as failure to do so leads to ineffective derivatives in this scope (**4.157**).

4.2.6.3 Bacterial efflux pump inhibition

Compounds **4.142** – **4.164** were evaluated for their EP inhibition in *S. aureus* 272123 and *S. Typhimurium* SL1344, through the real-time EtBr accumulation assay. Since none of the compounds presented antibacterial properties against the two strains in use for this assay, they were all applied at 50 μ M, while reserpine and CCCP, used as positive controls for *S. aureus* 272123 and *S. Typhimurium* SL1344, respectively, were used at 25 μ M. The results of this assay can be found in **Table 4.27**.

Table 4.27 RFI of the tested diarylpentanoids.

Compound	RFI $\pm$ SD	
	<i>S. aureus</i> 272123	<i>S. Typhimurium</i> SL1344
4.142	-0.16 $\pm$ 0.01	-0.08 $\pm$ 0.01
4.143	-0.25 $\pm$ 0.05	-0.13 $\pm$ 0.03
4.144	-0.08 $\pm$ 0.02	-0.04 $\pm$ 0.02
4.145	-0.11 $\pm$ 0.01	0.17 $\pm$ 0.02
4.146	-0.16 $\pm$ 0.04	0.04 $\pm$ 0.05
4.147	-0.12 $\pm$ 0.01	-0.24 $\pm$ 0.01
4.148	-0.05 $\pm$ 0.06	-0.02 $\pm$ 0.02
4.149	0.53 $\pm$ 0.03	0.06 $\pm$ 0.08
4.150	0.21 $\pm$ 0.05	0.16 $\pm$ 0.02
4.151	0.17 $\pm$ 0.04	0.05 $\pm$ 0.02
4.152	0.44 $\pm$ 0.01	0.12 $\pm$ 0.08
4.153	-0.19 $\pm$ 0.03	-0.15 $\pm$ 0.02
4.154	2.47 $\pm$ 0.24	-0.02 $\pm$ 0.02
4.155	0.25 $\pm$ 0.09	0.09 $\pm$ 0.02
4.156	0.02 $\pm$ 0.01	-0.05 $\pm$ 0.01
4.157	-0.23 $\pm$ 0.07	-0.08 $\pm$ 0.05
4.158	0.11 $\pm$ 0.02	-0.12 $\pm$ 0.02
4.159	-0.24 $\pm$ 0.04	-0.21 $\pm$ 0.01
4.160	0.48 $\pm$ 0.04	-0.31 $\pm$ 0.01
4.161	-0.02 $\pm$ 0.02	0.28 $\pm$ 0.06
4.162	0.01 $\pm$ 0.01	0.01 $\pm$ 0.03
4.163	-0.13 $\pm$ 0.06	0.21 $\pm$ 0.09
4.164	-0.39 $\pm$ 0.07	0.15 $\pm$ 0.04
Reserpine	0.35 $\pm$ 0.04	-
CCCP	-	0.30 $\pm$ 0.03

RFI: Relative fluorescence index; SD: Standard deviation; CCCP: carbonyl cyanide 3-chlorophenylhydrazine.

No diarylpentanoids were able to inhibit the efflux of EtBr in a higher extent than CCCP in *S. Typhimurium* SL1344. On the other hand, four compounds **4.149**, **4.152**, **4.154**, and **4.160** were more effective than reserpine towards *S. aureus* 272123. Due to this, the fluorescence of the effective compounds warranted no further analysis. Most of the compounds showed a negative RFI value, which means they show less EtBr efflux than the negative control.

The docking studies showed that the effective compounds were predicted to bind to NorA with more affinity in the binding core region. As such the four effective compounds were visualized in this site of the NorA homology model.

Firstly, a general view of derivatives **4.149**, **4.152**, **4.154**, and **4.160** shows that they bind approximately in the same region and seem to fit inside a pocket within this site (**Figure 4.25, A**). Concerning the specific residues with which the compounds bind, only for compound **4.160** could they be detected. In fact, it was predicted to establish a polar interaction between the carbonyl and Glu-222, and a hydrogen interaction between a methoxy oxygen and Thr-223, as can be seen in **Figure 4.25, B**. Even though the other compounds did not display polar interactions, they were able to establish weaker interactions with the target (not shown).

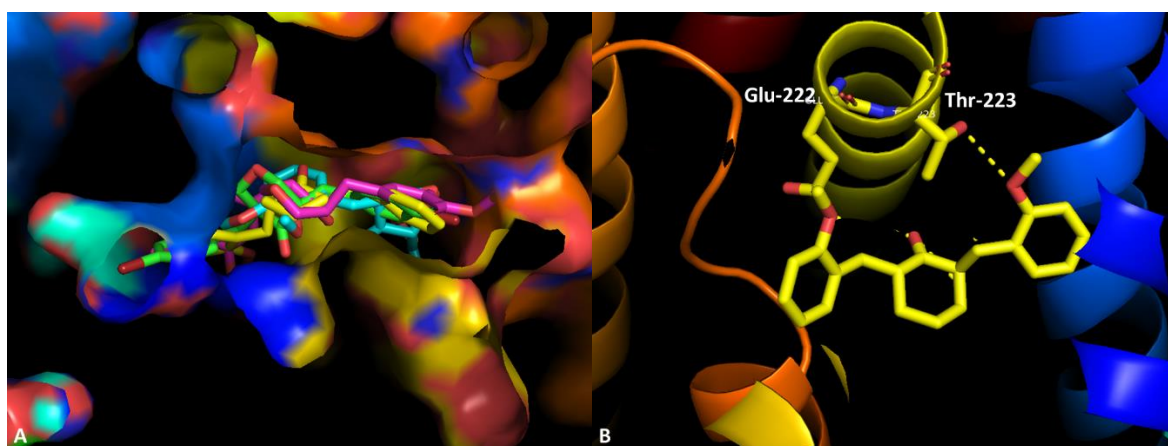


Figure 4.25 (A) General view of compounds **4.149**, **4.152**, **4.154**, and **4.160** in a pocket located within the binding core region of the NorA homology model; (B) Interactions between compound **4.160** and NorA.

Similar to what has been described so far, compounds **4.149**, **4.152**, **4.154**, and **4.160** have been studied for mechanisms of resistance related to EPs, namely biofilm and QS.

4.2.6.4 Inhibition of biofilm formation

The effective diarylpentanoids **4.149**, **4.152**, **4.154**, and **4.160** were evaluated for their activity against the formation of biofilm in *S. aureus* ATCC 25923 and *S. aureus* 272123.

They were tested at 100 μ M, as they did not show antibacterial activity for both strains in use, and reserpine was used as a positive control.

It was noticed that none of the compounds were able to influence the formation of biofilm in *S. aureus* ATCC 25923. In the oxacillin- and methicillin-resistant strain *S. aureus* 272123, compound **4.149** showed a decrease of 51.2 ± 0.11 % in biofilm formation, compound **4.152** decreased by 46.9 ± 0.30 %, and compound **4.160** showed an inhibition of 65.9 ± 0.04 % of biofilm formation. Compound **4.154** did not show inhibition at all. These results, however, are still below the inhibition of 72.1 ± 0.05 % showed by reserpine.

4.2.6.5 Inhibition of quorum-sensing

The same set of compounds were studied for the inhibition of QS in the three models previously described (wt85, *S. marcescens* AS-1, and EZF and CV026). The only compound which showed favorable activity in this regard was **4.152**, with an inhibition of 31 ± 0.5 mm, compared to 41 ± 0.5 mm of inhibition of PMZ, used herein as a positive control.

4.2.6.6 Antifungal activity

When tested against the yeast *C. albicans* ATCC 10231, the filamentous fungus *A. fumigatus* ATCC 204305, and the dermatophyte *T. rubrum* FF5, only compound **4.161** showed activity, out of the library of 23 diarylpentanoids. This compound could inhibit the growth of *C. albicans* ATCC 10231 at a concentration of 64 – 128 μ g/mL, and *T. rubrum* FF5 at 2 – 4 μ g/mL, with fungicidal activity for the latter at 4 μ g/mL.

4.2.6.7 Cytotoxicity

The four diarylpentanoids that showed EP inhibition, **4.149**, **4.152**, **4.154**, and **4.160**, were tested for their cytotoxicity on the NIH/3T3 cell line. The results are showed in **Table 4.28**.

Table 4.28 Cytotoxicity of the diarylpentanoids against NIH/3T3 cell line.

Compound	IC ₅₀ (μ M)
4.149	4.08 ± 0.66
4.152	4.99 ± 0.82
4.154	3.70 ± 0.41
4.160	64.8 ± 6.06
Doxorubicin	12.1 ± 0.81

IC₅₀: half maximal inhibitory effect.

It could be noticed that the IC_{50} of derivatives **4.149**, **4.152**, and **4.154** are very low, which compromises the use of these compounds as bacterial EPIs. Compound **4.160**, on the other hand, has a higher IC_{50} than the concentration needed for it to act as an EPI, which makes it safe in this instance.

4.2.7 Fiscalin derivatives

A library of five fiscalin derivatives were studied for their potential as bacterial EPIs and related mechanisms. The structures of derivatives **4.165** – **4.169** can be found in **Figure 4.26**.

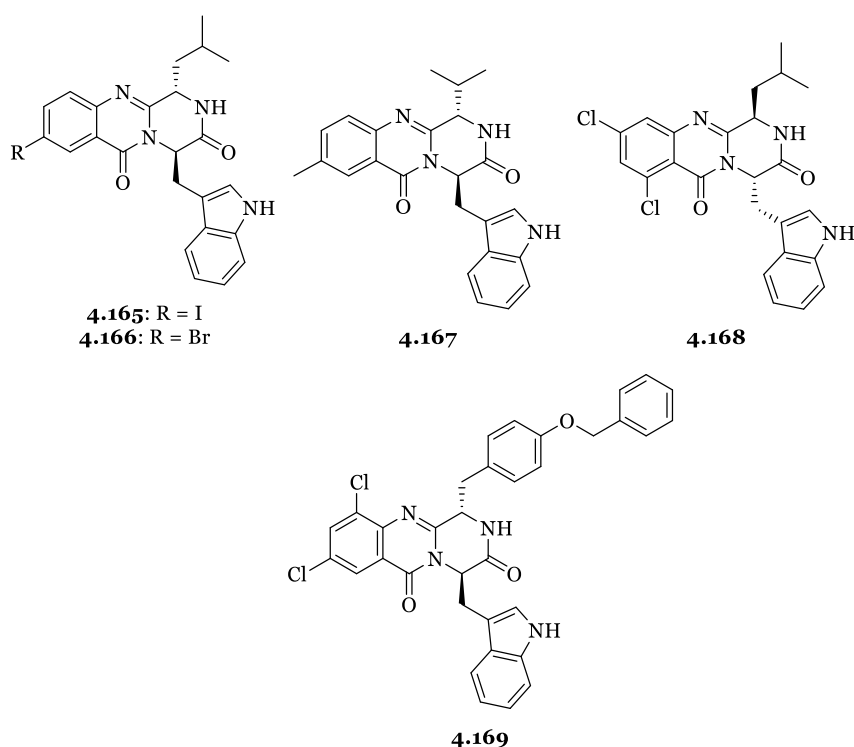


Figure 4.26 Structures of the fiscalin derivatives tested.

As these derivatives had already been studied for their antimicrobial activity and potentiation of antibiotics (146), herein they will only be evaluated for the bacteria used in the inhibition of EPs. Additionally, they have already been studied for their inhibition of biofilm formation by a reference strain of *S. aureus* and for antifungal activity (146), as well as cytotoxicity (139, 161). Therefore, they will not be evaluated for these activities in this instance.

4.2.7.1 Docking studies

The five fiscalin derivatives **4.165** – **4.169** were subject to docking studied in the AcrAB-TolC and NorA EPs, as has been done with the other classes of compounds throughout this project. The docking scores obtained are present in **Table 4.29**.

Table 4.29 Docking scores for the fiscalin derivatives in bacterial efflux pumps.

Compound	Docking score (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
4.165	-8.8	3.5	-7.0	-6.0	-7.8	-3.9	-7.2
4.166	-8.8	16.1	-7.2	-6.0	-7.8	-4.0	-7.3
4.167	-8.6	-5.1	-7.6	-6.0	-8.0	-3.3	-6.9
4.168	-8.7	6.6	-7.2	-5.9	-7.9	-6.2	-6.5
4.169	-9.7	30.2	-8.8	-7.2	-9.7	-3.5	-6.6
PAβN	-7.1	-4.7	-5.8	-4.9	-7.1	-	-
Reserpine	-	-	-	-	-	1.0	-4.6

SBS: Substrate binding site; **HT:** Hydrophobic trap; **HH:** Helical hairpin; **LD:** Lipoyl domain; **BCR:** Binding core region; **CS:** Cytoplasmic side; **PAβN:** Phenyl-arginine β-naphthylamide.

In terms of predicted affinity, fiscalin derivatives present better docking scores for the substrate binding site of AcrB. On the other hand, the scores for the hydrophobic trap are high, which makes the predicted affinity unfavorable.

Considering the NorA pump, the compounds are predicted to bind with more affinity towards the cytoplasmic side.

As compounds **4.165** – **4.169** showed better predicted affinities than the positive control, they were tested for their potential as EPIs.

4.2.7.2 Antibacterial activity

The fiscalins present in this study had been previously studied for their antibacterial activity against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, and *E. faecalis* ATCC 29212, as well as for their potentiation of antibiotics in clinically relevant strains (146).

Hence, they were only tested against *S. aureus* 272123 and *S. Typhimurium* SL1344, where only compound **4.167** displayed a MIC of 100 μM against *S. aureus* 272123.

4.2.7.3 Bacterial efflux pump inhibition

Derivatives **4.165** – **4.169** were explored for their potential as bacterial EPIs in the clinical strain *S. aureus* 272123 and *S. Typhimurium* SL1344, through the evaluation of the

accumulation of EtBr by means of fluorescence emission. Since compound **4.167** showed antibacterial activity, it was applied at 33 μ M, one-third of its MIC, while the other compounds were tested at 50 μ M. The Gram-positive positive control, reserpine, and Gram-negative positive control CCCP were applied at 25 μ M. **Table 4.30** comprises the results obtained, regarding the accumulation of EtBr.

Table 4.30 RFI of the tested fiscalin derivatives.

Compound	RFI $\pm$ SD	
	<i>S. aureus</i> 272123	<i>S. Typhimurium</i> SL1344
4.165	2.40 $\pm$ 0.10	9.09 $\pm$ 0.81
4.166	2.91 $\pm$ 0.86	1.53 $\pm$ 0.02
4.167	0.83 $\pm$ 0.09	0.94 $\pm$ 0.04
4.168	1.13 $\pm$ 0.20	-0.09 $\pm$ 0.06
4.169	0.19 $\pm$ 0.08	0.17 $\pm$ 0.07
Reserpine	0.35 $\pm$ 0.04	-
CCCP	-	0.33 $\pm$ 0.05

RFI: Relative fluorescence index; **SD**: Standard deviation; **CCCP**: carbonyl cyanide 3-chlorophenylhydrazone.

Derivatives **4.165** – **4.168** showed RFI values higher than reserpine in *S. aureus* 272123, while **4.165** – **4.167** showed the same activity in *S. Typhimurium* SL1344. In order to conclude whether these values are due to the inhibition of the efflux of EtBr or due to the fluorescence of the compound, compounds **4.165** – **4.167** were evaluated alone for their fluorescence. This can be seen in **Figure 4.27**.

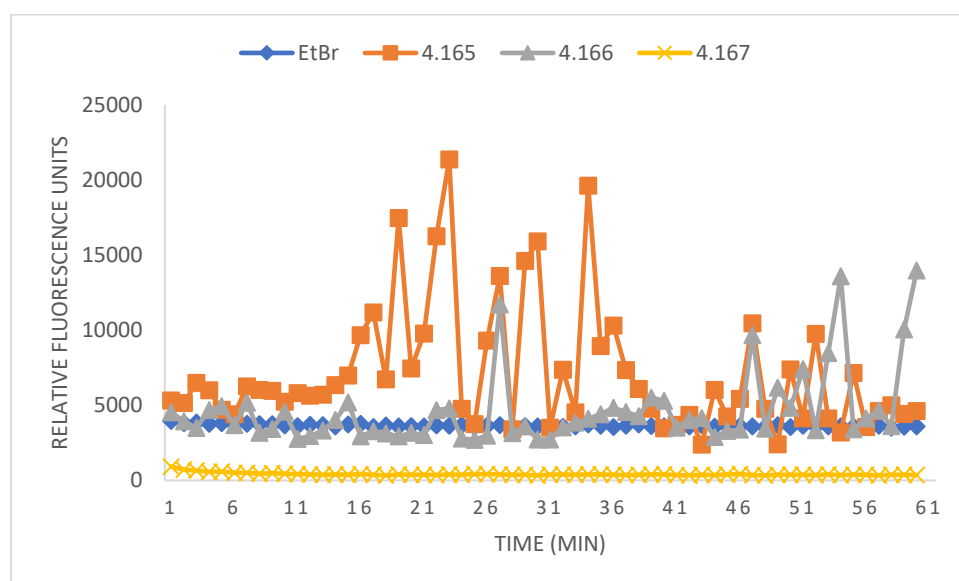


Figure 4.27 Fluorescence curves of EtBr and compounds **4.165**, **4.166**, and **4.167**.

The analysis of the curves shows that compounds **4.165** and **4.166** display both an erratic pattern and high relative fluorescence values. For this reason, these compounds could not be deemed as bacterial EPIs in this instance, unlike compound **4.167**, with a low and constant fluorescence throughout time.

Compounds **4.167** and **4.168** were then visualized in the cytoplasmic side of the NorA homology model, in order to understand how it is predicted to interact with this target. For the substrate binding site of AcrB, compound **4.167** was the one chosen to check the residues it was likely to interact.

In the cytoplasmic side of NorA, compound **4.167** was not predicted to establish any polar interaction with the neighboring residues, although weaker interactions, such as van der Waals, still occur. In the case of **4.168**, it is predicted to establish a polar interaction between a carbonyl and Ile-346, as can be seen in **Figure 4.28**.

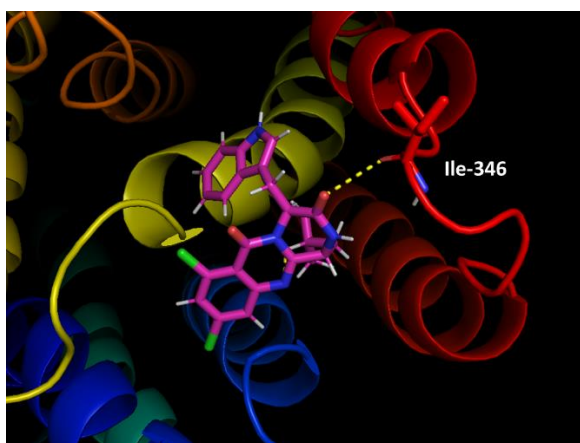


Figure 4.28 Interactions between compound **4.168** and NorA.

In the case of compound **4.167** and AcrB, it was shown that one carbonyl can interact with both Ser-46 and Gln-89. The nitrogen in the indole moiety can establish a hydrogen interaction with Glu-130, as can be observed in **Figure 4.29**.

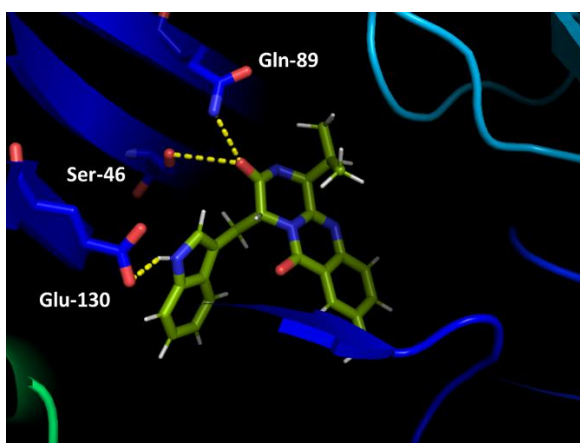


Figure 4.29 Interactions between compound **4.167** and AcrB.

Compounds **4.167** and **4.168** were tested for their potential of inhibiting biofilm formation in *S. aureus* 272123 and QS.

4.2.7.4 Inhibition of biofilm formation

Since these compounds had been tested for their capability of inhibiting biofilm formation in reference strains of *S. aureus*, they were only tested for *S. aureus* 272123. Compound **4.167** was applied at half its MIC, 50 μ M, while compound **4.168** was tested at 100 μ M, and reserpine, the positive control, at 25 μ M (inhibition of 90.3 ± 0.04 % of biofilm formation).

The results obtained were an inhibition of 81.0 ± 0.06 % by **4.167** and 91.4 ± 0.03 % by **4.168**, showing comparable and better results than reserpine, respectively.

4.2.7.5 Inhibition of quorum-sensing

None of the fiscalin derivatives were able to inhibit QS in wt85, *S. marcescens* AS-1, and in the pair EZF and CV026.

4.2.8 Metabolites from marine-derived fungi

A library of 19 structurally diverse metabolites isolated from marine-derived fungi was tested for the same relevant activities. These compounds can be further divided by the origin and chemical class of the compounds.

- Six compounds were obtained from the soil fungus *Neosartorya siamensis* (KUFC 6349) (162):
 - Five indole alkaloids:
 - Tryptoquivaline F (**4.170**);
 - Tryptoquivaline H (**4.171**);
 - Tryptoquivaline L (**4.172**);
 - Tryptoquivaline O (**4.173**);
 - 3'-(4-Oxoquinazolin-3-yl)spiro(1*H*-3,5'-oxolone)-2,2'-dione (**4.174**).
 - One meroditerpene:
 - Chevalone C (**4.175**).
- Four compounds were obtained from the marine-derived fungus *Neosartorya takakii* (KUFC 7898), which was isolated from the marine macroalga *Amphiroa* sp. (163):
 - Two meroditerpenes:
 - Chevalone B (**4.176**);

- Aszonapyrone A (**4.177**).
 - Two indole alkaloids:
 - Aszonalenin (**4.178**);
 - Acetyl aszonalenin (**4.179**).
- One compound was obtained from the diseased coral-derived fungus *Neosartorya laciniosa* (KUFC 7869) (164):
 - One meroditerpene:
 - Aszonapyrone B (**4.180**).
- Eight compounds were obtained from a marine-derived *Aspergillus elegans* (KUFA 0015), which was isolated from the marine sponge *Monanchora unguiculata* (165):
 - Three isochromones:
 - Ochratoxin A (**4.181**);
 - Ochratoxin A methyl ester (**4.182**);
 - Ochratoxin B (**4.183**).
 - Five dimeric naphthopyranones:
 - Rubrosulphin (**4.184**);
 - Rubrosulphin diacetate (**4.185**);
 - Vioxanthin (**4.186**);
 - Viomellein (**4.187**);
 - Xanthomegnin (**4.188**).

The structures of compounds **4.170** – **4.188** are comprised in **Figure 4.30**.

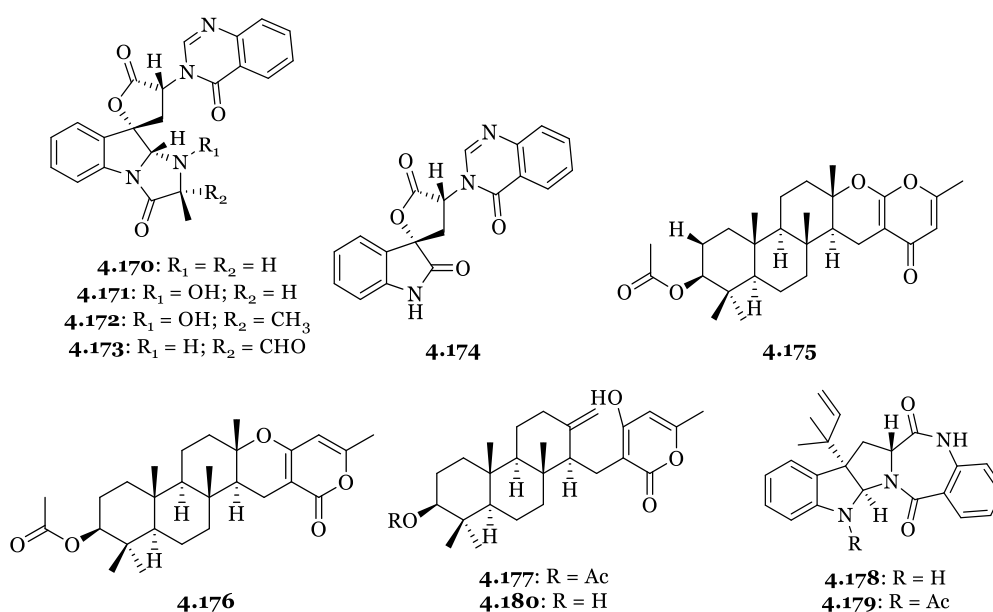


Figure 4.30 Structures of the metabolites from marine-derived fungi tested.

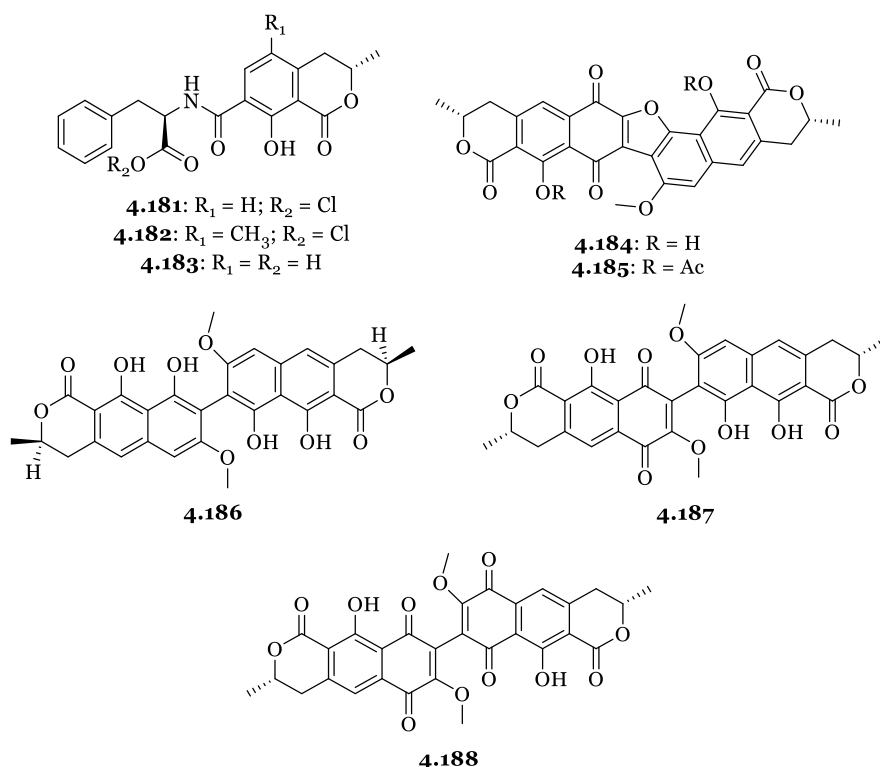


Figure 4.30 Structures of the metabolites from marine-derived fungi tested (cont.).

The antimicrobial activity of these compounds has previously been studied, and therefore it will not be discussed in this instance (162, 163, 165). The focus was then on the strains used to study the EP inhibition, which will be discussed in more detail.

4.2.8.1 Docking studies

The structurally diverse compounds **4.170** – **4.188** were studied for their predicted affinity to the AcrAB-TolC and NorA EPs, through docking studies. The docking scores obtained can be seen in **Table 4.31**.

Table 4.31 Docking scores for the metabolites from marine-derived fungi in bacterial efflux pumps.

Compound	Docking score (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
4.170	-9.2	-3.2	-7.9	-6.5	-8.5	0.1	-6.7
4.171	-9.6	17.4	-7.0	-6.2	-8.4	-0.3	-6.9
4.172	-9.3	1.7	-6.7	-6.1	-8.6	1.1	-6.9
4.173	-9.1	25.2	-6.9	-5.8	-8.5	1.7	-6.6
4.174	-8.9	0.7	-6.8	-6.2	-8.3	0.4	-6.8
4.175	-9.2	22.4	-6.7	-5.6	-9.0	-3.1	-6.7
4.176	-9.4	34.9	-6.9	-6.2	-9.4	-3.6	-7.4
4.177	-8.4	11.6	-6.8	-6.2	-8.4	-1.7	-5.8

Compound	Docking score (kcal/mol)						
	AcrB		AcrA		TolC	NorA	
	SBS	HT	HH	LD		BCR	CS
4.178	-8.3	2.9	-6.4	-5.5	-7.5	1.2	-6.7
4.179	-8.3	26.1	-6.4	-6.4	-7.7	2.8	-6.5
4.180	-8.5	11.0	-6.3	-6.2	-8.6	-2.3	-7.6
4.181	-8.0	-2.5	-6.3	-5.8	-7.5	-7.4	-7.4
4.182	-8.0	-5.8	-6.3	-5.6	-7.4	-7.5	-6.4
4.183	-8.0	-2.1	-6.8	-6.4	-8.0	-7.5	-5.9
4.184	-9.1	15.1	-6.8	-6.9	-8.5	7.3	19.8
4.185	-9.7	31.6	-6.7	-5.7	-8.1	4.8	28.0
4.186	-9.0	7.5	-6.2	-6.0	-9.3	3.6	-4.2
4.187	-9.3	40.5	-5.9	-5.9	-8.4	2.5	4.4
4.188	-10.9	29.9	-5.8	-5.2	-9.2	7.7	-5.3
PAβN	-7.1	-4.7	-5.8	-4.9	-7.1	-	-
Reserpine	-	-	-	-	-	1.0	-4.6

SBS: Substrate binding site; **HT:** Hydrophobic trap; **HH:** Helical hairpin; **LD:** Lipoyl domain; **BCR:** Binding core region; **CS:** Cytoplasmic side; **PAβN:** Phenyl-arginine β-naphthylamide.

The analysis of the docking scores in **Table 4.31** showed that, within the AcrAB-TolC efflux system, all the compounds are predicted to display more affinity towards the AcrB portion, and generally display better docking scores against the substrate binding site, similarly to the positive controls. On the other hand, the hydrophobic trap of AcrB is the site with the least predicted affinity, displaying very high docking scores. The periplasmic adaptor AcrA is a portion for which the compounds are predicted to globally display less affinity, which is in line with the results obtained for the other derivatives.

In the NorA homology model, there is a clear preference towards the cytoplasmic side, as does the alkaloid reserpine. Once again, the compounds display high docking scores towards the binding core region. One explanation for these values may be related to the bulkiness and rigidity of the compounds, which may lead to them not fitting in the regions described as potential binding sites.

Nevertheless, since the compounds displayed favorable results for at least one site of AcrB and NorA, and the similarity of their docking scores to those obtained for the positive controls, all the compounds were tested for the relevant activities regarding the circumvention of AMR.

4.2.8.2 Antibacterial activity

The antibacterial activity of compounds **4.170 – 4.188** was assessed in *S. aureus* 272123 and *S. Typhimurium* SL1344, since these were the strains to be used in the inhibition of

bacterial EPs assay, and they had already been tested for reference strains (162, 163, 165).

Table 4.32 comprises these results.

Table 4.32 Minimum inhibitory concentration of the metabolites from marine-derived fungi tested against bacteria.

Compound	MIC (μ M)	
	<i>S. aureus</i> 272123	<i>S. Typhimurium</i> SL1344
4.170	>100	>100
4.171	>100	>100
4.172	>100	>100
4.173	>100	>100
4.174	>100	>100
4.175	25	>100
4.176	>100	>100
4.177	12.5	>100
4.178	12.5	>100
4.179	100	>100
4.180	>100	>100
4.181	>100	>100
4.182	50	>100
4.183	50	>100
4.184	>100	>100
4.185	>100	>100
4.186	6.25	>100
4.187	6.25	>100
4.188	100	>100

MIC: Minimum inhibitory concentration.

None of the tested compounds is active against *S. Typhimurium* SL1344, while **4.175**, **4.177** – **4.179**, **4.182**, **4.183**, and **4.186** – **4.188** displayed growth inhibition against *S. aureus* 272123. Even though compounds **4.184** and **4.185** are structurally related to **4.186** – **4.188**, the first two compounds present a pyran ring between the two naphthopyranone moieties, which confers these compounds a rigid structure, which is absent in **4.186** – **4.188**, and could be related to the different profiles of these compounds. The fact that compounds **4.184** – **4.188** have already been tested for their antibacterial activity in susceptible and resistant strains, and the outcome was the same, with **4.186** – **4.188** exhibiting promising antibacterial activity, whereas **4.184** and **4.185** did not display any observable MIC, further emphasizes this point (165).

In this case, a few SAR conclusions could be gathered regarding the antibacterial activity in *S. aureus* 272123. For the alkaloids **4.178** and **4.179**, the substitution of the amine in the indoline moiety yields less potent compounds. For the dimeric naphthopyranones **4.184** –

4.188, substitution of the naphthodiol moiety with a naphthoquinone core, in **4.188**, also leads to a decrease of potency in the same model.

4.2.8.3 Bacterial efflux pump inhibition

As previously done, compounds **4.170** – **4.188** were studied for their potential to inhibit bacterial EPs through the evaluation of the accumulation of EtBr, a known EP substrate. The models chosen were *S. aureus* 272123, using reserpine as a positive control, and *S. Typhimurium* SL1344, using CCCP as a positive control. The compounds were used at 50 μ M, if they did not display bacterial growth inhibition, or one-third of their MIC for the tested strains, whereas the controls were applied at 25 μ M.

Since many compounds displayed MIC for *S. aureus* 272123, the concentration used for this assay, as well as the RFI values, are present in **Table 4.33**. All the compounds were tested at 50 μ M in *S. Typhimurium* SL1344.

Table 4.33 RFI of the tested metabolites from marine-derived fungi.

Compounds	<i>S. aureus</i> 272123		<i>S. Typhimurium</i> SL1344	
	Concentration (μ M)	RFI $\pm$ SD	Concentration (μ M)	RFI $\pm$ SD
4.170	50	-0.01 $\pm$ 0.04	50	0.03 $\pm$ 0.06
4.171		-0.21 $\pm$ 0.05		-0.15 $\pm$ 0.04
4.172		0.15 $\pm$ 0.05		0.07 $\pm$ 0.03
4.173		-0.02 $\pm$ 0.01		0.09 $\pm$ 0.04
4.174		0.07 $\pm$ 0.02		0.08 $\pm$ 0.02
4.175	8.33	0.99 $\pm$ 0.11		-0.02 $\pm$ 0.01
4.176	50	1.43 $\pm$ 0.20		0.22 $\pm$ 0.01
4.177	4.17	0.55 $\pm$ 0.01		3.38 $\pm$ 0.10
4.178		0.43 $\pm$ 0.07		2.38 $\pm$ 0.12
4.179	33.3	0.26 $\pm$ 0.06		0.22 $\pm$ 0.03
4.180	50	0.41 $\pm$ 0.02		-0.14 $\pm$ 0.01
4.181		0.29 $\pm$ 0.01		0.45 $\pm$ 0.02
4.182	16.67	-0.37 $\pm$ 0.02		-0.12 $\pm$ 0.02
4.183		-0.19 $\pm$ 0.01		-0.05 $\pm$ 0.01
4.184	50	-0.17 $\pm$ 0.03		-0.03 $\pm$ 0.01
4.185		1.09 $\pm$ 0.09		-0.11 $\pm$ 0.01
4.186	2.08	-0.07 $\pm$ 0.03		-0.72 $\pm$ 0.01
4.187		0.31 $\pm$ 0.09		-0.63 $\pm$ 0.01
4.188	33.3	-0.27 $\pm$ 0.13		-0.36 $\pm$ 0.01
Reserpine	25	0.59 $\pm$ 0.03	-	-
CCCP	-	-	25	0.33 $\pm$ 0.04

RFI: Relative fluorescence index; **SD**: Standard deviation; **CCCP**: carbonyl cyanide 3-chlorophenylhydrazone.

The marine-derived compounds **4.175**, **4.176**, and **4.185**, showed potential at inhibiting the efflux in *S. aureus* 272123, while compounds **4.177**, **4.178** and **4.181** showed the same potential for *S. Typhimurium* SL1344. As compounds **4.176** – **4.178**, and **4.181** showed a relatively high fluorescence, albeit lower than the positive control reserpine, they were examined for their autofluorescence, as a way to conclude if the effects obtained were due to the fluorescence of EtBr or the compound itself. The fluorescence curves can be seen in **Figure 4.31**.

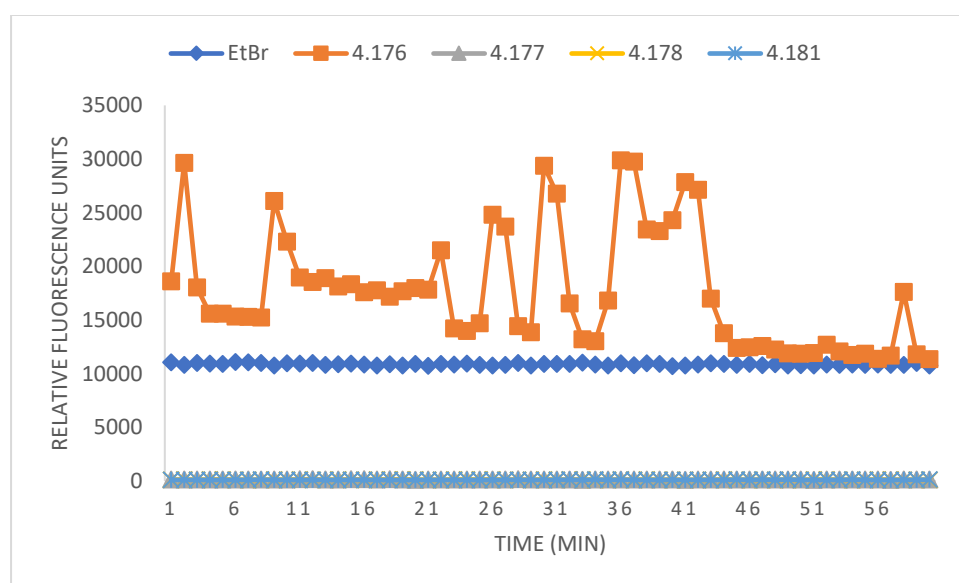


Figure 4.31 Fluorescence curves of EtBr and compounds **4.176** – **4.178** and **4.181**.

The results show that compound **4.176** has a high and irregular fluorescence pattern, and therefore no conclusions can be drawn about the potential of this compound to act as a bacterial EPI, unlike compounds **4.177**, **4.178** and **4.181**.

The compounds were then visualized in the respective site towards which they showed to have more predicted affinity. As such, compounds **4.175** and **4.185** were analyzed for their interactions with residues in the cavity in the cytoplasmic side of the NorA homology model, while compounds **4.177**, **4.178**, and **4.181** were visualized in the substrate binding site of NorA.

Considering NorA, compound **4.175** did not show any predicted polar interactions with this homology model, but weaker interactions still happen (not shown). Differently, compound **4.185** is predicted to establish interactions with Lys-31, Leu-224, and Ser-226, as it can be noted in **Figure 4.32**.

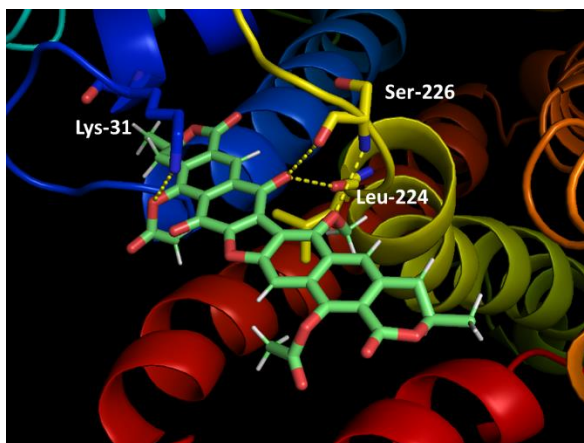


Figure 4.32 Interactions between compound **4.185** and the NorA homology model.

In the case of AcrB, compound **4.177** showed predicted polar interactions with Val-175 and Gln-176, as well as a hydrogen interaction with Asn-274 (**Figure 4.33, A**). In **Figure 4.33, B**, it can be seen that compound **4.178** can establish interactions with Ser-46, Gln-89, Lys-163, and Lys-770. Lastly, compound **4.181** was predicted to interact with Thr-87, Gln-176, Ser-180, Asn-274, and Arg-620 (**Figure 4.33, C**).

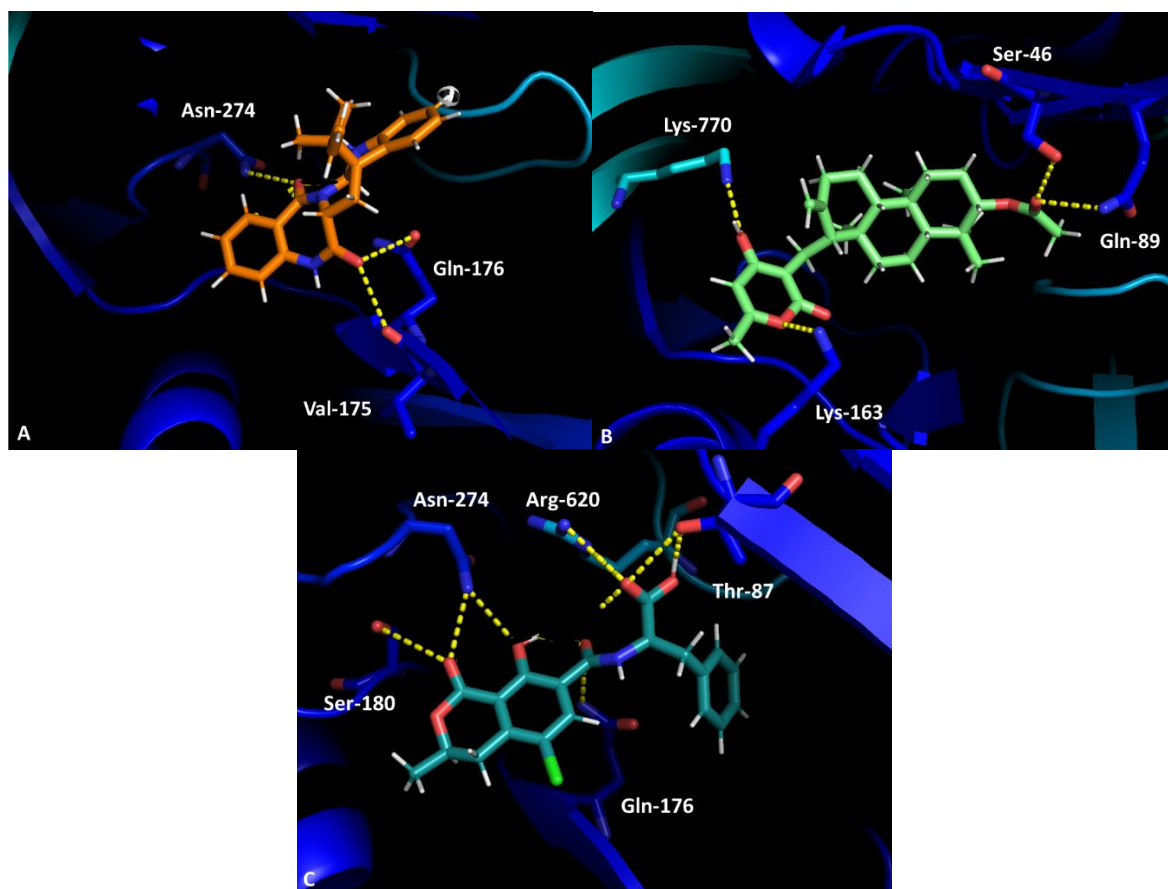


Figure 4.33 Molecular visualization of the metabolites from marine-derived fungi in the substrate binding site of AcrB. (A) Interactions between compound **4.177** and AcrB; (B) Interactions between compound **4.178** and AcrB; (C) Interactions between compound **4.181** and AcrB.

Given the results obtained, compounds **4.175**, **4.177**, **4.178**, **4.181**, and **4.185** were tested for the inhibition of biofilm formation and QS.

4.2.8.4 Inhibition of biofilm formation

The compounds that displayed EP inhibition, **4.175**, **4.177**, **4.178**, **4.181**, and **4.185**, were studied for their ability to inhibit the formation of biofilm. Reserpine was used herein as a positive control, at 50 μ M, and the results were expressed in the percentage of biofilm in comparison to the control group.

The compounds were tested at 100 μ M or half their MIC for the strains used, *S. aureus* ATCC 25923 and *S. aureus* 272123. The MIC of compounds **4.177**, **4.178**, **4.181**, and **4.185** had been previously determined for *S. aureus* ATCC 25923, with compound **4.177** showing a MIC of 18 μ M (8 μ g/mL), and **4.178**, **4.181** and **4.185** both showing a MIC >100 μ M (162, 165). Since compound **4.175** had not previously been studied in this strain, an assay was performed in order to know the MIC of this compound, which was 12.5 μ M.

The concentrations used in both strains, as well as the percentage of biofilm inhibition, are shown in **Table 4.34**.

Table 4.34 Inhibition of biofilm formation by metabolites from marine-derived fungi.

Compounds	<i>S. aureus</i> ATCC 25923		<i>S. aureus</i> 272123	
	Concentration (μ M)	Biofilm inhibition $\pm$ SD (%)	Concentration (μ M)	Biofilm inhibition $\pm$ SD (%)
4.175	6.25	0	12.5	78.5 $\pm$ 7.97
4.177	9	72.3 $\pm$ 2.29	6.25	93.4 $\pm$ 0.91
4.178	100	63.2 $\pm$ 2.42	6.25	93.5 $\pm$ 2.22
4.181	100	0	100	80.8 $\pm$ 5.27
4.185	100	0	100	92.6 $\pm$ 1.97
Reserpine	25	22.3 $\pm$ 5.10	25	72.1 $\pm$ 4.24

SD: Standard deviation.

The results showed that all the tested compounds were more effective at the inhibition of biofilm formation in *S. aureus* 272123, being even more effective than the positive control. Furthermore, compounds **4.177** and **4.178** were also effective against biofilm formed by *S. aureus* ATCC 25923, albeit at a lower extent.

It is noteworthy that compounds **4.177** and **4.178** were able to decrease biofilm produced by *S. aureus* 272123 at the low concentration of 6.25 μ M. Furthermore, the reduction of biofilm caused by compounds **4.175** and **4.185** could be related to the inhibition of efflux systems, as these compounds were effective EPIs in this strain and these mechanisms are closely related.

4.2.8.5 Inhibition of quorum-sensing

Compounds **4.175**, **4.177**, **4.178**, **4.181**, and **4.185** were tested in three models of QS inhibition: wt85, *S. marcescens* AS-1, and the pair EZF and CVO26. Only compound **4.177** was effective for the latter model, with a discoloration of 30 ± 0.1 mm, which was still lower than that of PMZ, the positive control, which showed 41 ± 0.5 mm of discoloration for the same system.

The fact that **4.177** is also an EPI for a Gram-negative strain could be related to these results, since both strains in this model are Gram-negative.

4.2.8.6 Cytotoxicity

The metabolites from marine-derived fungi **4.175**, **4.177**, **4.178**, **4.181**, and **4.185** were evaluated for their cytotoxicity in mouse embryonic fibroblasts (NIH/3T3 cell line), using doxorubicin as a positive control. The IC_{50} of the compounds are in **Table 4.35**.

Table 4.35 Cytotoxicity of the metabolites from marine-derived fungi against NIH/3T3 cell line.

Compound	IC_{50} (μ M)
4.175	31.0 ± 0.13
4.177	25.0 ± 2.37
4.178	16.7 ± 1.40
4.181	80.0 ± 3.66
4.185	34.5 ± 3.14
Doxorubicin	12.1 ± 0.81

IC_{50} : half maximal inhibitory effect.

The results show that all the compounds displayed cytotoxicity for the tested cell line, in different extents. The cytotoxicity of compounds **4.175**, **4.177** and **4.178** does not compromise the use of these compounds as antibacterials against *S. aureus* 272123, or their use as inhibitors of biofilm formation. The use of **4.175** as an EPI in the same strain is also safe. Compounds **4.177** and **4.178** were tested as EPIs for *S. Typhimurium* at a concentration higher than their IC_{50} , therefore studies with lower concentrations are suggested.

4.3 Concluding remarks

Throughout this chapter an extensive phenotypic screening of a library of 175 structurally diverse compounds was described. This study included docking studies, antimicrobial activity, potentiation of antibiotics in resistant strains, EP inhibition, and inhibition of mechanisms related to EPs, such as biofilm formation and QS, culminating with the

evaluation of the safety profile of these compounds, through cytotoxicity studies. As a way to have a better picture of how the different classes of compounds behaved face to the activities tested, they were percentually compared, as can be seen in **Figure 4.34**. This included the thioxanthenes described in **Chapter 3**.

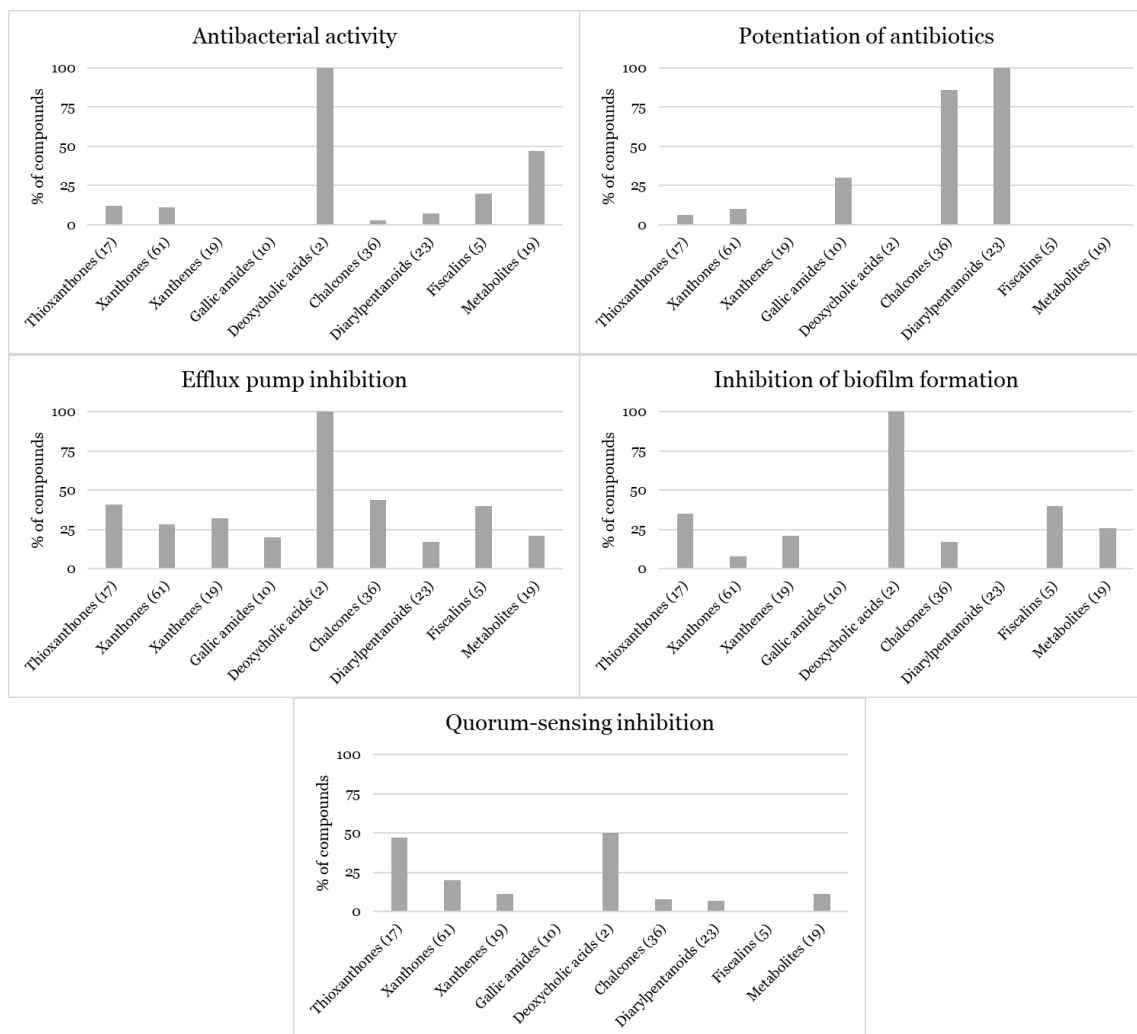


Figure 4.34 Diverse classes of compounds by activity, in percentage.

Overall, the deoxycholic acid derivatives appear to be the most effective by producing effects in most of the phenotypic studies performed. Regarding intrinsic antibacterial activity, approximately 50% of the metabolites from marine-derived fungi were active for the tested strains. This is not surprising, as the fungi from which they were isolated produce metabolites aimed to protect themselves from external threats, such as bacteria and higher organisms, which can also explain their high cytotoxicity.

When it comes to the potentiation of antibiotics, all the diarylpentanoids were able to show at least a 2-fold reduction in the MIC of the antibiotics used, as well as 90% of the structurally related chalcones. These results, however, do not seem to be related to the

inhibition of bacterial EPs, where the tricyclic scaffolds appeared to be among the most effective, with the xanthonenes, thioxanthonenes, and xanthenes displaying potential. The fiscalins also showed a good percentage of EP inhibition. Chalcones were also effective in this scope, but in a much lower number than that of the potentiation assay. For these classes, diarylpentanoids and chalcones, other mechanisms related to AMR rather than EPI deserve to be investigated.

Concerning the mechanisms related to EPs that were studied, inhibition of biofilm formation and QS, none of the classes studied in this chapter were better than thioxanthonenes. Since the scope of this work was finding compounds with potential in the circumvention of AMR mechanisms, thioxanthonenes and xanthonenes stand out. Nevertheless, hit compounds could be found within all the chemical classes studied: xanthonenes **4.37** and **4.67**, deoxycholic amide **4.105**, chalcones **4.129**, **4.131**, **4.132**, **4.134**, **4.136**, **4.137**, **4.139**, and **4.141**, diarylpentanoid **4.160**, fiscalins **4.167** and **4.168**, and the marine-derived **4.175**, **4.181**, and **4.185**.

Docking studies do not seem to be helpful in providing the most potent EPIs with the model used. On the other hand, there is no evidence that the compounds are interacting with the sites considered in the docking studies, as EPs of the RND and MFS family do not have a substrate binding site, and the compounds could act in other sites. It should also be noted that the use of a homology model of NorA could include errors, as it is not the real structure of the EP.

Since deoxycholic amide **4.105** showed potential as an antibacterial and an EPI, a library of derivatives was planned and evaluated in further studies. Thus, in the next chapter, the synthesis of new deoxycholic amides is proposed, and studies into their antibacterial activity and resistance mechanisms will be explored.

4.4 Experimental section

4.4.1 Docking studies

The crystal structure of the AcrB (PDB: 4DX5) (166), AcrA (PDB: 2F1M) (167), and TolC (PDB: 1EK9) (168) portions of the AcrAB-TolC bacterial efflux system, downloaded from the PDB (169), were used for this study. The known EPIs PA β N and reserpine, along with the tested compounds were drawn with ChemDraw (PerkinElmer Informatics) and minimized using ArgusLab. Docking was carried out using AutoDock Vina (Scripps, CA, USA) (170), in the sites described in (171, 172). The NorA efflux pump does not have an available crystal structure, and a homology model was prepared. The model was generated using the Swiss Model server (173) and the sequence deposited in Uniprot (Q5HHX4) (174), using the EmrD pump from *E. coli* (PDB: 2GFP) as the homolog, as described in (175). The

top nine poses were collected for each molecule and the lowest docking score value was associated with the most favorable binding conformation.

PyMOL (Schrödinger, NY, USA) (176) was used for visual inspection of results and graphical representations.

4.4.2 Biological activity

4.4.2.1 Culture media and chemicals

The culture media used in the experiments were the following: MHB II (Sigma-Aldrich, St- Louis, MO, USA and Biokar Diagnostics, Allone, Beauvais, France), LB-B (Sigma, St. Louis, MO, USA), TSB (Scharlau Chemie S. A., Barcelona), and TSA (Biokar Diagnostics, Allone, Beauvais, France), were purchased. SDA was purchased from bio-Mérieux, Marcy L'Etoile, France, RPMI-1640 broth medium from Biochrom AG, Berlin, Germany, which was buffered with MOPS, purchased from Sigma-Aldrich, St. Louis, MO, USA, to pH 7.0. The media LB*-A, used for the QS inhibition assays, was prepared in-house, according to the formula: 1.0 g yeast extract (Merck, Darmstadt, Germany), 10.0 g tryptone (Biolab, Budapest, Hungary), 10.0 g NaCl (Molar Chemicals, Halásztelek, Hungary), 1.0 g K₂HPO₄ (Biolab, Budapest, Hungary), 0.3 g MgSO₄ x 7H₂O (Reanal, Budapest, Hungary), 5 ml Fe-EDTA stock solution and 20.0 g of bacteriological agar (Molar Chemicals, Halásztelek, Hungary) per 1 L of media.

DMSO, MTT, SDS, PBS (pH 7.4), EtBr, reserpine, CCCP, PMZ, and CV were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Doxorubicin 2 mg/mL was purchased from Teva Pharmaceuticals, Budapest, Hungary. The antibiotic CTX was purchased from Duchefa Biochemie (Haarlem, The Netherlands) and VAN from Oxoid (Basingstoke, England). Bacteria were purchased from ATCC, and the cell line NIH/3T3 was purchased from Sigma (Steinheim, Germany).

4.4.2.2 Microorganisms

As Gram-positive strains, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, methicillin and ofloxacin-resistant *S. aureus* 272123 clinical isolate, and environmental isolate VRE *E. faecalis* B3/101 (177) were used. As Gram-negative strains, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, the *acrA* gene inactivated mutant *S. enterica* serovar Typhimurium SL1344, and clinical isolates of the ESBL *E. coli* SA/2 were investigated in this study.

For the QS tests, all the strains used were Gram-negative. The bacteria used were *C. violaceum* wild type 85 characterized by the AHL signal molecule-mediated production of the purple violacein pigment, capable of endogenous QS-signal molecule-production (*N*-

hexanoyl-L-HSL), *C. violaceum* CVO26, a Tn5 transposase-mutant, AHL-signal molecule indicator strain (produces purple violacein pigment in the presence of AHL), which is incapable of endogenous QS-signal molecule-production, but useful in the detection of external stimuli, *S. paucimobilis* Ezf 10-17, AHL-producing-strain (used with *C. violaceum* CVO26), and *S. marcescens* AS-1, characterized by the production AHL signal molecule-mediated production of the orange-red pigment prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin), capable of endogenous QS-signal molecule-production (*N*-hexanoyl-L-HSL) (178).

As yeast *C. albicans* ATCC 10231, and as filamentous fungus *A. fumigatus* ATCC 204305 and a dermatophyte (*T. rubrum* FF5, clinical isolate) were used. Cultures were obtained in SDA.

4.4.2.3 Antibacterial assay and potentiation of antibiotics

The MIC for each compound was determined by broth microdilution method in a 96-well plate according to the CLSI guidelines (179). The media used was MHB II. The concentrations tested ranged from 100 μ M to 0.195 μ M and were prepared from a stock solution of 10 mM in DMSO, for *S. aureus* 272123 and *S. Typhimurium* SL1344; for the other strains, the concentrations tested ranged from 64 μ g/mL to 4 μ g/mL and were prepared from a stock solution of 10 mg/mL in DMSO. DMSO was used in subinhibitory concentrations (1 % *v/v*). The MIC was determined by visual inspection.

The combined effect of the compounds and clinically relevant antimicrobial drugs was evaluated by determining the antibiotic MIC in the presence of each compound. Briefly, the MIC of CTX and VAN, for *E. coli* SA/2 and *E. faecalis* B3/101, respectively, were determined in the presence of the highest concentration of each compound tested that did not affect bacterial growth when the compound was used alone. The antibiotic tested was serially diluted whereas the concentration of each compound was kept fixed. The compounds that displayed antibacterial activity were tested at half their MIC. The compounds that did not show an observable MIC were tested at 64 μ g/mL. Antibiotic MICs were determined as described above.

4.4.2.4 Efflux pump inhibition assay

The compounds were evaluated for their ability to inhibit the efflux of EtBr, an efflux pump substrate in *S. enterica* serovar Typhimurium SL1344 and *S. aureus* 272123 strains, through real-time fluorimetry, monitoring the intracellular accumulation of EtBr, an efflux pump substrate.

This was determined by the automated method using a CLARIOstar Plus plate reader (BMG Labtech, Ortenberg, Germany). Reserpine and CCCP were applied at 25 μ M as positive controls, and the solvent DMSO was applied at 1 % *v/v*. The bacterial strains were incubated in an appropriate culture media (TSB – *S. aureus* 272123; LB-B – *S. Typhimurium* SL1344) at 37 °C until they reached an OD of 0.6 at 600 nm. The culture was centrifuged at 13,000 $\times$ g for 3 minutes, and the pellet washed and resuspended with PBS (pH 7.4). The suspension was centrifuged again in the same conditions and resuspended in PBS.

The compounds were applied at 50 μ M, or at one-third their MIC, in a solution of a non-toxic concentration of EtBr (1 μ g/mL) in PBS. Then, 50 μ L of this solution were transferred into a 96-well black microtiter plate (Greiner Bio-One Hungary Kft, Mosonmagyaróvár, Fertőszor, Hungary), and 50 μ L of bacterial suspension (OD₆₀₀ = 0.6) were added to each well. The plates were placed into the CLARIOstar plate reader, and the fluorescence was monitored at excitation and emission wavelengths of 530 nm and 600 nm, respectively, every minute for one hour in a real-time basis. From the real-time data, the activity of the compounds, namely the RFI of the last time point (minute 60) of the EtBr accumulation assay, was calculated according to the following equation:

$$RFI = \frac{RF_{\text{treated}} - RF_{\text{untreated}}}{RF_{\text{untreated}}}$$

where RF_{treated} is the RF at the last time point of EtBr accumulation curve in the presence of the compound, and RF_{untreated} is the RF at the last time point of the EB accumulation curve of the untreated control, having only the solvent (DMSO) control (180). The samples were tested in triplicate, and the RFI presented comes from the average of these three values. The SD was calculated automatically.

4.4.2.5 Inhibition of biofilm formation

The compounds were tested for their ability to decrease the formation of biofilm. The bacterial strains used were the Gram-positive *S. aureus* ATCC 25923 and *S. aureus* 272123.

The detection of the biofilm formation was carried out using the dye CV (0.1 % *v/v*). The initial inoculum was incubated in TSB overnight, and then diluted to an OD₆₀₀ of 0.1. Then, the bacterial suspension was added to 96-well microtiter plates and the compounds were added at a sub-MIC concentration. This meant that compounds with an observable MIC were added at half their MIC, while the rest of the compounds were added at the concentration of 100 μ M. The final volume in each well was 200 μ L. Reserpine was used as positive control, as it was the same compound used in the efflux pump inhibition assay and

it has shown activity in the inhibition of biofilm formation in *S. aureus* strains (181). The plates were incubated at 30 °C for 48 hours, with gentle stirring (100 rpm). After this incubation period, the TSB medium was discarded, and the plates were washed with tap water to remove unattached cells. Afterwards, 200 µL of a 0.1 % v/v CV solution were added to the wells and incubated for 15 minutes at room temperature. Then, the CV solution was removed from the wells, and the plates were washed again with tap water, and 200 µL of a 70 % ethanol solution were added to the wells. The biofilm formation was determined by measuring the OD₆₀₀ using a Multiscan EX ELISA plate reader (Thermo Labsystems, Cheshire, WA, USA). The anti-biofilm effect of the compounds was expressed in the percentage (%) of decrease of biofilm formation.

4.4.2.6 Inhibition of quorum-sensing

The QS inhibitory effect of the compounds was examined on the EZF and the sensor CVO26 strains, on the wt85 strain, and on *S. marcescens*. The method used was the parallel inoculation method, where pair combinations of the used sensor strain CVO26 and the AHL producing strain EZF were inoculated directly onto the LB*-A agar surface in parallel, at an approximate distance of 5 mm from each other. *S. marcescens* AS-1 and wt85 were inoculated as a single line.

Filter paper disks (7 mm in diameter) were placed on the center of the inoculated line(s) and impregnated with 8 µL of a solution of 10 mM of the compounds. PMZ was used as positive control, as previous results have demonstrated its activity as a QS inhibitor (182).

The agar plates were incubated at room temperature (20 °C) for 24 – 48 hours. The QS inhibition was accessed visually, through the inhibition of pigment production. The discolored, but intact, bacterial colonies were measured with a ruler (182).

4.4.2.7 Antifungal activity

The susceptibility tests for yeasts were performed based on the CLSI description for the broth microdilution method on the reference document M27A-3 for yeasts (183) and M38-A2 (184) for filamentous fungi, in RPMI-1640 broth culture media. The concentrations tested ranged from 128 µg/mL to 32 µg/mL.

The MIC was considered the lowest concentration that was able to totally inhibit the growth when comparing to control (non-treated microorganism in culture medium). A DMSO control (microorganism in RPMI with DMSO (1% v/v) was included.

4.4.2.8 Cytotoxicity in NIH/3T3 cell line

A 10.0 mM stock solution of each compound was prepared in DMSO. All stock solutions were stored at -80°C and freshly diluted on the day of the experiment in fresh cell culture medium (ensuring that DMSO did not exceed 0.1 % DMSO concentration of the exposure media).

Mouse fibroblasts (NIH/3T3, ATCC CRL-1658TM) were cultivated in DMEM (Gibco 52100-039) and supplemented with 10 % heat-inactivated fetal bovine serum (Biowest, VWR International Kft, Debrecen, Hungary), 2 mM of L-glutamine, 1 mM Na pyruvate, 100 U/L and 10 mg/L penicillin/streptomycin mixture (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), respectively, and 0.1 % nystatin (8.3 g/L in ethylene glycol). The adherent cells were detached using a combination of 0.25 % Trypsin-Versene (EDTA) solution for 5 minutes at 37°C . Before each cytotoxicity assay using this cell line, cells were seeded in untreated 96-well flat-bottom microtiter plates, following a 4-hour incubation period in a humidified atmosphere (5 % CO_2 , 95 % air) at 37°C (185).

The cytotoxicity of the most promising compounds was assessed in NIH/3T3 cells, using MTT assay. Prior to the assay, the cells were seeded for 4 hours using 1×10^4 cells/well. The compounds were added by 2-fold serial dilutions to the cells distributed into 96-well flat bottom microtiter plates starting with 100 μM . The plates were incubated for 24 hours, after which a solution of MTT in PBS was added to each well and incubated for another 4 hours. After this, 100 μL of SDS (10% in a 0.01 M HCl solution) were added to each well and incubated overnight at 37°C . Doxorubicin was used as positive control. Cell growth was determined in quadruplicate by measuring OD at $\lambda=540$ nm (reference 630 nm) in a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). The percentage of inhibition of cell growth was determined according to the equation.

$$100 - \left(\frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{medium control}}}{\text{OD}_{\text{cell control}} - \text{OD}_{\text{medium control}}} \right) \times 100$$

The results of the cytotoxicity in the NIH/3T3 cell line were expressed as the mean $\pm$ SD, and the IC_{50} values were obtained by best fitting the dose-dependent inhibition curves in GraphPad Prism 5.03 for Windows software.

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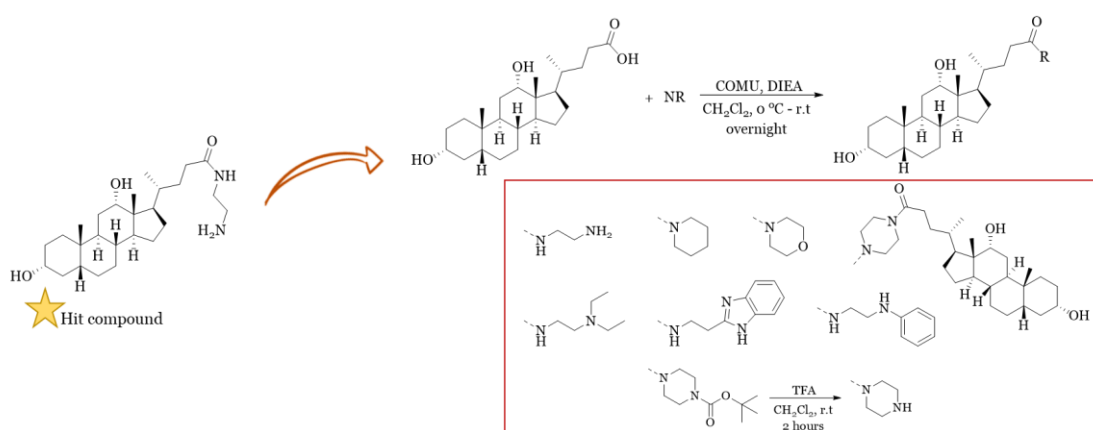
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Chapter 5 – Deoxycholic amides: synthesis and antimicrobial activities



Antibacterial activity

- Reference strains
- Clinical isolates
- Environmental isolates
- Animal isolates
- Fish pathogens

Efflux pump inhibition

- Gram-negative
- Gram-positive

Inhibition of related mechanisms

- Biofilm formation
- Quorum-sensing

Antifungal activity

- Reference strains
- Clinical isolates
- Fish pathogens

Cytotoxicity

- Mouse embryonic fibroblasts

Author contributions: The synthesis of compounds **5.15** – **5.22** was performed by Fernando Durães, under the supervision of Prof. Emília Sousa and Prof. Madalena Pinto. The synthesis of compound **5.12** was performed by Ana Rita Neves, under the supervision of Prof. Marta Correia da Silva and Prof. Emília Sousa. The antibacterial assays in *E. coli* AG100, *E. coli* AG100A, *S. aureus* ATCC 25923, and *S. aureus* 27212, as well as the efflux pump inhibition, inhibition of biofilm formation, and cytotoxicity assays were performed by Nikoletta Szemerédi, under the supervision of Prof. Gabriella Spengler. The antibacterial assays in the remaining bacteria were performed by Fernando Durães, under the supervision of Prof. Paulo Martins da Costa. The antifungal assays were performed by Joana Freitas da Silva and Joana Cardoso, under the supervision of Prof. Eugénia Pinto.

5.1 Introduction

Bile acids are naturally synthesized in the hepatocytes of vertebrates, as metabolites of cholesterol. As such, these compounds are acidic steroids, composed of 24 carbon atoms arranged in a rigid steroidal scaffold and an acidic side chain, presenting varying hydroxy groups, which confers bile acids amphiphilic properties (**Figure 5.1**) (1, 2).

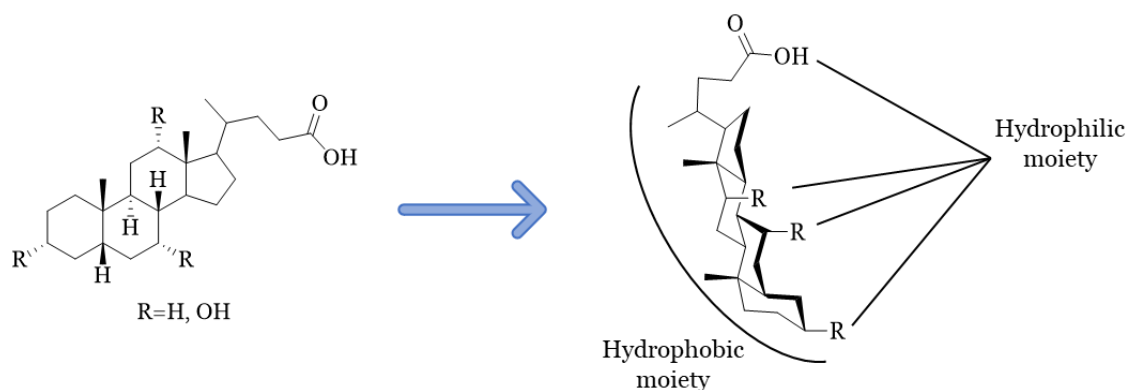


Figure 5.1 General structure of bile acids.

Bile acids carry out fundamental physiological functions, namely in the digestive process. They are secreted into bile in their conjugated form with taurine or glycine, where they play a role in the absorption of fat and hydrophobic vitamins obtained through nutrition. Then, most of them are absorbed in the ileum and returned to the liver, where this process will happen repeatedly (3). However, some bile acids remain in the intestine and undergo further transformations, due to intestinal bacteria, and in some cases a further modification in the liver, hence originating the classification of primary, secondary, and tertiary bile acids (4-6).

The primary bile acids in humans are cholic acid (**5.1, Figure 5.2**) and chenodeoxycholic acid (**5.2, Figure 5.2**), synthesized in the liver directly from the enzymatic metabolization of cholesterol. These are the ones conjugated with taurine and glycine, which can be cleaved in the colon by hydrolases. Then, by the action of intestinal bacteria, they can originate the secondary bile acids, deoxycholic acid (**5.3, Figure 5.2**) and lithocholic acid (**5.4, Figure 5.2**). Ursodeoxycholic acid (**5.5, Figure 5.2**) is obtained after a secondary bile acid undergoes liver reabsorption and suffers redox metabolism by host enzymes, being this bile acid classified as tertiary (7-9).

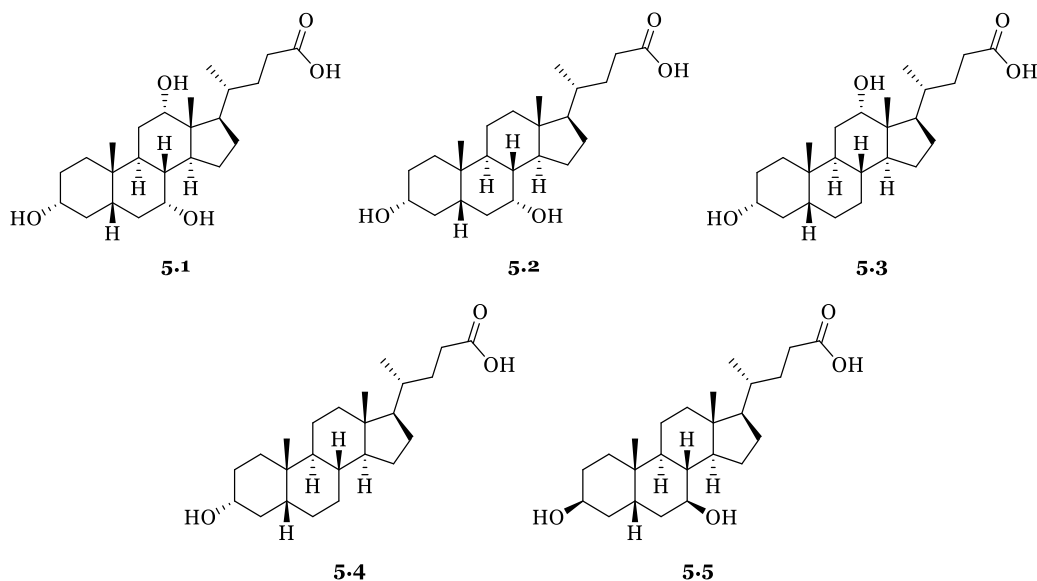


Figure 5.2 Structures of human bile acids **5.1** – **5.5**.

Over the years, bile acids have caught the attention of medicinal chemists, not only for their commercial availability and for the presence of functional groups prone to chemical modifications, but also due to the existence of transport systems that recognize this scaffold within the human body. In fact, bile acids are part of physiological processes, with several of them already approved in therapeutics (10-13).

There are transport mechanisms that ensure these compounds to successfully reach their targets. Their crossing of cellular membranes happens both through active and passive transport (14, 15). In terms of active transporters, there are Na^+ -dependent transport systems for bile acids, in the hepatocytes, ileocytes, and kidney cells, as well as organic anion transport proteins. These ensure that bile acids circulate efficiently between the liver and the intestines. These active transporters are promiscuous in the liver, being responsible for the transport of several amphiphilic organic compounds. However, transporters are more specific in the ileum, where they have certain sites that recognize different moieties of bile acids, increasing their selectivity: a region of recognition for the steroid scaffold, another for the acidic side chain and a nearby binding site for Na^+ (8, 16).

Taking these factors into account, it can be seen why bile acids pose an attractive and interesting choice as scaffolds for medicinal chemistry. Apart from the obvious application of bile acid and their derivatives for the treatment of liver and metabolic diseases (17-20), these compounds have been studied as anti-inflammatory (21, 22), antitumor (22-33), anti-osteoporosis (34, 35), anti-neurodegenerative diseases (36), inhibitors of efflux by P-glycoprotein (37), among others (38, 39).

Bile acid derivatives have also been studied for their potential to act as antibacterial agents. In fact, it was in the beginning of the 20th century that bile acids were described as

bacteriostatic against *S. aureus*. It was also shown that bile solubility occurs due to an activation of the regular autolytic process, and that bile acids exert their activity without a significant injury to cell walls (40).

A more recent study regarding the mechanism of action of bile acids in *S. aureus* showed that these compounds can decrease intracellular pH in a dose-dependent manner, as they are weak acids. This decrease was sharper with unconjugated bile acids, such as deoxycholic and cholic acids, which can be linked to the higher pKa values of these compounds in comparison to conjugated bile acids, meaning they are less dissociated at physiological pH, and therefore are more able to cross cell membranes. Because of this ease to penetrate membranes, unconjugated bile acids ultimately lead to a pH reduction, which in turn leads to a reduction in the transmembrane potential, and subsequent dissipation of the proton motive force and inhibits bacterial growth (41).

The same study noticed a marked difference between the MIC of cholic (**5.1, Figure 5.2**) and deoxycholic acid (**5.3, Figure 5.2**), with the latter showing much greater effects in bacterial growth, internal pH, and membrane potential. This results from the fact that compound **5.3** has two hydroxy groups, which makes it travel passively through small vesicles much faster than the less hydrophobic **5.1**, gifting **5.3** with a greater potency. It also increases membrane permeability, due to membrane damage. However, all things considered, the MIC of **5.3** was still high, at 1 mM (41).

Furthermore, it was shown that at a low concentration, bile acids can dissipate the membrane of the pH gradient and loss of transmembrane electric potential, displaying bacteriostatic effects. At higher concentrations, bactericidal activity occurs, with membrane damage and leakage of content (41-43). Nevertheless, these compounds are not as prone to the appearance of resistance, as their interaction is biophysical and not towards a specific membrane target, which can be considered an advantage (44).

However, since these compounds target the membrane, it is vital to know if the compounds also target eukaryotic membranes, which is possible by using erythrocytes to determine the minimum hemolytic concentration, and subsequently calculate membrane selectivity by comparing with the MIC obtained for bacteria, being the desired combination a high minimum hemolytic concentration and a low MIC. This can be achieved by taking advantage of the fact that bacterial membranes are negatively charged. Thus, cationic moieties, such as polyamines, will show more affinity towards bacterial cells than human cells, as charge recognition happens (45, 46).

In terms of efflux, it was shown that bile acids are prone to be transported by efflux systems, which include, but are not limited to, the AcrAB-TolC efflux system. The nature of the porins involved in the transport of bile acids is also determinant, as strains which

produce porins with wider channel diameters are more prone to the effects of bile acids than strains which produce smaller channels (47).

With this knowledge in mind, bile acid derivatives have been synthesized, with the aim of finding new antibacterial compounds. Ceragenins, or cation steroid antibiotics, come as an alternative to antimicrobial peptides, as they present an amphiphilic nature and displayed the same mechanism of action, while being easier to synthesize (48). Notably, cholic acid derivative **5.6 (Figure 5.3)**, has showed a broad spectrum of antibacterial activity, showing potential against *S. pneumoniae*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *S. aureus*, and *Helicobacter pylori*. The fact that it contains a large hydrophobic chain, allows it to cross the membrane of Gram-negative bacteria (49-57).

Cholic acid (**5.1, Figure 5.2**) was also the template for the synthesis of hydrazone derivatives. This yielded compounds with antibacterial activity against *E. coli*, *S. aureus*, and *E. faecalis* strains, which emphasizes the potential that lies within this scaffold, even though the antibacterial activity was lower than the drugs used as reference. Compound **5.7 (Figure 5.3)** exemplifies the derivatives present in this work (58).

The synthesis of cholic acid dimers, such as compound **5.8 (Figure 5.3)**, has also showed potential at inhibiting a methicillin- and ciprofloxacin-resistant strain of *S. aureus*, and another *S. aureus* strain that showed additional resistance to chloramphenicol, with a MIC of 4 µg/mL and 8 µg/mL, respectively. Furthermore, compound **5.8** also showed a 33-fold reduction in the MIC of CTX and an impressive 67-fold reduction in the MIC of amikacin, at the concentration of 2 µg/mL, as well as inhibition of biofilm formation (59). Imidazolium salt-derivatives of lithocholic acid (**5.4, Figure 5.2**) were also prepared and evaluated for their antibacterial properties. It was shown that derivatives with longer alkyl chains were more effective towards Gram-positive bacteria, with the most promising compound (**5.9, Figure 5.3**) showing a MIC of 0.8 µM against a reference strain of *S. aureus* and 26 µM against a reference strain of *E. coli* (60). A series of deoxycholic acid (**5.3, Figure 5.2**) derivatives was prepared with the aim of discovering new antimicrobial compounds. In this scope, compound **5.10 (Figure 5.3)** emerged as a hit compound, as it displayed IC₅₀ values of 7.5 and 15 µM against a reference strain of *S. aureus* and *E. faecalis*, respectively, emphasizing the potential this scaffold can provide in the synthesis of new antibacterials (61). Conjugation of deoxycholic acid with chiral amino-alcohols also yielded promising antibacterial compounds, with compound **5.11** showing bactericidal activity against reference strains of *S. aureus* and *E. coli* (62).

Deoxycholic acid derivative **5.12 (Figure 5.3)** has been described in **Chapter 4**, section 4.2.4.2, as having a broad spectrum of antibacterial and antifungal activity, as well as efflux pump inhibition, inhibition of biofilm formation, and inhibition of QS. Moreover, compounds with this scaffold have shown potential to act as bacterial EPIs. This is the case

of ginsenoside **5.13**, which has shown efficacy at decreasing the MIC of ciprofloxacin in clinical strains of *S. aureus*. Studies on the expression of the *norA* gene show that there is a correlation between the MIC decrease and the inhibition of the NorA pump (63).

The cardiac glycoside lanatoside C (**5.14**, **Figure 5.3**), containing a steroid scaffold, has also showed potentiation of the antibiotics carbenicillin and levofloxacin against *E. coli* and *P. aeruginosa* strains. It has also showed accumulation of EtBr in the same strains, suggesting that EPs are being inhibited (64).

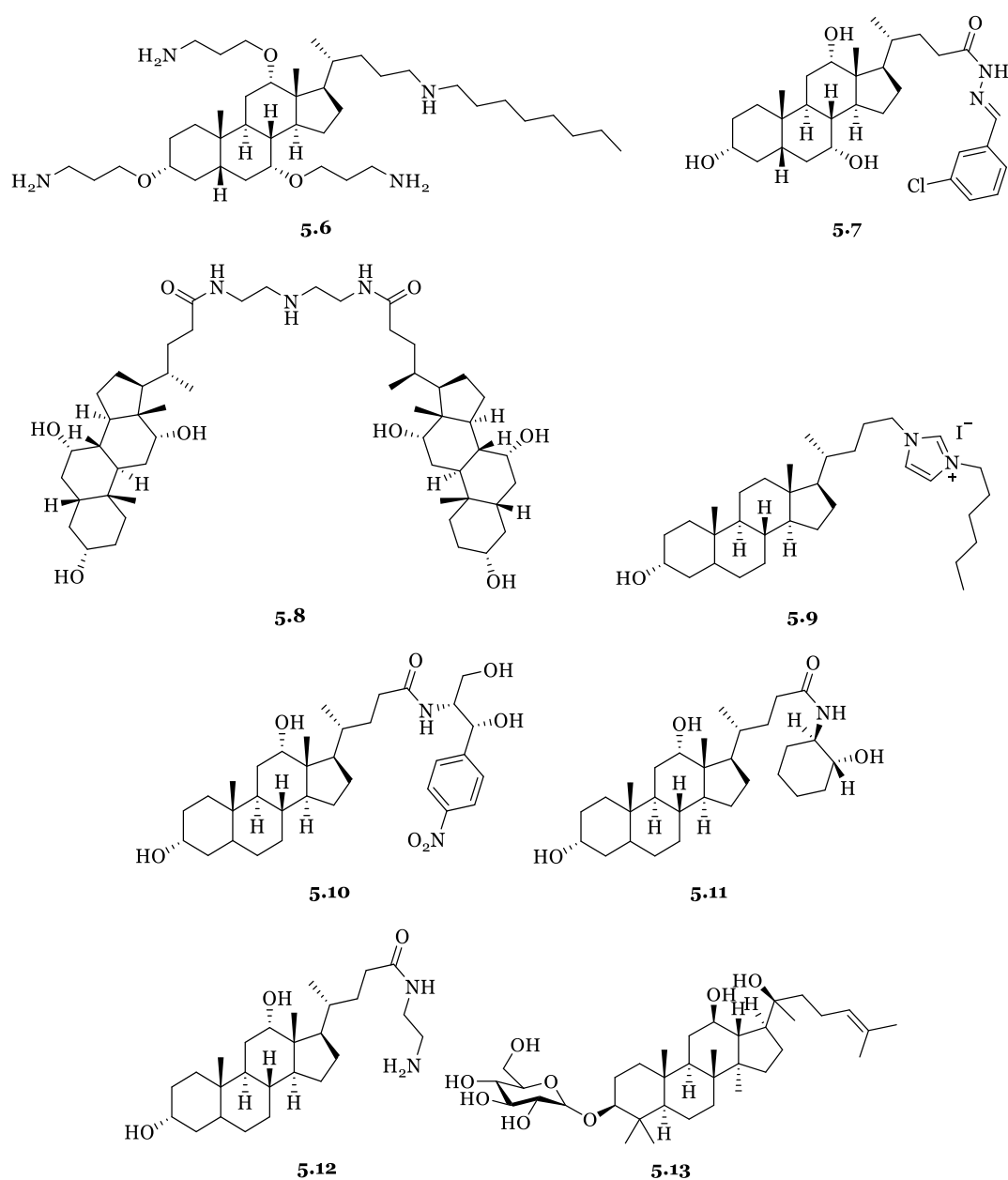


Figure 5.3 Structures of antibacterial (**5.6 – 5.12**) and EPI (**5.12 – 5.14**) bile acid derivatives.

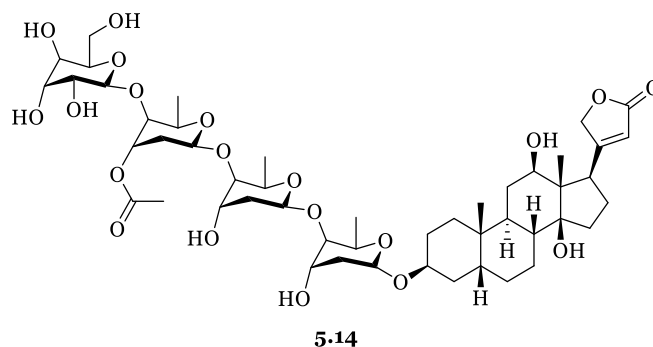


Figure 5.3 Structures of antibacterial (5.6 – 5.12) and EPI (5.12 – 5.14) bile acid derivatives (cont.).

Based on the fact that the steroid scaffold has shown potential to afford antibacterial agents, as well as in the circumvention of resistance mechanisms while not being prone to the appearance of AMR, it was chosen as a template for the synthesis of derivatives with dual antibacterial and EP inhibition activity.

Considering the results obtained with **5.12**, a small library of deoxycholic acid derivatives was planned and synthesized, using polyamines or other moieties that showed activity in other derivatives as substituents, to increase the cationic character of the compound and thus provide more affinity towards prokaryotic membranes. Eight derivatives were synthesized and studied for their potential as antimicrobials in a broad spectrum of bacterial and fungal strains, as well as their inhibition of bacterial EPs, biofilm formation, and cytotoxicity.

5.2 Synthesis of deoxycholic acid derivatives

Starting from deoxycholic acid (**5.3**, **Figure 5.2**), a library of amide derivatives was planned, based on moieties that have either shown potential as bacterial EPIs, or polyamines, like compound **5.12** (**Figure 5.3**).

To obtain these derivatives, a direct coupling reaction between compound **5.3** (**Figure 5.2**) and a suitable amine was employed, using (1-cyano-2-ethoxy-2-oxoethylidenaminoxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU), a uronium coupling reagent, *N,N*-diisopropylethylamine (DIEA) as a base, and dichloromethane as a solvent. The reaction was stirred at 0 °C for 30 minutes, and then allowed to reach room temperature and stirred overnight. The compounds obtained, **5.12** – **5.21**, and the respective yields, can be seen in **Table 5.1**.

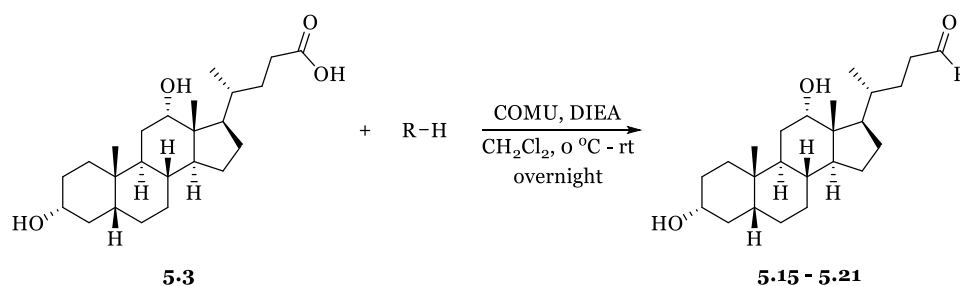
The piperidine introduction (**5.15**, **Table 5.1**) yielded an effective bacterial EPI. In fact, piperidine-substituted thioxanthenes, xanthenes, and xanthenes in different positions were shown to display EP inhibition in the models tested, *S. aureus* 272123 and *S. Typhimurium* SL1344 (65). Morpholine (**5.16**, **Table 5.1**) and piperazine were chosen as substituents due to their structural similarity to piperidine. However, reaction with piperazine originated a

dimer (**5.17**, **Table 5.1**), similar to compound **5.8** (**Figure 5.3**), and a piperazine protected with *tert*-butyloxycarbonyl (Boc) was synthesized (**5.18**, **Table 5.1**).

The polyamine derivatives were inspired in derivative **5.12**, in an attempt to evaluate the effect of different substitution patterns. As such, the amines planned included *N,N*-diethylethylenediamine (**5.19**, **Table 5.1**), a tertiary amine substituted with aliphatic chains, 2-(2-aminoethyl)benzimidazole (**5.20**, **Table 5.1**), which included a nitrogen heterocyclic aromatic system, and *N*-phenylethylenediamine (**5.21**, **Table 5.1**), a moiety containing an aromatic amine.

Compounds **5.15** – **5.19** were purified by flash chromatography [chloroform and methanol (90:10)], and compounds **5.20** and **5.21** were crystallized from dichloromethane.

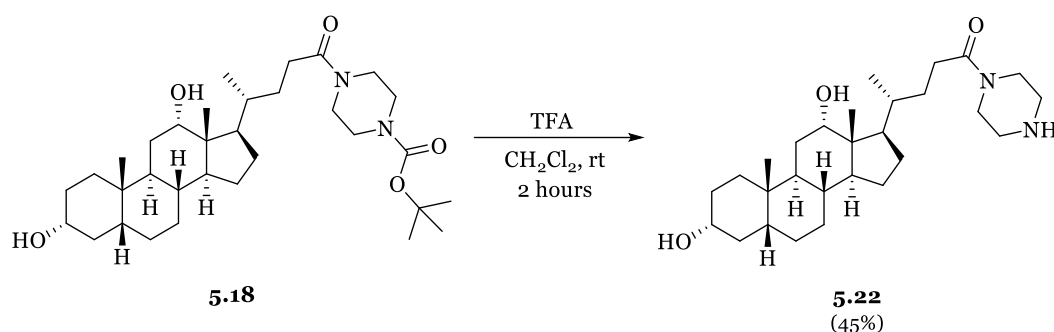
Table 5.1 Reaction used, and compounds obtained (**5.15** – **5.21**).



Compound	R	Yield
5.15		88%
5.16		82%
5.17		32%
5.18		66%
5.19		89%
5.20		26%

Compound	R	Yield
5.21		42%

In order to obtain the piperazine derivative, compound **5.18** underwent a Boc-deprotection, using trifluoroacetic acid (TFA), and dichloromethane as a solvent, yielding compound **5.22** (**Scheme 5.1**), which was purified by crystallization from methanol.



Scheme 5.1 Synthetic pathway to obtain compound **5.22**.

The structure elucidation was achieved by ^1H NMR, ^{13}C NMR and HRMS. The ^1H NMR spectra showed the presence of signals characteristic of both deoxycholic acid and the steroid scaffold. In fact, the two doublets present at approximately 4.0 ppm and 3.8 ppm correspond to the hydroxyls in C3 and C7, respectively, and the multiplet between approximately 1.0 and 2.0 ppm corresponds to the steroidal scaffold. The success of the reaction can also be inferred by the lack of a signal at approximately 10 ppm, which is due to the absence of the carboxylic acid moiety (66).

Concerning compounds **5.15** – **5.18** and **5.22**, it was not possible to distinguish the signals of the moieties coupled to the deoxycholic acid scaffold. However, for compounds **5.19** – **5.21**, the signals corresponding to the amines, the aliphatic chains and aromatic moieties can be found. These are present in **5.5.1.2**.

Through the analysis of mass spectra, it was clear that the compounds obtained corresponded to the structures that were originally planned, being this analysis crucial for the structure elucidation of derivatives **5.15** – **5.22**.

5.3 Biological activity

5.3.1 Antibacterial activity

Compounds **5.12** and **5.15** – **5.22** were tested for their antibacterial activity in 25 bacterial strains. These included reference strains, clinical isolates, animal isolates,

environmental isolates, and fish pathogens, and covered all the pathogens in the priority list of bacteria for which new antibiotics are urgently needed (67).

The Gram-negative bacteria used were reference strains *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13883, *S. enterica* serovar Typhimurium Colección Española de Cultivos Tipo (CECT) 443, *P. aeruginosa* ATCC 27853, *A. baumannii* ATCC 19606, *C. jejuni* ATCC 33560; clinical isolates *E. coli* SA/2, an ESBL-producing strain and *P. aeruginosa* 33b, a pan-drug-resistant isolate; and animal isolates *C. jejuni* P5/4, a ciprofloxacin-resistant strain, *C. jejuni* 4432, *C. jejuni* 4433, *C. jejuni* 4448, and *E. coli* 2252, a colistin-resistant strain (68). Additionally, wild-type *E. coli* K-12 AG100, which expresses AcrAB-TolC at a basal level, and its AcrAB-TolC deleted mutant, *E. coli* AG100A were also used (69).

Gram-positive strains included reference strains *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, and *Streptococcus pyogenes* ATCC 19615, environmental isolates methicillin-resistant *S. aureus* 66/1 (70), and VRE *E. faecalis* B3/101 (71), and the methicillin- and oxacillin-resistant clinical strain *S. aureus* 272123.

Gram-negative fish pathogens *Yersinia ruckeri* ATCC 29473, *Listonella anguillarum* ATCC 1924, and *Tenacibaculum maritimum* ATCC 43397 were also included. *Y. ruckeri* is a rod-shaped Gram-negative bacteria, responsible for enteric red mouth disease, an hemorrhagic pathology that affects several species of salmonids worldwide (72). *L. anguillarum* (formerly *Vibrio anguillarum*), a Gram-negative, arcuate-rod bacteria with one flagella, is the causative agent of vibriosis, a lethal pathology that is devastating in an aquaculture setting, characterized by ulceration, necrosis, hemorrhages, ultimately leading to death (73). Lastly, *T. maritimum* is a Gram-negative filamentous bacterium, responsible for tenacibaculosis, that manifests through skin ulcers, fin necrosis and tail rot (74).

The results obtained are seen in **Table 5.2**. It should be mentioned that, regarding *T. maritimum* ATCC 43397, CLSI guidelines determine that MIC determination should be performed through the disk diffusion assay. Therefore, the results obtained for this strain will be provided as the measure, in mm, of the inhibition halo. The MICs determined for compound **5.12** in the scope of the screening described in **Chapter 4** will also be present in **Table 5.2**.

Table 5.2 Minimum inhibitory concentration of deoxycholic amides **5.12** and **5.15 – 5.22** against bacteria.

Strain		5.12	5.15	5.16	5.17	5.18	5.19	5.20	5.21	5.22
<i>E. coli</i> ATCC 25922	MIC ¹	74	>140	>138	>77	>114	>130	>119	>125	>139
	MBC ¹	147	>140	>138	>77	>114	>130	>119	>125	>139
<i>E. coli</i> SA/2	MIC ¹	74	>140	>138	>77	>114	>130	>119	>125	>139
	MBC ¹	74	>140	>138	>77	>114	>130	>119	>125	>139
<i>E. coli</i> 2252	MIC ¹	147	>140	>138	>77	>114	>130	>119	>125	>139
	MBC ¹	ND	>140	>138	>77	>114	>130	>119	>125	>139

Strain		5.12	5.15	5.16	5.17	5.18	5.19	5.20	5.21	5.22
<i>E. coli</i> AG100	MIC ¹	50	>140	>138	>77	>114	>130	>119	>125	100
	MBC ¹	ND	ND	ND	ND	ND	ND	ND	ND	ND
<i>E. coli</i> AG100A	MIC ¹	50	>140	>138	>77	>114	>130	>119	>125	100
	MBC ¹	ND	ND	ND	ND	ND	ND	ND	ND	ND
<i>K. pneumoniae</i> ATCC 13883	MIC ¹	147	>140	>138	>77	>114	>130	>119	>125	>139
	MBC ¹	147	>140	>138	>77	>114	>130	>119	>125	>139
<i>S. Typhimurium</i> CECT 443	MIC ¹	37	>140	>138	77	>114	>130	>119	>125	139
	MBC ¹	37	>140	>138	77	>114	>130	>119	>125	139
<i>P. aeruginosa</i> ATCC 27853	MIC ¹	147	>140	>138	>77	>114	>130	>119	>125	>139
	MBC ¹	147	>140	>138	>77	>114	>130	>119	>125	>139
<i>P. aeruginosa</i> 33b	MIC ¹	147	>140	>138	>77	>114	>130	>119	>125	>139
	MBC ¹	>147	>140	>138	>77	>114	>130	>119	>125	>139
<i>A. baumannii</i> ATCC 19606	MIC ¹	147	>140	>138	74	>114	130	>119	>125	139
	MBC ¹	147	>140	>138	74	>114	130	>119	>125	139
<i>C. jejuni</i> ATCC 33560	MIC ¹	147	140	>138	>77	>114	>130	>119	>125	>139
	MBC ¹	147	140	>138	>77	>114	>130	>119	>125	>139
<i>C. jejuni</i> P5/4	MIC ¹	74	>140	>138	>77	>114	>130	>119	>125	>139
	MBC ¹	74	>140	>138	>77	>114	>130	>119	>125	>139
<i>C. jejuni</i> 4432	MIC ¹	147	140	>138	>77	>114	>130	>119	>125	>139
	MBC ¹	147	>140	>138	>77	>114	>130	>119	>125	>139
<i>C. jejuni</i> 4433	MIC ¹	147	>140	>138	>77	>114	>130	>119	>125	>139
	MBC ¹	147	>140	>138	>77	>114	>130	>119	>125	>139
<i>C. jejuni</i> 4448	MIC ¹	147	>140	>138	>77	>114	>130	>119	>125	>139
	MBC ¹	147	>140	>138	>77	>114	>130	>119	>125	>139
<i>S. aureus</i> ATCC 29213	MIC ¹	37	70	>138	77	29	130	15	16	139
	MBC ¹	37	>140	>138	>77	>114	130	>119	>125	139
<i>S. aureus</i> ATCC 25923	MIC ¹	37	12.5	50	>77	6.25	6.25	6.25	3.13	6.25
	MBC ¹	37	ND	ND	ND	ND	ND	ND	ND	ND
<i>S. aureus</i> 66/1	MIC ¹	74	70	>138	77	114	130	30	31	139
	MBC ¹	74	>140	>138	>77	>114	130	>119	>125	139
<i>S. aureus</i> 272123	MIC ¹	12.5	>140	>138	>77	6.25	50	3.13	>125	50
	MBC ¹	ND	ND	ND	ND	ND	ND	ND	ND	ND
<i>E. faecalis</i> ATCC 29212	MIC ¹	37	70	>138	77	>114	130	>119	>125	139
	MBC ¹	74	>140	>138	77	>114	130	>119	>125	139
<i>E. faecalis</i> B3/101	MIC ¹	74	>140	>138	77	>114	>130	>119	>125	70
	MBC ¹	74	>140	>138	>77	>114	>130	>119	>125	>139
<i>S. pyogenes</i> ATCC 19615	MIC ¹	147	70	>138	>77	>114	>130	>119	>125	>139
	MBC ¹	147	70	>138	>77	>114	>130	>119	>125	>139

Strain		5.12	5.15	5.16	5.17	5.18	5.19	5.20	5.21	5.22
<i>Y. ruckeri</i> ATCC 29473	MIC ¹	37	>140	>138	77	>114	>130	>119	>125	>139
	MBC ¹	74	>140	>138	77	>114	>130	>119	>125	>139
<i>L. anguillarum</i> ATCC 1924	MIC ¹	37	>140	>138	38	>114	130	>119	>125	139
	MBC ¹	74	>140	>138	38	>114	130	>119	>125	139
<i>T. maritimum</i> ATCC 43397	DD ²	20	18	0	10	10	10	9	12	9

MIC: Minimum inhibitory concentration; **MBC:** Minimum bactericidal concentration; **DD:** Disk diffusion; **ND:** Not determined; ¹ The MIC and MBC are expressed in μM ; ² The results of the disk diffusion assay are expressed in mm.

In terms of antibacterial activity, the compound with the broadest spectrum was **5.12**, as it showed bactericidal activity for every bacterial strain tested, except for *P. aeruginosa* 33b. On the opposite side of the spectrum is compound **5.16**, which was only active against the reference strain *S. aureus* ATCC 25923.

Some SAR conclusions could be drawn from these results. Compound **5.12** presents the broader spectrum, as it is active in every bacterial strain tested. It is also the only active compound in the *E. coli*, *K. pneumoniae* and *P. aeruginosa* strains tested. It was the most effective compound in every strain tested, except for the *S. aureus* strains, where **5.20** displayed the lowest MIC. The activity of the compounds was, overall, greater for the reference strains. It was also shown that most compounds were active against Gram-positive strains, specifically *S. aureus*. *A. baumannii* ATCC 19606 was the Gram-negative bacteria towards which more compounds were active.

Regarding antibacterial activity, SAR studies suggested that the presence of a primary amine favors Gram-negative activity, as evidenced by compound **5.12**. The presence of an electron withdrawing group hinders the antibacterial activity, as observed in the case of compound **5.16**. The presence of an oxygen atom in the morpholine moiety may also be a hindrance of the activity of this compound, as it possesses a partial negative charge, which could prevent interaction with the negatively charged membranes. It was also shown that the presence of a dimer can promote antibacterial activity, as compound **5.17** was active in nine of the tested strains. The presence of an amine at the terminal of the molecule can also be a favorable feature, as compound **5.22** also shows a broad spectrum of activity. And lastly, aliphatic substituents seem to be preferable over aromatic.

5.3.2 Bacterial efflux pump inhibition

The group of deoxycholic amides **5.12** and **5.15** – **5.22** were tested for their potential to inhibit bacterial efflux pumps in *E. coli* AG100, *E. coli* AG100A, and *S. aureus* 272123. The

pair *E. coli* AG100 and *E. coli* AG100A allow the determination of AcrAB-TolC inhibition, as the first strain expresses this EP at its basal level and the latter has the *acrA* and *acrB* genes deleted. The Gram-positive *S. aureus* 272123 was used to compare the activity of these derivatives to other compounds previously studied in this scope (65, 75-79).

As positive controls, CCCP was used for the *E. coli* strains at 50 μ M, and reserpine was used for *S. aureus* 272123 at 25 μ M. The compounds were tested at 50 μ M or half their MIC for the respective strain. Specifically, for *E. coli* AG100 and *E. coli* AG100A, compounds **5.12** and **5.22** were tested at 25 and 50 μ M, respectively. Concerning *S. aureus* 272123, compound **5.18** was tested at 3.13 μ M, compounds **5.19** and **5.22** were tested at 25 μ M, and compound **5.20** was tested at 1.6 μ M. The results obtained are present in **Table 5.3**. Compound **5.12** had previously been assayed for EP inhibition in *S. aureus* 272123, **Chapter 4**, section 4.2.4.3, and these results are displayed for comparison.

Table 5.3 RFI of the tested deoxycholic amides.

Compound	RFI $\pm$ SD (μ M)		
	<i>E. coli</i> AG100	<i>E. coli</i> AG100A	<i>S. aureus</i> 272123
5.12	1.22 $\pm$ 0.05	1.16 $\pm$ 0.06	0.15 $\pm$ 0.04
5.15	0.46 $\pm$ 0.08	0.31 $\pm$ 0.02	2.24 $\pm$ 0.01
5.16	0.22 $\pm$ 0.05	0.12 $\pm$ 0.07	1.85 $\pm$ 0.07
5.17	-0.04 $\pm$ 0.07	0.02 $\pm$ 0.03	0.80 $\pm$ 0.20
5.18	0.27 $\pm$ 0.08	0.16 $\pm$ 0.03	0.15 $\pm$ 0.04
5.19	0.77 $\pm$ 0.09	0.22 $\pm$ 0.05	0.63 $\pm$ 0.01
5.20	0.36 $\pm$ 0.03	0.31 $\pm$ 0.08	0.09 $\pm$ 0.01
5.21	0.45 $\pm$ 0.07	0.36 $\pm$ 0.05	0.74 $\pm$ 0.01
5.22	0.35 $\pm$ 0.01	0.83 $\pm$ 0.08	0.36 $\pm$ 0.01
CCCP	1.17 $\pm$ 0.07	1.25 $\pm$ 0.01	-
Reserpine	-	-	0.35 $\pm$ 0.05

RFI: Relative fluorescence index; SD: Standard deviation; CCCP: carbonyl cyanide 3-chlorophenylhydrazone.

The analysis of the data obtained shows that only compound **5.12** was active against Gram-negative EPs. It also leads to the conclusion that this compound is acting on EPs other than AcrAB-TolC, as the RFI remains practically unchanged between the wild-type and the AcrAB-TolC deleted mutant.

It could also be seen that a few other compounds inhibit the efflux of EtBr in the Gram-negative strains tested, albeit in a lower extent than CCCP, the positive control, with compounds **5.15**, **5.16**, and **5.18** – **5.22** showing moderate RFI values.

Compound **5.19** shows results that are in agreement with the inhibition of the AcrAB-TolC efflux system, since its RFI is much higher in the wild-type strain than in the mutant. Oppositely, compound **5.22** appears to be both a substrate and an inhibitor of EPs. The results obtained with the wild-type *E. coli* AG100 suggest moderate inhibition, while a

higher inhibition is noticed in *E. coli* AG100A. This is likely due to efflux of the compound in the first strain by AcrAB-TolC, which makes its intracellular concentration lower and thus decreases its activity as an EPI.

In the case of *S. aureus* 272123, six compounds showed a higher RFI than reserpine. In fact, compounds **5.15** – **5.17**, **5.19**, **5.21**, and **5.22** showed potential at inhibiting EPs. This is a clinical strain and expresses the *norA* gene at a basal level (80, 81), but the lack of a NorA-deleted mutant in this specific case prevents a better vision of which EP is being inhibited.

5.3.3 Inhibition of biofilm formation

The library of deoxycholic amides synthesized were studied for the inhibition of biofilm formation in the pair used previously: *S. aureus* ATCC 25923 and *S. aureus* 272123. The compounds were tested at 100 μ M or half their MIC for these strains, and reserpine, used as a positive control, was tested at 25 μ M. The concentrations used and the percentage of biofilm inhibition by deoxycholic amides **5.12** and **5.15** – **5.22** are present in **Table 5.4**.

Table 5.4 Inhibition of biofilm formation by deoxycholic amides **5.12** and **5.15** – **5.22**.

Compounds	<i>S. aureus</i> ATCC 25923		<i>S. aureus</i> 272123	
	Concentration (μ M)	Biofilm inhibition $\pm$ SD (%)	Concentration (μ M)	Biofilm inhibition $\pm$ SD (%)
5.12	19	91.4 $\pm$ 2.18	6.25	0
5.15	6.25	96.2 $\pm$ 0.02	100	43.8 $\pm$ 1.97
5.16	25	1.20 $\pm$ 0.06	100	38.6 $\pm$ 0.09
5.17	100	0	100	70.7 $\pm$ 0.53
5.18	3.125	2.40 $\pm$ 0.01	3.125	0
5.19	3.125	1.49 $\pm$ 0.03	25	7.73 $\pm$ 0.17
5.20	3.125	1.73 $\pm$ 0.03	1.57	2.32 $\pm$ 0.32
5.21	1.57	2.45 $\pm$ 0.03	100	27.61 $\pm$ 0.11
5.22	3.125	0	25	0
Reserpine	25	22.3 $\pm$ 4.21	25	73.6 $\pm$ 0.23

SD: Standard deviation.

The results obtained showed that compounds **5.12** and **5.15** were able to inhibit biofilm formation in *S. aureus* ATCC 25923. Compound **5.15** also displayed a mild decrease in biofilm formation in *S. aureus* 272123. The dimer **5.17** was the most effective compound at inhibiting the formation of biofilm in *S. aureus* 272123, which was still beneath the positive control.

The other compounds were not shown to be promising at inhibiting the formation of biofilm in these strains, despite **5.15** – **5.17**, **5.19**, **5.21**, and **5.22** having displayed inhibition of EPs in *S. aureus* 272123.

5.3.4 Antifungal activity

The library of deoxycholic amides was tested against the yeast *C. albicans* ATCC 10231, the filamentous fungus *A. fumigatus* ATCC 204305, and the dermatophyte *T. rubrum* FF5. If the compounds showed antifungal activity for any of the fungal strains used, they would be tested to a broader spectrum of yeasts, filamentous fungi, and dermatophytes, accordingly.

The additional yeasts used were the azole-resistant *C. albicans* H37, *Candida krusei* ATCC 6258, *Candida glabrata* 10R, and *Cryptococcus neoformans* CECT 1078. As filamentous fungi, the azole-resistant *A. fumigatus* C111, *Aspergillus niger* ATCC 16404, *Aspergillus flavus* F44, *Fusarium solani* FF125, *Fusarium oxysporum* FF115, *Mucor* spp., *Lichtheimia* spp., and *Scedosporium* spp. The dermatophytes used if the compound was active against *T. rubrum* FF5 were *Trichophyton mentagrophytes* FF7 and *Nannizzia gypsea* FF3. A reference strain of the oomycete fish pathogen *Saprolegnia parasitica* CBS 223.65 was also included. This oomycete is the causative agent of saprolegniasis, an endemic disease for salmon and catfish, that causes lethal ulceration and hemorrhage (82).

The first screening showed that only compounds **5.19** and **5.22** were active against the three strains tested, apart from **5.12** whose antifungal activity has previously been described for these three strains (MIC *C. albicans* ATCC 10231 = 64 µg/mL; MIC *A. fumigatus* ATCC 204305 = 128 µg/mL; MIC *T. rubrum* FF5 = 32 µg/mL). In this scope, compound **5.19** showed a MIC of 128 µg/mL against *C. albicans* ATCC 10231 and *T. rubrum* FF5, with fungicidal activity for both strains at the same concentration. Compound **5.22** was active for all the strains tested, with a MIC of 64 µg/ml for *C. albicans* ATCC 10231, 128 µg/mL for *A. fumigatus* ATCC 204305, and 32 µg/mL for *T. rubrum* FF5, all with fungicidal properties.

As such, compounds **5.12** and **5.22** were tested for all the additional fungal strains, while compound **5.19** was tested for the additional yeast and dermatophyte strains proposed. Given the potential that compound **5.12** demonstrated against the bacteria pathogenic to fish, it was also tested against *S. parasitica* CBS 223.65. The results of these assays can be seen in **Table 5.5**.

Table 5.5 Minimum inhibitory concentration of the deoxycholic amides tested against fungi.

Strain		5.12	5.19	5.22
Yeasts				
<i>C. albicans</i> H37	MIC (µg/mL)	64	128-256	64
	MFC (µg/mL)	64	128-256	64
<i>C. krusei</i> ATCC 6258	MIC (µg/mL)	64	64	32
	MFC (µg/mL)	64	128	32
<i>C. glabrata</i> 10R	MIC (µg/mL)	64	256	64
	MFC (µg/mL)	64	256	64
<i>C. neoformans</i> CECT 1078	MIC (µg/mL)	32	64	32
	MFC (µg/mL)	64	64-128	32
Filamentous fungi				
<i>A. fumigatus</i> C111	MIC (µg/mL)	128	-	128
	MFC (µg/mL)	>128	-	128
<i>A. niger</i> ATCC 16404	MIC (µg/mL)	64	-	64
	MFC (µg/mL)	128	-	>128
<i>A. flavus</i> FF44	MIC (µg/mL)	>128	-	>128
	MFC (µg/mL)	>128	-	>128
<i>F. solani</i> FF125	MIC (µg/mL)	64	-	64
	MFC (µg/mL)	>128	-	64
<i>F. oxysporum</i> FF115	MIC (µg/mL)	128	-	128
	MFC (µg/mL)	>128	-	128
<i>Mucor</i> spp.	MIC (µg/mL)	64	-	32
	MFC (µg/mL)	64	-	32-64
<i>Lichtheimia</i> spp.	MIC (µg/mL)	64	-	64
	MFC (µg/mL)	>128	-	64
<i>Scedosporium</i> spp.	MIC (µg/mL)	64	-	64
	MFC (µg/mL)	128	-	64
Dermatophytes				
<i>T. mentagrophytes</i> FF7	MIC (µg/mL)	32	64	32
	MFC (µg/mL)	64	64	32
<i>N. gypsea</i> FF3	MIC (µg/mL)	32	128	32
	MFC (µg/mL)	32	128	32
Fish pathogen				
<i>S. parasitica</i> CBS 223.65	MIC (µg/mL)	32-64	-	-
	MFC (µg/mL)	32-64	-	-

MIC: Minimum inhibitory concentration; **MFC:** Minimum fungicidal concentration.

Except for the filamentous fungus *A. flavus* FF4, all the compounds showed at least fungistatic activity against all the strains tested. Compound **5.22** displayed the overall best results, even though they were quite similar to the ones displayed by compound **5.12**, showing the broad spectrum of these compounds against fungi.

Compound **5.12** also showed activity against the fish pathogen *S. parasitica* CBS 223.65, which, adding the results obtained with the bacteria pathogenic to fish, makes it a possible choice for the treatment of diseased fish in aquaculture settings.

5.3.5 Cytotoxicity

The derivatives **5.15** – **5.22** were investigated for their cytotoxicity in NIH/3T3 cell line (mouse embryonic fibroblasts). The IC₅₀ of the compounds, as well as the previously determine IC₅₀ of compound **5.12**, are present in **Table 5.6**. Doxorubicin was used as a positive control.

Table 5.6 Cytotoxicity of the deoxycholic amides against NIH/3T3 cell line.

Compound	IC ₅₀ (μM)
5.12	56.75 ± 1.87
5.15	32.75 ± 4.52
5.16	47.98 ± 3.16
5.17	16.93 ± 1.66
5.18	25.42 ± 3.78
5.19	23.08 ± 0.30
5.20	35.86 ± 1.51
5.21	12.75 ± 0.60
5.22	45.37 ± 0.56
Doxorubicin	26.89 ± 1.02

IC₅₀: half maximal inhibitory effect.

The compounds show cytotoxicity towards the tested cell line, which compromises their use for the activities mentioned above. However, given the promising results regarding fish pathogens, the focus of future studies should lie in this potential. However, future work concerning safety studies in fish species affected by these microorganisms are warranted. Nevertheless, the IC₅₀ values of the compounds is higher than their MIC, which is unlikely to compromise their activity against few bacterial strains.

5.4 Concluding remarks

In this chapter, a library of deoxycholic amides was planned and synthesized, bearing in mind previous moieties that have proved effective at inhibiting bacterial EPs, and the deoxycholic acid scaffold, as derivative **5.12** had previously proved to be a broad spectrum antibacterial and an EPI for a Gram-negative strain.

The eight newly synthesized compounds **5.15** – **5.22**, along with the hit compound **5.12**, were tested for a broad variety of bacterial strains, including clinical strains, animal pathogens, and reference strains. Compound **5.12** proved to have a broad spectrum of

antibacterial activity, showing antibacterial activity against strains that included pan-drug-resistant bacteria. These results could make this compound an interesting candidate for *in vivo* testing, even though future studies, such as hemolysis, should be carried out, in order to conclude about its selectivity.

Even though compounds **5.19** – **5.22** were planned around the structural features of this hit compound, they did not meet the expectations when it came to antibacterial activity, leading to the assumption that a primary amine is essential for broad spectrum antibacterial activity. Compound **5.17**, structurally related to the antibacterial **5.8**, was also beneath the expected activity, which can be explained by the piperazine linker, which has a lower length than the linker presented in **5.8**.

Most of the compounds displayed antibacterial activity in reference strains, which was encouraging. However, these strains are not representative of the worrisome bacteria present in clinical and environmental settings. Nevertheless, it should be emphasized that compounds **5.18** and **5.20** displayed a very low MIC against the methicillin- and oxacillin-resistant *S. aureus* 272123.

Concerning the EP inhibition assay, compounds **5.12**, **5.15**, and **5.19** – **5.22** showed inhibition of EPs in the Gram-negative strains used, albeit lower than the positive control. Among these, compound **5.19** stands out as it likely inhibits the AcrAB-TolC efflux system.

The dimer **5.17** also showed effectiveness against the Gram-positive strain studied. Compound **5.18** did not show any potential as an EPI, but this compound was an intermediate for the synthesis of compound **5.22** and was not planned for this activity. The moiety presented in compound **5.19** was present in a thioxanthone EPI, active on *S. aureus* 272123, and displayed a broader activity in this scope, with EP inhibition in Gram-positive and Gram-negative bacteria. The results obtained emphasized the potential of deoxycholic amides as potential EPIs, and compounds **5.15**, **5.19**, and **5.22** seem to have a larger scope of activity than **5.12**, as they can inhibit EPs in both strains used.

Concerning the studies on the inhibition of biofilm formation, the compounds did not seem as promising. In fact, only compounds **5.12** and **5.15** showed significant biofilm inhibition against *S. aureus* ATCC 25923, and **5.17** was the only active compound regarding biofilm formed by *S. aureus* 272123. **Figure 5.4** compares the overall results obtained with these derivatives and the most represented classes for each phenotypic study described in **Chapter 3** and **4**.

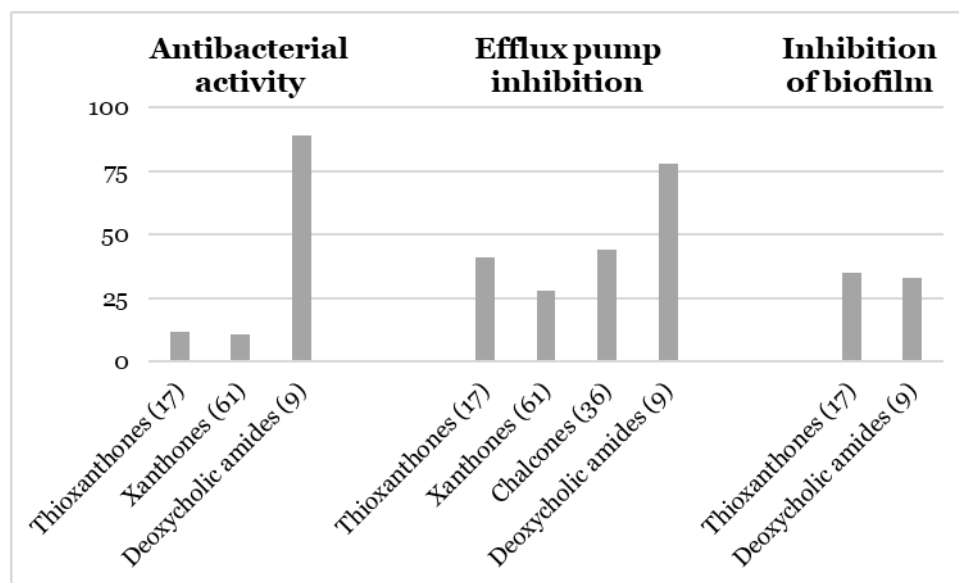


Figure 5.4 Comparison of the results obtained with the deoxycholic amides, and the best classes previously described.

The results obtained show that deoxycholic amides provided the greatest percentage of antibacterial and EPIs. However, these compounds are more useful as Gram-positive EPIs, while xanthones and thioxanthones showed more affinity towards inhibiting Gram-negative EPs. Chalcones had a more even distribution between both. However, the Gram-negative model used was different between deoxycholic amides and the other classes. Concerning biofilm inhibition, thioxanthones are still the class with the most effective compounds.

Compounds **5.12**, **5.19** and **5.22** showed a broad range of activity when it comes to fungi. Moreover, compounds **5.12** and **5.22** were active against yeasts, filamentous fungi, and dermatophytes, which could mean that an amine must be in the extremity of the derivatives in order for them to show broad spectrum antifungal activity. It is also noteworthy that the antifungal activity demonstrated by these derivatives is quite remarkable comparing to those obtained in the previous chapters, which highlights the potential of these compounds as broad spectra antimicrobials.

However, all the compounds showed some cytotoxicity for the cell line used. This cytotoxic effect was influenced by the amine introduction, as methyl deoxycholate (**4.104**, **Figure 4.18**), which was studied in **Chapter 4**, showed no cytotoxicity for this cell line. The synthesis of safer derivatives could be a challenge to be tackled in future studies with this scaffold. When it comes to biological activity, future assays could include testing the compounds at sub-cytotoxic concentrations, in order to see if the activity is kept under safer concentrations. As these compounds were better EPIs in the Gram-positive model, models more specific to the study of EPs could be used. Genetic assays could also be performed in the case of compound **5.19**, in order to prove if the AcrAB-TolC EP is being inhibited.

5.5 Experimental section

5.5.1 Chemistry

5.5.1.1 General

All reagents and solvents used for the synthesis were purchased from Sigma-Aldrich (Sigma-Aldrich Co. Ltd., Gillingham, UK) and no further purification process was implemented. Solvents were evaporated using a rotary evaporator under reduced pressure, Buchi Waterchath B-480. All reactions were monitored by TLC carried out on precoated plates with 0.2 mm of thickness using Merck silica gel 60 (GF₂₅₄) with appropriate mobile phases. As the compounds could not be detected at 254 nm or 345 nm, a 20% solution of sulfuric acid in methanol was pulverized onto the plate, followed by heating at 100 °C for five minutes in an oven.

Flash column chromatography using silica gel 60 (0.040–0.063 mm, Merck, Darmstadt, Germany) was used in the purification of the synthesized compounds. Mp were measured in a Köfeler microscope (Wagner and Munz, Munich, Germany) and are uncorrected. ¹H- and ¹³C-NMR spectra were recorded at the CEMUP, University of Porto, in CDCl₃ or DMSO-*d*₆ (Deutero GmbH, Ely, UK) at room temperature on a Bruker Avance III 400 spectrometer (400.14 MHz for ¹H and 100.14 MHz for ¹³C, Bruker Biosciences Corporation, Billerica, MA, USA). Chemical shifts are expressed in δ (ppm) values relative to TMS as an internal reference. Coupling constants are reported in Hz. HRMS spectra were measured on a LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) at CEMUP, University of Porto, Portugal.

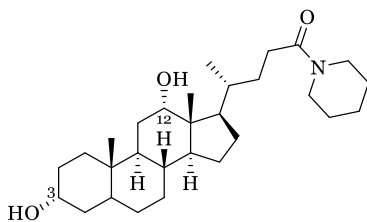
The peak purity of the compounds was determined by HPLC. The HPLC system consisted of Thermo Scientific SpectraSystem P4000 pump, equipped with a degasser, a Thermo Scientific SpectraSystem AS3000 autosampler fitted with a maximum volume 100 μ L loop, and a Thermo Scientific SpectraSystem UV8000 DAD detector (Thermo Fisher Scientific, San Jose, CA, USA). Data acquisition was performed using ChromQuest 5.0 software, version 3.2.1. The column used in this study was ACE - C18 (150 $\times$ 4.6 mm I.D., particle size 5 μ m) manufactured by Advanced Chromatography Technologies Ltd (Aberdeen, Scotland, UK). The mobile phase composition was water and acetonitrile (30:70 *v/v*) or water, acetonitrile and diethylamine (30:70:0.1 *v/v/v*) (for **5.19**), all of which were HPLC grade solvents obtained from Merck Life Science S.L.U. (Darmstadt, Germany). The flow rate was 1.0 mL/minute and the UV detection wavelength was set at 221 nm. Analyses were performed at room temperature in an isocratic mode in a 30-60 minute run. Peak purity index was determined by total peak UV-Vis spectra between 210-650nm with a step of 4nm.

5.5.1.2 General procedure for the synthesis of deoxycholic amides **5.15** – **5.21**

Compounds **5.15** – **5.21** were obtained following the procedure in **Table 5.1**. To a suspension of deoxycholic acid (0.250 g, 0.6 mmol) in 5 mL of dichloromethane, DIEA (1.3 mmol, 2eq.) was added dropwise until dissolution. Then, the reaction was cooled to 0 °C and COMU (1.3 mmol, 2 eq.) was added. The reaction stirred for 30 minutes, after which the appropriate amine was added. Then, the reaction was gradually heated up to room temperature and was left stirring overnight.

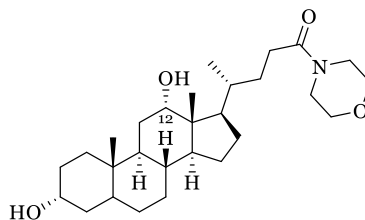
The products of the reaction were first extracted with an aqueous solution of hydrochloric acid (1 M), and the aqueous layer was alkalinized with a saturated solution of sodium bicarbonate until basic pH, followed by extraction with dichloromethane (3 × 20 mL). The organic layers were concentrated via rotary evaporator and, in the case of compounds **5.15** – **5.19**, the crude product was purified by flash chromatography using chloroform and methanol (90:10) as a mobile phase. In the case of compounds **5.20** and **5.21**, the crude product was purified by washing with dichloromethane and/or crystallization. All the reactions performed were analyzed by TLC, using the mobile phase chloroform, methanol and triethylamine (80:20:0.1), and the stationary phase precoated plates with 0.2 mm of thickness using Merck silica gel 60 (GF₂₅₄). A 20% solution of sulfuric acid in methanol was used as a visualization reagent.

(*R*)-4-((3*R*,5*R*,8*R*,9*S*,10*S*,12*S*,13*R*,14*S*,17*R*)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-1-(piperidin-1-yl)pentan-1-one (**5.15**)



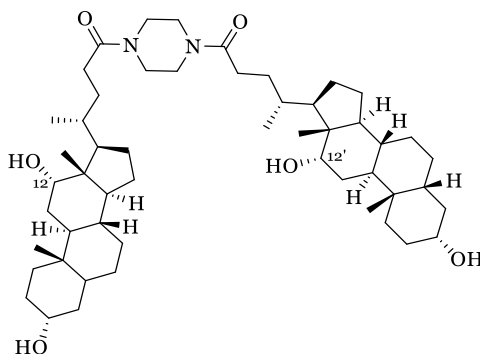
88% as white solid. mp 78.1 - 79.8 °C (chloroform:metanol). IR (KBr) ν_{max} : 3423, 2935, 2861, 1753, 1739, 1694, 1627, 1606, 1458, 1373, 1307, 1255, 1223, 1192, 1136, 1094, 1066, 1044, 1014, 969, 943, 919, 755, 668. ¹H NMR (CDCl₃, 400.14 MHz) δ (ppm): 3.98 (-OH (12) 1H, d, *J* = 3.03 Hz), 3.61 (-OH (3), 1H, m), 2.39 (1H, m), 2.24 (1H, m), 2.17 (1H, d, *J* = 4.89 Hz), 1.81 (8H, m), 1.64 (8H, m), 1.52 (8H, m), 1.40 (5H, m), 1.29 (2H, m), 1.24 (2H, m), 1.09 (2H, m), 0.99 (3H, d, *J* = 6.36 Hz), 0.90 (3H, s), 0.68 (3H, s). ¹³C NMR (CDCl₃, 100.63 MHz) δ (ppm): 172.1 (C=O), 73.3, 72.0, 48.4, 47.4, 46.6, 42.8, 42.2, 36.6, 36.2, 35.5, 35.3, 34.2, 33.8, 31.6, 30.6, 30.4, 29.8, 28.8, 27.6, 27.3, 26.3, 25.6, 24.7, 23.8, 23.3, 17.7, 12.9, 11.4 (**Figure S5.1**). HRMS (ESI⁺): *m/z* [C₂₉H₄₉NO₃ + H]⁺ calcd. for [C₂₉H₅₀NO₃]: 460.3785; found 460.3779 (**Figure S5.2**). Peak purity [ACE – C18; water and acetonitrile (30:70 *v/v*)]: 99.2% (**Figure S5.3**).

(*R*)-4-((3*R*,5*R*,8*R*,9*S*,10*S*,12*S*,13*R*,14*S*,17*R*)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-1-morpholinopentan-1-one (**5.16**)



82% as white solid. mp 166.0 - 167.8 °C (chloroform:metanol); IR (KBr) ν_{max} : 3495, 3388, 2988, 2922, 2857, 1621, 1479, 1466, 1440, 1391, 1372, 1361, 1302, 1277, 1253, 1111, 1095, 1063, 1047, 1014, 966, 580. ^1H NMR (CDCl_3 , 400.14 MHz) δ (ppm): 3.98 (-OH (12) 1H, d, $J = 3.02$ Hz), 3.63 (7H, m), 3.46 (2H, m), 2.37 (1H, m), 2.21 (1H, m), 1.80 (8H, m), 1.62 (5H, s), 1.52 (4H, m), 1.38 (6H, m), 1.24 (1H, s), 1.10 (2H, m), 0.99 (3H, d, $J = 6.33$ Hz), 0.90 (3H, s), 0.68 (3H, s). ^{13}C NMR (CDCl_3 , 100.63 MHz) δ (ppm): 172.5 (C=O), 73.3, 71.9, 67.1, 66.8, 48.4, 47.3, 46.6, 46.2, 42.2, 42.0, 36.5, 36.1, 35.4, 35.3, 34.2, 33.8, 31.3, 30.6, 30.0, 28.8, 27.6, 27.2, 26.2, 23.8, 23.3, 17.7, 12.9 (**Figure S5.4**). HRMS (ESI⁺): m/z [$\text{C}_{28}\text{H}_{47}\text{NO}_4 + \text{H}$]⁺ calcd. for [$\text{C}_{28}\text{H}_{48}\text{NO}_4$]: 462.3578; found 462.3577 (**Figure S5.5**). Peak purity [ACE – C18; water and acetonitrile (30:70 v/v)]: 99.8% (**Figure S5.6**).

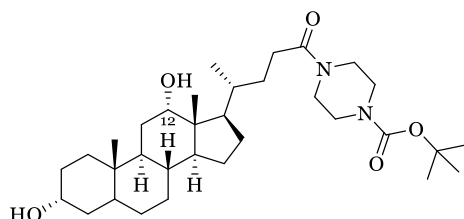
(*R*)-4-((3*R*,5*R*,8*R*,9*S*,10*S*,12*S*,13*R*,14*S*,17*R*)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-1-(4-((*S*)-4-((3*S*,5*S*,8*S*,9*R*,10*R*,12*R*,13*S*,14*R*,17*S*)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentan-oyl)piperazin-1-yl)pentan-1-one (**5.17**)



32% as white solid. mp 237.5 - 238.4 °C (chloroform:metanol). IR (KBr) ν_{max} : 3423, 2921, 2861, 1560, 1468, 1446, 1375, 1255, 1051. ^1H NMR (CDCl_3 , 400.14 MHz) δ (ppm): 3.98 (-OH (12 and 12'), 2H, d, $J = 3.2$ Hz), 3.61 (6H, m), 3.46 (4H, m), 2.40 (2H, m), 2.25 (2H, m), 1.80 (28H, m), 1.52 (10H, m), 1.41 (9H, m), 1.26 (5H, m), 1.00 (6H, d, $J = 6.4$ Hz), 0.91 (6H, s), 0.68 (6H, s). ^{13}C NMR (CDCl_3 , 100.63 MHz) δ (ppm): 172.6 (C=O), 73.3, 72.0, 48.4, 47.3, 46.6, 45.5, 42.2, 41.7, 36.5, 36.1, 35.4, 34.2, 33.8, 31.3, 30.6, 29.8, 28.8, 27.7, 27.2, 26.2, 23.8, 23.3, 17.7, 17.6, 12.9 (**Figure S5.7**). HRMS (ESI⁺): m/z [$\text{C}_{52}\text{H}_{86}\text{N}_2\text{O}_6 + \text{H}$]⁺ calcd.

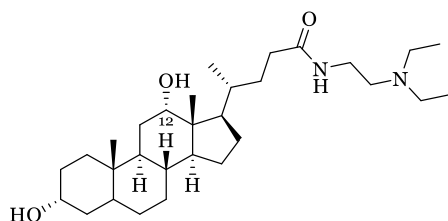
For [C₅₂H₈₇N₂O₆]: 835.6559; found 835.6566 (**Figure S5.8**). Peak purity [ACE – C18; water and acetonitrile (30:70 v/v)]: 95.3% (**Figure S5.9**).

tert-butyl 4-((*R*)-4-((3*R*,5*R*,8*R*,9*S*,10*S*,12*S*,13*R*,14*S*,17*R*)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoyl)piperazine-1-carboxylate (**5.18**)



66% as white solid. mp 100.3 - 101.7 °C (chloroform:methanol). IR (KBr) ν_{max} : 3442, 2935, 2863, 1700, 1633, 1459, 1421, 1366, 1286, 1252, 1169, 1129, 1090, 1045, 997. ¹H NMR (CDCl₃, 400.14 MHz) δ (ppm): 3.98 (-OH (12), 1H, d, *J* = 2.99 Hz), 3.61 (3H, m), 3.40 (7H, m), 2.39 (1H, m), 2.23 (1H, m), 1.79 (8H, m), 1.59 (4H, s), 1.52 (5H, m), 1.47 (10H, s), 1.27 (4H, m), 1.09 (3H, m), 0.99 (3H, d, *J* = 6.3 Hz), 0.90 (3H, s), 0.68 (3H, s). ¹³C NMR (CDCl₃, 100.63 MHz) δ (ppm): 172.4 (-NC=O), 154.7 (-OC=O), 80.4, 73.3, 71.9, 48.4, 47.3, 46.6, 45.6, 42.2, 41.5, 36.6, 36.2, 35.4, 35.3, 34.2, 33.8, 31.4, 30.6, 30.3, 28.8, 28.5, 27.7, 27.2, 26.2, 23.8, 23.3, 17.7. 12.9 (**Figure S5.10**). HRMS (ESI⁺): *m/z* [C₃₃H₅₆N₂O₅ + H]⁺ calcd. For [C₃₃H₅₇N₂O₅]: 561.4267; found 561.4258 (**Figure S5.11**). Peak purity [ACE – C18; water and acetonitrile (30:70 v/v)]: 99.9% (**Figure S5.12**).

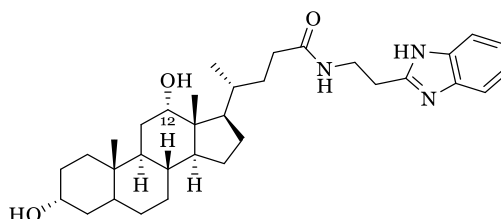
(*R*)-*N*-(2-(diethylamino)ethyl)-4-((3*R*,5*R*,8*R*,9*S*,10*S*,12*S*,13*R*,14*S*,17*R*)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanamide (**5.19**)



89% as white solid. mp 95–5 - 96.4 °C (chloroform:methanol); IR (KBr) ν_{max} : 3404, 2937, 2858, 1625, 1560, 1467, 1446, 1385, 1375, 1090, 1045, 850, 559. ¹H NMR (CDCl₃, 400.14 MHz) δ (ppm): 7.63 (-CONH-, 1H, s), 3.96 (-OH (12), 1H, d, *J* = 2.97 Hz), 3.57 (3H, m), 3.00 (7H, m), 2.32 (1H, m), 2.17 (1H, m), 1.77 (10H, m), 1.47 (13H, m), 1.29 (8H, t, *J* = 7.23 Hz), 0.99 (3H, d, *J* = 5.97 Hz), 0.89 (3H, s), 0.66 (3H, s). ¹³C NMR (CDCl₃, 100.63 MHz) δ (ppm): 175.3 (C=O), 73.2, 71.8, 52.7, 48.4, 47.5, 46.9, 46.7, 42.2, 36.5, 36.1, 35.6, 35.5, 35.4, 34.3, 33.7, 33.1, 31.5, 30.6, 28.7, 27.7, 27.3, 26.3, 23.9, 23.3, 17.6, 12.8, 9.6 (**Figure S5.13**). HRMS (ESI⁺): *m/z* [C₃₀H₅₄N₂O₃ + H]⁺ calcd. for [C₃₀H₅₅N₂O₃]: 491.4212;

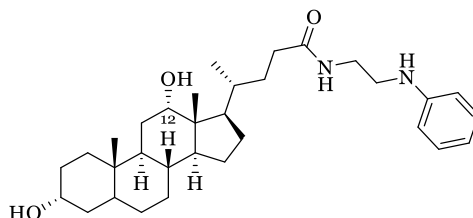
found 491.4206 (**Figure S5.14**). Peak purity [ACE – C18; water, acetonitrile and diethylamine (30:70:0.1 v/v/v)]: 95.0% (**Figure S5.15**).

(*R*)-*N*-(2-(1*H*-benzo[*d*]imidazol-2-yl)ethyl)-4-((3*R*,5*R*,8*R*,9*S*,10*S*,12*S*,13*R*,14*S*,17*R*)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanamide (**5.20**)



26% as white solid. mp 152.7 – 154.6 °C (methanol). IR (KBr) ν_{max} : 3411, 3096, 2926, 2861, 1671, 1525, 1448, 1416, 1369, 1308, 1273, 1252, 1225, 1087, 1052, 1014, 769, 754, 736. ^1H NMR (DMSO-*d*₆, 400.14 MHz) δ (ppm): 12.30 (-CNHC-, 1H, s), 7.93 (-CONH-, 1H, t, J = 4.26 Hz), 7.46 (*H*_{ar}, 2H, m), 7.10 (*H*_{ar}, 2H, dd, J = 2.34 and 6.45 Hz), 4.44 (1H, d, J = 3.15 Hz), 4.15 (1H, d, J = 3.09 Hz), 3.77 (-OH (12), 1H, d, J = 2.76 Hz), 3.48 (2H, q, J = 5.45 Hz), 2.93 (2H, t, J = 5.45 Hz), 2.08 (1H, m), 1.84 (6H, m), 1.55 (7H, m), 1.25 (12H, m), 0.89 (3H, d, J = 4.80 Hz), 0.84 (3H, s), 0.57 (3H, s). ^{13}C NMR (DMSO-*d*₆, 100.63 MHz) δ (ppm): 172.8 (C=O), 152.8 (*C*_{ar}), 110.7 (*C*_{ar}), 79.3 (*C*_{ar}), 79.1 (*C*_{ar}), 78.9 (*C*_{ar}), 78.6 (*C*_{ar}), 71.0, 69.9, 47.4, 46.2, 45.9, 41.6, 37.2, 36.3, 35.6, 35.1, 35.0, 33.8, 32.9, 32.5, 31.6, 30.6, 30.2, 29.0, 28.6, 27.1, 26.7, 26.1, 23.5, 23.1, 17.1, 12.4 (**Figure S5.16**). HRMS (ESI⁺): m/z [*C*₃₃*H*₄₉*N*₃*O*₃ + H]⁺ calcd. For [*C*₃₃*H*₅₀*N*₃*O*₃]: 536.3852; found 536.3843 (**Figure S5.17**). Peak purity [ACE – C18; water and acetonitrile (30:70 v/v)]: 96.7% (**Figure S5.18**).

(*R*)-4-((3*R*,5*R*,8*R*,9*S*,10*S*,12*S*,13*R*,14*S*,17*R*)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-(2-(phenylamino)ethyl)pentanamide (**5.21**)



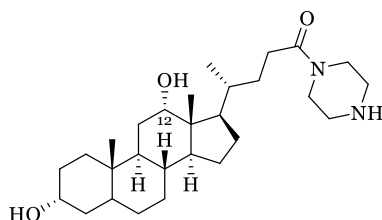
42% as white solid. mp 180.1 – 181.9 °C (methanol). IR (KBr) ν_{max} : 3615, 3293, 3083, 3019, 2931, 2864, 1659, 1605, 1553, 1513, 1499, 1447, 1377, 1335, 1298, 1233, 1194, 1152, 1114, 1083, 1063, 1043, 1013, 747, 693, 604. ^1H NMR (DMSO-*d*₆, 400.14 MHz) δ (ppm): 8.30 (-CH₂NHC-, 1H, d, J = 2.64 Hz), 7.87 (*H*_{ar}, 1H, dd, J = 4.86 and 8.12 Hz), 7.06 (*H*_{ar}, 2H, td, J = 2.39 and 7.94 Hz), 6.54 (2H, m), 5.54 (1H, m), 4.45 (1H, t, J = 3.38 Hz), 4.17 (1H, t, J = 3.18 Hz), 3.78 (-OH (12), 1H, d, J = 3.36 Hz), 3.20 (2H, m), 3.04 (2H, m), 1.93 (7H, m), 1.56 (7H, m), 1.26 (11H, m), 1.03 (2H, m), 0.92 (3H, d, J = 4.12 Hz), 0.84 (3H, s), 0.58

(3H, s). ^{13}C NMR (DMSO- d_6 , 100.63 MHz) δ (ppm): 172.9 (C=O), 148.6 (C_{ar}), 128.9 (C_{ar}), 115.6 (C_{ar}), 111.9 (C_{ar}), 79.2 (C_{ar}), 71.0, 69.9, 47.4, 46.2, 46.0, 42.6, 41.6, 38.0, 36.3, 35.6, 35.1, 35.0, 33.8, 32.9, 32.5, 31.6, 30.2, 28.6, 27.2, 27.0, 26.1, 23.5, 23.1, 17.1, 12.4 (**Figure S5.19**). HRMS (ESI $^{+}$): m/z [$\text{C}_{32}\text{H}_{50}\text{N}_2\text{O}_3 + \text{H}$] $^{+}$ calcd. For [$\text{C}_{32}\text{H}_{51}\text{N}_2\text{O}_3$]: 511.3900; found 511.3913 (**Figure S5.20**). Peak purity [ACE – C18; water and acetonitrile (30:70 v/v)]: 99.9% (**Figure S5.21**).

5.5.1.3 Synthesis of deoxycholic amide **5.22**

To a solution of **5.18** (100.3 mg, 0.18 mmol) in dichloromethane, 1.58 mL of TFA (114 eq.) was added dropwise, and the reaction was stirred at room temperature for 2 hours. The reaction was quenched with saturated sodium bicarbonate and extracted with dichloromethane. The crude product obtained after solvent evaporation was washed with methanol, furnishing compound **5.22**. The reaction was analyzed by TLC, using the mobile phase chloroform, methanol and triethylamine (80:20:0.1), and the stationary phase precoated plates with 0.2 mm of thickness using Merck silica gel 60 (GF $_{254}$). A 20% solution of sulfuric acid in methanol was used as a visualization reagent.

(*R*)-4-((3*R*,5*R*,8*R*,9*S*,10*S*,12*S*,13*R*,14*S*,17*R*)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-1-(piperazin-1-yl)pentan-1-one (**5.22**)



45% as white solid. mp 229.3 – 230.8 °C (methanol). IR (KBr) ν_{max} : 3417, 2936, 2862, 1602, 1470, 1445, 1254, 1052. ^1H NMR (CDCl_3 , DMSO- d_6 , 400.14 MHz) δ (ppm): 7.62 (– CH_2NHCH_2 –, 1H, m), 3.93 (–OH (12), 1H, m), 3.49 (5H, m), 3.37 (1H, m), 2.86 (12H, m), 2.60 (1H, m), 2.38 (1H, m), 2.22 (1H, m), 1.81 (7H, m), 1.60 (4H, m), 1.26 (4H, m), 1.12 (1H, m), 1.00 (3H, d, $J = 4.36$ Hz), 0.89 (3H, s), 0.66 (3H, s). ^{13}C NMR (CDCl_3 , DMSO- d_6 , 100.63 MHz) δ (ppm): 171.4 (C=O), 71.7, 70.3, 47.3, 46.2, 46.1, 45.7, 45.7, 45.1, 41.9, 41.4, 35.8, 35.3, 34.8, 34.7, 33.5, 32.8, 30.7, 29.7, 29.5, 28.1, 26.9, 26.6, 25.5, 23.1, 22.6, 16.7, 12.0 (**Figure S5.22**). HRMS (ESI $^{+}$): m/z [$\text{C}_{28}\text{H}_{48}\text{N}_2\text{O}_3 + \text{H}$] $^{+}$ calcd. For [$\text{C}_{28}\text{H}_{49}\text{N}_2\text{O}_3$]: 461.3743; found 461.3747 (**Figure S5.23**). Peak purity [ACE – C18; water and acetonitrile (30:70 v/v)]: 95.5% (**Figure S5.24**).

5.5.2 Biological activity

5.5.2.1 Culture media and chemicals

The culture media used in the experiments were the following: MHB II (Sigma-Aldrich, St- Louis, MO, USA and Biokar Diagnostics, Allone, Beauvais, France), LB-B (Sigma, St. Louis, MO, USA), TSB (Scharlau Chemie S. A., Barcelona), and TSA (Biokar Diagnostics, Allone, Beauvais, France), were purchased. SDA was purchased from bio-Mérieux, Marcy L'Etoile, France, RPMI-1640 broth medium from Biochrom AG, Berlin, Germany, which was buffered with MOPS, purchased from Sigma-Aldrich, St. Louis, MO, USA, to pH 7.0. Corn Meal Agar plates (CMA; BD, Le Pont-de-Claix, France) was purchased.

DMSO, MTT, SDS, PBS (pH 7.4), EtBr, reserpine, CCCP, and CV were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Doxorubicin 2 mg/mL was purchased from Teva Pharmaceuticals, Budapest, Hungary. Bacteria were purchased from ATCC, and the cell line NIH/3T3 was purchased from Sigma (Steinheim, Germany).

5.5.2.2 Microorganisms

Thirteen reference bacterial strains, eleven MDR clinically relevant bacterial strains, and one mutant *E. coli* strain were used in this study. Gram-negative bacteria comprised reference strains *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13883, *Salmonella enterica* serovar Typhimurium CECT 443, *P. aeruginosa* ATCC 27853, *A. baumannii* ATCC 19606, *C. jejuni* ATCC 33560; clinical isolates *E. coli* SA/2, an ESBL-producing strain and *P. aeruginosa* 33b, a pan-drug-resistant isolate; and animal isolates *C. jejuni* P5/4, a ciprofloxacin-resistant strain, *C. jejuni* 4432, *C. jejuni* 4433, *C. jejuni* 4448, and *E. coli* 2252, a colistin-resistant strain. The wild-type *Escherichia coli* K-12 AG100 strain [*argE3 thi-1 rpsL xyl mtl* Δ (*gal-uvrB*) *supE44*], expressing AcrAB-TolC at its basal level, and its AcrAB-TolC deleted mutant *E. coli* AG100A strain were used in the study.

Gram-positive strains included *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *S. pyogenes* ATCC 19615, environmental isolates methicillin-resistant *S. aureus* 66/1, and *E. faecalis* B3/101, and clinical isolate *S. aureus* 272123, resistant to methicillin and oxacillin.

Gram-negative fish pathogens *Y. ruckeri* ATCC 29473, *L. anguillarum* ATCC 1924, and *T. maritimum* ATCC 43397 were also included.

Strains were kept in TSA slants and, before each assay, were sub-cultured in Müller-Hinton Agar. *Y. ruckeri* ATCC 29473 and *L. anguillarum* ATCC 1924 were kept in Nutrient Agar (Condalab, Madrid, Spain) and *T. maritimum* ATCC 43397 was kept in TMM agar (Condalab, Madrid, Spain), and were sub-cultured in the respective culture media before

each assay. For *S. pyogenes* ATCC 19615, MHB II was supplemented with 3.75% lysed horse blood (Oxoid, Basingstoke, England), whereas for *C. jejuni* it was supplemented with 2.5% lysed horse blood.

Fifteen fungal strains were used in this study, including reference strains and clinical isolates of yeasts and filamentous fungi. Yeast strains included reference strains and clinical isolates: *C. albicans* ATCC 10231, *C. krusei* ATCC 6258, *C. albicans* H37, *C. glabrata* 10R, and *C. neoformans* CECT 1078.

Filamentous fungi included *A. fumigatus* ATCC 240305, *A. fumigatus* C111, *A. niger* ATCC 16404, *A. flavus* F44, *F. solani* FF125, *F. oxysporum* FF115, and dermatophytes *T. rubrum* FF5, *T. mentagrophytes* FF7, and *N. gypsea* FF3 (formerly *Microsporum gypseum*) and a species of genera *Mucor*, *Lichetheimia* and *Scedosporium*. *S. parasitica* CBS 223.65, a reference strain of an oomycete fish pathogen, was also included.

All microorganisms were kept in Sabouraud Dextrose Broth (BioMérieux, Marcy L'Etoile, France) plus glycerol (20%) at -80 °C. The strains were kept in SDA (BioMérieux, Marcy L'Etoile, France) slants and were sub-cultured in SDA before each test. *S. parasitica* was kept in CMA and was sub-cultured in CMA before each experiment.

5.5.2.3 Antibacterial assay

The MIC for each compound was determined by broth microdilution method in a 96-well plate according to the CLSI guidelines (83). The media used was MHB II. The concentrations tested ranged from 100 µM to 0.195 µM and were prepared from a stock solution of 10 mM in DMSO, for *S. aureus* 272123, *E. coli* AG100 and *E. coli* AG100A; for the other strains, the concentrations tested ranged from 64 µg/mL to 4 µg/mL and were prepared from a stock solution of 10 mg/mL in DMSO. DMSO was used in subinhibitory concentrations (1 % v/v).

Colony-forming unit counts of the inoculum were conducted to determine the initial inoculum size (which should be approximately 5×10^5 CFU/mL). Sterility and growth controls were included in each assay. The 96-well U-shaped untreated polystyrene microtiter plates were incubated for 20 h at 37 °C (42 °C for *C. jejuni*, in a microaerophilic atmosphere) and the MIC was determined as the lowest concentration of compound that prevented visible growth. The MBC was determined by spreading 10 µL of the content of the wells with no visible growth on MH plates. The MBC was determined as the lowest concentration of compound that killed 99.9% of the initial inoculum after overnight incubation at 37 °C.

For the Gram-negative fish pathogens, the conditions needed to be adapted. According the CLSI guidelines, the broth microdilution assay was fit to be used for MIC determination in *Y. ruckeri* and *L. anguillarum*. The medium used was MHB II and were incubated at 22

°C for 48 hours. For *T. maritimum*, the disk diffusion assay was used, in *Flexibacter maritimus* media (FMM), supplemented with salt to make the medium similar to salt water. After inoculation with the bacteria, blank filter paper disks (7 mm in diameter; Oxoid, Basingstoke, England) were placed in the plate, and impregnated with 15 µg of each compound were placed on seeded FMM plate. A blank disk impregnated with DMSO was also placed, as a negative control.

Two independent assays were performed in quadruplicate for all strains that were assayed by the broth microdilution assay, and two independent assays were performed for the disk diffusion assay.

5.5.2.4 Efflux pump inhibition assay

The efflux pump inhibiting activity of the compounds was tested on *S. aureus* MRSA 272123, *E. coli* AG100 and *E. coli* AG100A strains by real-time fluorimetry monitoring the intracellular accumulation of the efflux pump substrate EtBr, using a CLARIOstar Plus plate reader (BMG Labtech, Ortenberg, Germany).

Reserpine was applied at 25 µM and CCCP was applied at 50 µM, as positive controls; the solvent DMSO was applied at 1% *v/v*. The examined bacterial strains were cultured at 37 °C in a shaking incubator until they reached an OD of 0.6 at 600 nm. The bacterial cells were then washed with 500 µL PBS and centrifuged at 13,000× g for 2 minutes, then the pellet was re-suspended in 1 mL PBS.

The compounds were applied at half their MIC and, where the MIC was >100 µM, the tested concentration was 50 µM in PBS supplemented with a non-toxic concentration of EtBr (2 µg/mL). Then, the solutions were pipetted into a 96-well black microtiter plate (Greiner Bio-One Hungary Kft, Hungary) in 50 µL, and 50 µL of bacterial suspension (OD₆₀₀ 0.6) was pipetted to the wells.

The plates were then measured with a CLARIOstar plate reader, and the fluorescence was recorded at excitation and emission wavelengths of 530 nm and 600 nm, respectively, every minute for 1 hour. From the real-time data, the relative RFI of the last time point (minute 60) was calculated according to the subsequent equation:

$$RFI = \frac{RF_{\text{treated}} - RF_{\text{untreated}}}{RF_{\text{untreated}}}$$

where RF_{treated} is the RF at the last time point of EtBr accumulation curve in the presence of the compound, and $RF_{\text{untreated}}$ is the RF at the last time point of the EB accumulation curve of the untreated control, having only the solvent (DMSO) control (75). The samples were

tested in triplicate, and the RFI presented comes from the average of these three values. The SD was calculated automatically.

5.5.2.5 Inhibition of biofilm formation

The compounds were tested for their ability to decrease the formation of biofilm. The bacterial strains used were the Gram-positive *S. aureus* ATCC 25923 and *S. aureus* 272123.

The detection of the biofilm formation was carried out using the dye CV (0.1 % v/v). The initial inoculum was incubated in TSB overnight, and then diluted to an OD₆₀₀ of 0.1. Then, the bacterial suspension was added to 96-well microtiter plates and the compounds were added at a sub-MIC concentration. This meant that compounds with an observable MIC were added at half their MIC, while the rest of the compounds were added at the concentration of 100 µM. The final volume in each well was 200 µL. Reserpine was used as positive control, as it was the same compound used in the efflux pump inhibition assay and it has shown activity in the inhibition of biofilm formation in *S. aureus* strains (84).

The plates were incubated at 30 °C for 48 hours, with gentle stirring (100 rpm). After this incubation period, the TSB medium was discarded, and the plates were washed with tap water to remove unattached cells. Afterwards, 200 µL of a 0.1 % v/v CV solution were added to the wells and incubated for 15 minutes at room temperature. Then, the CV solution was removed from the wells, and the plates were washed again with tap water, and 200 µL of a 70 % ethanol solution were added to the wells.

The biofilm formation was determined by measuring the OD₆₀₀ using a Multiscan EX ELISA plate reader (Thermo Labsystems, Cheshire, WA, USA). The anti-biofilm effect of the compounds was expressed in the percentage (%) of decrease of biofilm formation.

5.5.2.6 Antifungal activity

Antifungal activity was evaluated by determining the MIC of the compounds by the broth microdilution method, according to CLSI guidelines (85, 86). Briefly, cell or spore suspensions were prepared in RPMI-1640 broth medium (Biochrom, Berlin, Germany) buffered with MOPS (Sigma-Aldrich, St. Louis, MO, USA) (henceforth referred to as RPMI) from fresh cultures of the different strains of fungi.

For yeasts, the inoculum was adjusted to $0.5\text{--}2.5 \times 10^3$ CFU/mL. For filamentous fungi, the inoculum was adjusted to $1\text{--}3 \times 10^3$ CFU/mL for dermatophytes, and $0.4\text{--}5 \times 10^4$ CFU/mL for all other strains. Two-fold serial dilutions of the compound were prepared in RPMI, within the concentration range of 8-128 µg/mL. Sterility and growth controls were included in each assay.

The 96-well flat-bottomed untreated polystyrene microtiter plates which were incubated for 48 hours at 35 °C, except for *Lichtheimia* spp., which was incubated at 25 °C for 48 hours, and the dermatophyte strains, which incubated for 5-7 days at 25 °C. MICs were recorded as the lowest concentrations that completely inhibited growth in comparison to the compound-free controls. The MIC of voriconazole (kindly provided by Pfizer Ltd., UK) for *C. krusei* ATCC 6258 was used as quality control and the assays were validated when the results obtained were within the recommended limits.

The MFC was determined by spreading 20 µL of culture collected from wells showing no visible growth on SDA plates. The MLC was determined as the lowest concentration showing complete growth inhibition after 48 h at 35 °C, 48 h at 25 °C (*Lichtheimia* spp.) or 5-7 days at 25 °C (dermatophytes). At least two independent assays were performed for all tested strains.

Antifungal activity of compound **5.12** against *S. parasitica* CBS 223.65 was evaluated by determining the MIC by a broth microdilution method carried out in Glucose Yeast Broth (10 g/L D-(+)-Glucose [Merck, Darmstadt, Germany]; 2g/L yeast extract [Liofilchem, Roseto Degli Abruzzi, Italy]).

Briefly, two-fold serial dilutions of the compound were prepared in Glucose Yeast broth, within the concentration range of 8-128 µg/mL and 200 µL of each concentration were distributed in the wells of 96-well flat-bottomed untreated polystyrene microtiter plates. Each well was then inoculated with approximately 1 mm² of mycelium. Sterility and growth controls were included in each assay.

After 5-7 days of incubation at 24 °C, MIC was recorded as the lowest concentration that prevented obvious mycelial growth in comparison to the compound-free controls. Subsequently, the portions of the inocula in which growth was not observed were transferred to Glucose Yeast agar (Glucose Yeast broth with 15 g/L of agar [Agar Bios Special LL, Biolife, Milano, Italy]), after being washed two times in saline. The plates were incubated 5-7 days at 24 °C, to evaluate fungal viability and determine the MFC. At least four independent assays were performed.

5.5.2.7 Cytotoxicity in NIH/3T3 cell line

A 10.0 mM stock solution of each compound was prepared in DMSO. All stock solutions were stored at –80°C and freshly diluted on the day of the experiment in fresh cell culture medium (ensuring that DMSO did not exceed 0.1 % DMSO concentration of the exposure media).

Mouse fibroblasts (NIH/3T3, ATCC CRL-1658TM) were cultivated in DMEM (Gibco 52100-039) and supplemented with 10 % heat-inactivated fetal bovine serum (Biowest, VWR International Kft, Debrecen, Hungary), 2 mM of L-glutamine, 1 mM Na pyruvate, 100

U/L and 10 mg/L penicillin/streptomycin mixture (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), respectively, and 0.1 % nystatin (8.3 g/L in ethylene glycol). The adherent cells were detached using a combination of 0.25 % Trypsin-Versene (EDTA) solution for 5 minutes at 37 °C. Before each cytotoxicity assay using this cell line, cells were seeded in untreated 96-well flat-bottom microtiter plates, following a 4-hour incubation period in a humidified atmosphere (5 % CO₂, 95 % air) at 37 °C (87).

The cytotoxicity of the most promising compounds was assessed in NIH/3T3 cells, using MTT assay. Prior to the assay, the cells were seeded for 4 hours using 1×10^4 cells/well. The compounds were added by 2-fold serial dilutions to the cells distributed into 96-well flat bottom microtiter plates starting with 100 µM. The plates were incubated for 24 hours, after which a solution of MTT in PBS was added to each well and incubated for another 4 hours. After this, 100 µL of SDS (10% in a 0.01 M HCl solution) were added to each well and incubated overnight at 37 °C. Doxorubicin was used as positive control. Cell growth was determined in quadruplicate by measuring OD at $\lambda=540$ nm (reference 630 nm) in a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). The percentage of inhibition of cell growth was determined according to the equation.

$$100 - \left(\frac{OD_{\text{sample}} - OD_{\text{medium control}}}{OD_{\text{cell control}} - OD_{\text{medium control}}} \right) \times 100$$

The results of the cytotoxicity in the NIH/3T3 cell line were expressed as the mean $\pm$ SD, and the IC₅₀ values were obtained by best fitting the dose-dependent inhibition curves in GraphPad Prism 5.03 for Windows software.

5.6 Supporting information

The ¹H and ¹³C NMR spectra for the newly synthesized deoxycholic acid derivatives **5.15** – **5.22**, as well as the HRMS spectra, and HPLC chromatograms will be displayed in **Figure S5.1 – Figure S5.24**.

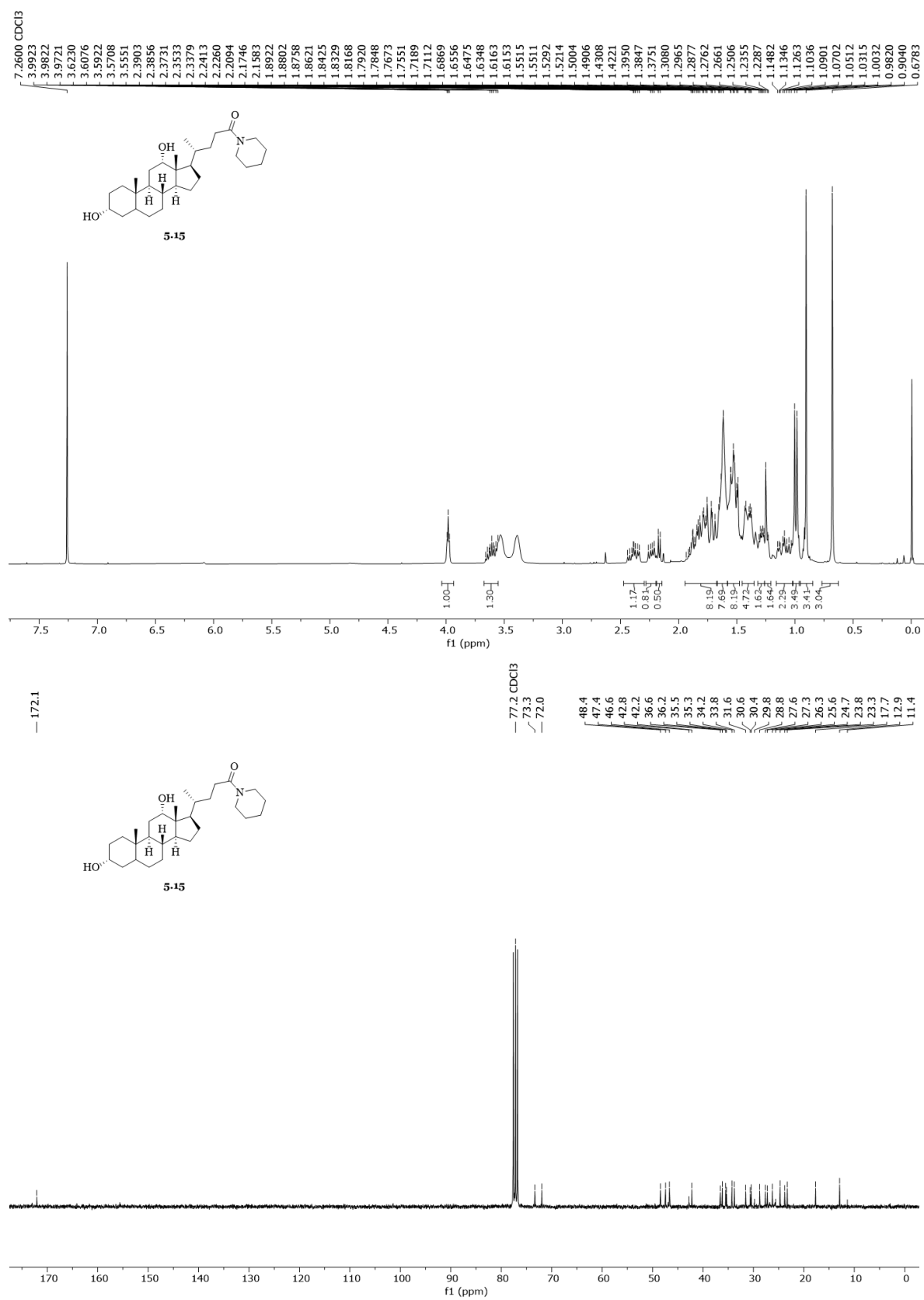


Figure S5.1 ¹H NMR (top, 400.14 MHz, CDCl₃) and ¹³C NMR (bottom, 100.63 MHz, CDCl₃) for compound **5.15**.

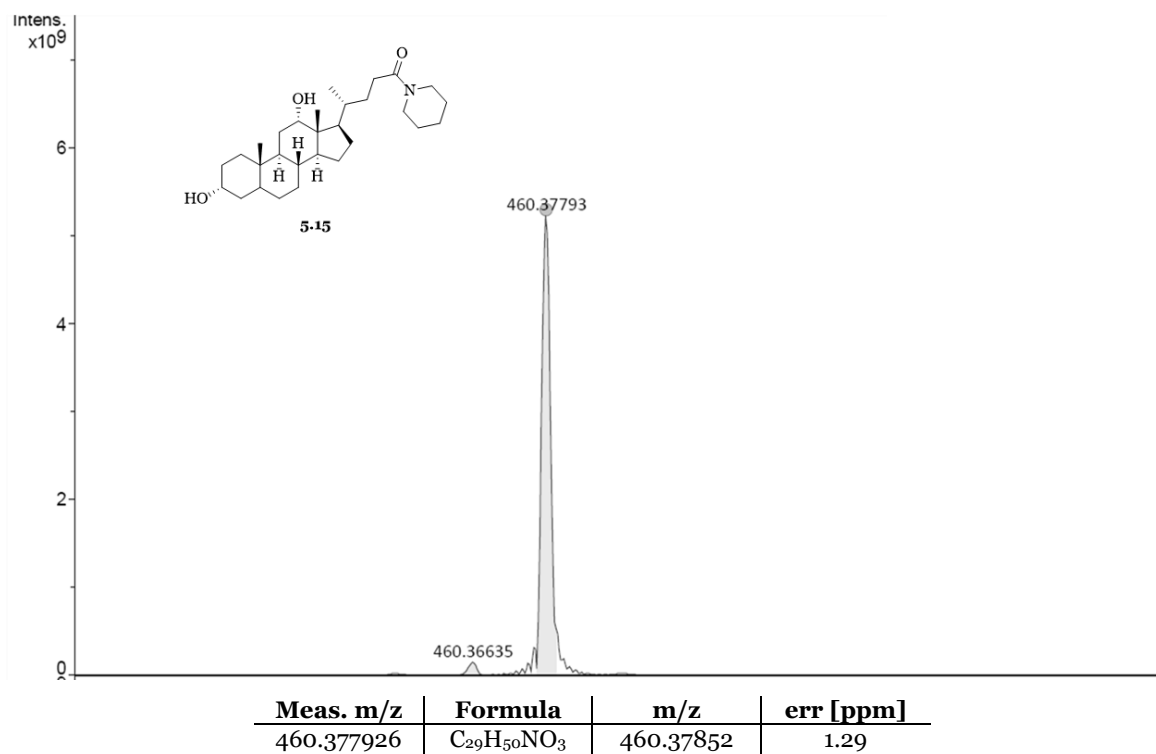


Figure S5.2 ESI data for compound **5.15**.

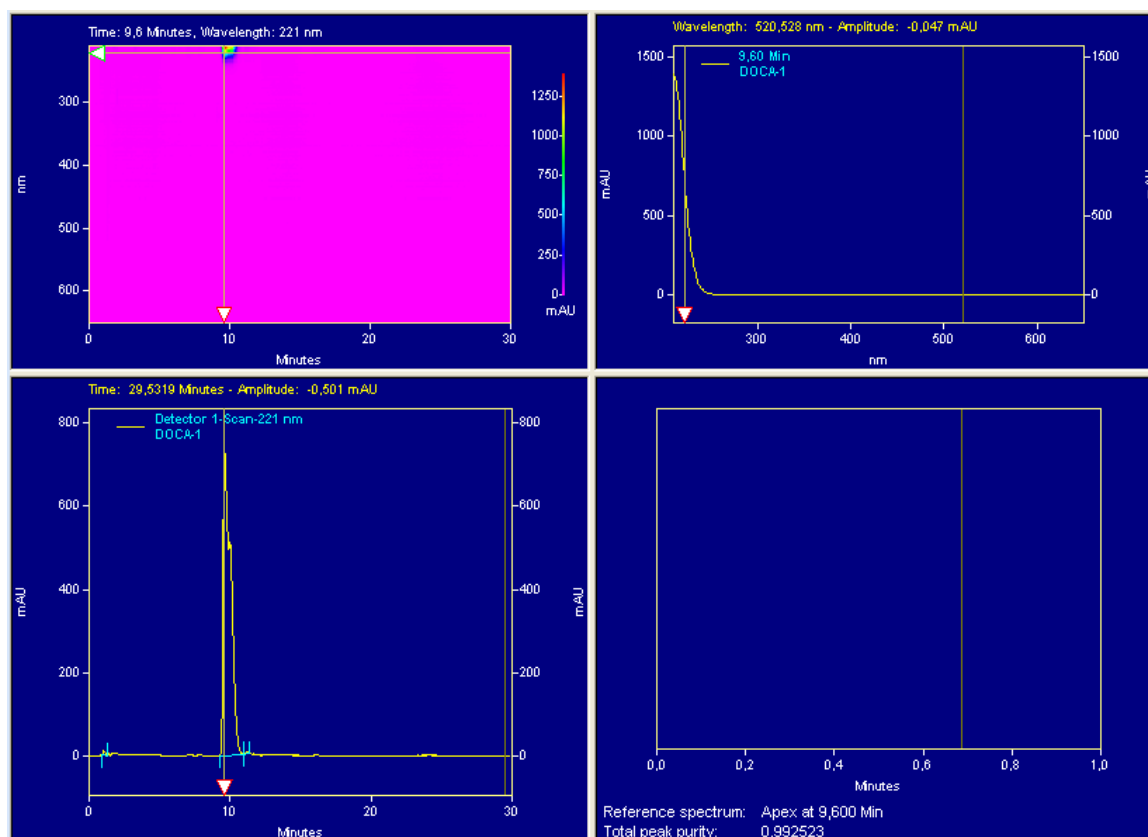


Figure S5.3 Peak purity of compound **5.15**.

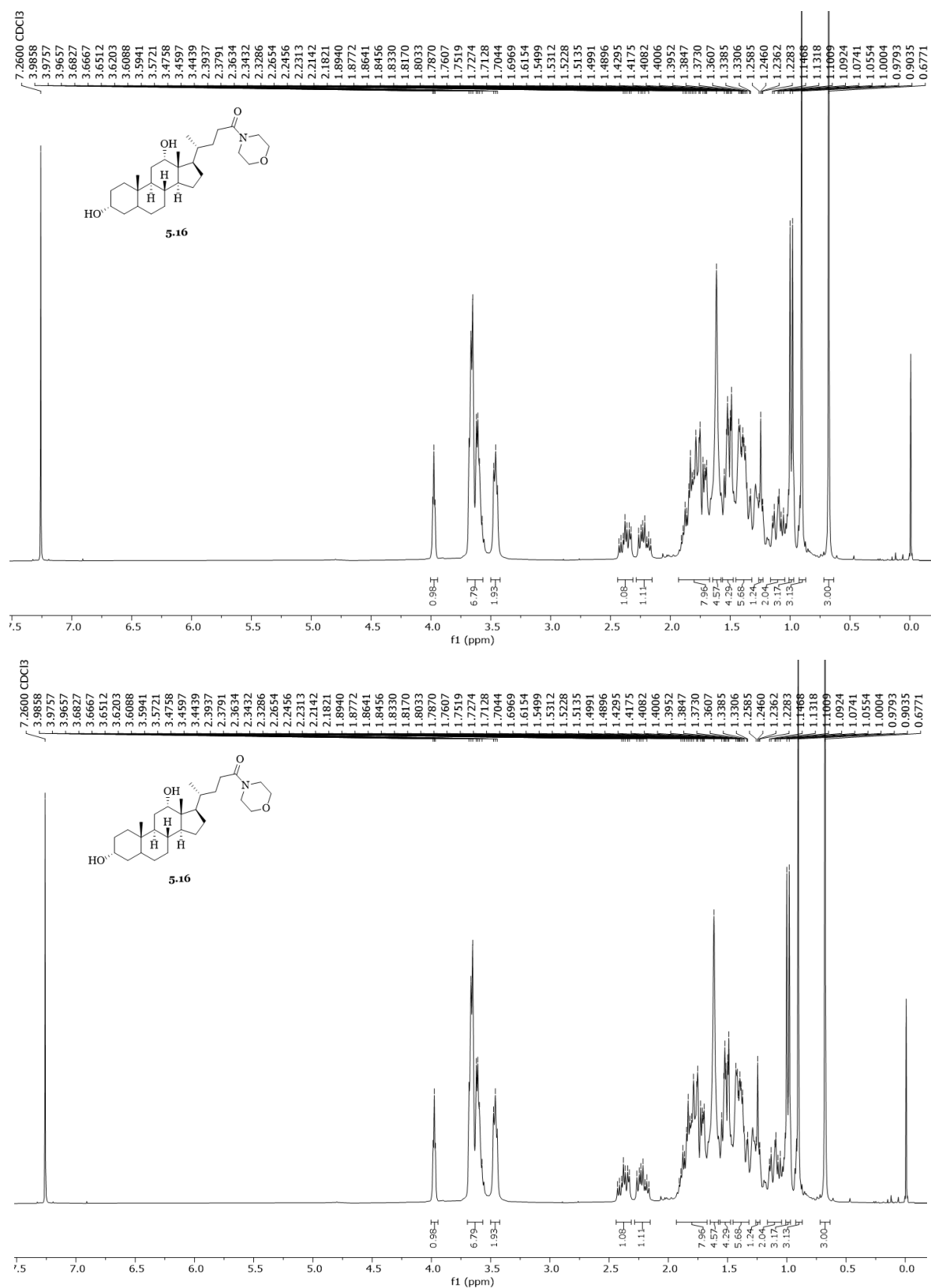


Figure S5.4 ¹H NMR (top, 400.14 MHz, CDCl₃) and ¹³C NMR (bottom, 100.63 MHz, CDCl₃) for compound **5.16**.

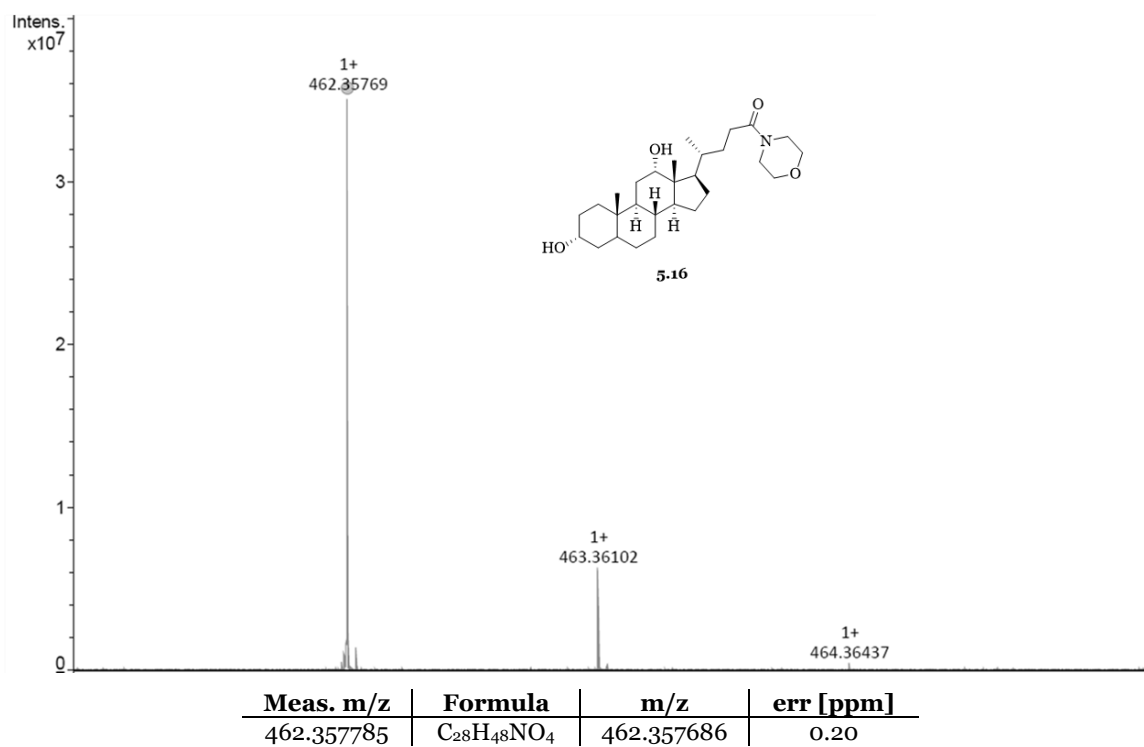


Figure S5.5 ESI data for compound **5.16**.

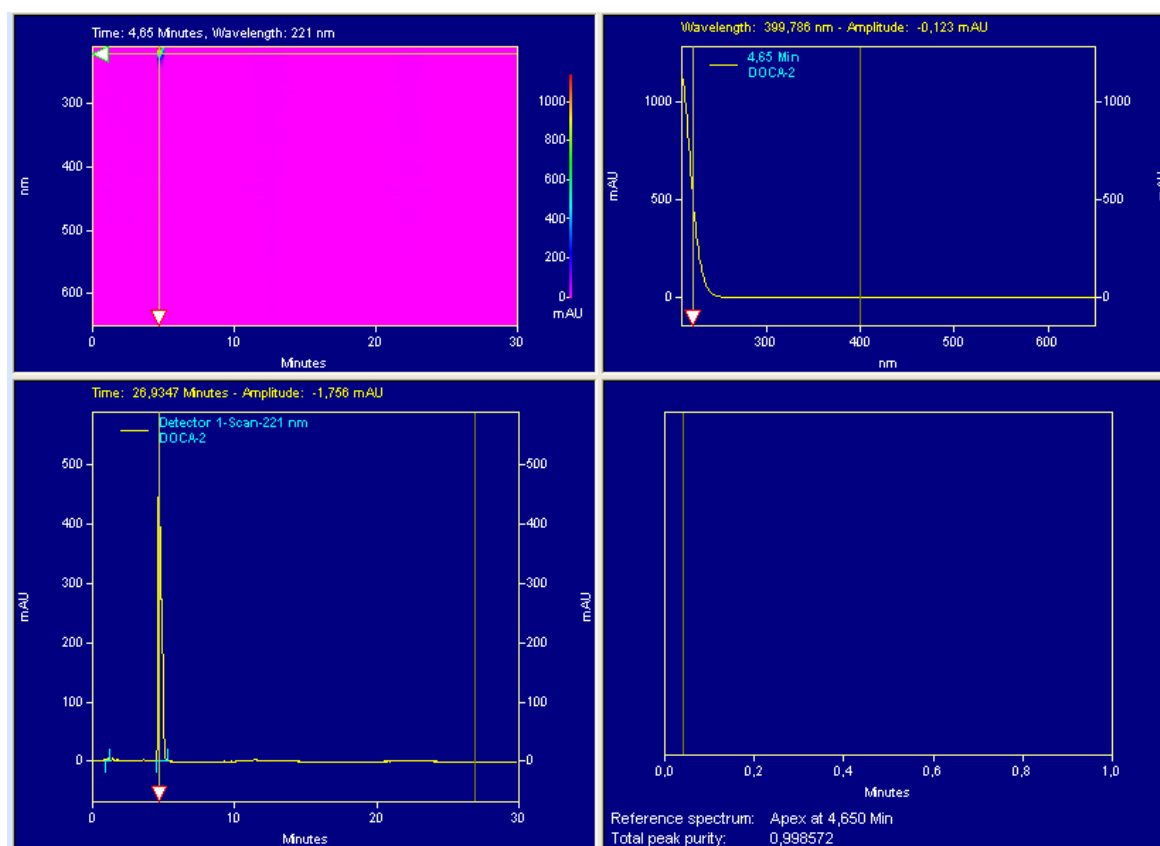


Figure S5.6 Peak purity of compound **5.16**.

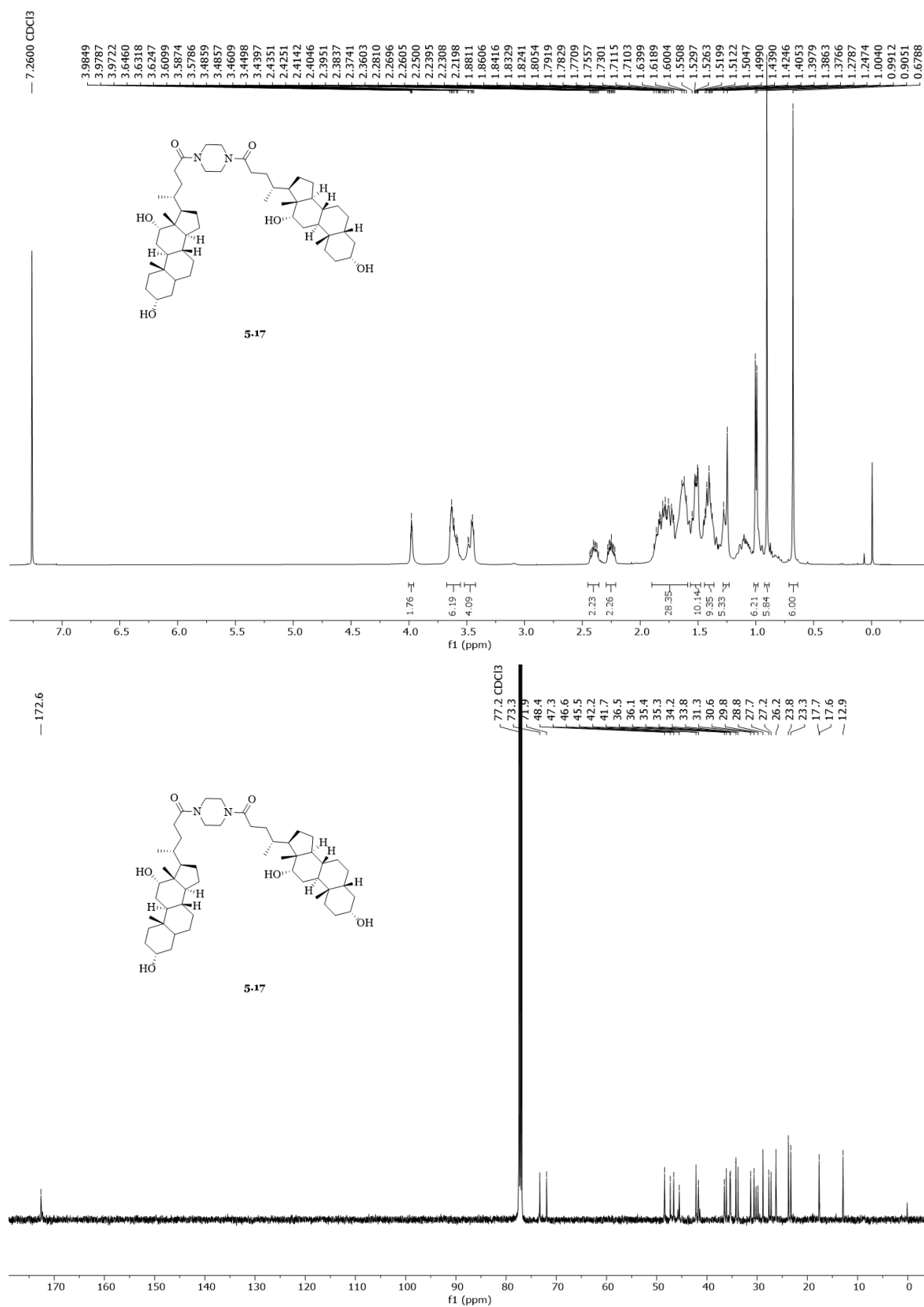


Figure S5.7 ¹H NMR (top, 400.14 MHz, CDCl₃) and ¹³C NMR (bottom, 100.63 MHz, CDCl₃) for compound **5-17**.

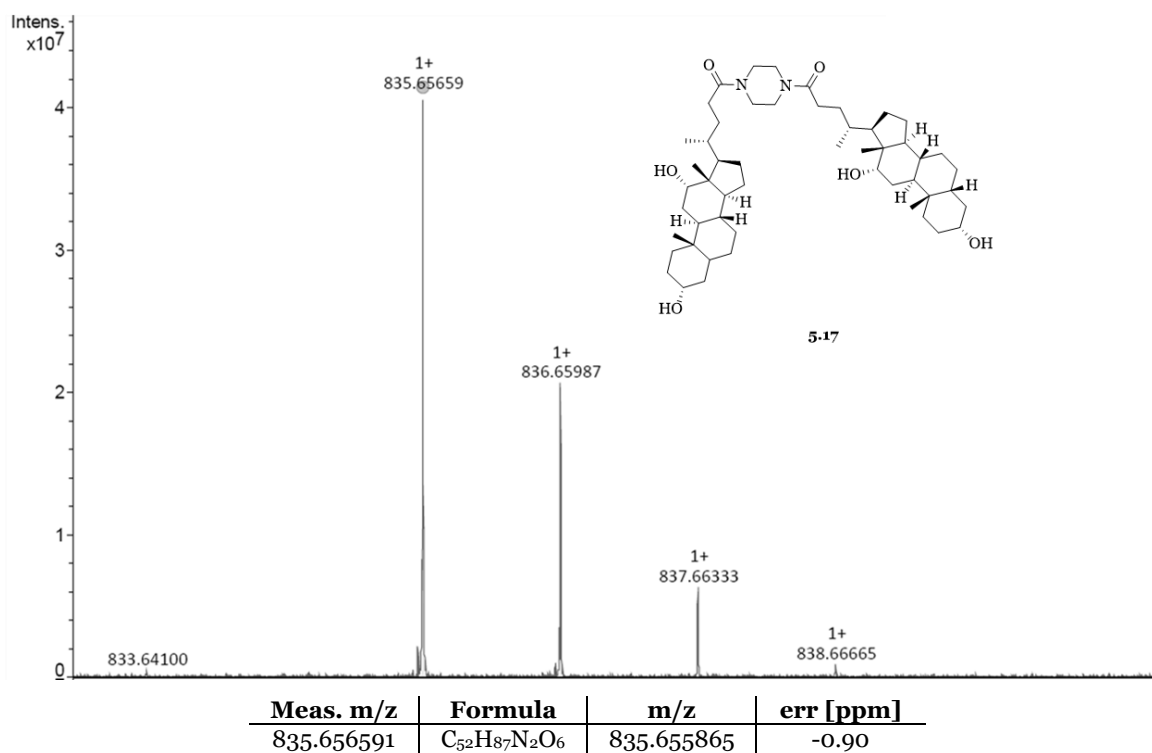


Figure S5.8 ESI data for compound 5.17.

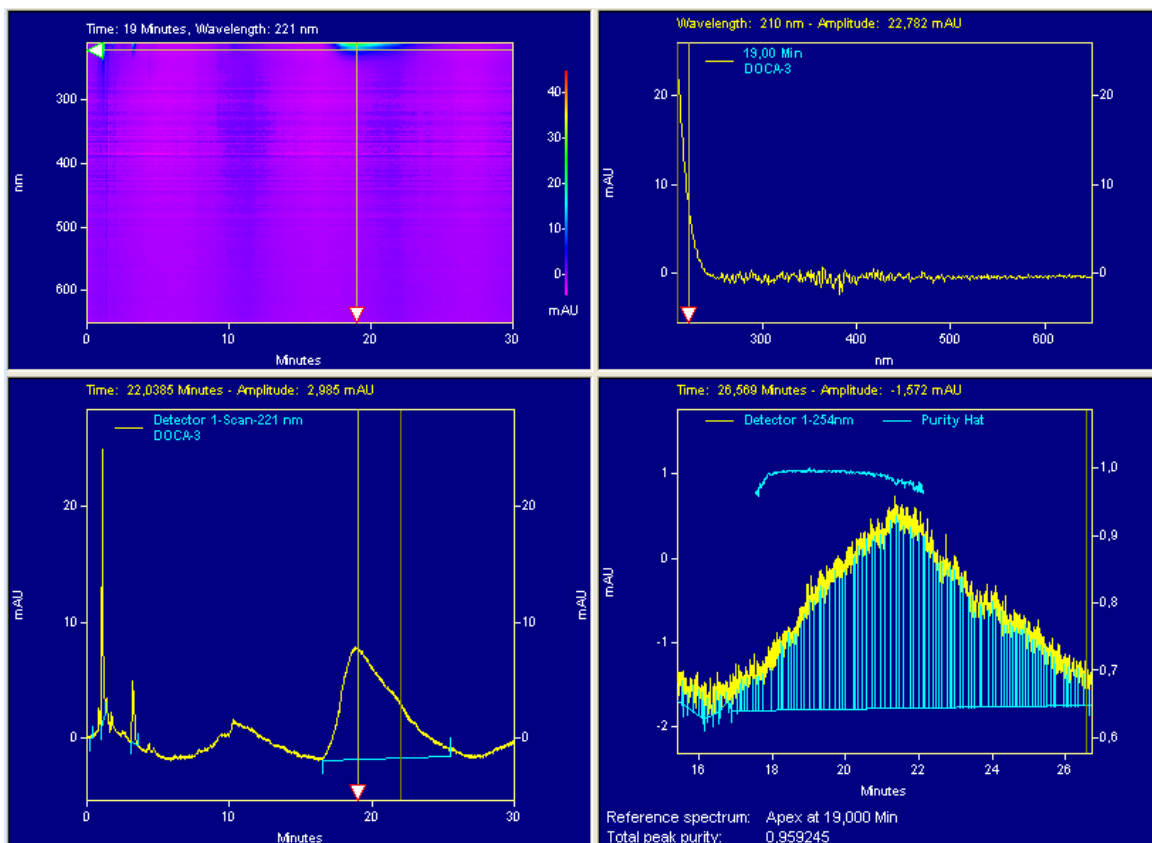


Figure S5.9 Peak purity of compound 5.17.

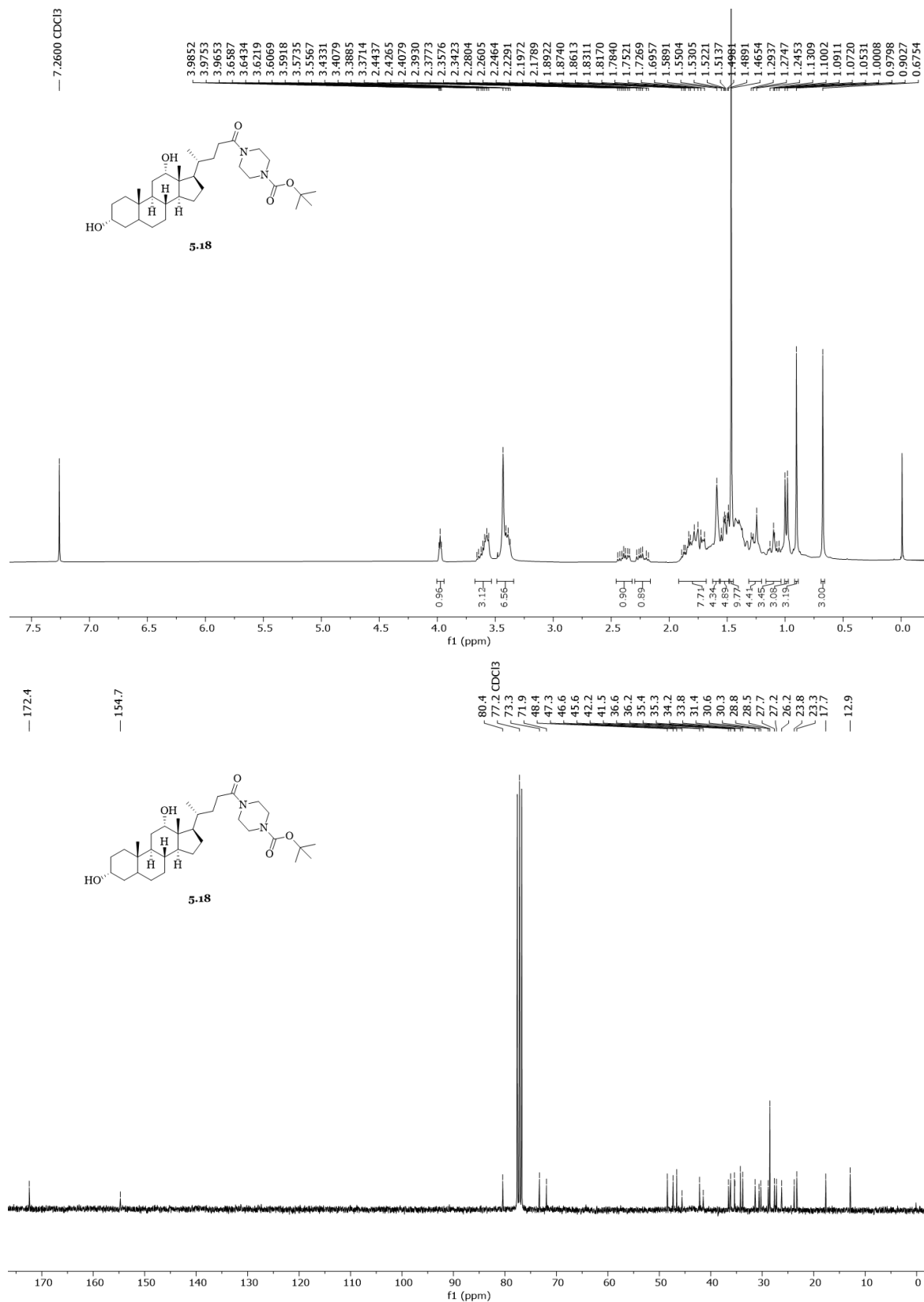


Figure S5.10 ¹H NMR (top, 400.14 MHz, CDCl₃) and ¹³C NMR (bottom, 100.63 MHz, CDCl₃) for compound **5.18**.

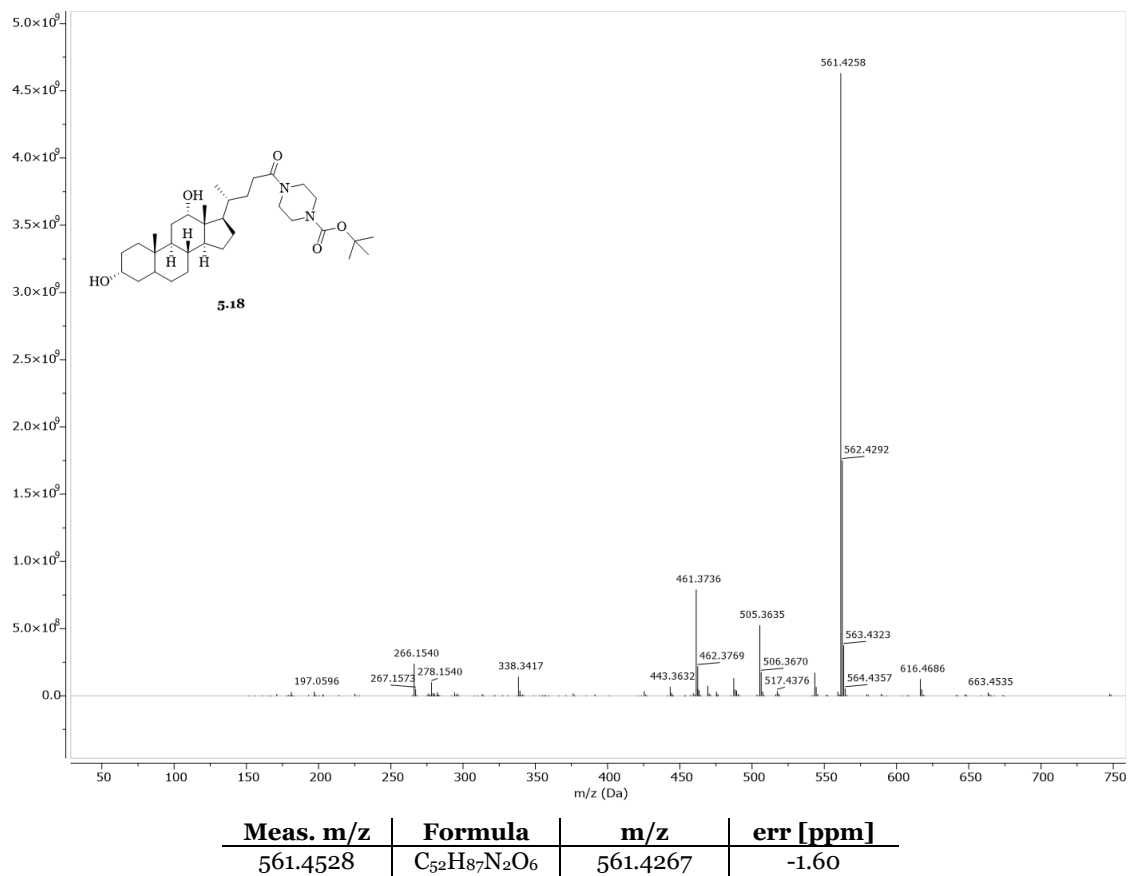


Figure S5.11 ESI data for compound **5.18**.

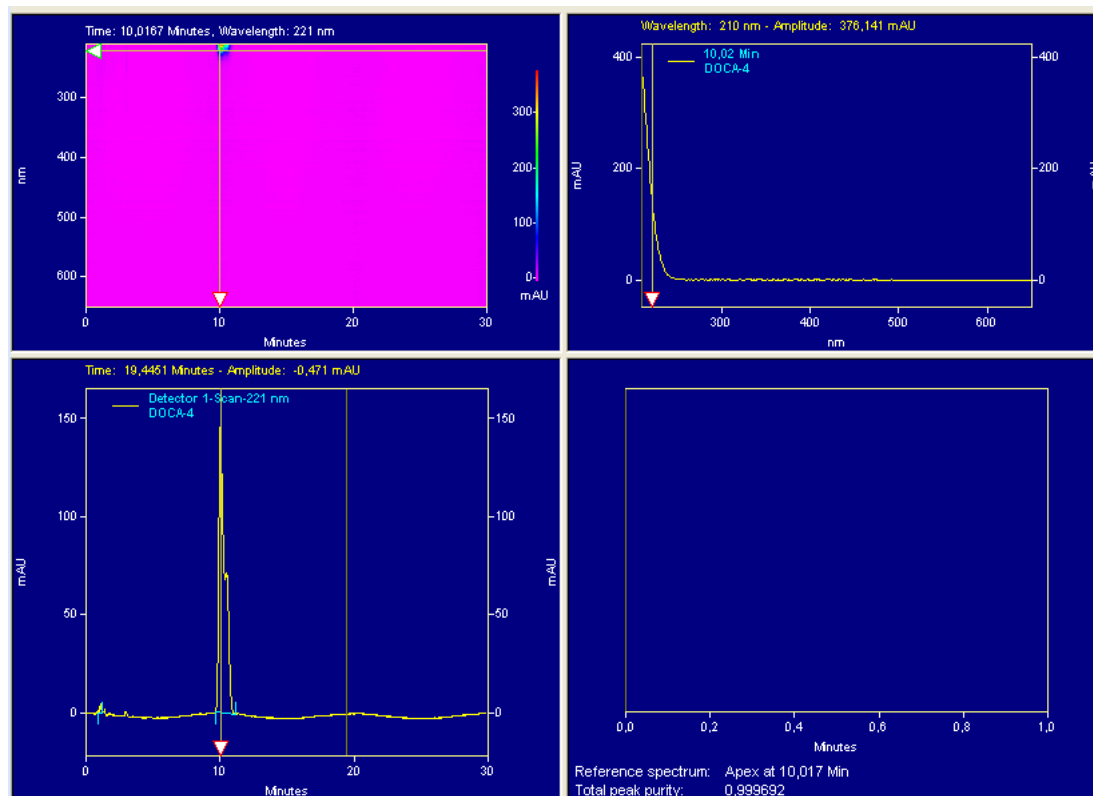


Figure S5.12 Peak purity of compound **5.18**.

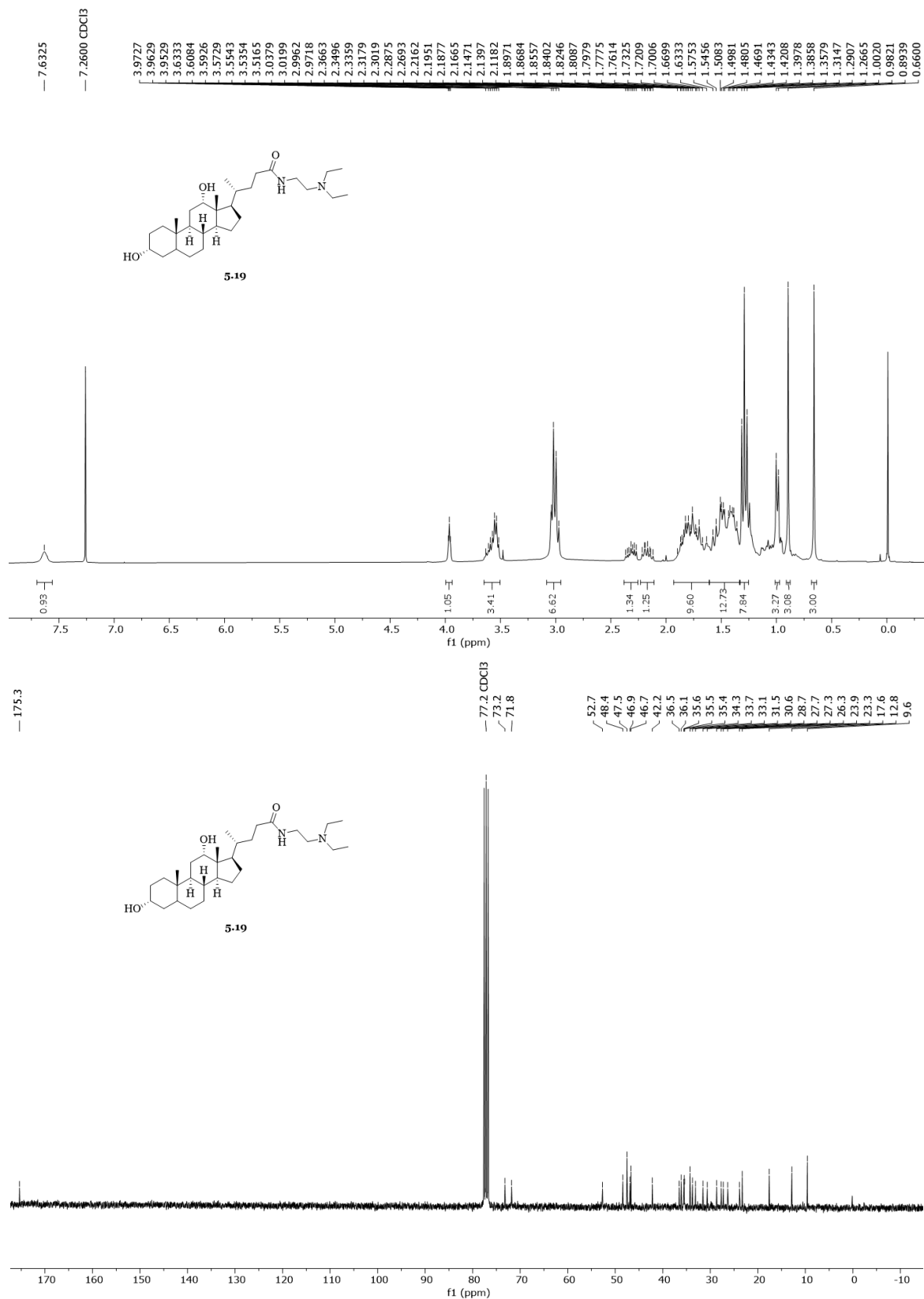


Figure S5.13 ¹H NMR (top, 400.14 MHz, CDCl₃) and ¹³C NMR (bottom, 100.63 MHz, CDCl₃) for compound **5-19**.

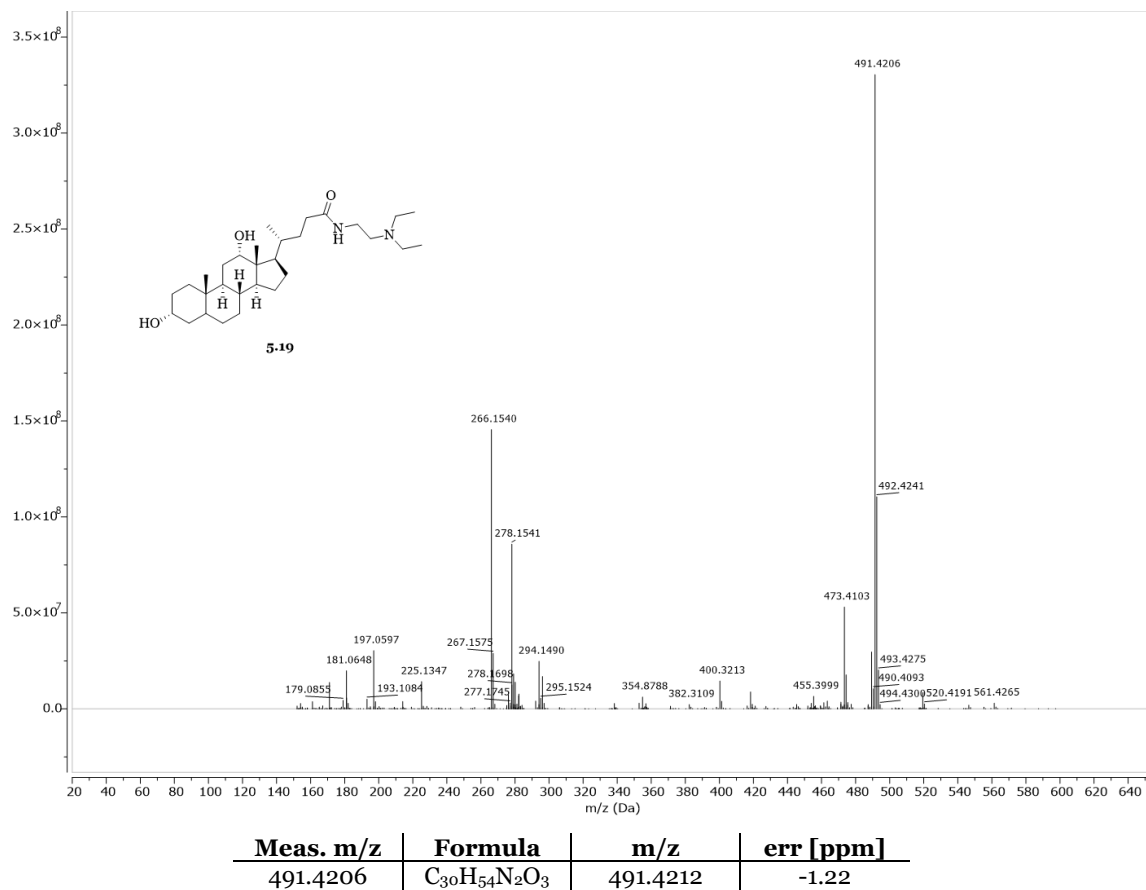


Figure S5.14 ESI data for compound 5.19.

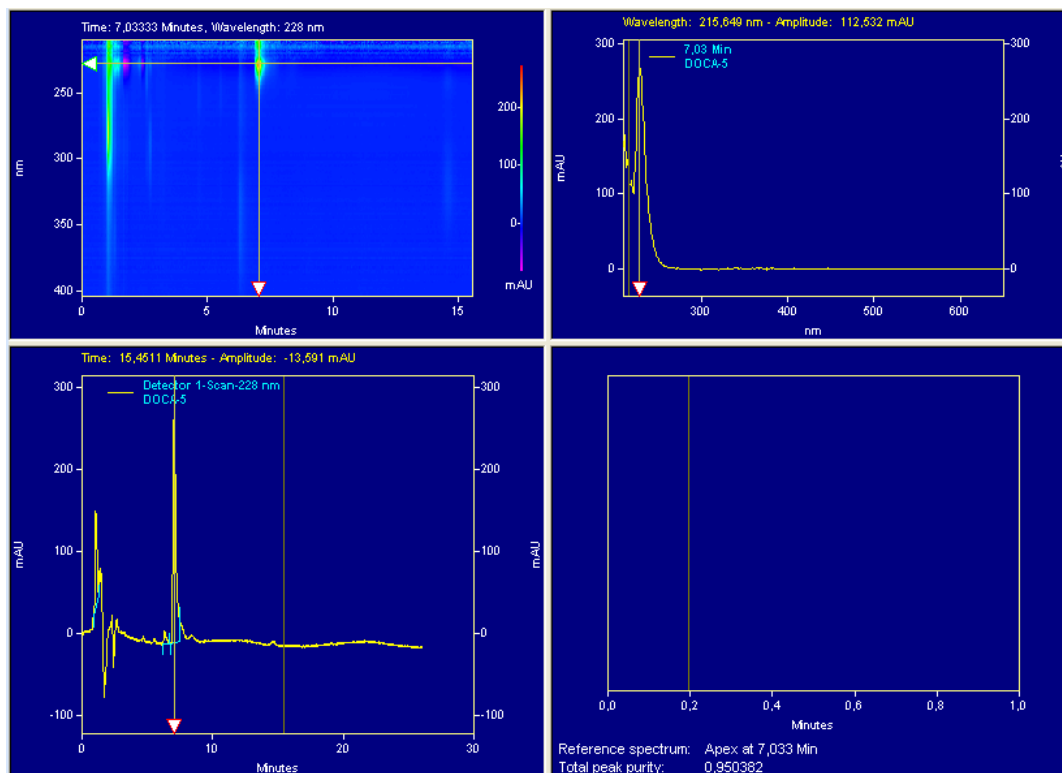


Figure S5.15 Peak purity of compound 5.19.

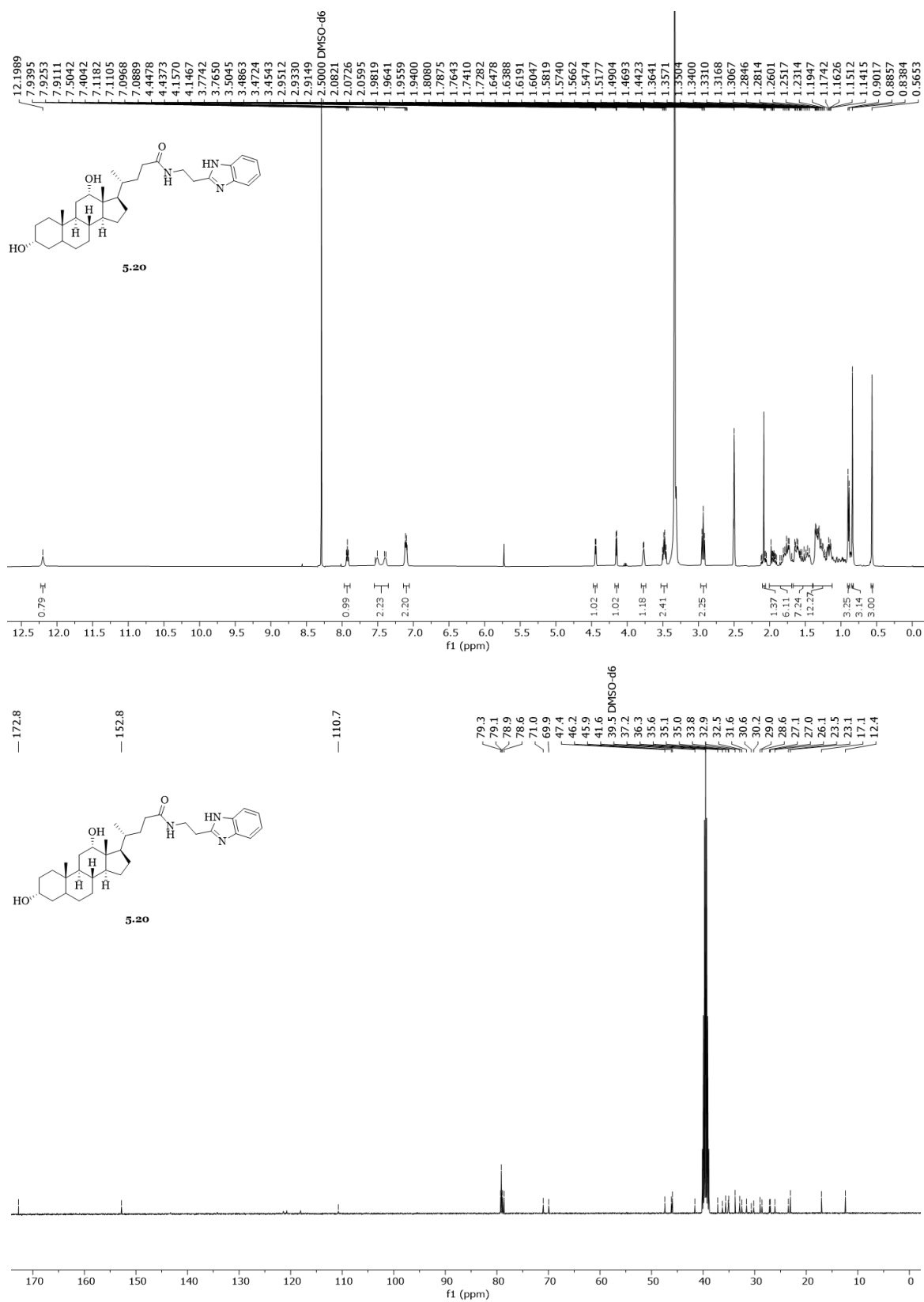


Figure S5.16 ¹H NMR (top, 400.14 MHz, CDCl₃) and ¹³C NMR (bottom, 100.63 MHz, CDCl₃) for compound **5.20**.

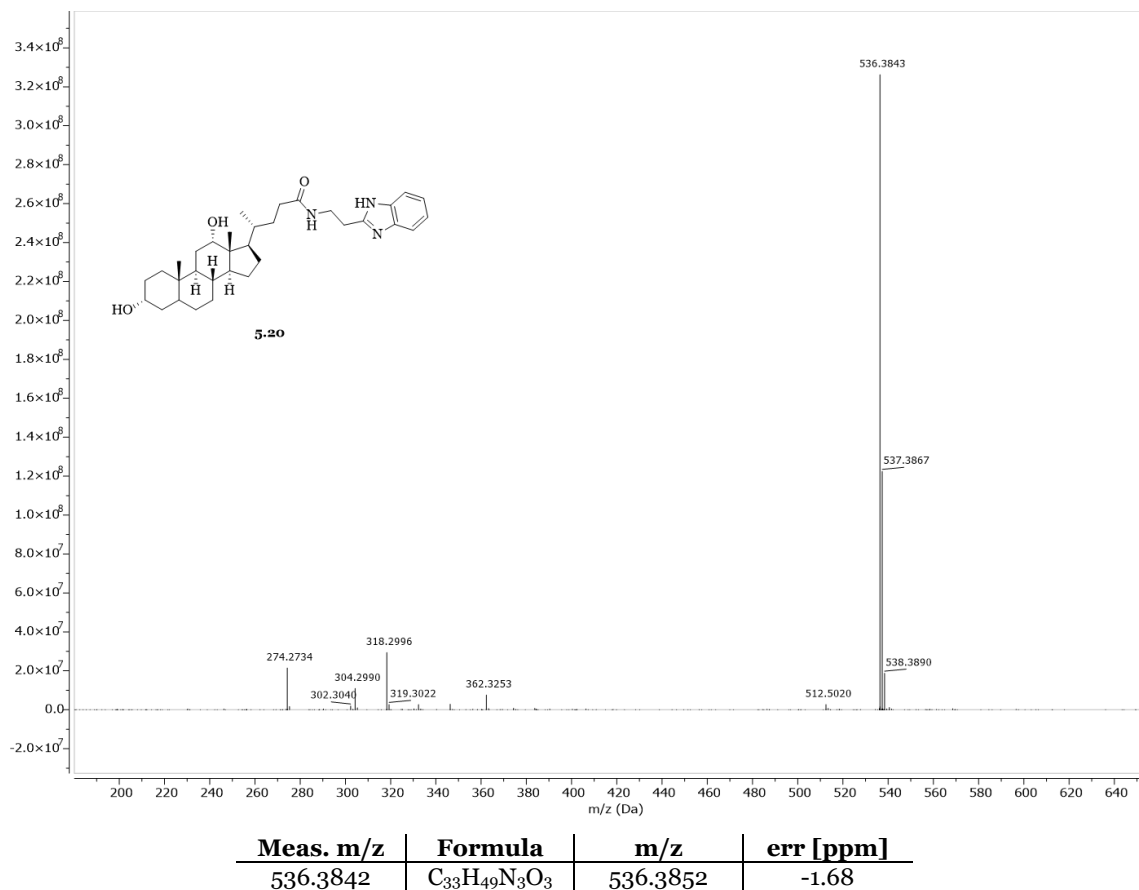


Figure S5.17 ESI data for compound **5.20**.

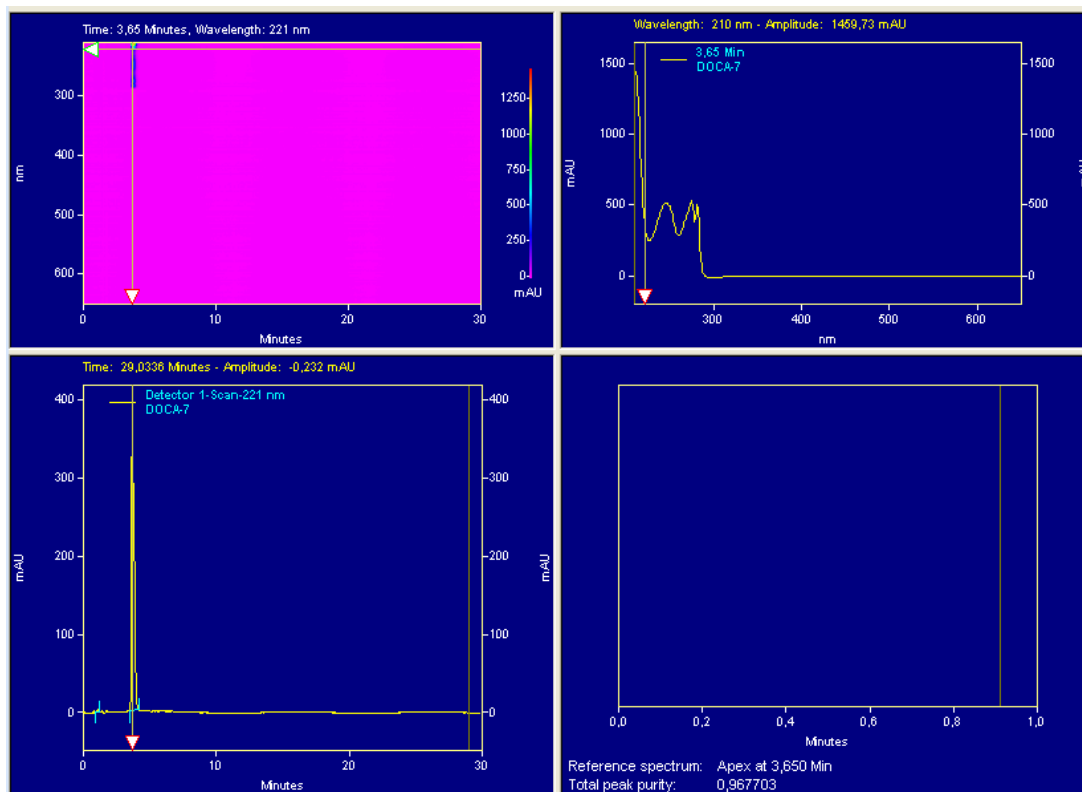


Figure S5.18 Peak purity of compound **5.20**.

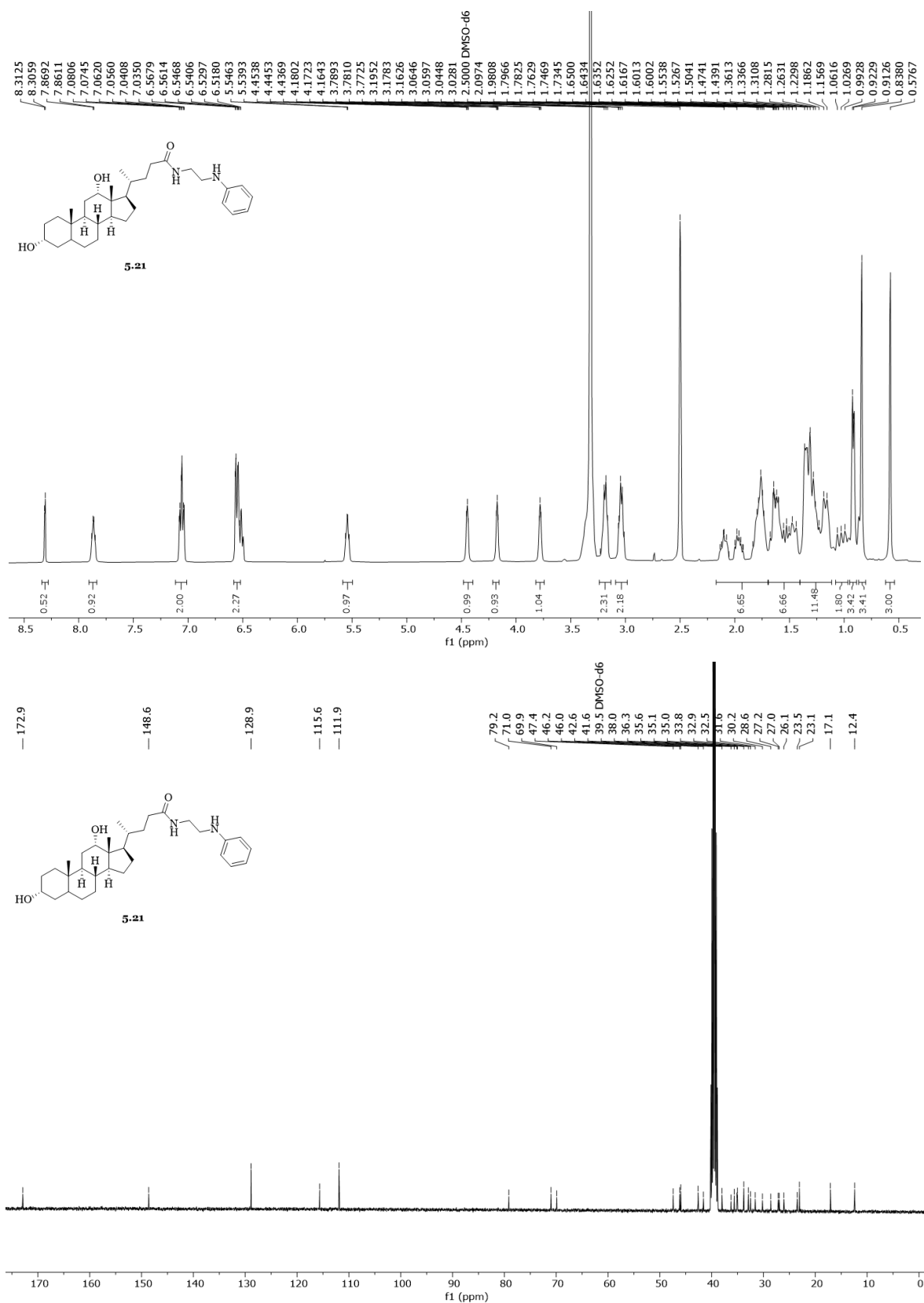


Figure S5.19 ¹H NMR (top, 400.14 MHz, CDCl₃) and ¹³C NMR (bottom, 100.63 MHz, CDCl₃) for compound **5.21**.

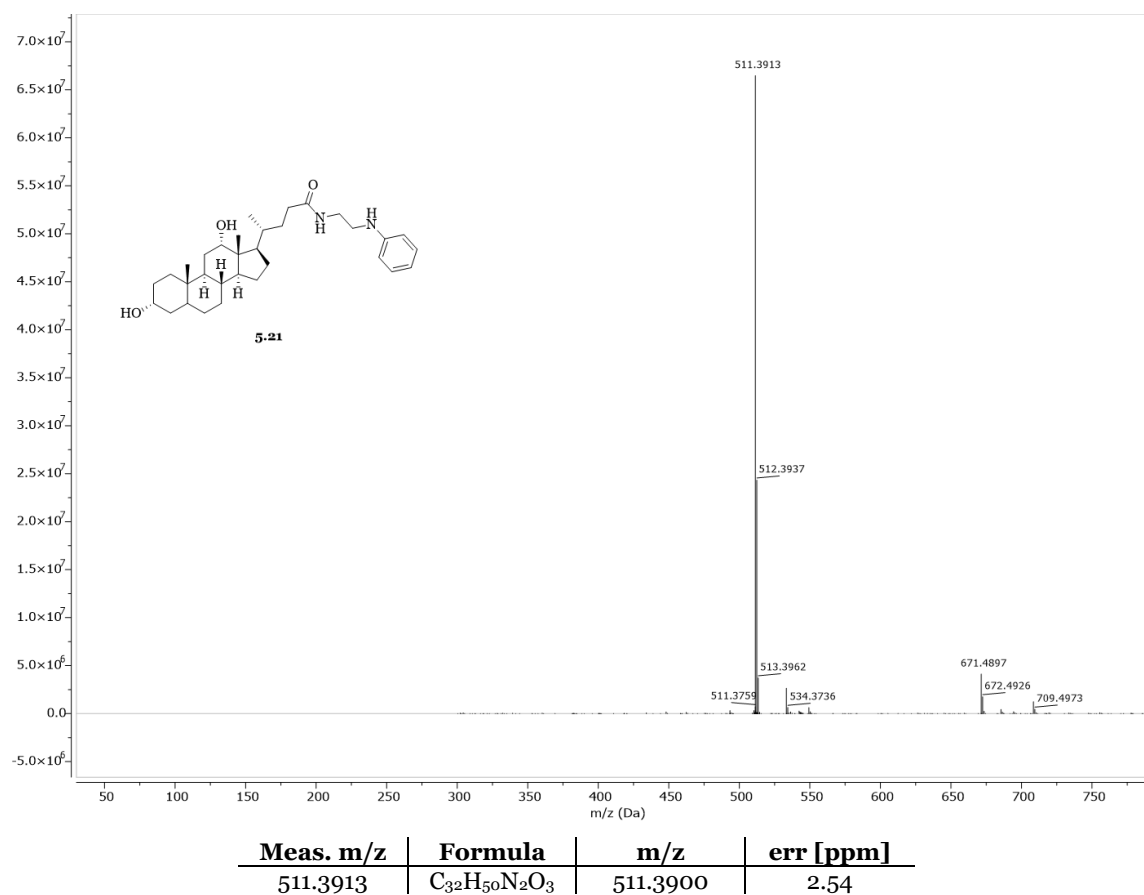


Figure S5.20 ESI data for compound **5.21**.

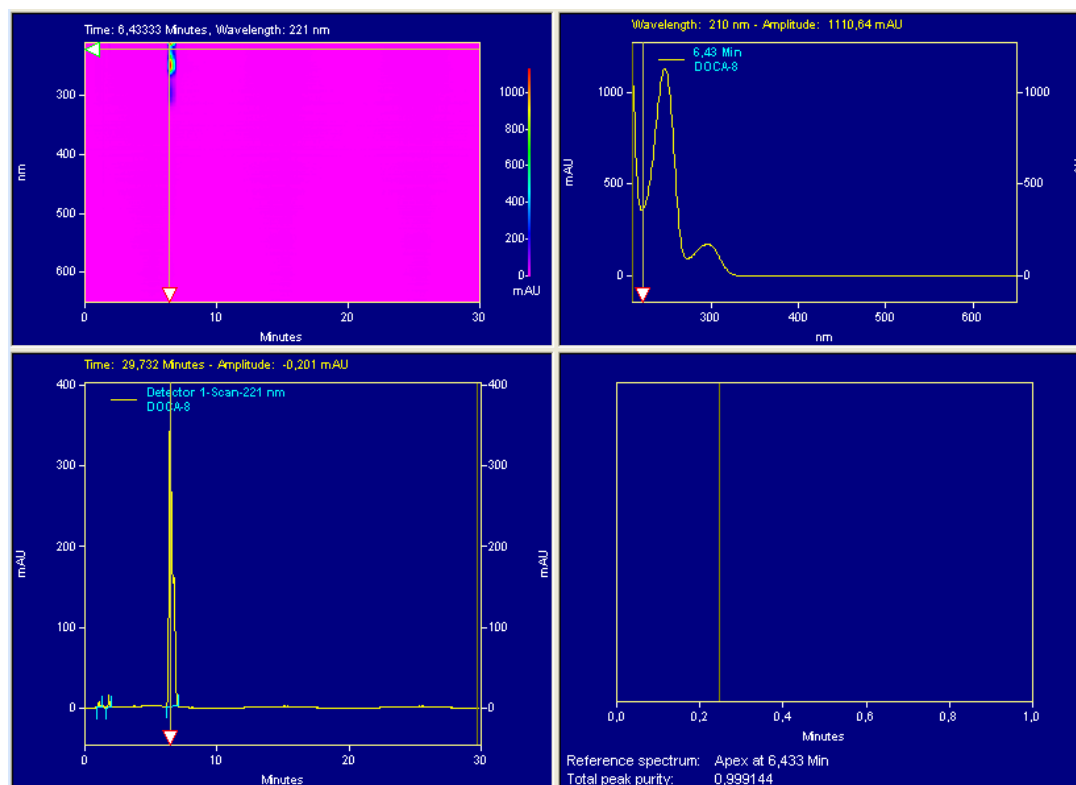


Figure S5.21 Peak purity of compound **5.21**.

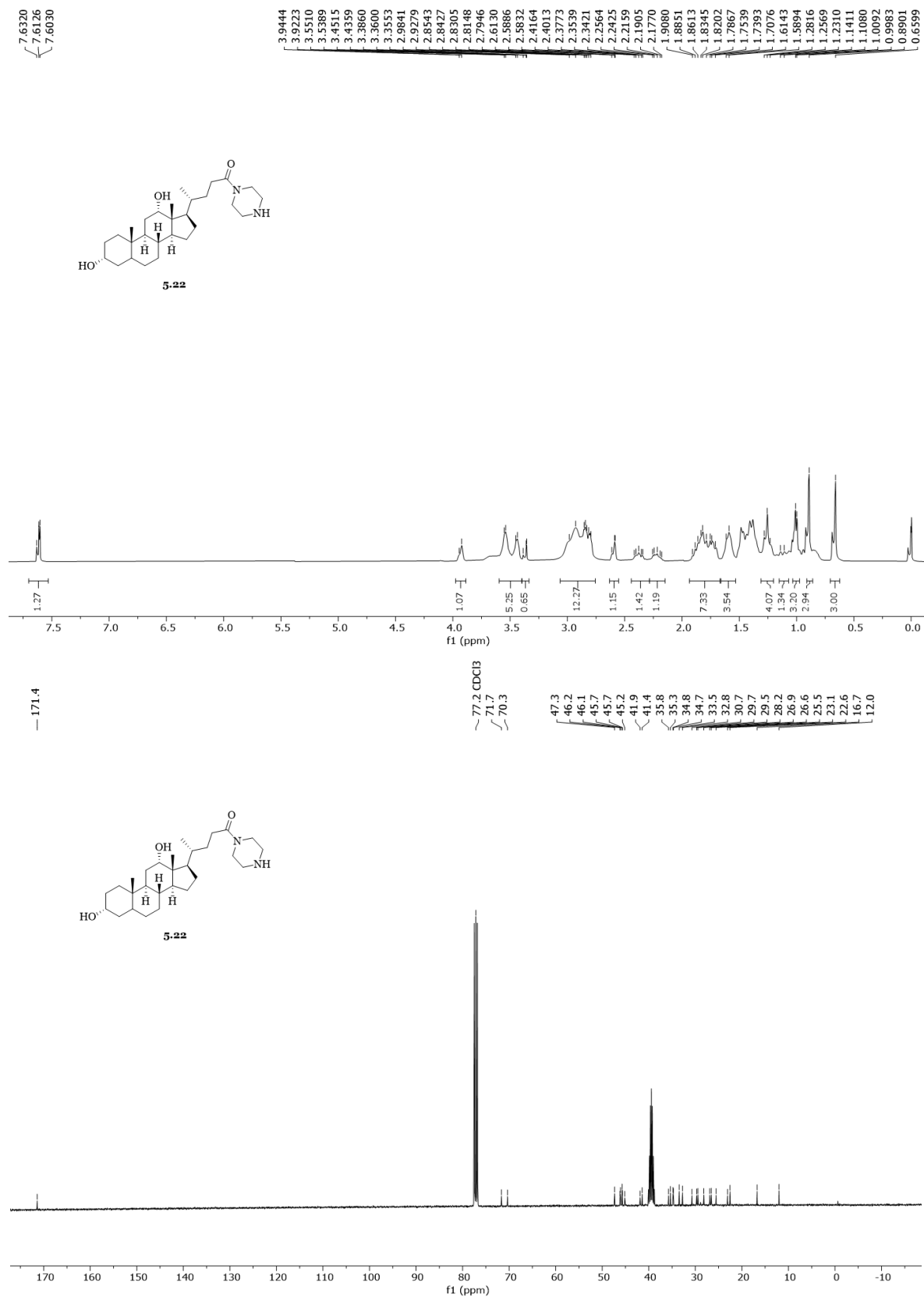
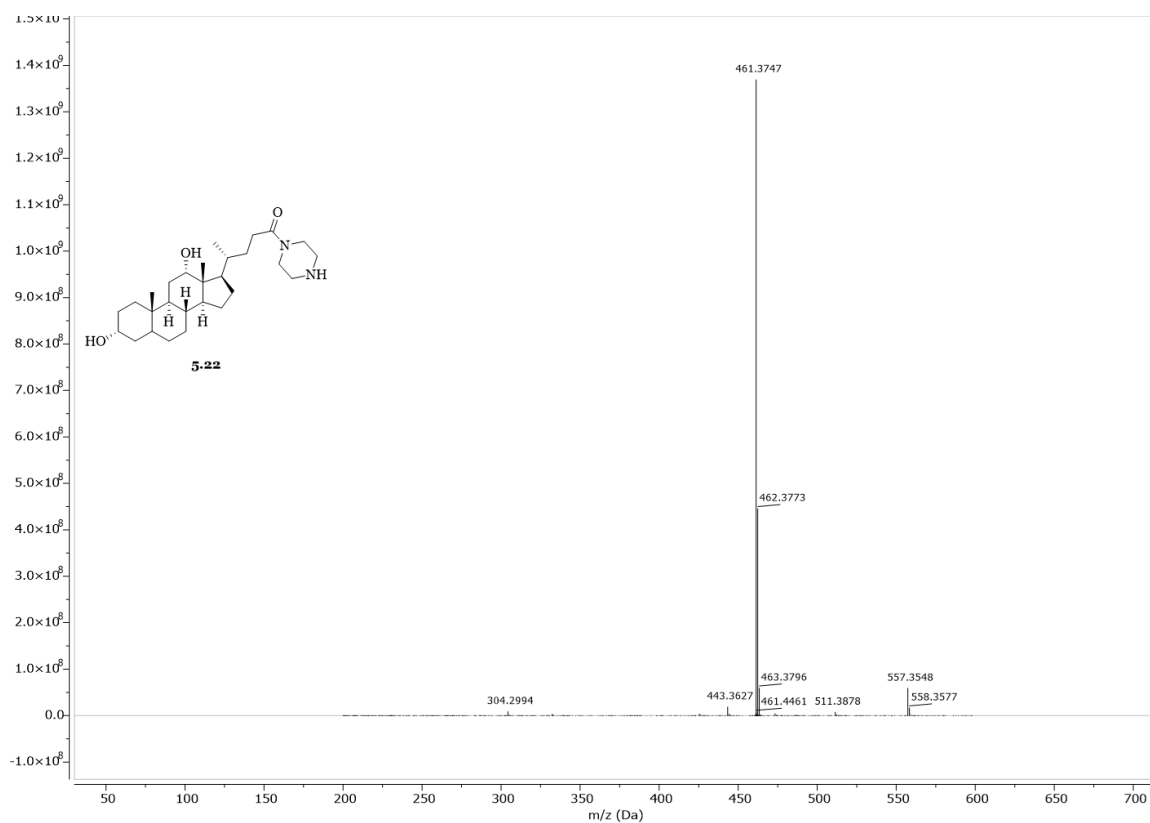


Figure S5.22 ¹H NMR (top, 400.14 MHz, CDCl₃) and ¹³C NMR (bottom, 100.63 MHz, CDCl₃) for compound **5.22**.



Meas. m/z	Formula	m/z	err [ppm]
461.3747	C ₂₈ H ₄₉ N ₂ O ₃	461.3743	0.87

Figure S5.23 ESI data for compound **5.22**.

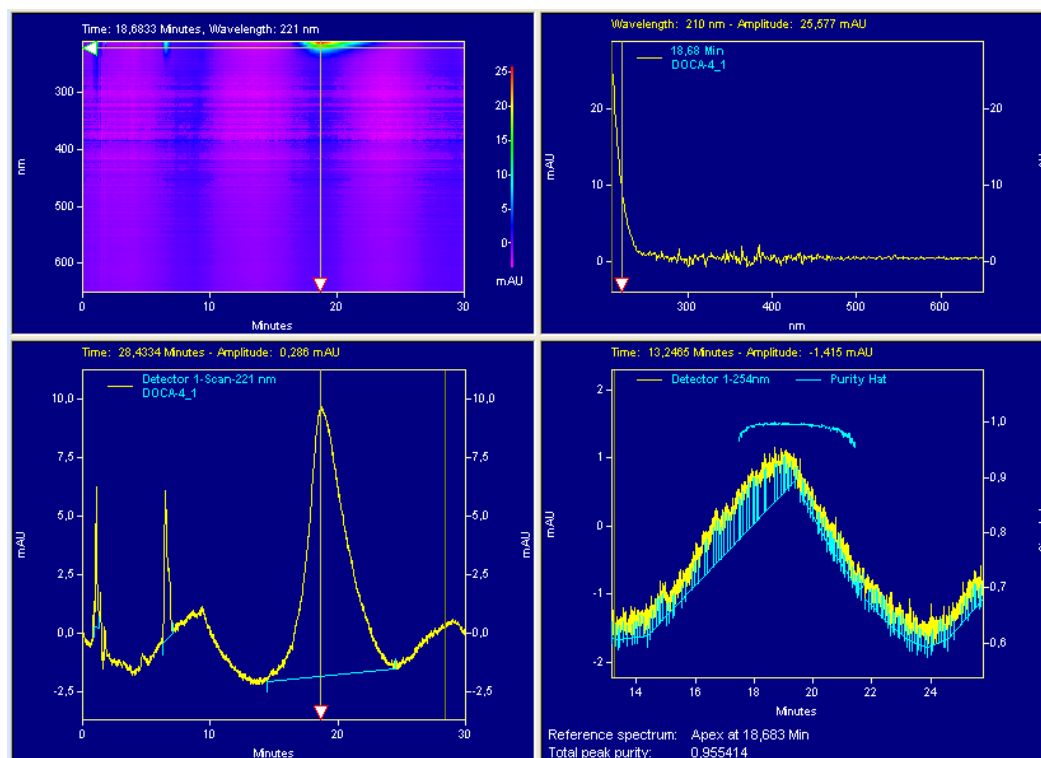


Figure S5.24 Peak purity of compound **5.22**.

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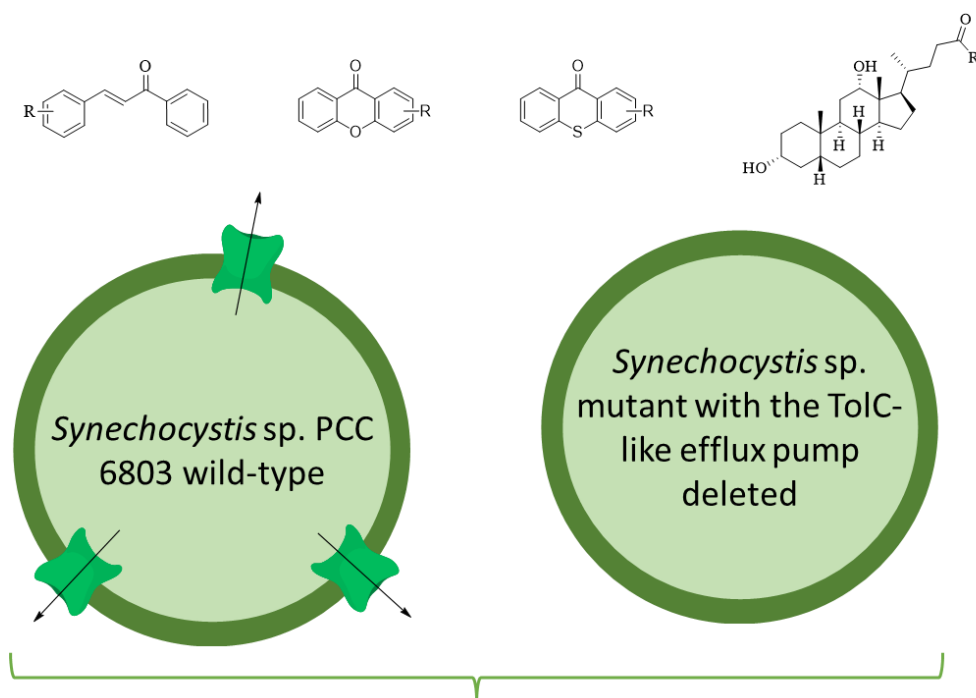
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Chapter 6 – Inhibitory studies in cyanobacteria

Library of 15 structurally diverse compounds



↓ Growth inhibition in the wild-type

↑ Growth inhibition in the mutant strain

Reporter compounds of efflux in cyanobacteria

Author contributions: Compound **6.1** was synthesized by Daniela Pereira, under the supervision of Prof. Honorina Cidade and Prof. Marta Correia da Silva. Compounds **6.2** and **6.3** were synthesized by Diana Resende, under the supervision of Prof. Emília Sousa and Prof. Madalena Pinto. Compound **6.5** was synthesized by Andreia Palmeira, under the supervision of Prof. Emília Sousa and Prof. Madalena Pinto. Compounds **6.6** and **6.7** were synthesized by Ana Rita Neves, under the supervision of Prof. Marta Correia da Silva and Prof. Emília Sousa. Compounds **6.4** and **6.8 – 6.15** were synthesized by Fernando Durães, under the supervision of Prof. Emília Sousa and Prof. Madalena Pinto. The antibacterial assay in the *Synechocystis* sp. strains were performed by Fernando Durães, under the supervision of Dr. Paulo Oliveira.

6.1 Introduction

Aquatic pollution is a major problem of current days, whose lack of management may jeopardize future sustainability. This issue affects not only aquatic biodiversity, but also the quality of rain and drinking water, as well as the climate and even the quality of the air (1). The causes are mostly linked to human activity, being pesticides, metals, plastics, and pharmaceuticals the most found pollutants (2). As such, measures and initiatives are being developed in order to maintain water sustainability, with this matter being covered across the United Nations Sustainable Development Goals (3).

One particularly concerning phenomenon is that of cyanobacterial harmful algal blooms (HABs), caused by climate changes and presence of excessive nutrients in marine and freshwater environments. These HABs are produced in eutrophic conditions by toxigenic Gram-negative cyanobacteria, which are capable of secreting cyanotoxins, and of creating a dense film of cells that accumulates on the surface of aquatic bodies, where these photoautotrophic microorganisms benefit from better light availability (4, 5). Besides the clear decrease in water quality, HABs can also be detrimental to human health, and affect the balance in aquatic ecosystems. In fact, their accumulation on the surface is responsible for decreasing the amount of light irradiation that reaches water environments, leading to changes in water temperature that ultimately result in disturbances to biodiversity (6-8).

The unicellular, freshwater cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) is a globally accepted model for the study of these microbial primary producers. This comes from the fact that it is easily cultivated under laboratory conditions, its genome has been fully sequenced and annotated, and the organism is amenable to genetic modifications (9). Therefore, *Synechocystis* has been widely used to probe various aspects of cyanobacterial physiology, including those related to key processes of primary metabolism, such as photosynthesis, but also mechanisms that contribute to cell fitness and survival, such as secretion mechanisms (10-12).

In the last few years, the interest in studying the profile of compounds that can be recognized and secreted by the cyanobacterial secretory systems, as well as the function of their efflux mechanisms, has been rising steeply, not only due to the important environmental impact of these microorganisms, but also related to the increasing interest on their use for biotechnological applications (13-17). One of the most well-described secretion systems of *Synechocystis* is that dependent on the outer membrane protein Slr1270, which has been shown to mediate the export of a wide array of biomolecules, including proteins, antibiotics, and fatty acids (13, 14, 18). Given its functional roles in secretion, it has been proposed that Slr1270 is the *Synechocystis* outer membrane component of the classical type I secretion and RND systems, homologous to the TolC channel present in Gram-negative bacteria, specifically *E. coli* (11, 13, 14). Even though its

protein structure remains to be elucidated, it is known that Slr1270 displays many structural similarities with TolC, including α -helical properties and a trimer fold (19). Additionally, it is also known that Slr1270 is the only outer membrane protein to be permeable to organic solutes (12), albeit being less abundant than major outer membrane porins (20, 21).

Considering this, a small library of compounds was tested against *Synechocystis* with the aim of evaluating the potential of the compounds to be used as antibacterial agents against noxious cyanobacteria. Moreover, considering the presence of a TolC-like secretion system, the effect of the compounds was also evaluated in a TolC-deletion *Synechocystis* mutant, to gain a better understanding of the interaction of different chemical classes of compounds with the Slr1270 outer membrane protein.

6.2 Library of compounds

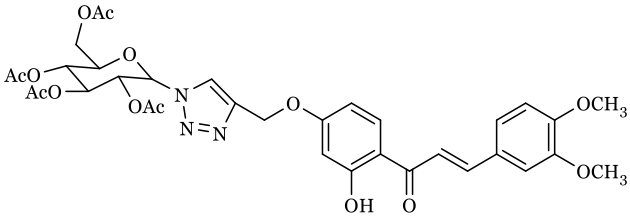
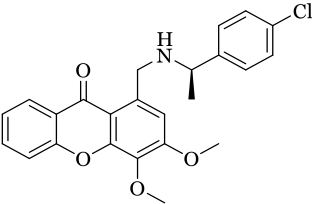
In order to evaluate the effects of the compounds in *Synechocystis* and its TolC-deletion strain, a library of 15 compounds was assembled. These compounds were chosen according to their previous results on bacterial efflux pump inhibition of Gram-negative strains and structural features, meaning the deoxycholic amides synthesized were all tested, due to their potential to interact with Gram-negative bacteria.

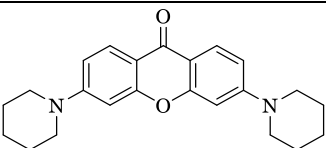
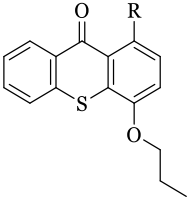
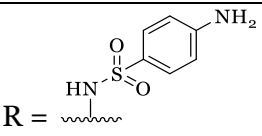
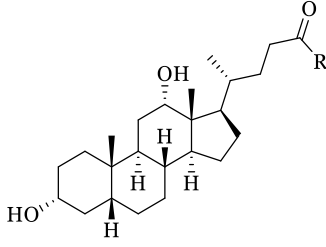
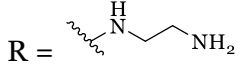
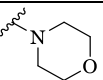
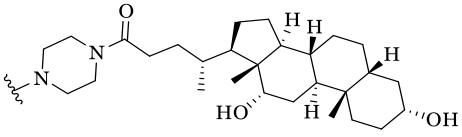
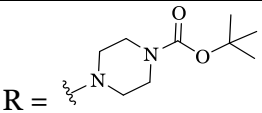
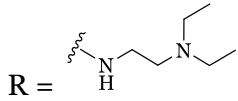
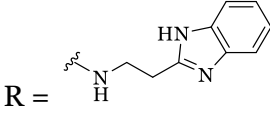
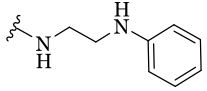
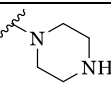
As such, the investigated library was as follows:

- One chalcone (**6.1**);
- Two xanthenes (**6.2** and **6.3**);
- Two thioxanthenes (**6.4** and **6.5**);
- Ten deoxycholic acid derivatives (**6.6** – **6.15**).

The structures of the compounds can be seen in **Table 6.1**.

Table 6.1 Structure of the studied compounds **6.1** – **6.15**.

Compound	Structure
6.1	
6.2	

Compound	Structure	
6.3		
6.4		 $R =$
6.5		 $R =$
6.6		$R = \text{OCH}_3$
6.7		 $R =$
6.8		 $R =$
6.9		 $R =$
6.10		 $R =$
6.11		 $R =$
6.12		 $R =$
6.13		 $R =$
6.14		 $R =$
6.15		 $R =$

6.3 Antibacterial activity

The library of structurally diverse compounds **6.1** – **6.15** was explored for its effect in microorganisms from a different ecological niche than the previously reported, particularly photosynthetic microbes with cosmopolitan distribution.

To that end, the cyanobacterium *Synechocystis* was used. For this study, both wild-type, expressing the TolC-like efflux pump Slr1270, and a mutant, in which the gene coding for this pump is deleted (ΔtolC), were investigated. The compounds were tested at four concentrations, 1, 4, 16, and 64 $\mu\text{g/mL}$, and the OD_{730} of the *Synechocystis* cultures was monitored daily for four days. Based on the collected data, the percentage of growth inhibition on the last measured time-point was calculated based on the DMSO control (**Table 6.2**). The graphs regarding the growth inhibition of the compounds over time can be seen in **Figure S6.1 – Figure S6.15**.

Table 6.2 Percentage of growth inhibition of *Synechocystis* and its TolC-deletion mutant on the fourth day.

Compound	Growth inhibition $\pm$ standard deviation (%)							
	<i>Synechocystis</i> sp. 6803 (wild-type)				<i>Synechocystis</i> ΔtolC			
	1 $\mu\text{g/mL}$	4 $\mu\text{g/mL}$	16 $\mu\text{g/mL}$	64 $\mu\text{g/mL}$	1 $\mu\text{g/mL}$	4 $\mu\text{g/mL}$	16 $\mu\text{g/mL}$	64 $\mu\text{g/mL}$
6.1	0	2.13 $\pm$ 0.07	8.79 $\pm$ 0.08	17.9 $\pm$ 0.09	0	0	3.93 $\pm$ 0.05	32.7 $\pm$ 0.03
6.2	0	1.87 $\pm$ 0.08	6.17 $\pm$ 0.10	10.8 $\pm$ 0.09	0.40 $\pm$ 0.03	92.0 $\pm$ 0.02	91.7 $\pm$ 0.02	91.2 $\pm$ 0.05
6.3	0	0	4.29 $\pm$ 0.09	63.6 $\pm$ 0.09	6.52 $\pm$ 0.02	18.0 $\pm$ 0.07	94.0 $\pm$ 0.03	94.1 $\pm$ 0.01
6.4	0	0	0	0	0	0	0	0
6.5	0	0	54.8 $\pm$ 0.05	73.0 $\pm$ 0.07	21.5 $\pm$ 0.04	94.7 $\pm$ 0.05	93.9 $\pm$ 0.01	88.2 $\pm$ 0.01
6.6	2.78 $\pm$ 0.08	5.51 $\pm$ 0.11	6.86 $\pm$ 0.05	5.64 $\pm$ 0.02	1.78 $\pm$ 0.05	9.97 $\pm$ 0.01	4.25 $\pm$ 0.01	26.7 $\pm$ 0.01
6.7	0	0	21.9 $\pm$ 0.07	90.1 $\pm$ 0.04	2.03 $\pm$ 0.04	91.7 $\pm$ 0.03	94.3 $\pm$ 0.01	90.7 $\pm$ 0.01
6.8	7.31 $\pm$ 0.20	2.87 $\pm$ 0.20	6.11 $\pm$ 0.18	28.9 $\pm$ 0.11	6.87 $\pm$ 0.15	74.8 $\pm$ 0.15	99.4 $\pm$ 0.01	90.7 $\pm$ 0.03
6.9	9.69 $\pm$ 0.22	8.86 $\pm$ 0.24	6.44 $\pm$ 0.15	10.3 $\pm$ 0.14	4.14 $\pm$ 0.21	48.9 $\pm$ 0.07	93.0 $\pm$ 0.03	90.8 $\pm$ 0.03
6.10	5.93 $\pm$ 0.16	6.21 $\pm$ 0.19	1.80 $\pm$ 0.15	0	3.28 $\pm$ 0.16	49.6 $\pm$ 0.09	94.7 $\pm$ 0.01	92.1 $\pm$ 0.06
6.11	8.52 $\pm$ 0.19	4.39 $\pm$ 0.17	4.08 $\pm$ 0.15	6.93 $\pm$ 0.12	7.87 $\pm$ 0.13	99.8 $\pm$ 0.01	93.6 $\pm$ 0.03	89.8 $\pm$ 0.03
6.12	11.2 $\pm$ 0.07	21.7 $\pm$ 0.09	92.7 $\pm$ 0.01	93.2 $\pm$ 0.01	12.8 $\pm$ 0.04	94.6 $\pm$ 0.01	95.1 $\pm$ 0.01	95.7 $\pm$ 0.01
6.13	21.0 $\pm$ 0.09	32.7 $\pm$ 0.05	33.8 $\pm$ 0.05	41.6 $\pm$ 0.11	7.40 $\pm$ 0.03	53.7 $\pm$ 0.04	90.6 $\pm$ 0.04	86.6 $\pm$ 0.01
6.14	3.85 $\pm$ 0.06	7.69 $\pm$ 0.06	30.8 $\pm$ 0.07	39.2 $\pm$ 0.02	2.12 $\pm$ 0.24	52.9 $\pm$ 0.20	86.8 $\pm$ 0.15	82.4 $\pm$ 0.17
6.15	4.72 $\pm$ 0.03	5.40 $\pm$ 0.09	5.58 $\pm$ 0.02	8.39 $\pm$ 0.06	0	17.3 $\pm$ 0.07	52.9 $\pm$ 0.01	82.6 $\pm$ 0.01

Synechocystis ΔtolC : strain with the TolC-like efflux system impaired.

From the analysis of the results, it can be noted that compounds are more effective towards the mutant strain than the wild-type. The general greater potency against the mutant strain supports the possibility that the compounds presented herein are substrates of Slr1270 and are therefore exported through this channel. It could also be noted that the chalcone derivative **6.1** and the thioxanthone **6.4** do not present promising results for either strain tested.

Xanthone derivative **6.2**, thioxanthone **6.5**, and deoxycholic amides **6.7**, **6.11**, and **6.12** showed growth inhibition of the TolC-deleted mutant higher than 90% at concentrations as low as 4 µg/mL, even though no structural feature can be held responsible for this activity. Thioxanthone **6.5** and deoxycholic amides **6.7** and **6.12** were found to be active on both strains. This could mean that linear aliphatic chains containing amines favor the activity of deoxycholic amides in this scope.

The fact that compounds **6.7** and **6.12** show high growth inhibition in the wild-type strain at 64 and 16 µg/mL, respectively, can lead to the hypothesis that these compounds, although being substrates, do not present the same affinity as the rest of the library tested herein for the TolC-like secretion system. It could also mean that these compounds are simply more toxic for this model, since in the $\Delta tolC$ strain they inhibit over 90% of bacterial growth at the low concentration of 4 µg/mL. They can, therefore, be used as growth inhibitors of cyanobacteria.

Overall, considering the results obtained with this model, no compound can unequivocally be deemed as an EPI. However, the differences observed between the wild-type and TolC-deleted strains, show that these compounds can be used as “reporters” of the good functioning of secretion in this model, except for compounds **6.1** and **6.4**. They can also be used as substrates in future assays aiming at finding new EPIs in this setting, as a safer option to EtBr, a known substrate, with mutagenic properties (22, 23).

6.4 Concluding remarks

In this section, studies on the cyanobacterial model *Synechocystis* are described, as a way to study the interaction of a small library of compounds with a very sparsely studied secretion system, resembling TolC. The library of compounds chosen spanned four chemical classes and included compounds able to inhibit EtBr efflux in models of clinically relevant bacteria. It also included the library of deoxycholic amides approached in the previous chapter, due to their structural potential to selectively interact with bacterial membranes.

The initial aim of finding EPIs acting on secretion systems of cyanobacteria was unsuccessful. However, it was noticed that Slr1270, similar to the TolC channel, is definitely involved in the resistance of *Synechocystis* against the compounds tested. As such, compounds **6.2**, **6.10**, **6.11**, and **6.15** can be used as “reporter” compounds for the activity

of this secretion system, as they barely present any activity in the wild-type strain, but display high growth inhibition in the *ΔtolC* strain.

For antibacterial activity against the wild-type strain, compound **6.12** arises as a hit compound, with a high rate of growth inhibition at 16 µg/mL, which could become useful for the treatment of water bodies in eutrophic conditions. Further studies could contemplate the inclusion of other strains of cyanobacteria in antibacterial assays. Future studies in this line could include the enlargement of the library of compounds tested, with the addition of more chemical classes and/or derivatives from the chemical classes tested herein. Derivatives of compounds **6.5**, **6.7**, and **6.12** may also be planned, in order to draw conclusions in terms of SAR for the antibacterial activity of these compounds. Furthermore, studies regarding the synergistic effects of these compounds with known cyanobacterial growth inhibitors could be worthwhile.

6.5 Experimental section

6.5.1 Microorganisms and culture media

The cyanobacterium *Synechocystis* sp. 6803 was grown in BG11 (24), and the *Synechocystis tolC*-deletion mutant strain was cultivated in the same medium supplemented with 100 µg kanamycin/mL.

6.5.2 Antibacterial activity

The *Synechocystis* strains used herein were cultivated in a volume of 50 mL in 100 mL Erlenmeyer flasks. The cultures were kept in an orbital shaker (90 rpm), at 28 °C, in cycles of 16 hours of light (40 µmol photons/m²/s) and 8 hours of darkness.

From this stock solution, a fresh culture would be prepared for each assay performed. Both strains used, wild-type and *tolC*-mutant, were inoculated in 200 mL of liquid BG11 medium, in glass gas-washing bottles with bubbling of 1 L of ambient air per minute, at an initial optical density at 730 nm (OD₇₃₀) of 0.05.

After three to four days, the cultures would reach an OD₇₃₀ of 0.8-1.0, and would be diluted in BG11 to an OD₇₃₀ of 0.4. Then, 150 µL of bacterial suspension would be transferred onto transparent microtiter 96-well plates, which would be supplemented with 50 µL of a solution of each compound in BG11, at 0 (control, containing only the same amount of DMSO as the other conditions), 1, 4, 16, or 64 µg/mL of compounds **6.1** – **6.15**.

The plates were maintained in an orbital shaker at 150 rpm, at 30 °C, under continuous light (40 µmol photons/m²/s). The cell growth was monitored daily for four days, by measuring the OD₇₃₀ after suspending the cells by pipeting, in a Synergy Mx Microplate

reader (BioTek). Each assay was performed three times with independent cultures, each with three technical replicates. The results are presented as growth inhibition $\pm$ SD, and the graphs were made using Microsoft Excel®.

6.6 Supporting information

The graphs of the growth inhibition of the compounds over time can be seen in **Figure S6.1 – Figure S6.15**.

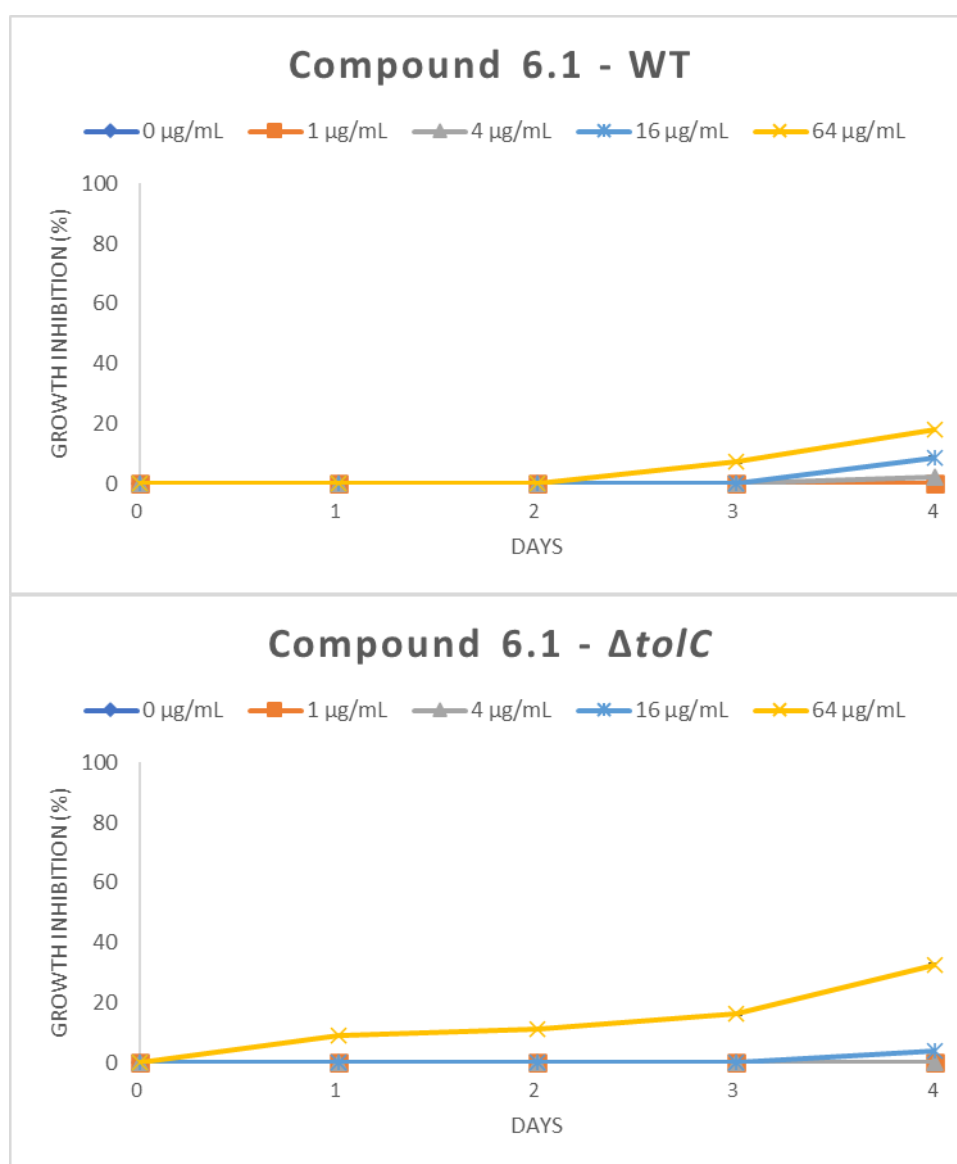


Figure S6.1 Growth inhibition of *Synechocystis* wild-type (top) and $\Delta tolC$ (bottom) over time - compound 6.1.

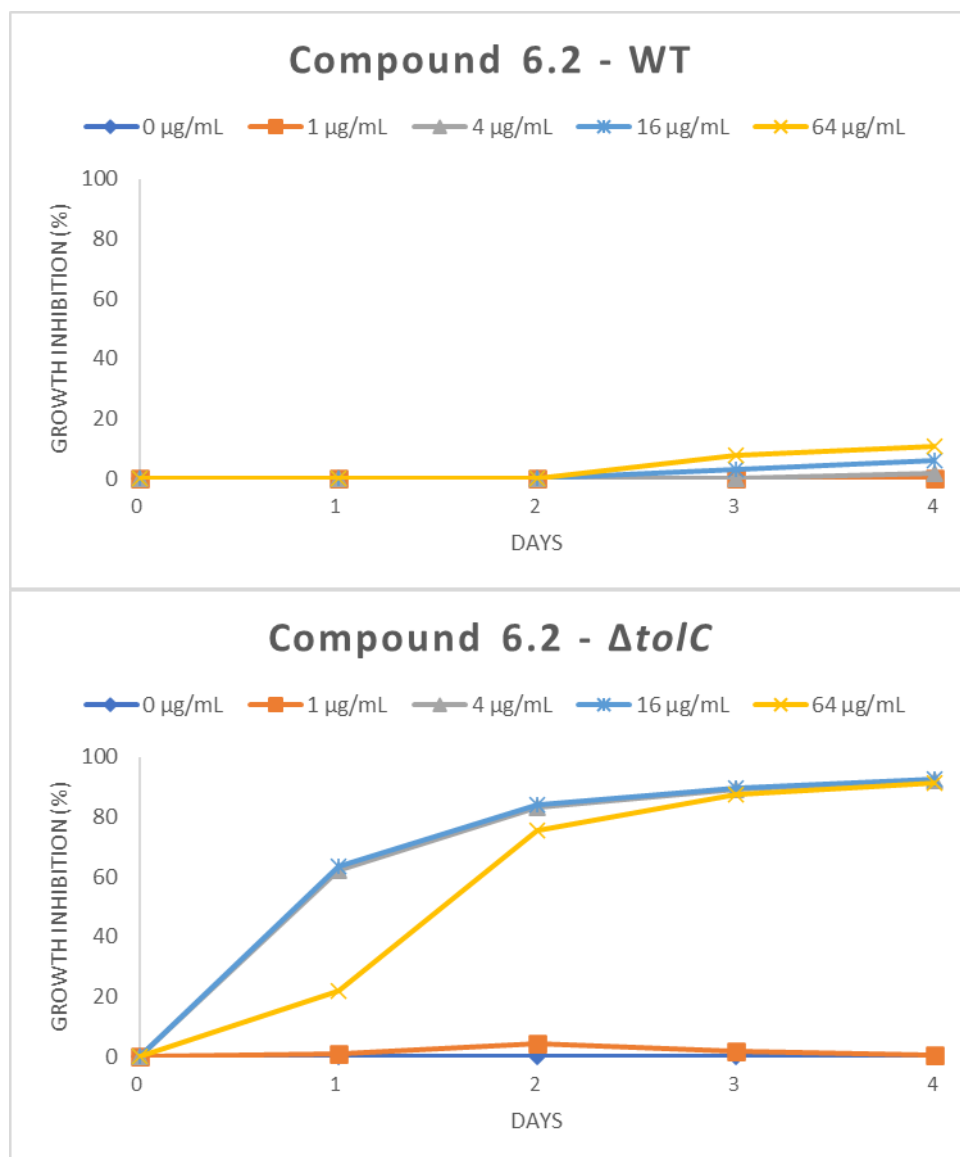


Figure S6.2 Growth inhibition of *Synechocystis* wild-type (top) and ΔtolC (bottom) over time - compound 6.2.

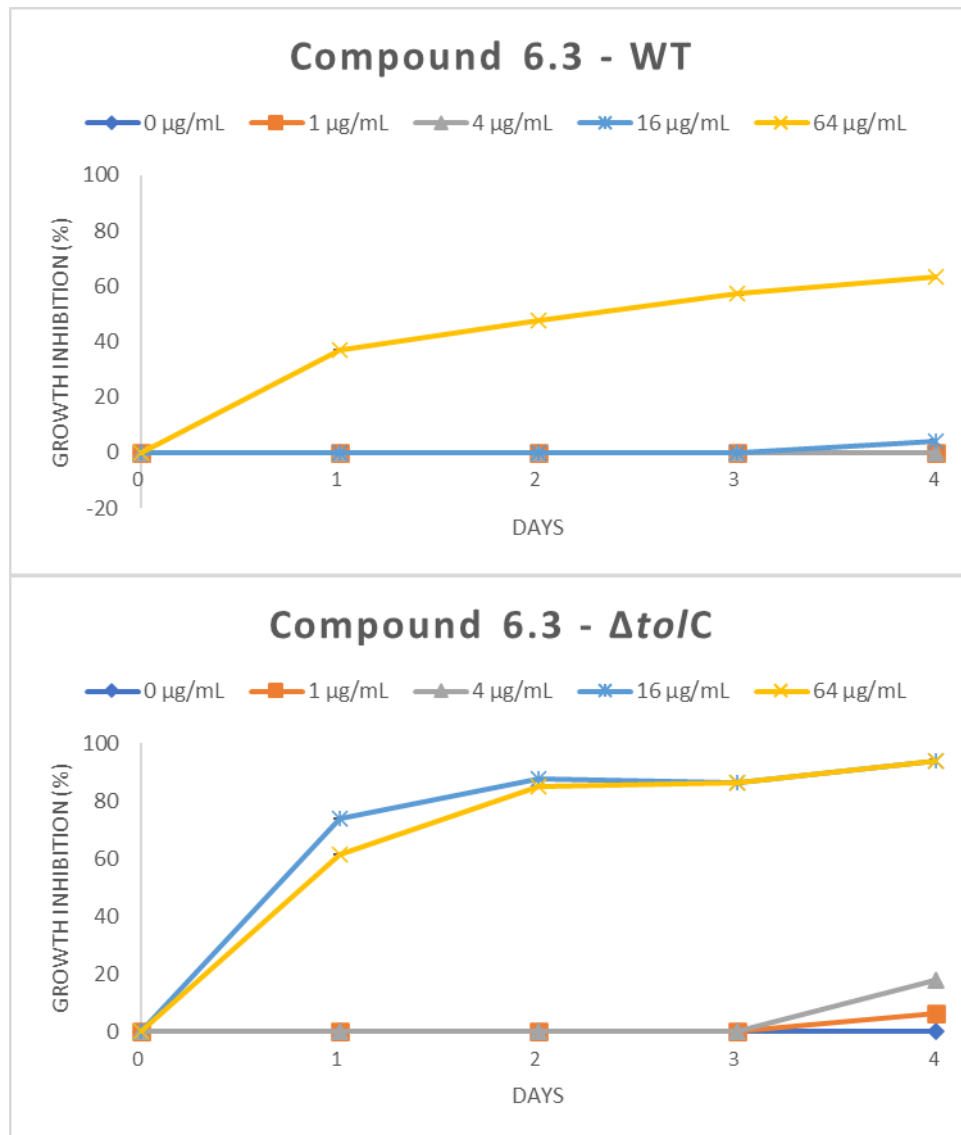


Figure S6.3 Growth inhibition of *Synechocystis* wild-type (top) and ΔtolC (bottom) over time - compound 6.3.

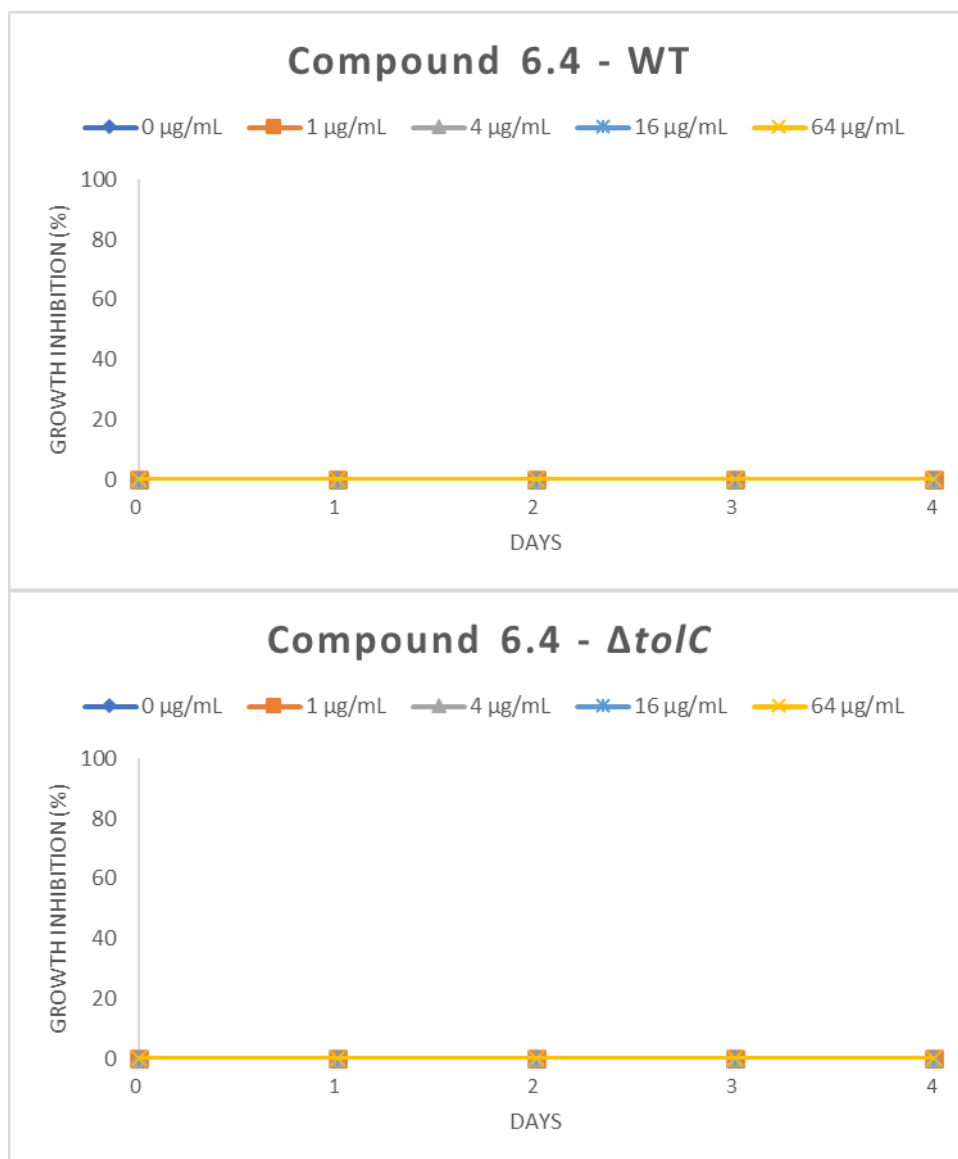


Figure S6.4 Growth inhibition of *Synechocystis* wild-type (top) and $\Delta tolC$ (bottom) over time - compound 6.4.

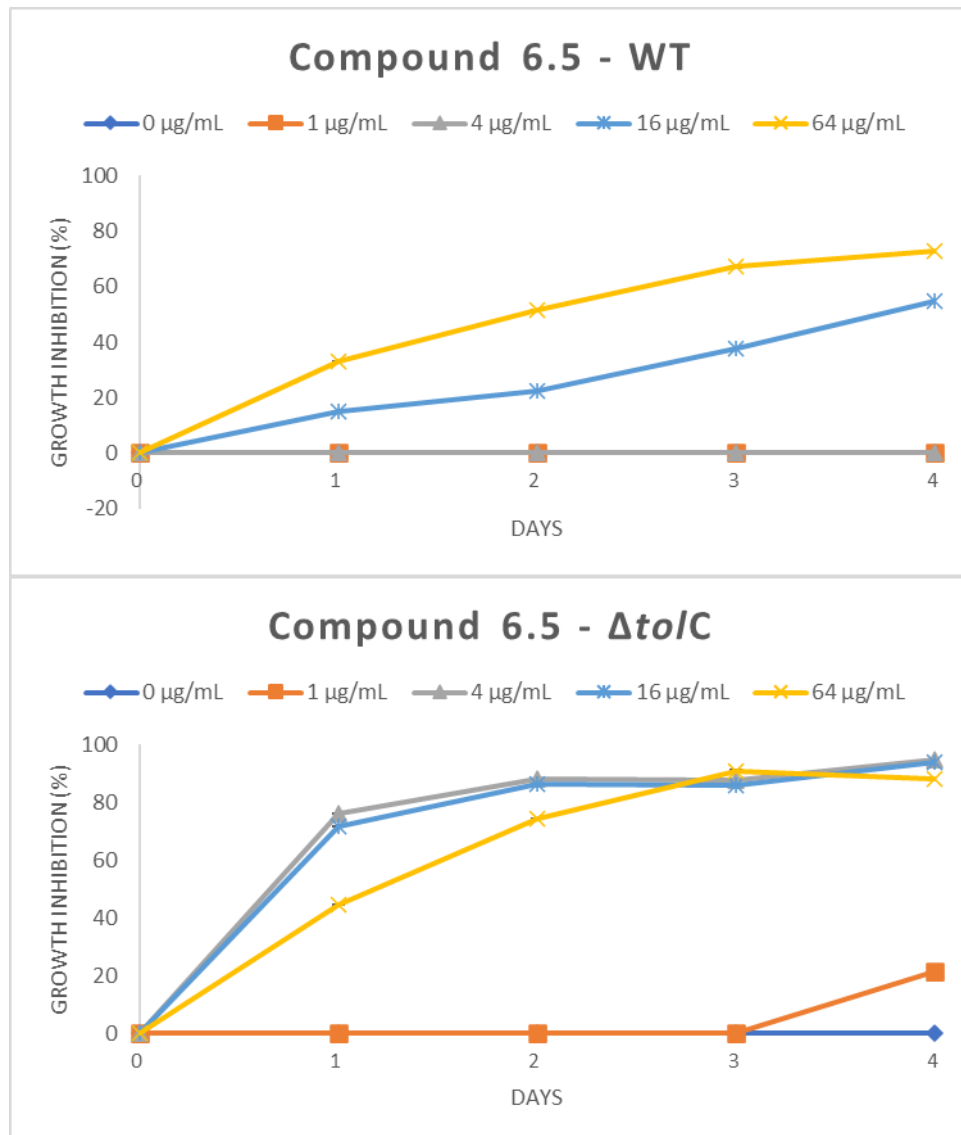


Figure S6.5 Growth inhibition of *Synechocystis* wild-type (top) and $\Delta tolC$ (bottom) over time - compound 6.5.

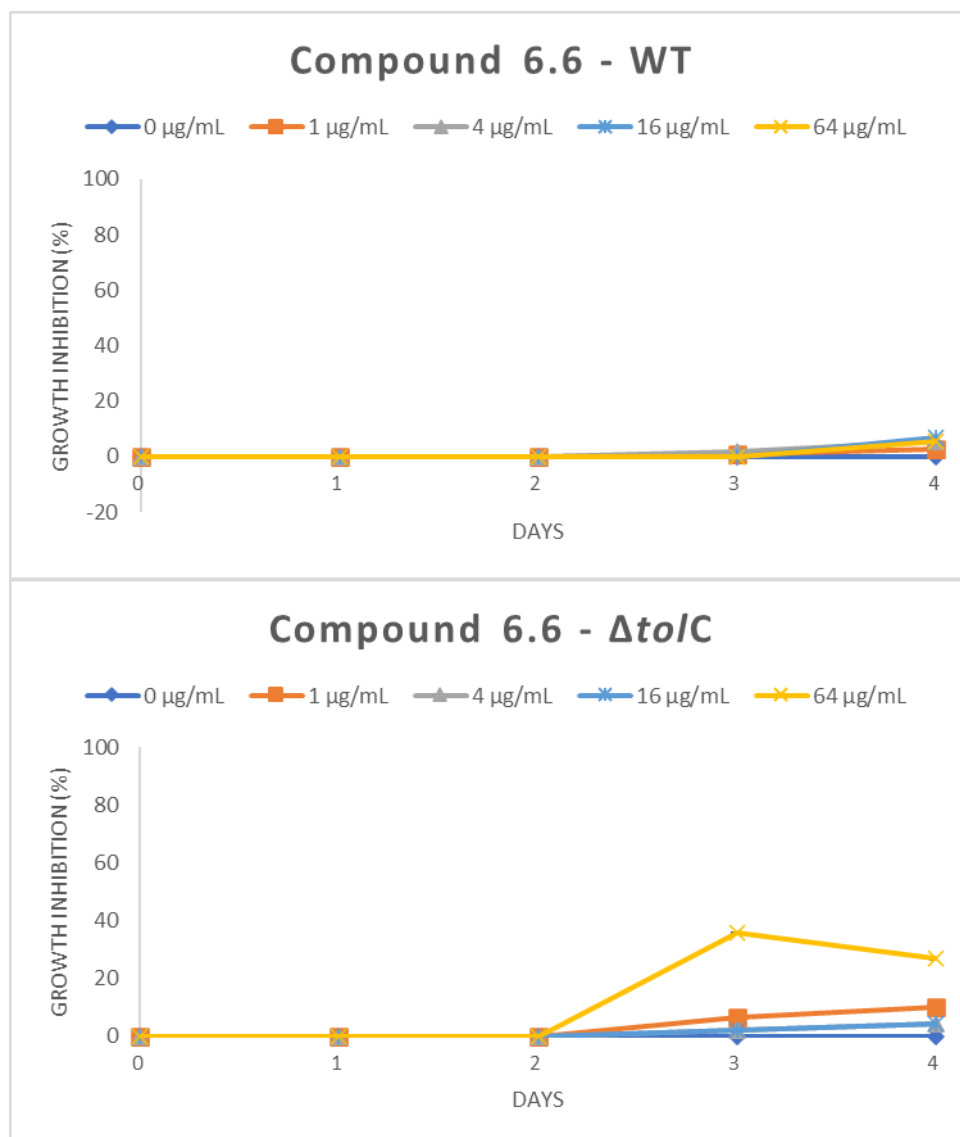


Figure S6.6 Growth inhibition of *Synechocystis* wild-type (top) and $\Delta tolC$ (bottom) over time - compound 6.6.

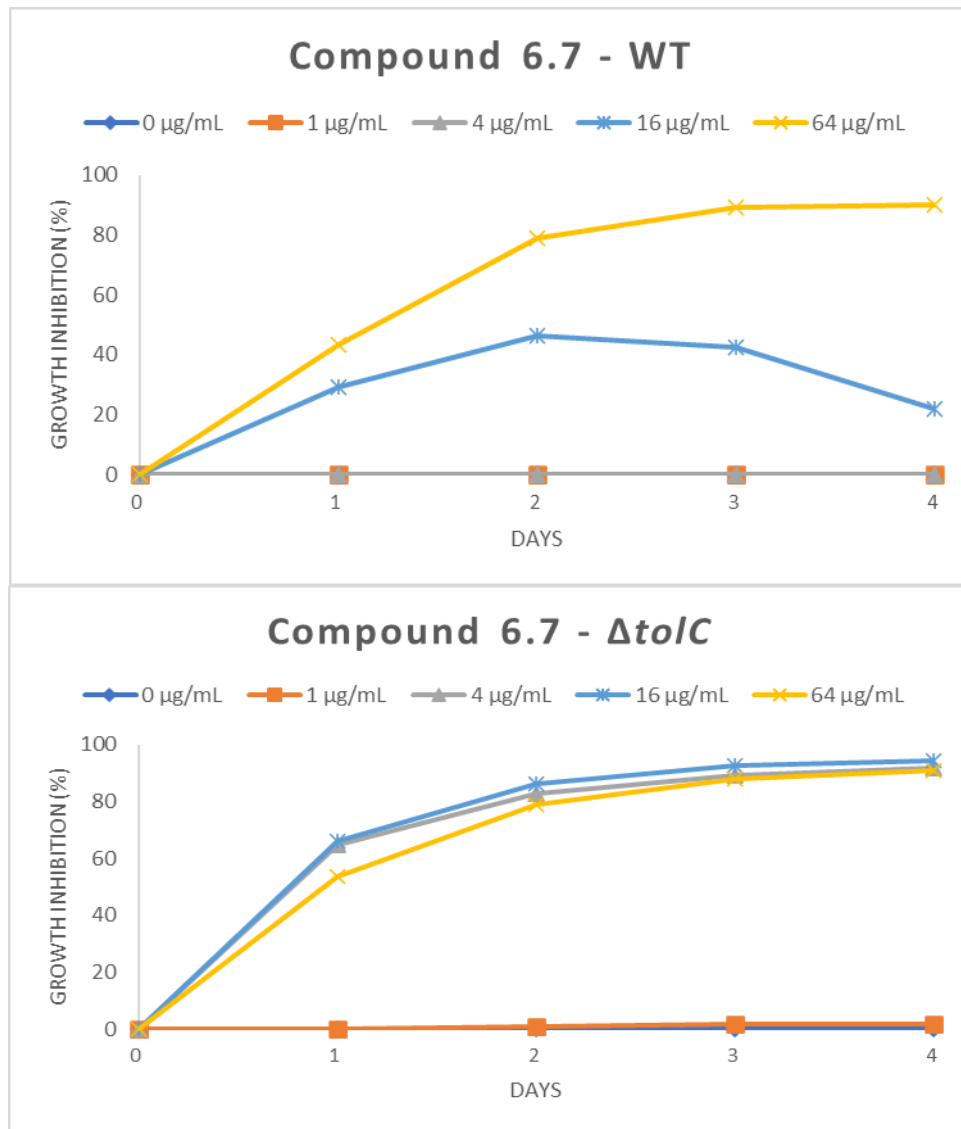


Figure S6.7 Growth inhibition of *Synechocystis* wild-type (top) and ΔtolC (bottom) over time - compound 6.7.

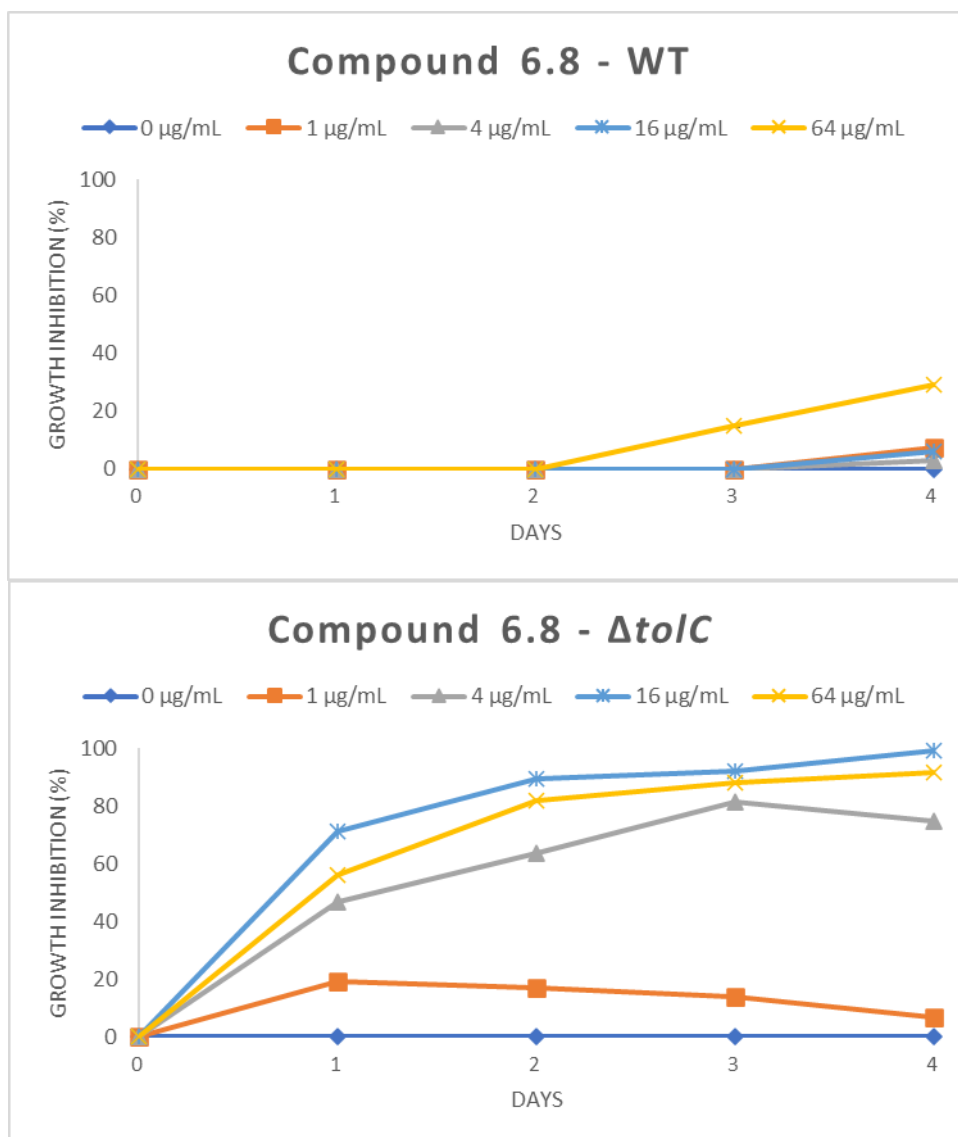


Figure S6.8 Growth inhibition of *Synechocystis* wild-type (top) and ΔtolC (bottom) over time - compound 6.8.

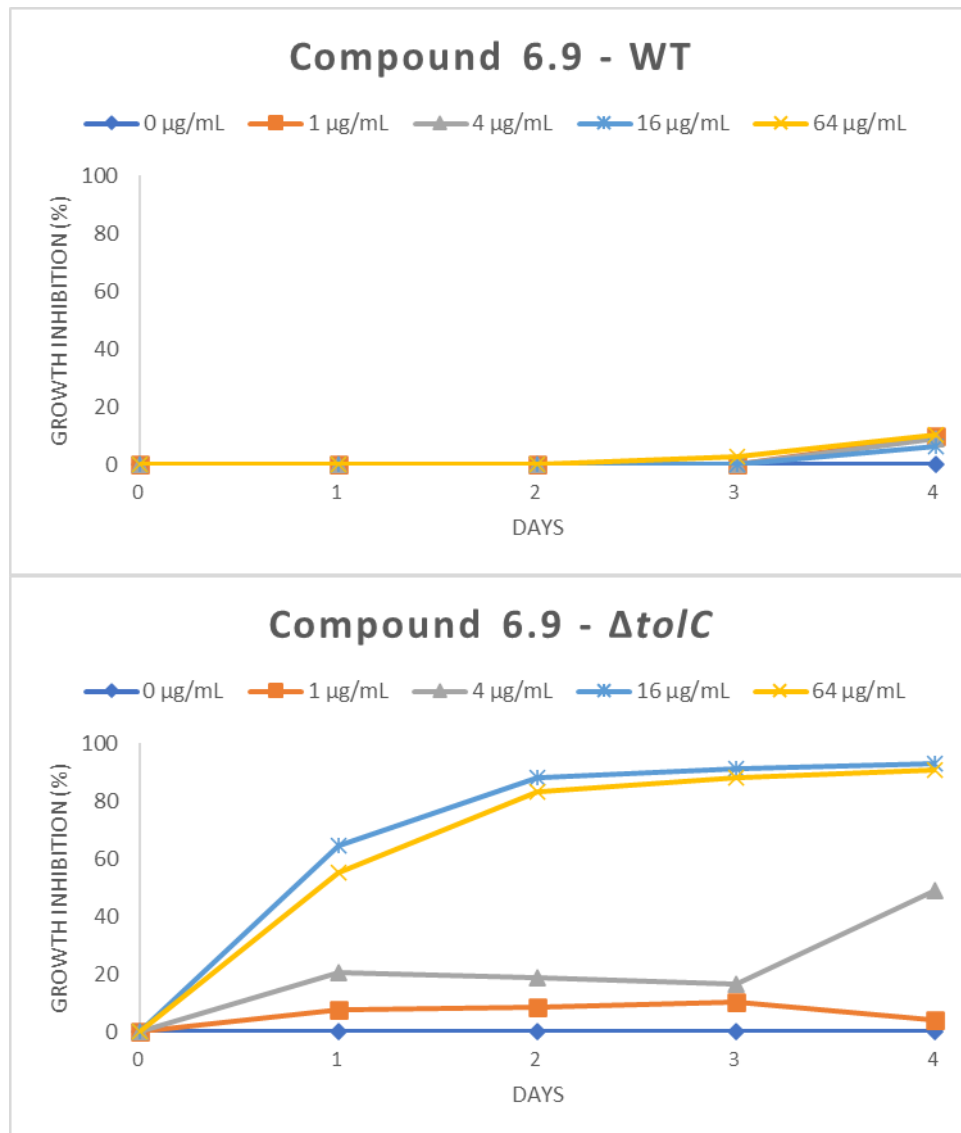


Figure S6.9 Growth inhibition of *Synechocystis* wild-type (top) and $\Delta tolC$ (bottom) over time - compound 6.9.

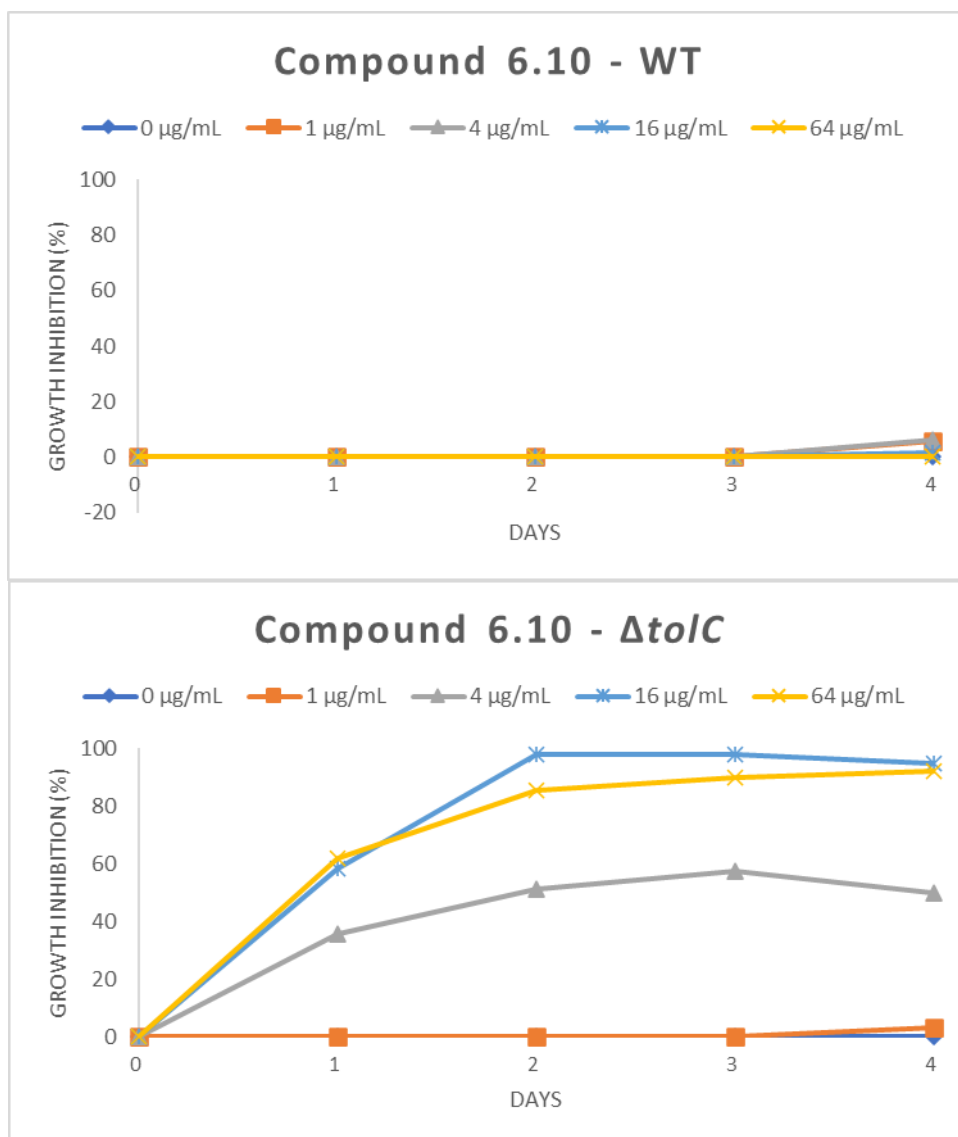


Figure S6.10 Growth inhibition of *Synechocystis* wild-type (top) and $\Delta tolC$ (bottom) over time - compound **6.10**.

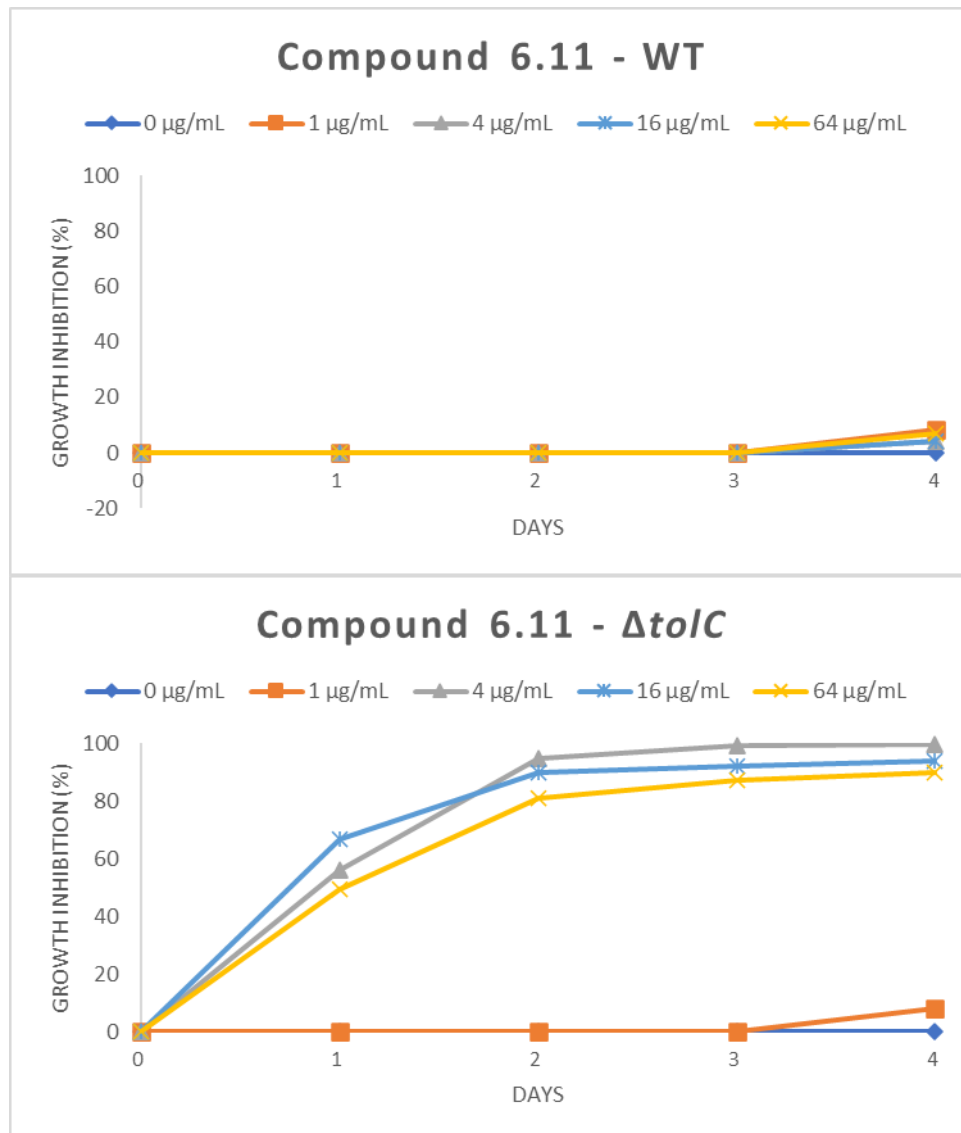


Figure S6.11 Growth inhibition of *Synechocystis* wild-type (top) and $\Delta tolC$ (bottom) over time - compound 6.11.

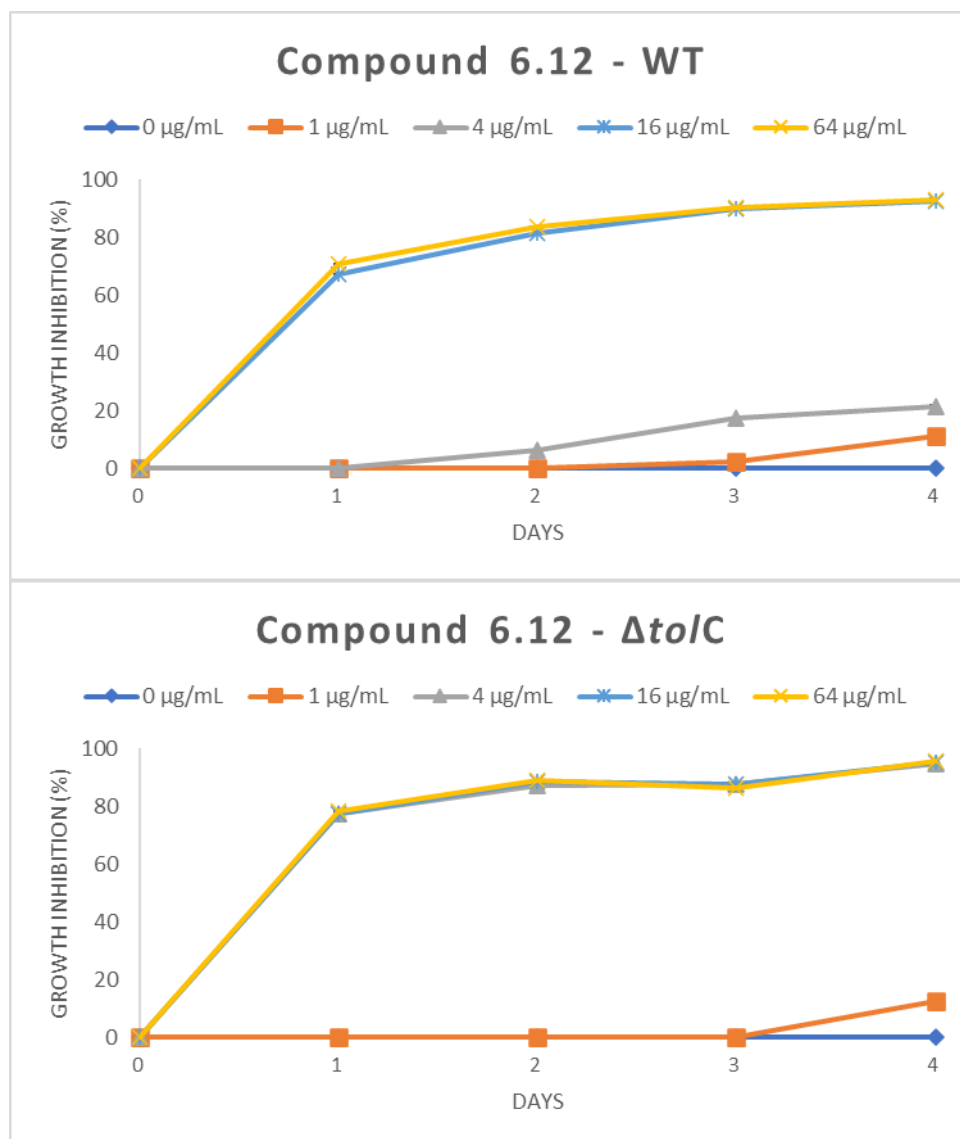


Figure S6.12 Growth inhibition of *Synechocystis* wild-type (top) and $\Delta tolC$ (bottom) over time - compound **6.12**.

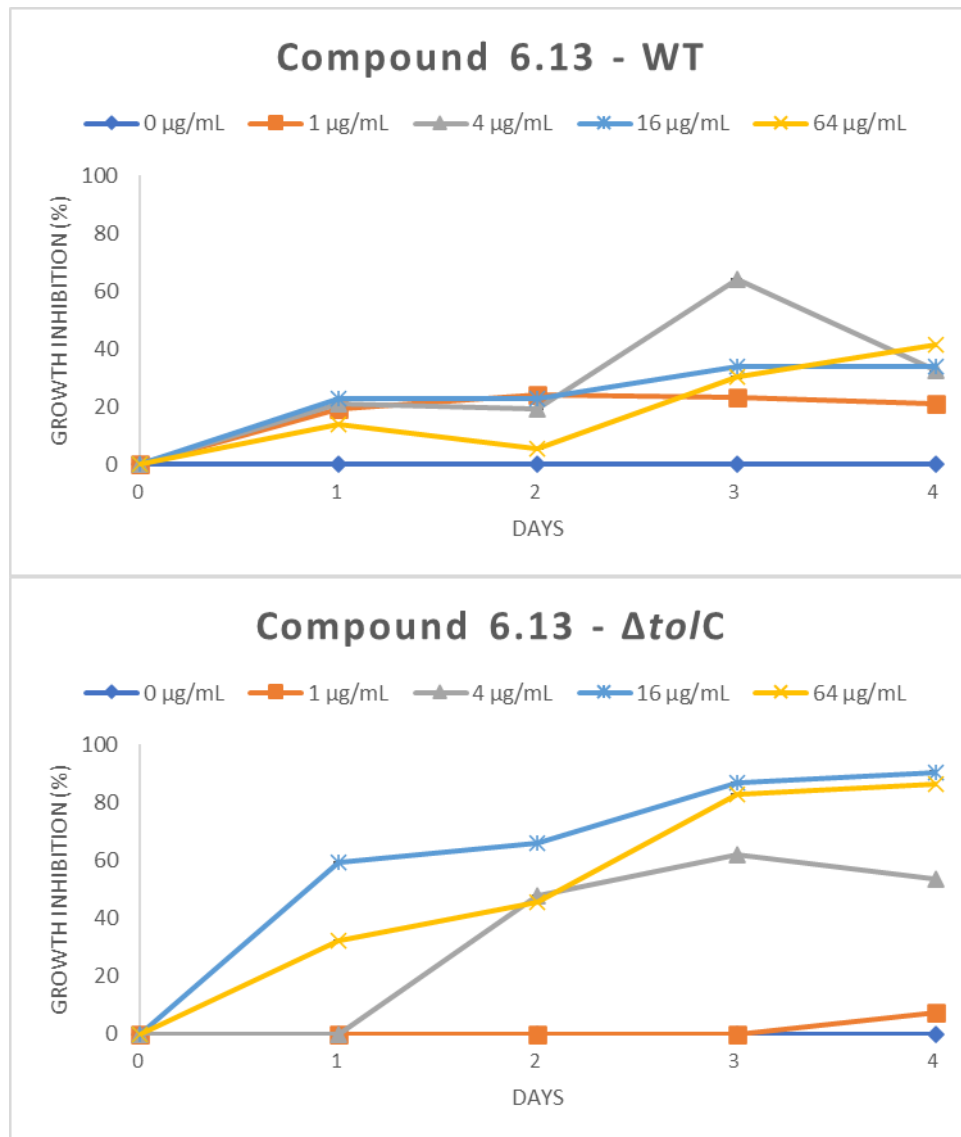


Figure S6.13 Growth inhibition of *Synechocystis* wild-type (top) and ΔtolC (bottom) over time - compound 6.13.

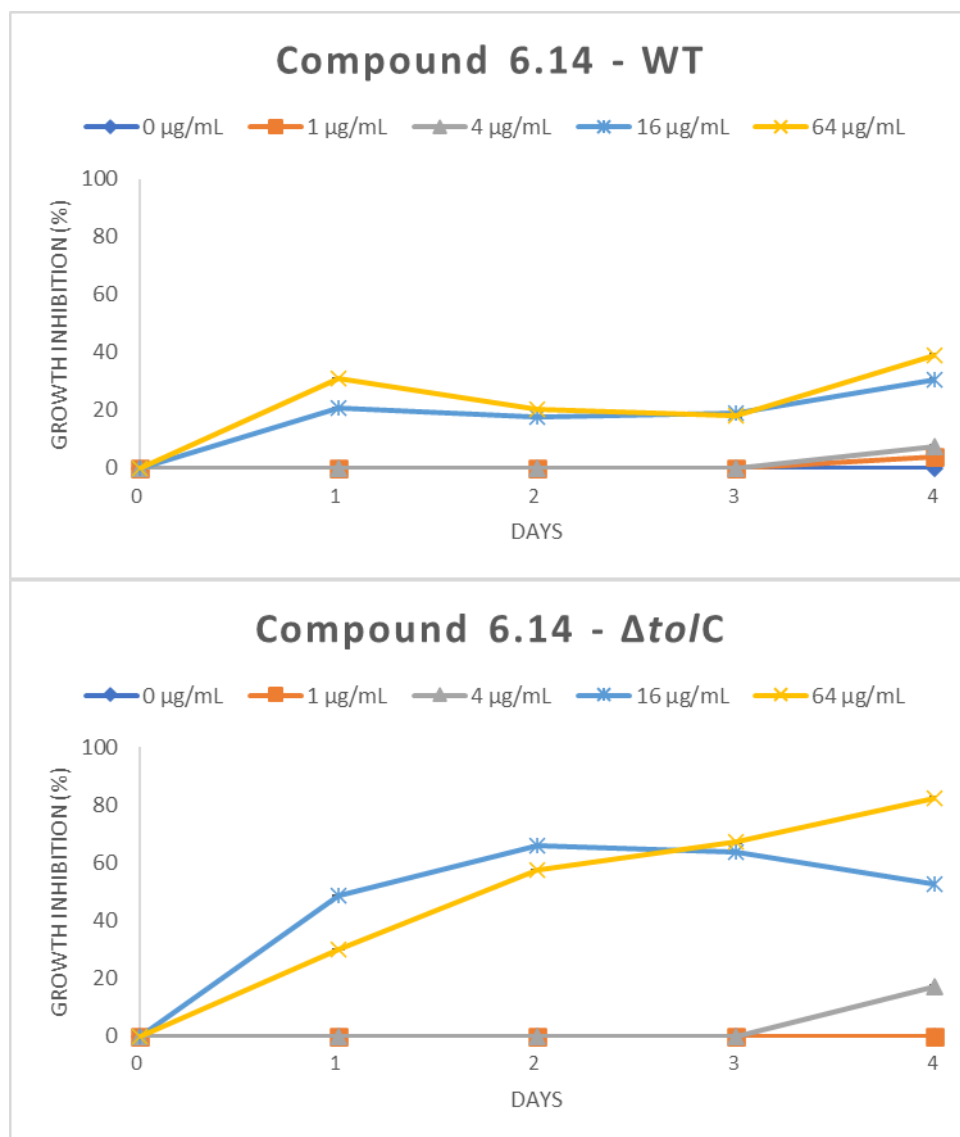


Figure S6.14 Growth inhibition of *Synechocystis* wild-type (top) and $\Delta tolC$ (bottom) over time - compound 6.14.

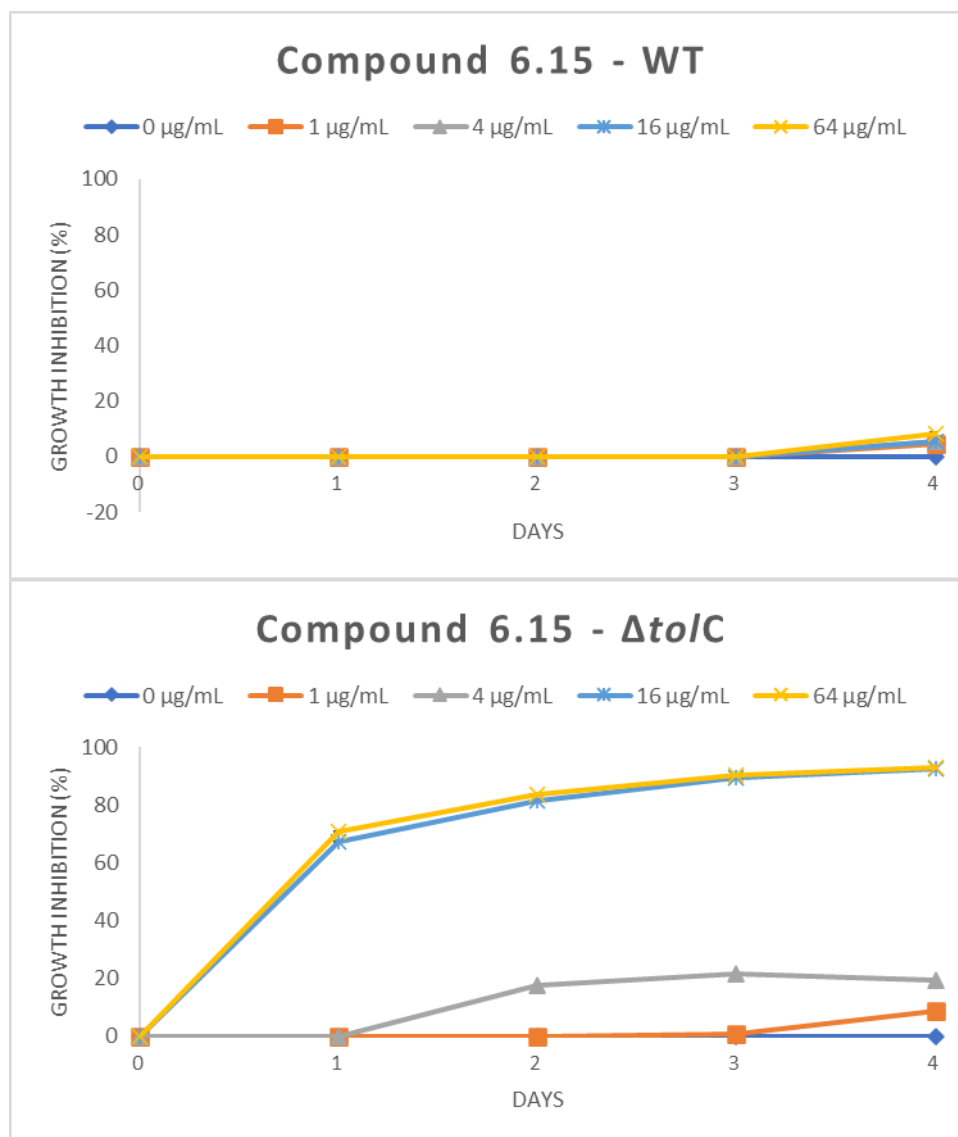


Figure S6.15 Growth inhibition of *Synechocystis* wild-type (top) and $\Delta tolC$ (bottom) over time - compound 6.15.

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Chapter 7 – Conclusions

The problematic of AMR is one of the greatest concerns of public health, and a timely and unique response seems unlikely to come from the discovery of new antibiotics. As such, new strategies should be planned, taking into account the resistance mechanisms used by bacteria to escape the action of antibacterial drugs. Among the resistance mechanisms, the overexpression of EPs acquires particular importance, since they are not only a resistance mechanism, but are also propellers of the development of every other mechanism, by reducing the concentration of drugs to a non-toxic level, allowing bacteria to thrive. Considering this, the inhibition of bacterial EPs appears to be a good strategy to not only reverse already existing resistance, but also to prevent new resistant strains to arise. Hence, this project was developed with the aim of finding new compounds that could inhibit these membrane transporters.

As such, a screening was conducted with an in-house library of structurally diverse derivatives, as well as a library of thioxanthenes structurally designed for this effect, through docking studies of a virtual library of 1000 compounds against protein efflux pumps. The hit compounds comprise one thioxanthone (7.1), two xanthenes (7.2 and 7.3), one deoxycholic amide (7.4), three chalcones (7.5 – 7.7), one diarylpentanoid (7.8), two fiscalins (7.9 and 7.10), and three metabolites from marine-derived fungi (7.11 – 7.13). Their structures can be seen in **Figure 7.1**.

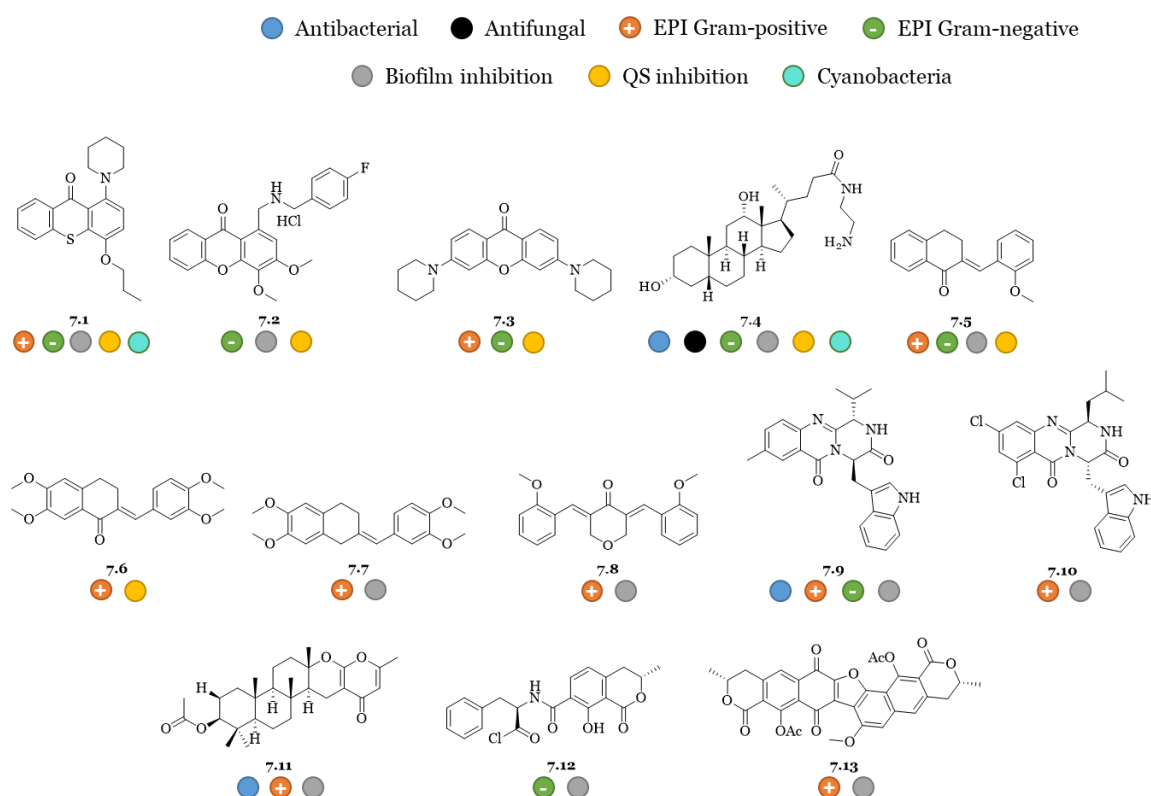


Figure 7.1 Structures of hit compounds 7.1 – 7.13.

The first activity to be studied was antibacterial. It could be noticed that, out of hundreds of compounds tested, only a few showed antibacterial activity, with even fewer being active against relevant strains, which emphasizes the difficulty that is finding new antibiotics, particularly for Gram-negative bacteria. Structural diversity among the antibacterial compounds was noted, with the only common feature being bulky substituents. The presence of an amphiphilic scaffold, specifically in compound **7.4**, yielded a broad spectrum of activity, as it was active against all the strains used, including clinical, animal, and environmental isolates, and fungi. However, this is accompanied by cytotoxicity and lack of selectivity, and further studies on this compound are needed.

The promising results obtained with **7.4** and the fact that the appearance of resistance is unlikely with cationic steroids led to the design of a library of derivatives, with the possibility of yielding dual antibacterial-EPI compounds. A narrowing of the spectrum of activity could be noticed, with the derivatives showing mostly mild activity against Gram-positive strains. However, the new derivatives showed EPI activity against *S. aureus* 272123, unlike **7.4**. The problem of cytotoxicity persisted, nevertheless. A class of compounds that also showed to be promising in the inhibition of Gram-positive EPs was chalcones, specifically dihydrochalcones and diarylpropanes, and showed no cytotoxicity for the tested cell line.

Regarding EP inhibition in Gram-negative bacteria, xanthonones and thioxanthonones yielded the most promising derivatives, including **7.1** and **7.2**, which did not show cytotoxicity in the tested cell line. There are a few other compounds that were EPIs in both Gram-positive and Gram-negative bacteria, with the deoxycholic amides being the class with the most broad-spectrum compounds, even though some of them showed a lower EtBr accumulation than the positive control in the *E. coli* strains used. It should, however, be noted that the compounds that were able to quantitatively accumulate more EtBr were xanthonones, albeit they did not show any remarkable biofilm formation and QS inhibition.

Resistance mechanisms related to the inhibition of EPs were also studied, namely biofilm formation and QS. In this scope, the class with the higher number of effective compounds was thioxanthonones, followed by xanthonones. These results should be studied in more detail, as a way to infer if there is a relationship with the inhibition of EPs or if it is a multitargeted mechanism.

The employment of the docking studies was to guide the synthesis of thioxanthonones that would be selective against bacterial EPs. It had been shown that these compounds could modulate human EPs, and, despite the structural and biochemical differences between P-gp and RND and MFS bacterial EPs, it was hard to dissociate these activities *in silico*. Nevertheless, the docking studies were useful at selecting thioxanthonones for synthesis.

Considering the studies in cyanobacteria, thioxanthone **7.1** and deoxycholic amide **7.4** were promising cyanobacteria antibacterials. Herein, the aim was to assess the interaction

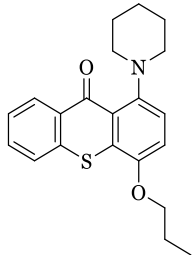
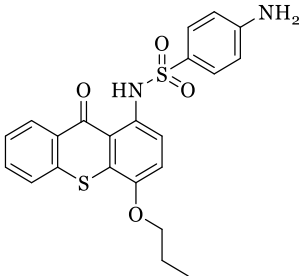
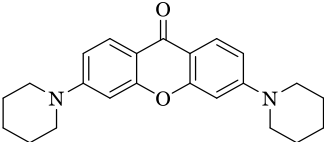
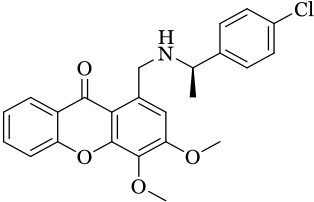
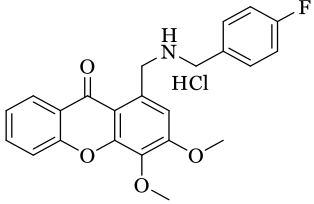
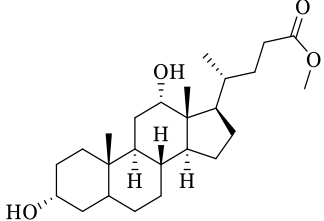
of the compounds with a TolC-like channel, however the results obtained showed that they are likely substrates of the efflux pump, as most of them were only active in the mutant strain, without the TolC-like channel. This could also be an opportunity, as there are not many studies regarding these models and this could be helpful in the design of future experiments, or even as a possible substitute for EtBr.

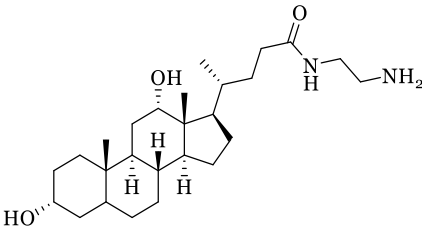
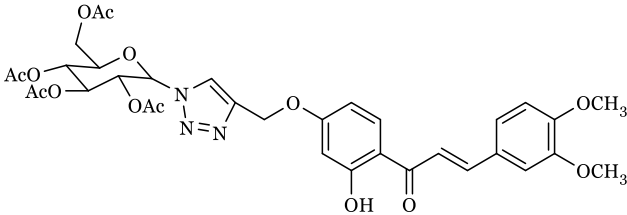
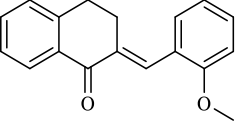
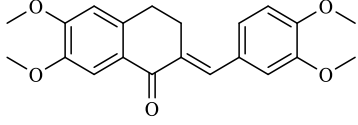
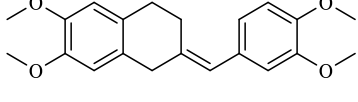
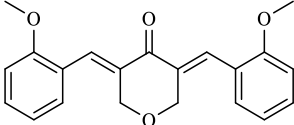
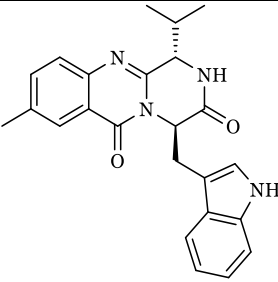
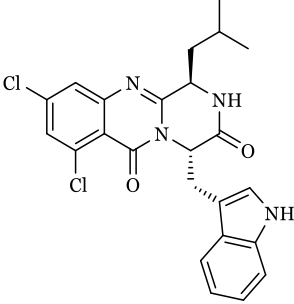
Future work in this scope, biologically, could include screenings in other models of bacteria expressing EPIs, or even genetic assays, in order to study the bacterial genetic response to efflux inhibition, and unequivocally point out which EP is being inhibited. Concerning the cyanobacterial studies, a larger screening would be desired, or a different assay, such as the EtBr real-time accumulation, which could be useful in understanding the real behavior of the bacteria when in presence of the compounds. However, cyanobacteria have a slower growth rate than the other models used, which is also a hurdle in this scenario.

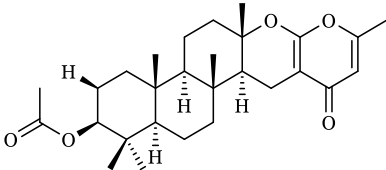
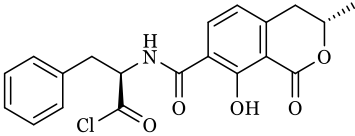
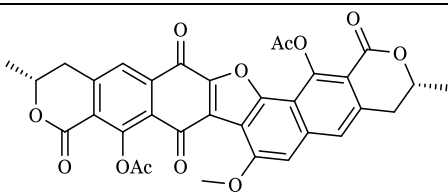
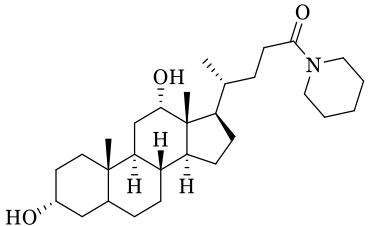
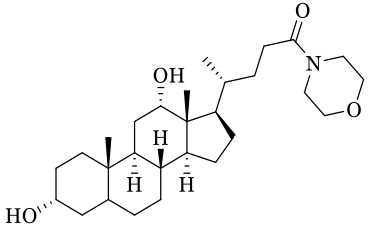
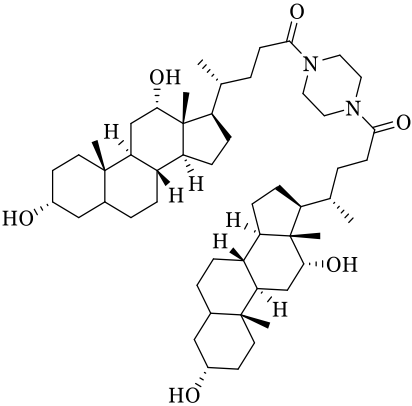
Future studies may diverge among chemical classes. Derivatives of compound **7.4** could be a choice if the objective is finding new antibacterial agents, keeping in mind that its mechanism of action is likely related to membrane destabilization, which is not an ideal mechanism as hemolytic effects must be dissociated. However, endogenous substances with this scaffold are easily transported in the human body, and could also be used as carriers, for instance. When it comes to resistance circumvention, the best classes to develop appear to be tricyclic compounds, such as xanthenes and thioxanthenes, which are overall more effective on the set of resistance mechanisms studied herein and were described for the first time within the scope of this work.

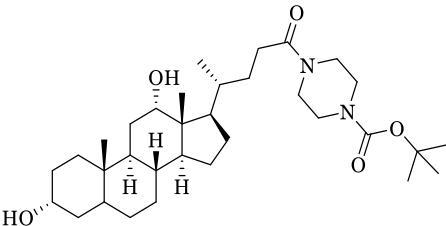
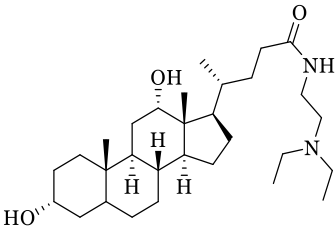
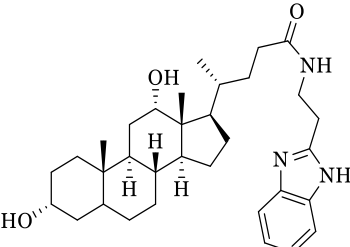
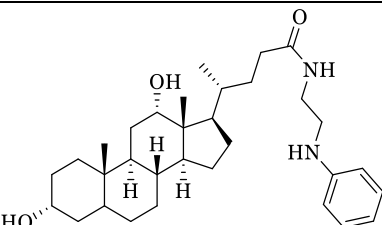
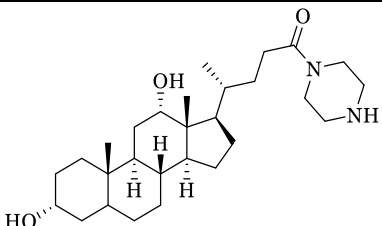
In conclusion, it is known that developing new antibacterials is a hard task, evidenced by the lack of new approvals over the past few years. Perhaps the focus could be shifted to finding new compounds to be used in combination with the antibacterials already approved, capable of restoring their activity. In this scope and given that an antimicrobial adjuvant is not made by any specific structural feature, the possibilities are endless.

Appendix – Chemical structures by chapter

Structure	Chapter				
	3	4	5	6	7
	3.7	-	-	6.5	7.1
	3.34	-	-	6.4	-
	-	4.37	-	6.3	7.3
	-	4.66	-	6.2	-
	-	4.67	-	-	7.2
	-	4.104	-	6.7	-

Structure	Chapter				
	3	4	5	6	7
	-	4.105	5.12	6.8	7.4
	-	4.108	-	6.1	-
	-	4.129	-	-	7.5
	-	4.130	-	-	7.6
	-	4.141	-	-	7.7
	-	4.150	-	-	7.8
	-	4.167	-	-	7.9
	-	4.168	-	-	7.10

Structure	Chapter				
	3	4	5	6	7
	-	4.175	-	-	7.11
	-	4.181	-	-	7.12
	-	4.185	-	-	7.13
	-	-	5.15	6.8	-
	-	-	5.16	6.9	-
	-	-	5.17	6.10	-

Structure	Chapter				
	3	4	5	6	7
	-	-	5.18	6.11	-
	-	-	5.19	6.12	-
	-	-	5.20	6.13	-
	-	-	5.21	6.14	-
	-	-	5.22	6.15	-

