RESISTANCE REVERSING ACTIVITY OF PLANT-DERIVED AND NOVEL SYNTHETIC COMPOUNDS IN BACTERIAL MODELS

Ph.D. Thesis

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CONTENTS

PUBLICATIONS

1. Publications related to the thesis

I. **Kincses A**, Szabó ÁM, Saijo R, Watanabe G, Kawase M, Molnár J, Spengler G. Fluorinated β-diketo phosphorus ylides are novel efflux pump inhibitors in bacteria. *In Vivo*. **30**: 813-17, 2017.
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II. **Kincses A**, Varga B, Csonka A, Sancha S, Mulhovo S, Madureira AM, Ferreira MJU, Spengler G. Bioactive compounds from the African medicinal plant *Cleistochlamys kirkii* as resistance modifiers in bacteria. *Phytother Res*. **32**: 1039-46, 2018.
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III. Mosolygó T, **Kincses A**, Csonka A, Tönki ÁS, Witek K, Sanmartín C, Marć MA, Handzlik J, Kieć-Kononowicz K, Domínguez-Álvarez E, Spengler G. Selenocompounds as novel antibacterial agents and bacterial efflux pump inhibitors. *Molecules.* **24**: E1487, 2019.
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2. Publications not related to the thesis

I. Żesławska E, **Kincses A**, Spengler G, Nitek W, Wyrzuc K, Kieć-Kononowicz K, Handzlik J. The 5-aromatic hydantoin-3-acetate derivatives as inhibitors of the tumour multidrug resistance efflux pump P-glycoprotein (ABCB1): Synthesis, crystallographic and biological studies. *Bioorg Med Chem.* **24**: 2815-22, 2016.
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XII. Mouwakeh A, **Kincses A**, Nové M, Mosolygó T, Mohácsi-Farkas C, Kiskó G, Spengler G. *Nigella sativa* essential oil and its bioactive compounds as resistance modifiers against *Staphylococcus aureus*. *Phytother Res.* **33**: 1010-18, 2019.
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XIII. Csonka A**, Kincses A**, Nové M, Vadas Zs, Sanmartín C, Domínguez-Álvarez E, Spengler G. Selenoesters and selenoanhydrides as novel agents against resistant breast cancer. *Anticancer Res.* **39**: 3777-83, 2019.
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ABBREVATIONS

ABC	ATP-binding cassette family
ABCB1	ATP-binding cassette subfamily B member 1
AGE	aminoglycoside-modifying enzyme
AHL	N-acyl-homoserine lactone
AI	autoinducer
AMR	antimicrobial resistance
ATP	adenosine triphosphate
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazone
CI	combination index
CIP	ciprofloxacin
CV	crystal violet
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EB	ethidium bromide
ED_{90}	effective dose for 90% inhibition of bacterial growth
EP	efflux pump
EPI	efflux pump inhibitor
EPS	extracellular polymeric substance
ESBL	extended-spectrum-β-lactamase
HGT	horizontal gene transfer
IM	inner membrane
LB	Luria-Bertani
MATE	multidrug and toxic compound extrusion family
MDR	multidrug resistant
MFS	major facilitator superfamily
MH	Mueller Hinton
MIC	minimum inhibitory concentration
MRSA	methicillin resistant *Staphylococcus aureus*
NBD	nucleotide-binding domain
OD	optical density
OM	outer membrane

PACE	proteobacterial antimicrobial compound efflux family
PBS	phosphate buffered saline
PMF	proton motive force
PMZ	promethazine
PPB	potassium phosphate buffer
QS	quorum sensing
RF	relative fluorescence
RFI	relative fluorescence index
RNA	ribonucleic acid
RND	resistance-nodulation-division family
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SMR	small multidrug resistance family
TET	tetracycline
TMD	transmembrane domain
TSB	tryptic soy broth

INTRODUCTION

1. Antimicrobial resistance

In ancient cultures bacterial infections were treated using different materials such as animal faeces, herbs and honey and one of the greatest successes was the use of moldy bread. The beneficial effects of mold were also published in 1640 by John Parkinson. Besides organic compounds, in the ancient Asian and Mediterranean civilizations the antimicrobial properties of silver in wound care were known and applied[1]. In the 1600s silver clips were used in surgery to prevent infection, then in the 1880s silver nitrate solution was applied to the eyes of newborns to reduce the incidence of ophthalmia neonatorum[2]. A major break-through in the treatment of bacterial infections was the discovery of antibiotics. Penicillin was the first antibiotic discovered by Sir Alexander Fleming in 1928 and that became widespread in 1944. With the introduction of penicillin began the golden age of antibiotics. However, just a few new antibiotics had been discovered in recent decades (for example linezolid in 2000, daptomycin in 2013 and ceftaroline in 2010)[3]. The evolution of resistant pathogens and antimicrobial resistance (AMR) have begun to increase dramatically after the introduction of antibiotics[4–6]. AMR is the ability of a microorganism to survive treatment with an antibiotic or antimicrobial agent to which it was previously sensitive. The rapid spread of AMR is a consequence of the inappropriate use of antibiotics, in particular in medical practice, aquaculture, and agriculture[7,8]. Resistant bacteria are able to withstand the attack of antimicrobials, consequently the standard therapy becomes ineffective leading to increased treatment costs and may cause fatal outcomes[9]. The seriousness of AMR is corroborated by several facts: according to the report of The European Centre for Disease Prevention and Control (ECDC) *Escherichia coli* isolates resistant to aminopenicillins show an alarming tendency in Latvia: ratio of resistant isolates was 48.4% in 2014, and three years later was already 60.4%. In contrast the number of invasive isolates of methicillin resistant *Staphylococcus aureus* (MRSA) experienced decreasing trend between 2014 (19.6%) and 2017 (16.9%)[10]. The O'Neill report estimates that by 2050 10 million lives and 100 trillion USD of economic output are at risk from AMR[11].

Multidrug resistant (MDR) bacteria show resistance against at least three different classes of antibiotics that is also a serious problem for the treatment of bacterial infections[3]. In Europe, 28.4% of the *Acinetobacter* isolates were resistant to aminoglycosides, carbapenems and fluoroquinolones in 2017. Even challenging is the MDR *Klebsiella*

pneumoniae in Europe whereas 15% of the total number of tested isolates were resistant to three antimicrobial groups[10]. One more fact from the United States of America: about 26,000 health-care acquired extended-spectrum-β-lactamase (ESBL)-producing Enterobacteriaceae infections result in 1,700 deaths every year[12].

Bacteria have evolved different mechanisms to prevent the harmful effect of antibacterial drugs as a result of chromosomal mutations or horizontal gene transfer (HGT)[13]. AMR may be acquired, adaptive or intrinsic[14]. Acquired resistance may develop through the following mechanisms: inactivation, increased efflux, reduced uptake and target alteration of antibiotics[15,16]. One of the most important resistance mechanisms is the presence of MDR efflux pumps (EPs). EPs can transport antimicrobial agents out of the bacterial cells[17]. Adaptive resistance means that bacteria are able to adapt to different environmental conditions by differential gene expression pattern[14]. Examples of adaptive resistance are the persister cells that can change their metabolism, stop their growth and thus tolerate the antimicrobial treatments[18]. All Gram-negative bacteria have intrinsic resistance due to the outer membrane (OM) that is a permeability barrier and prevents the entrance of antibiotics[19].

The spread of AMR is a global challenge and to overcome this problem the combination therapy could be a solution (antibiotic plus adjuvants: for example EP inhibitor) in order to improve the efficacy of antibacterial therapy, prevent the emergence of MDR bacteria and decrease the costs of therapy[20].

2. Horizontal gene transfer mechanisms

Emergence of AMR has become through the chromosomal mutations or the HGT between bacterial species[21]. In the course of HGT bacteria acquire genetic information (for example antibiotic resistance genes) from the environment by three canonical routes: transformation, transduction and conjugation (Figure 1)[9,21,22].

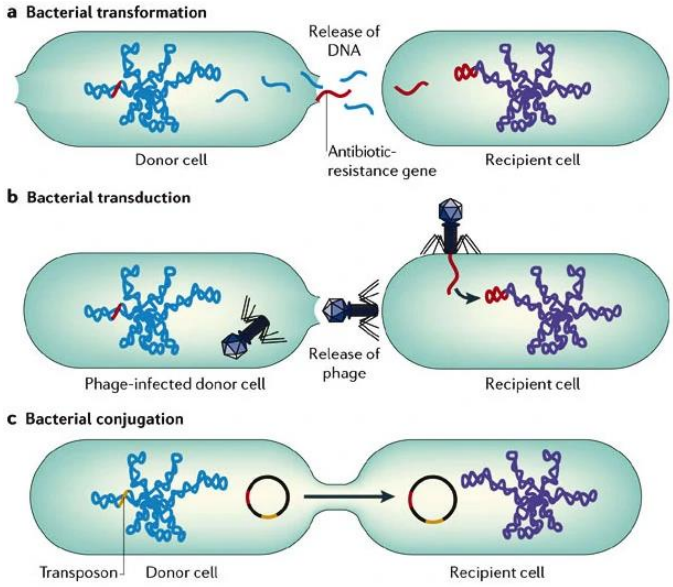


Figure 1 Mechanisms of horizontal gene transfer (HGT)[21]

Bacterial transformation

Transformation means that the bacterium is able to be competent namely it is able to take up extracellular DNA from the environment and after that stably integrate it into its genome using homologous recombination[23] or autonomously replicating element[22–24]. For example, *Neisseria gonorrhoeae* acquired *penA* (penicillin-binding protein 2) gene via transformation which is implicated in ceftriaxone resistance[25].

Bacterial transduction

Through transduction bacteriophages can transfer DNA from phage-infected donor cell into a recipient bacterium during infection. After the infection, the transferable DNA can be integrated into the genome of the new host. The DNA sequence encapsulated in the virus particle may be chromosomal DNA molecules, plasmids, or large chromosomal regions such as genomic islands[9,22,26]. Colomer-Llunch and his co-workers found that *mecA* gene is present in bacteriophage from cattle farm slurry, furthermore pigs and poultry wastewater abattoir. In addition *mecA* gene plays a role in the dissemination of MRSA[27].

Bacterial conjugation

Conjugation is the progress when DNA is transferred on plasmids from a donor cell to a recipient cell via a small tube namely pilus[28]. Conjugation is the most studied HGT process[29] and the most significant mechanism of AMR spreading[30]. Plasmids are responsible for the dissemination of ESBL[31] and New Delhi metallo-β-lactamase producing bacteria[32].

3. Mechanisms of acquired antimicrobial resistance

Bacteria are able to become resistant to antibiotics by the following mechanisms: over-expression of EPs, antibiotic inactivation, modification of target molecule, and reduced influx of antibiotics (Figure 2)[16,33,34].

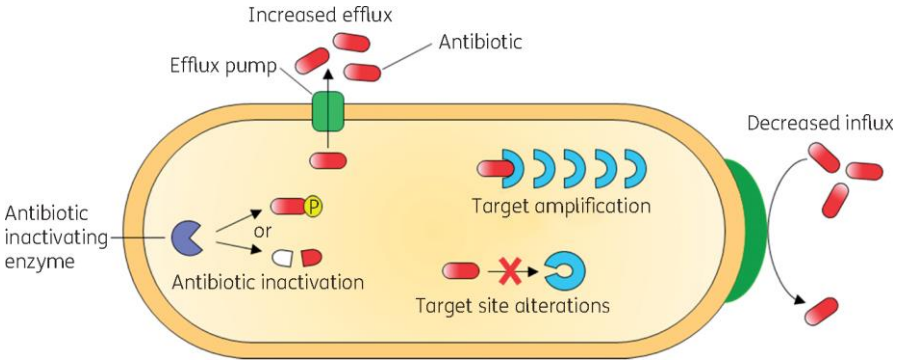


Figure 2 Mechanisms of antimicrobial resistance (AMR) in bacteria[35]

Over-expression of efflux pumps

EPs are cytoplasmic membrane proteins which extrude various, structurally unrelated agents from the bacterial cell to the environment. EPs are found in Gram-negative and Gram-positive bacteria as well and these pumps contribute significantly to the development of AMR because they can be involved in intrinsic and acquired resistance, furthermore they can be responsible for transient, non-inheritable phenotypic resistance[36].

Antibiotic inactivation

Bacteria can inactivate antibiotics by enzymes such as aminoglycoside-modifying enzymes (AGE's), β-lactamases and chloramphenicol acetyltransferases[37]. AGE's have already been described in *Enterococcus faecalis*, *S. aureus* and *Streptococcus pneumoniae*[38], furthermore they can provide resistance to fluoroquinolones and aminoglycosides[39]. The members of β-lactamases (about 300) can develop AMR in Gram-negative bacteria by hydrolyzing β-lactam antibiotics[40]. Clinically, the most important enzymes are the ESBLs that can hydrolyze cephalosporins and penicillins in Enterobacteriaceae[41]. Furthermore, resistance enzymes are the chloramphenicol acetyltransferases found in some Gram-negative and Gram-positive strains. These enzymes acetylate the hydroxyl groups of chloramphenicol and as a result chloramphenicol is not able to bind to the 50S subunit of ribosome[42].

Modification of target molecule

The antibiotics have targets and bind to them with high affinity, however if a spontaneous chromosomal mutation occurs in the target, the antibiotic is not able to bind[37].

One example is the methicillin and oxacillin resistance in *S. aureus*. If the *mecA* resistance gene is integrated into the chromosome of *S. aureus*, this gene codes a new penicillin-binding protein contributing to antibiotic resistance in this strain[43].

Reduced influx of antibiotics

Gram-negative bacteria have selective diffusion barriers that reduce the penetration of the antibiotics into the cell, for example these barriers are the porins[44–46] and the composition of cell wall (can be responsible for the lower penetration rate of antibiotics)[47]. The porins are located in the OM and through them the small hydrophilic drugs get into the cells. The permeability of molecules decreases with the number of porin channels[37].

4. Transporters in bacteria

Transporter proteins are common in bacterial species and their normal physiological and protective function is to expel the noxious agents out of the cells[48,49]. Bacterial EPs are located in the cytoplasmic membrane[50]. One of the most important mechanisms of multidrug resistance is the over-expression of EPs which are able to export antimicrobials agents outside the bacterium before they reach their targets within the bacterial cell[50]. In bacteria six transporter families are distinguished: the ATP (adenosine triphosphate)-binding cassette (ABC) family, the multidrug and toxic compound extrusion (MATE) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the proteobacterial antimicrobial compound efflux (PACE) family, and the resistance-nodulation-division (RND) family[51–53]. Based on their energy source transporters can be divided into two types: the primary EPs namely ABC transporters can bind and hydrolyze ATP to remove small molecules while the secondary EPs use electrochemical gradients namely proton motive force (PMF) or sodium ions to drive efflux (Figure 3)[36,54]. The EPs are able to transport structurally unrelated molecules; they can drop one or more substrates to the environment of bacteria[55].

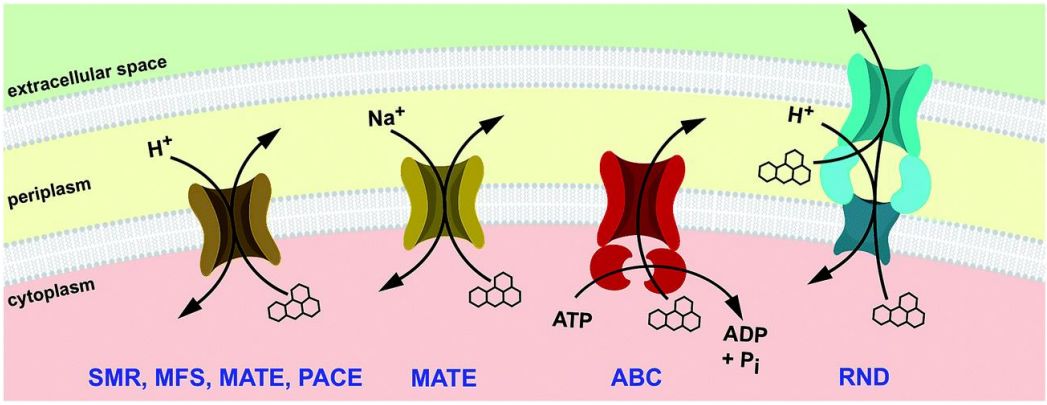


Figure 3 Schematic representation of transporter families based on energy sources used for export[55]

ABC transporters

ABC transporters have been found in Gram-negative and Gram-positive bacteria[56]. It is the only transporter family that derives energy from ATP hydrolysis for the removal of substrates[52,57]. These four partite EPs consists of two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs). NBDs are able to bind and hydrolize ATP. The function of TMDs is the recognition and export of wide range of compounds[58–60]. EfrAB from *E. faecalis* displays resistance to norfloxacin, ciprofloxacin (CIP) and doxycycline[61]. Additional ABC pumps have been described in the literature such as MacB from *E. coli*[62], PatAB from *S. pneumoniae*[63], and LmrA from *Lactococcus lactis*[64].

MATE transporters

The EPs of the MATE family are present in both Gram-negative and Gram-positive bacteria. MATE transporters comprise 12 α-helix transmembrane regions. MATE family members use the PMF (H^+) and the sodium ion (Na^+) gradient to extrude noxious compounds[65]. MATE pumps are capable of transporting a wide group of substrates as ethidium bromide (EB), metformin, cimetidine and the following antibiotics: norfloxacin, CIP, kanamycin, ampicillin, among others[66]. Multidrug EP members of the MATE include NorM from *Vibrio parahaemolyticus*[67], YdhE from *E. coli*[68], HmrM from *Haemophilus influenzae*[69], PmpM from *Pseudomonas aeruginosa*[70] and MepA from *S. aureus*[71].

MFS transporters

The members of MFS are widespread in both Gram-positive and Gram-negative bacteria. These proteins use the PMF in order to transport diverse substrates such as ions, sugars, Krebs-cycle intermediates as well as tetracycline (TET) and fluoroquinolones[72,73].

The MFS superfamily contains 12 or 14 transmembrane helices[74]. The EmrAB-TolC tripartite EP[75], EmrD[76] and MdfA from *E. coli* are structurally characterized. MdfA promotes the efflux of CIP, chloramphenicol and erythromycin[77,78]. Other members of this family are NorA[79], QacA, QacB[80] and LmrS[81] of *S. aureus*, LmrP[82] of *L. lactis*, PmrA[83] of *S. pneumoniae*.

SMR transporters

SMR family is found in both Gram-positive and Gram-negative bacteria and these proteins are the smallest known transporters. SMR transporters have a predicted function as homodimers that contain 4 transmembrane helices. The efflux of noxious agents is mediated by the PMF[57,65]. One of the best studied pump is EmrE[84] from *E. coli* that is able to extrude streptomycin, tobramycin[85], erythromycin and TET[86]. Other representatives of SMR family: EbrAB[87] from *Bacillus subtilis*, QacC[88] from *S. aureus*, YnfA[89] from *E. coli.*

PACE transporters

PACE is the newest family of bacterial transporters, which has been described in Gram-negative bacteria. It has 4 transmembrane helices based on its predicted topology. The transporter AceI is the prototype protein from *Acinetobacter baumannii* and the homologue of this transporter has been found in the genomes of *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas* and *Salmonella* species. AceI and its homologues can mediate extrusion of chlorhexidine, benzalkonium, acriflavine and proflavine[53,90].

RND transporters

RND transporters are widely distributed and they form a tripartite efflux system from the inner membrane (IM) to OM in Gram-negative bacteria[91]. The family uses PMF to accelerate the extrusion of broad range of antibiotics and biocides[92]. In Gram-positive bacteria (*S. aureus*, *B. subtilis*, *Corynebacterium glutamicum* and *Clostridium difficile*) only a few RND pump monomers have been described[54,93,94]. The most well characterized RND pump is the AcrAB-TolC multidrug transporter that consists of an IM transporter AcrB, a periplasmic adaptor protein AcrA and an OM channel called TolC (Figure 4)[95,96]. The AcrB EP consists of 12 transmembrane α-helices[97].

AcrZ is 49 amino-acid length IM protein that associates with the AcrAB-TolC system: it is directly bound to the AcrB in order to regulate the substrate specificity of AcrB[98]. The hexamer protein AcrA stabilizes the assembly of the pump and the homotrimer TolC provides a channel to expel the substrates from bacteria to the milieu[99,100]. The system can transport EB, crystal violet (CV), erythromycin, fusidic acid, novobiocin, fluoroquinolones, macrolides, chloramphenicol, TET, rifampicin; detergents such as bile salts and disinfectants[101,102]. Other RND drug transporters have been discovered such as MexAB-OprM[103], MexD, MexF and MexY[104] from *P. aeruginosa*, AcrD[105] from *E. coli*, KexD[106] from *K. pneumoniae* and MtrD[107] from *N. gonorrhoeae*.

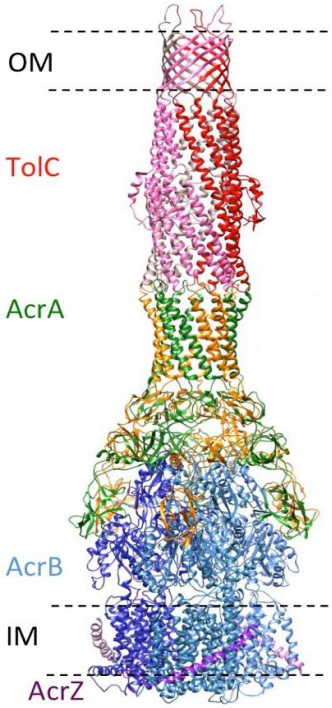


Figure 4 Structure of the AcrAB-TolC complex[108]

5. Biofilm formation and quorum sensing

Bacterial biofilm is a microbial community consisting of sessile bacterial cells attached to each other and to a surface and is embedded in a self-produced extracellular matrix[109]. Bacteria have the ability to form biofilms on both biotic (epithelial cells, tooth enamel etc.) and abiotic (plastic, glass, medical devices such as pacemakers, implants, catheters etc.) surfaces[110,111]. Biofilm-associated bacteria cause numerous infections, including endocarditis, urinary tract infections, periodontitis and nosocomial infections[112]. Biofilm formation has five stages: attachment, microcolonies and early biofilm formation, maturation and dispersion (Figure 5)[113]. In the first stage the planktonic cells attach to the surface. At the beginning, this attachment is initial and reversible and later becomes irreversible by flagella, pili, curli, and fimbriae[114]. After adherence, the bacterial cells proliferate and form a microcolony. The cells begin to grow and mature when these commence to synthesize extracellular polymeric substance (EPS) matrix[113], which includes exopolysaccharides, proteins, nucleic acids[115] and serves as a protective and diffusion barrier against antimicrobial agents. When conditions become unfavorable (lack of nutrients, competition, number of the bacterial population), a part of the biofilm will disperse and planktonic cells will be released from the biofilm so they can colonize other surfaces[116].

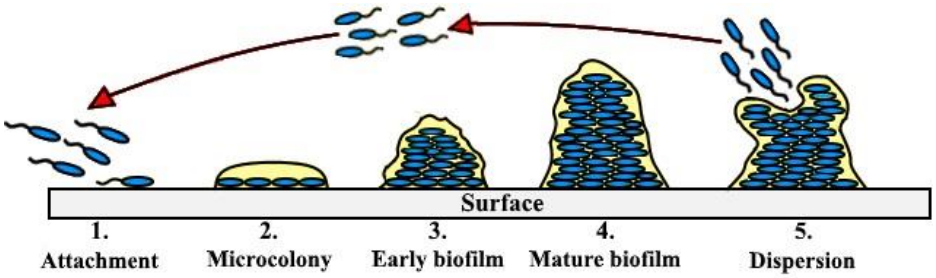


Figure 5 Schematic representation for the main stages of biofilm formation[117]

In the biofilm bacterial cells adapt to environmental conditions and bacteria show increased (about 10-1000 times) resistance against antimicrobial agents compared to planktonic bacteria[118]. Some factors play a role in the AMR of biofilm such as poor penetration of the antibiotic due to the EPS[119], slow growth and metabolism of biofilm cells[120], furthermore the transfer of AMR genes by conjugation is higher in biofilms[121]. Another reason is the presence of EPs. In some bacterial strains, EP genes are up-regulated showing that they contribute to biofilm formation[35] and reduce susceptibility of the biofilm[122]. EPs during biofilm formation extrude EPS and quorum sensing (QS) molecules. Transporters influence genes important for biofilm formation indirectly, furthermore they can pump out antibiotics and intermediates of metabolism[35].

The expression of the genes required for biofilm formation have to be coordinated and bacteria achieve this using the QS signal-response process. In bacteria the QS is a cell-to-cell communication and regulatory mechanism that controls gene expression depending on bacterial cell density[123–125]. For example, Gram-negative bacteria synthesize the extracellular signaling molecules namely autoinducers (AIs) like N-acyl-homoserine lactone (AHL) by Lux-I-type synthase enzymes. In the next step AHLs diffuse or are transported into the environment to bind to their LuxR-type receptors (transcriptional regulator) in other nearby cells. The AI and regulator complex binds to DNA to influence the expression of target genes[126].

In *E. coli* SdiA is a LuxR homolog that detects AHL signals from other bacteria and has a great impact on the colonization of *E. coli*. SdiA represses the expression of virulence genes by interacting with unknown stationary-phase signals in *E. coli* O157:H7[127], and enhances multidrug resistance by activating MDR EPs in *E. coli*[109].

6. Background of resistance modulators applied in this study

Resistance to antibiotics has become a serious problem in the treatment of infectious diseases because of the rapid spread of AMR. Consequently, there is a need to develop

alternative compounds that may be useful alone or in combination therapy[129]. The discovery of plant-derived antimicrobials is advantageous because these plants are widely distributed in nature[130] and they could be used in order to overcome drug resistance in bacteria by blocking QS, biofilm formation[131] and multidrug EPs[132]. There are several studies in the literature providing evidence of the EP inhibitory potential of plant-derived compounds. It has been described that reserpine from the roots of *Rauwolfia serpentina* and *R. vomitoria* inhibits TET efflux by Bmr transporter of *B. subtilis*[133]. Piperine isolated from black pepper (*Piper nigrum*) has been identified as an inhibitor of NorA pump of MRSA[134]. Falcarindiol, linoleic and oleic acids of *Levisticum officinale* reduce the efflux of AcrAB-TolC EP of *Salmonella enterica* serovar Typhimurium[135]. *Nigella sativa* essential oil has shown activity against the EP systems of methicillin sensitive *S. aureus* and MRSA strains[136]. *Cleistochlamys kirkii* (Benth) Oliv. (Annonaceae) is an African medicinal plant traditionally used in Mozambique for the treatment of wound infections, tuberculosis and rheumatism[137]. Based on preliminary studies *C. kirkii* derivatives have a broad antimicrobial spectrum: they had antifungal activity against *Candida albicans* and exhibited antibacterial effect against methicillin sensitive *S. aureus* and MRSA[138,139].

Organic compounds of phosphorus ylides (P-ylides) are a fascinating class of compounds in organic chemistry[140]. The EP modulating activity of P-ylides has already been described regarding the ATP-binding cassette subfamily B member (ABCB1) pump of MDR mouse T-lymphoma cells[141] and the further biological aspects of the phosphorus ylides in bacteria have not been yet investigated in the literature.

Selenium is an important trace element, which plays a role in the prevention of inflammatory diseases and cancer[142]. The antibacterial activity of selenium-containing compounds has been found by many studies: research was mostly done using bacteria that cause nosocomial infections. *E. coli*[143] and *S. aureus*[144] are often responsible for infections during hospitalization[145]. Infections caused by these bacteria are difficult to treat due to their biofilm forming ability. It has been demonstrated by a research group that a perihydroselenoxantine compound showed antibacterial activity on *S. aureus* strain[146]. Sodium selenite has been found to eradicate *Helicobacter pylori* and it showed antibacterial effect against *H. pylori* at a low concentration in rats[147]. In another study, three polymers (polyvinyl chloride, polyurethane and silicone) were coated with selenium nanoparticles. The growth of *S. aureus* was prevented on selenium-coated polymers compared to the uncoated materials[148]. The novel selenocompounds tested in this study have been previously described as anticancer compounds against T-lymphoma and colon cancer cell lines[149,150].

AIMS OF THE STUDY

Resistant bacteria are able to withstand the attack of antimicrobials, so that standard treatments become ineffective and infections persist and may spread to others. Efflux mechanisms of bacteria, namely, pumping of antimicrobial agents out of the cells play an important role in antimicrobial resistance of pathogenic bacteria. The aim of our study was to investigate the bioactive compounds of *Cleistochlamys kirkii*, synthesized fluorinated β-diketo phosphorus ylides and selenocompounds on Gram-positive and Gram-negative model bacterial strains in order to find resistance modifiers which could be applied later alone or in combination with antibiotics.

The main goals of the study were the following:

1. **Determination of the antibacterial activity of the compounds** (five natural compounds, ten fluorinated β-diketo phosphorus ylides and eleven selenocompounds) on Gram-positive (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *S. aureus* 272123) and Gram-negative strains (*Escherichia coli* AG100, AG100 A, *Salmonella enterica* serovar Typhimurium SL1344 (wild-type), L644 (ΔacrB strain), 14028s (biofilm producing strain), *Chromobacterium violaceum* 026 and *Enterobacter cloacae* 31298 by microdilution method.

2. **Investigation of the efflux pump (EP) inhibitory effect of the compounds** (five natural compounds, ten fluorinated β-diketo phosphorus ylides and eleven selenocompounds) on Gram-positive (*S. aureus* ATCC 25923, *S. aureus* 272123) and Gram-negative strains (*E. coli* AG100 expressing the AcrAB-TolC EP and its AcrAB-TolC deleted mutant AG100 A strain) using real-time ethidium bromide (EB) accumulation assay.

3. **Evaluation of the EP inhibitory effect of eleven selenocompounds** on Gram-negative wild-type *S.* Typhimurium SL1344 strain expressing the AcrAB-TolC EP and its AcrB deleted mutant L644 strain using EB efflux assay.

4. **Determination of the anti-biofilm activity of eleven selenocompounds** on Gram-negative, biofilm producing *S.* Typhimurium 14028s strain using crystal violet (CV).

5. **Characterization of the activity of five natural compounds of *C. kirkii* as adjuvants** in the presence of tetracycline (TET) and ciprofloxacin (CIP) on *S. aureus* ATCC 25923 and *S. aureus* 27213 strains by checkerboard method.

6. **Evaluation of the adjuvant role of eleven selenocompounds** on *E. coli* AG100 strain by a two-fold broth microdilution method in the presence of TET and CIP.

7. **Quorum sensing (QS) inhibition analysis of five natural compounds of *C. kirkii* and ten fluorinated β-diketo phosphorus ylides** using the sensor strain *C. violaceum* 026 and the AHL producer strain *E. cloacae* 31298 by agar diffusion method.

8. **Monitoring the changes in relative gene expression of** efflux (*norA*, *mepA*, *acrA*, *acrB*), antibiotic resistance (*marR*) and QS (*sdiA*) **genes in the presence of the most effective EP inhibitors** (natural compounds isolated from *C. kirkii*, fluorinated β-diketo phosphorus ylides and selenocompounds) investigated by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR).

MATERIALS AND METHODS

1. Compounds studied

Five natural compounds (**CK1-5**) were isolated from the methanol extract of the root barks of *Cleistochlamys kirkii* (Benth.) Oliv. (Annonaceae)*:* triterpene polycarpol (**CK1**), *C*-benzylated flavanones chamanetin (**CK2**), isochamanetin (**CK3**), dichamanetin (**CK4**) and the heptane derivative acetylmelodorinol (**CK5**) were kindly provided by Prof. Dr. Maria-José U. Ferreira (Universidade de Lisboa, Lisbon, Portugal; Figure 6). The stock solutions (in 10 mM concentration) of compounds were prepared in dimethyl sulfoxide (DMSO).

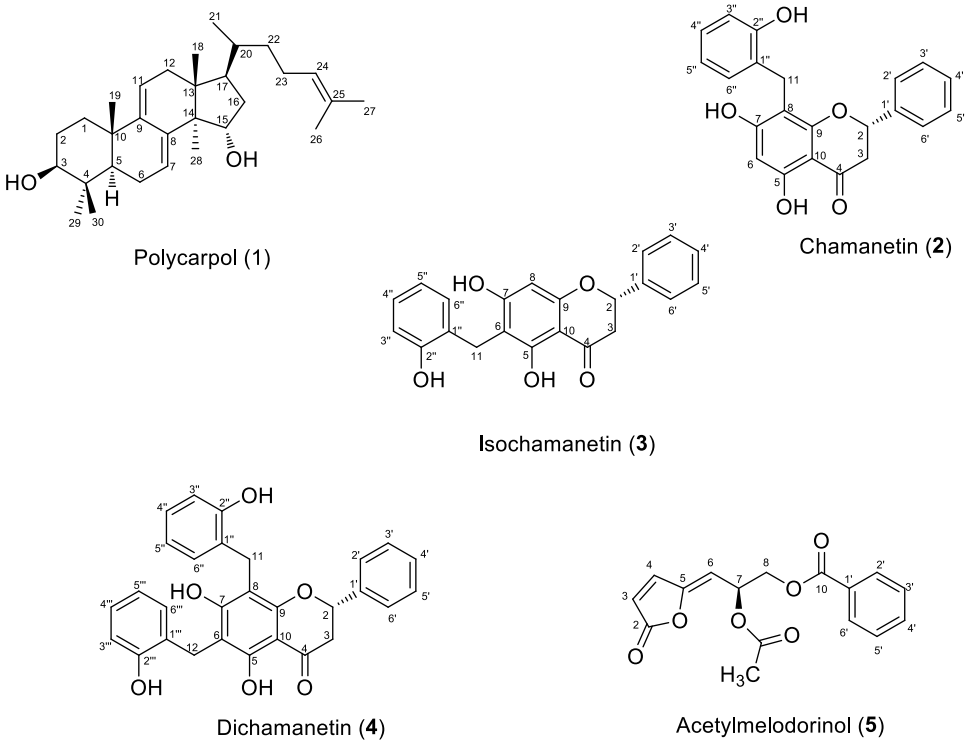
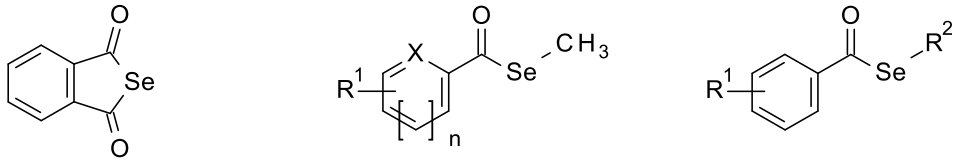


Figure 6 Chemical structures of bioactive compounds (**CK1**–**5**)

Ten synthesized fluorinated β-diketo phosphorus ylides (P-ylides; **PY1-10**) were kindly provided by Prof. Dr. Masami Kawase (Matsuyama University, Matsuyama, Japan) (See Appendix 1 and 2). The stock solutions (in 10 mg/mL concentration) of compounds were prepared in DMSO.

Eleven selenocompounds including a cyclic selenoanhydride (**EDA1**), heteroaryl selenoesters (**EDA2**-**3**) and aryl selenoesters (**EDA4**-**11**) were kindly provided by Dr. Enrique Domínguez-Álvarez (Consejo Superior de Investigaciones Científicas, Madrid, Spain) and Prof. Dr. Carmen Sanmartín (University of Navarra, Pamplona, Spain)[149,151]

(Figure 7; See Appendix 3 and 4). The stock solutions (in 10 mM concentration) of compounds were prepared in DMSO.



A B C

Figure 7 Chemical structures of tested selenoanhydride (**EDA1**) (A), heteroaryl selenoesters (**EDA2-3**) (B) and aryl selenoesters (**EDA4-11**) (C)

2. Reagents and media

Promethazine (PMZ; EGIS), ethidium bromide (EB), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), verapamil, crystal violet (CV), tetracycline-hydrochloride (TET), ciprofloxacin-hydrochloride (CIP), DMSO, Luria-Bertani (LB) broth, and LB agar were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The modified LB medium (LB*) was prepared from yeast extract 5 g/L, tryptone 10 g/L, NaCl 10 g/L, K_2HPO_4 1 g/L, $MgSO_4$ x $7H_2O$ 0.3 g/L and FeNaEDTA 36 mg/L. In case of modified LB* agar, the LB* medium was supplemented with agar 20 g/L (Difco, Detroit, USA). pH was adjusted to 7.2. Tryptic soy broth (TSB), tryptic soy agar (TSA), and Mueller Hinton (MH) broth were purchased from Scharlau Chemie S. A. (Barcelona, Spain).

3. Bacterial strains

Wild-type *Escherichia coli* K-12 AG100 strain [argE3 thi-1 rpsL xyl mtl Δ(gal-uvrB) supE44], expressing the AcrAB-TolC efflux pump (EP) at its basal level and its AcrAB-TolC deleted mutant *E. coli* AG100 A strain were used in the study. These strains were kindly provided by Prof. Dr. Hiroshi Nikaido (University of California, Berkeley, CA, USA).

Wild-type *Salmonella enterica* serovar Typhimurium SL1344 expressing the AcrAB-TolC EP and its *acrB* gene inactivated mutant *S.* Typhimurium strain (L644)[152], furthermore, the biofilm producing *S.* Typhimurium 14028s strain were used in the study. These strains were kindly provided by Dr. Jessica M. A. Blair (University of Birmingham, Birmingham, United Kingdom).

Staphylococcus aureus ATCC (American Type Culture Collection) 25923, was used as the methicillin susceptible reference strain, and the methicillin and ofloxacin resistant *S. aureus* 272123 clinical isolate was kindly provided by Prof. Dr. Leonard Amaral (Institute of Hygiene and Tropical Medicine, Lisbon, Portugal). In addition, *Enterococcus faecalis* ATCC 29212 strain was used in the assays.

For quorum sensing (QS) tests, the following strains were used: *Chromobacterium violaceum* 026 (CV026) as sensor strain and *Enterobacter cloacae* 31298 as N-acyl-homoserine lactone (AHL) producer strain (a clinical isolate from a wound)[153].

4. Determination of minimum inhibitory concentrations by microdilution method

The minimum inhibitory concentrations (MICs) of all tested compounds were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines in three independent assays[154]. The solvent DMSO had no antibacterial effect.

5. Real-time accumulation assay of ethidium bromide

The activity of compounds isolated from *C. kirkii* (**CK1**-**5**), P-ylides (**PY1**-**10**) and selenocompounds (**EDA1**-**11**) on the real-time accumulation of EB was assessed by the automated EB method[155] using a LightCycler real-time thermocycler (LightCycler 1.5, Roche, Indianapolis, USA). Briefly, an aliquot of an overnight culture of *S. aureus* strains (ATCC 25923 and MRSA 272123) in TSB medium was transferred to fresh TSB medium, and it was incubated until it reached an optical density (OD) of 0.6 at 600 nm. In case of *E. coli* AG100 and AG100 A, the medium used in the assay was LB broth; the preparation of the inoculum was similar to the one of *S. aureus*. The cells were washed with phosphate buffered saline (PBS; pH 7.4) and centrifuged at 13,000 × *g* for 3 minutes, the pellets were re-suspended in PBS (pH 7.4), and the OD was adjusted to 0.6 at 600 nm. The compounds were added individually at different concentrations at ½ MIC, ⅓ MIC, ¼ MIC or ⅕ MIC (in double concentrated form) to the EB solution in PBS. The final concentration of EB was based on the MIC and the lowest fluorescent signal produced by this sub-MIC concentration of EB. In case of *S. aureus* strains, the concentration of EB was 0.5 μg/mL, for *E. coli* AG100 1 μg/mL, and in case of *E. coli* AG100 A it was 0.25 μg/mL. Then, 10 μL of the EB solution containing the compound were transferred into standard glass capillary tubes of 20 μL maximum volume (Roche, Indianapolis, IN, USA), and 10 μL of bacterial suspension (OD of 0.6 at 600 nm) were added to the capillaries. The capillaries containing the samples were placed into the carousel (Roche), and the fluorescence was monitored at the FL-2 channel in every minute on a real-time basis.

From the real-time data, the activity of the compounds, namely the relative fluorescence index (RFI) of the last time point (minute 30) of the EB accumulation assay, was calculated according to the following formula:

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

Where $RF_{treated}$ is the relative fluorescence (RF) at the last time point of EB retention curve in the presence of an inhibitor, and $RF_{untreated}$ is the RF at the last time point of the EB retention curve of the untreated control having the solvent control (DMSO). Verapamil was applied as a positive control on Gram-positive strains and PMZ was used on Gram-negative strains.

6. Efflux assay using ethidium bromide

The activity of selenocompounds (**EDA1-11**) on the efflux of EB was determined on wild-type (SL1344) and *acrB* gene inactivated (L644) *S.* Typhimurium strains. Briefly, an aliquot of an overnight culture of *S.* Typhimurium strain in LB medium was transferred to fresh LB medium, and it was incubated at 37°C with shaking at 150 rpm until it reached an OD of 0.4 at 600 nm. The cultures were centrifuged at 3500 rpm for 10 min at 21°C. The supernatant was removed, and the pellet was re-suspended in 20 mM potassium phosphate buffer (PPB; pH 7.0) with 1 mM $MgCl_2$ and the OD was adjusted to 0.2 at 600 nm. 100 μM of CCCP was added to de-energize the cells and therefore EB was added at 50 μg/mL concentration. The cultures were incubated at 23°C with shaking at 150 rpm for 1 h. After the incubation period the cultures were centrifuged at 3500 rpm for 10 min at 21°C. The supernatant was decanted, and the pellet was re-suspended in 20 mM PPB buffer (pH 7.0) with 1 mM $MgCl_2$ and 5% glucose to energize the cells. Following energization, 200 μL of the bacterial culture were transferred into black 96-well microtiter plate (Corning, Amsterdam) containing the compounds at 50 μM (½ MIC) and the fluorescence of EB was measured over 2 hours at excitation and emission wavelengths of 530 and 600 nm, respectively, using a FLUOstar Optima plate reader (BMG Labtech, United Kingdom)[152]. During the evaluation the exact time was determined when the fluorescence dropped by 25% and 50% of the starting value. CCCP was applied as a positive control and DMSO was used as a negative control.

7. Measuring biofilm formation using crystal violet

The biofilm forming ability of *S.* Typhimurium 14028s strain was studied in 96-well microtiter plates using LB broth without salt in the presence of selenocompounds (**EDA1-11**). Initially, overnight cultures were diluted to an OD of 0.1 at 600 nm and then added to each well with the exception of the medium control wells and compounds were added at 50 µM (½ MIC) concentration. The final volume was 200 µL in each well. Plates were incubated at 30°C for 48 h with gentle agitation (100 rpm). After the incubation period the medium was discarded, and the plate was washed with tap water to remove unattached cells. 200 µL CV (0.1% [v/v]) was added to the wells and incubated for 15 minutes at room temperature. CV was removed from the wells and the plate was washed again with tap water. 200 µL of 70% ethanol was added to each well and the biofilm formation was determined by measuring the OD at 600 nm using a FLUOstar Optima plate reader. The anti-biofilm effect of selenocompounds was expressed in the percentage (%) of decrease in biofilm formation. The results were analyzed using t-test and *p*-values of <0.05 were considered significant.

8. Interaction between antibiotics and resistance modifiers using checkerboard method

The combined effect of chamanetin (**CK2**) and dichamanetin (**CK4**) and antibiotics on the growth inhibition of *S. aureus* ATCC 25923 and methicillin resistant *S. aureus* 272123 strains was evaluated by checkerboard method. Two-fold serial dilutions of antibiotics were prepared in MH broth on the horizontal rows of microtiter plate and then cross-diluted vertically by two-fold serial dilutions of the compounds[156]. For this assay, only the compounds with well-defined MIC values could be used. The dilutions of the antibiotics (TET or CIP) were made in a horizontal direction in 100 µL, and the dilutions of compounds were made vertically in the microtiter plate in 50 µL. After the dilution of an overnight culture, bacterial cells were re-suspended in MH medium containing 1×10^4 cells and distributed into each well. The plates were incubated for 18 h at 37°C. The cell growth rate was determined after MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) staining (See Appendix 5), as described elsewhere (Figure 8)[156]. The combination index (CI) values at 90% growth inhibition (ED_{90}) were determined by CompuSyn software to plot 4 or 5 data points for each ratio (www.combosyn.com, ComboSyn, Inc., Paramus, NJ. 07652 USA). CI values were calculated by means of the median-effect equation, where CI < 1, CI = 1 and CI > 1 represent synergism, an additive effect (or no interaction) and antagonism, respectively[157].

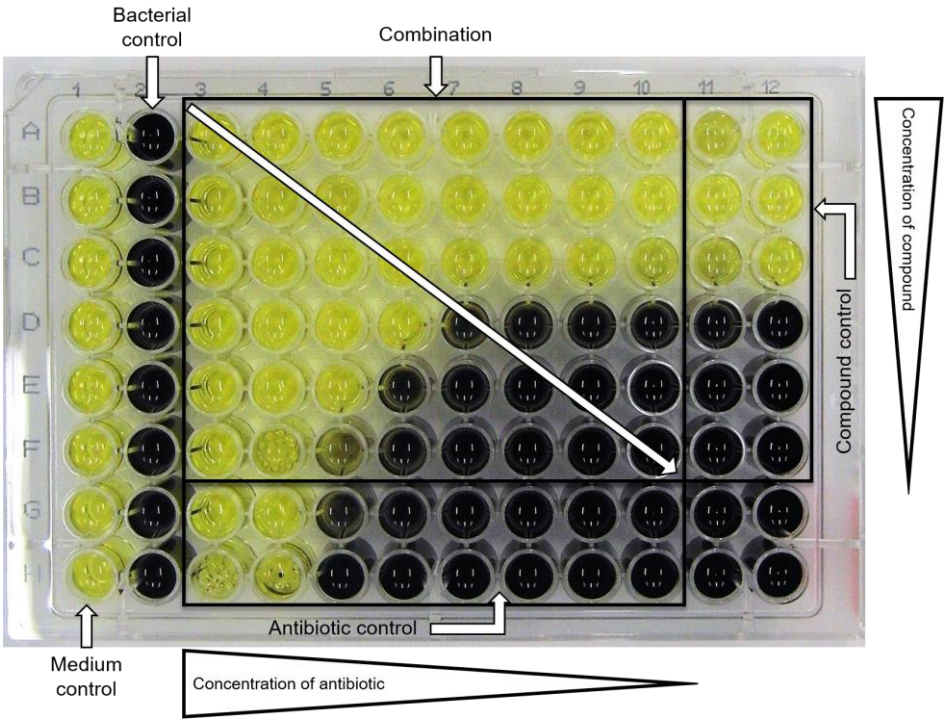


Figure 8 The layout of checkerboard plates

9. Interaction between antibiotics and resistance modifiers using minimum inhibitory concentration reduction assay

The chemosensitizing effect of the selenocompounds (**EDA1**-**11**) was evaluated by the determination of the MIC values of the antibiotics (TET and CIP), in the presence of sub-inhibitory concentrations of the compounds (½ MIC) in *E. coli* AG100 strain by a two-fold broth microdilution method in the 96-well plates, using serial dilutions of TET and CIP. The first four rows contained two-fold dilutions of antibiotics, and the combinations of the antibiotics and tested compounds were added into the last four rows. 10^{-4} dilution of an overnight bacterial culture in 50 µL of MH was then added to each well, with the exception of the medium control wells. The plates were then incubated at 37°C for 18 h. MIC values of the antibiotics and their combination with the tested compounds were determined by naked eyes.

10. Assay for quorum sensing inhibition

LB* was used for these experiments. The sensor strain *C. violaceum* 026 and the AHL producer strains *E. cloacae* 31298 were inoculated as parallel lines and incubated at room temperature (20°C) for 24–48 h. QS inhibition was monitored by agar diffusion method. Filter paper discs (7.0 mm in diameter) were impregnated with 10 µL of stock solutions (10 mM or 10 mg/mL) of the **CK** and **PY** compounds in DMSO. The discs were

placed between the parallel lines of the sensor and the AHL producer strains on the surface of the nutrient agar (Figure 9). The plates were incubated at room temperature for another 24–48 h, and the interactions between the strains and compounds were evaluated for the reduction in the size of the zone of pigment production and the zone of growth inhibition of the affected strains, in millimeters[153]. PMZ was applied as a positive control.

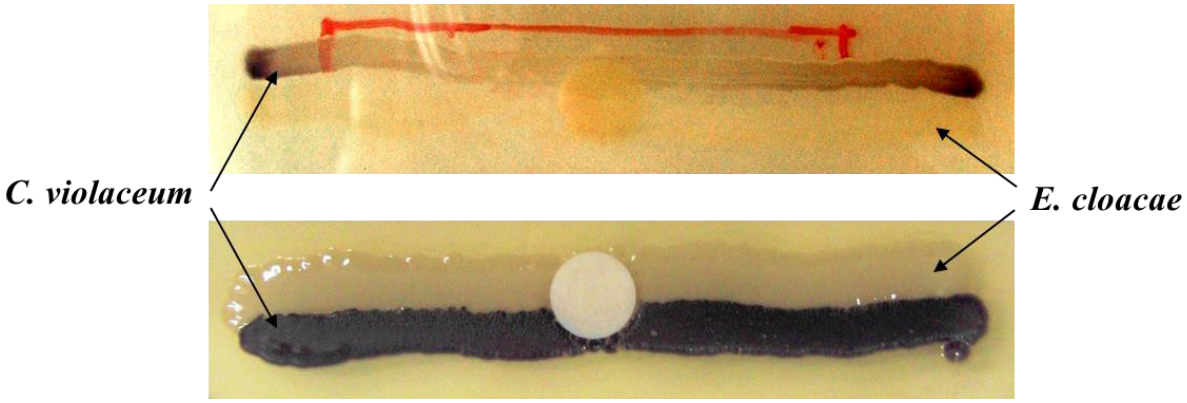


Figure 9 Positive (promethazine (PMZ; top)) and negative (DMSO; bottom) controls of quorum sensing (QS) on *C. violaceum* 026 and *E. cloacae* 31298

11. Expression analyses of genes by RT-qPCR reaction

E. coli AG100 strain was cultured in LB broth, *S. aureus* ATCC 25923 and MRSA 272123 strains were cultured in TSB broth and were incubated overnight at 37°C with shaking. On the day of RNA isolation, the bacterial suspensions (OD of 0.6 at 600 nm) were transferred to 10 mL tubes in 3 mL aliquots, and the compounds (**CK2**, **-4**; **PY2**, -**4**, -**5** and **EDA1**, **-4**, **-7**) were added to the tubes at 50 µM (for **EDA1**, -**4**, -**7**), 50 µg/mL (for **PY2**, -**4**, -**5**), 5 µM (for **CK2**) and 0.5 µM (for **CK4**) concentrations, which were incubated at 37°C. After 4 hours (for **CK2** and -**4**) or 4 and 18 hours (for **PY2**, -**4**, -**5** and **EDA1**, **-4**, **-7**) of culturing, the tubes were centrifuged at 12,000 × *g* for 2 min. Pellets were re-suspended in 100 µL Tris-EDTA buffer containing 1 mg/mL lysozyme by vigorous vortexing, and they were incubated at 37°C for 10 min. The total RNA was isolated in an RNase-free environment using NucleoSpin RNA kit (Macherey Nagel, Germany) according to the manufacturer's instructions. Purified RNA was stored in RNase-free water in nuclease-free collection tubes and was maintained at −20°C until quantification was performed. The concentration of the extracted RNA templates was assessed by SmartSpec™ Plus Spectrophotometer at 260 nm (Bio-Rad, USA).

Expression of the EP genes *norA* and *mepA* in the presence of chamanetin (**CK2**) and dichamanetin (**CK4**) was studied by reverse transcription of the total RNA of *S. aureus*

ATCC 25923 and MRSA 272123 strains. The data obtained for gene targets were normalized against the *S. aureus 16S ribosomal RNA* gene measured in the same sample.
The effect of **PY2**, -**4**, -**5** and **EDA1**, -**4**, -**7** on the relative expression of the EP (*acrA*, *acrB*), antibiotic resistance (*marR*) and QS (*sdiA*) genes were studied in *E. coli* AG100. The data obtained for gene targets were normalized against the *E. coli* house-keeping gene glyceraldehide-3-phosphate-dehydrogenase (*gapdh)* measured in the same sample. The full names of the tested genes are presented in Table 1. The forward and reverse primers used in this assay are shown in Appendix 6.

Gene	Full name	Reference
acrA	Acridine resistance protein A	158
acrB	Acridine resistance protein B	
marR	Multiple antibiotic resistance protein R	
sdiA	Quorum-sensing transcriptional activator	This study
gapdh	Glyceraldehyde-3-phosphate dehydrogenase	158
norA	Quinolone resistance protein NorA	159
mepA	Multidrug export protein MepA	
16S rRNA	16S Ribosomal RNA	

Table 1 Full name of the tested genes investigated in the RT-qPCR assay

Real-time quantification of the RNA templates by one-step RT-qPCR was performed in a CFX96 Touch real-time PCR detection system (Bio-Rad, USA) strictly adhered to the manufacturer's recommendations of the SensiFAST^{TM} SYBR No-ROX One-Step Kit (Bioline GmbH, Luckenwalde, Germany). Briefly, each well of the 96-well microtiter plates in a final volume of 20 µL contained 10 µL of the 2x SensiFAST^{TM} SYBR No-ROX One-Step Mix, 0.2 µL reverse transcriptase, 0.4 µL ribosafe RNAase inhibitor, 5.4 µL diethylpyrocarbonate-treated water, 500 nM of each primer and approximately 20 ng of the

total RNA in RNAase-free water. Thermal cycling was initiated with a denaturation step of 5 min at 95°C, followed by 40 cycles, each of 10 s at 95°C, 30 s at 57°C and 20 s at 72°C.

The relative quantities of the mRNA of each gene of interest were determined by the $\Delta\Delta C_T$ method[160]. Gene transcript levels were normalized against the previously mentioned housekeeping genes. The formula $2^{-\Delta\Delta C_T}$ allows the relative quantification of differences of each gene's expression level between two samples, the sample of interest and a calibrator or reference sample.

RESULTS

1. *In vitro* antibacterial activity of compounds

1.1. Bioactive compounds from *C. kirkii*

Compounds (**CK1**–**CK5**) were assessed for their antibacterial activity against methicillin susceptible *Staphylococcus aureus* ATCC 25923, and the methicillin and ofloxacin resistant *S. aureus* 272123 clinical isolate. Wild-type *Escherichia coli* K-12 AG100 and *E. coli* AG100 A strains, over-expressing and lacking the AcrAB-TolC efflux pump (EP) system, respectively, were used as Gram-negative models. In addition, the antibacterial activity of the compounds was tested on quorum sensing (QS) strains *Chromobacterium violaceum* 026 and *Enterobacter cloacae* 31298.

Concerning the antibacterial effect of the compounds, chamanetin (**CK2**) and dichamanetin (**CK4**) had a potent antibacterial effect on both *S. aureus* strains. Minimum inhibitory concentration (MIC) of compound **CK2** was 12.5 µM on reference *S. aureus* strain; however, the MIC in case of the methicillin and ofloxacin resistant strain was 25 µM. Compound **CK4** was the most effective flavanone because its MIC on *S. aureus* ATCC 25923 strain was 0.8 µM; furthermore, on the methicillin resistant strain it exhibited the MIC of 1.56 µM. The compounds had no antibacterial effect on the Gram-negative *E. coli* AG100, AG100 A, *C. violaceum,* and *E. cloacae* strains (MIC: >100 or 100 µM) (Table 2).

Compounds	***MIC (µM)***					
	S. aureus ATCC 25923	*S. aureus* 272123	*E. coli* AG100	*E. coli* AG100 A	*C. violaceum* 026	*E. cloacae* 31298
CK1	>100	>100	>100	>100	>100	>100
CK2	**12.5**	**25**	>100	>100	>100	>100
CK3	100	>100	>100	>100	>100	>100
CK4	**0.8**	**1.56**	>100	>100	>100	>100
CK5	>100	>100	>100	>100	>100	>100

Table 2 Minimum inhibitory concentrations (MICs) of the **CK** compounds isolated from *C. kirkii* on Gram-positive and Gram-negative bacteria

1.2. Fluorinated β-diketo phosphorus ylides

Compounds (**PY1-10**) were investigated for their antibacterial activity against wild-type *E. coli* K-12 AG100 strain, and the AcrAB-TolC pump mutant *E. coli* AG100 A strain. Furthermore, the antibacterial activity of the compounds was tested on QS strains *C. violaceum* 026 and *E. cloacae* 31298.

Compounds **PY1-10** did not have any antibacterial effect on the AcrAB-TolC expressing *E. coli* AG100, *C. violaceum* and *E. cloacae* strain and the AcrAB-TolC deleted *E. coli* AG100 A strain (MIC: >100 μg/mL), except for ethyl-4,4,4-trifluoro-3-oxo-2-(triphenyl phosphoranylidene)butanoate (**PY6**), which had a mild effect on the EP deleted strain (MIC: 50 μg/mL) (See Appendix 7).

1.3. Selenocompounds

The antibacterial effect of selenocompounds (**EDA1-11**) were tested on Gram-negative *E. coli* AG100, *E. coli* AG100 A, *Salmonella enterica* serovar Typhimurium SL1344, *acrB* inactivated *S.* Typhimurium L644 and *S.* Typhimurium 14028s strains. Furthermore, the following Gram-positive strains were used: *S. aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212.

The ketone-containing selenoesters (**EDA9**–**11**) showed a potent antibacterial activity against the Gram-positive *S. aureus* ATCC 25923. The methylketone selenoester **EDA9** was the most active compound with noteworthy MIC value (3.12 μM). The chloro-substituted *tert*-butylketone selenoester (**EDA10**) and dimethoxy-substituted *tert*-butylketone selenoester (**EDA11**) showed lower antibacterial activity than **EDA9** (25 and 50 μM, respectively). The selenoanhydride **EDA1** and the remaining selenoesters **EDA2**–**8** evaluated were inactive as their MIC was equal or above 100 μM on *S. aureus* ATCC 25923. **EDA9** showed antibacterial activity towards *E. faecalis* (MIC: 12.5 μM). The compounds had no antibacterial effect (MIC: 100 μM or >100 μM) on Gram-negative strains (See Appendix 8).

2. Efflux pump inhibiting activity (accumulation assay)

2.1. Bioactive compounds from *C. kirkii*

The ethidium bromide (EB) accumulation assay provides information about the intracellular accumulation of the general EP substrate EB. A potential efflux pump inhibitor (EPI) increases the fluorescence level of EB because of its accumulation within the bacterial cell. The EP inhibiting activity of the compounds was compared based on the relative fluorescence index (RFI) of the real-time accumulation curves in *E. coli* AG100, *E. coli*

AG100 A, *S. aureus* ATCC 25923 and *S. aureus* 272123. In case of real-time EB accumulation by the LightCycler thermocycler, the amount of EB accumulated by cells is higher if the difference between $RF_{treated}$ and $RF_{untreated}$ is greater; therefore, the degree of inhibition of the EP system by the compound becomes greater.

As shown in Figure 10, **CK2** and **CK4** had EP inhibiting activity compared to verapamil (RFI: 0.13) on the *S. aureus* ATCC 25923 strain, and the most active compound was **CK2**. However, compounds **CK1–5** had no EP inhibitory activity on the methicillin resistant *S. aureus* strain at the concentrations applied in the assay (See Appendix 9).

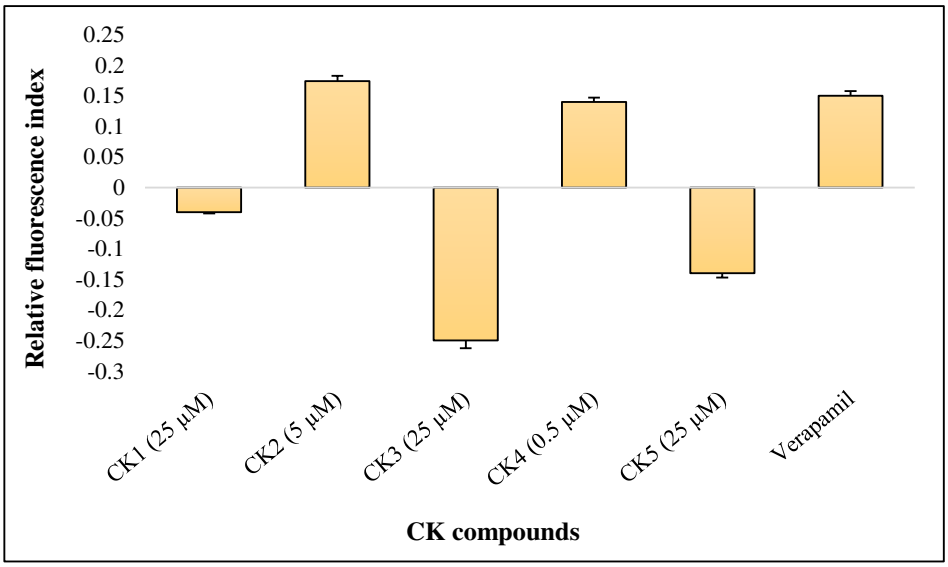


Figure 10 Relative final fluorescence index (RFI) of compounds (**CK1-5** isolated from *C. kirkii*) on *S. aureus* ATCC 25923 at different concentrations

Concerning the inhibitory activity on Gram-negatives, triterpene polycarpol (**CK1**) and **CK5** could inhibit the AcrAB-TolC system of *E. coli* AG100 compared to promethazine (PMZ; RFI: 0.15). **CK1** proved to be the most effective EPI (Figure 11). Based on the real-time accumulation data, **CK1–5** had no effect on the *E. coli* AG100 A strain lacking the AcrAB-TolC pump (See Appendix 10).

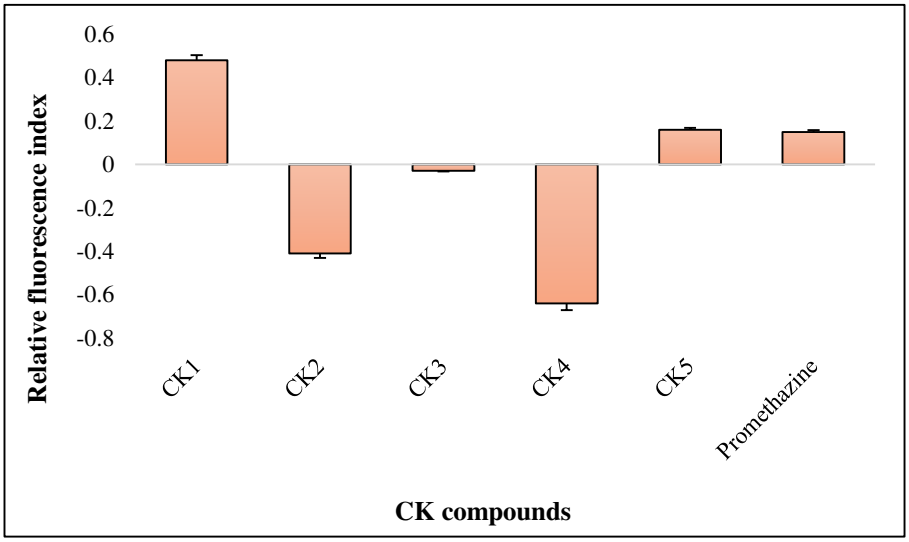


Figure 11 Relative final fluorescence index (RFI) of compounds (**CK1**-**5** isolated from *C. kirkii*) on *E. coli* AG100 strain at 50 µM

2.2. Fluorinated β-diketo phosphorus ylides

The EP inhibitory effect was tested on the AcrAB-TolC expressing *E. coli* AG100 and the mutant AG100 A strains. The majority of the P-ylides were found to inhibit the AcrAB-TolC system of *E. coli* except trifluoro-1-phenyl-2-(triphenylphosphoranylidene)butane-1,3-dione (**PY3**), 4,4,5,5,5-pentafluoro-1-phenyl-2-(triphenylphosphoranylidene)pentane-1,3-dione (**PY7**) and 4,4,5,5,6,6,6-heptafluoro-1-phenyl-2-(triphenylphosphoranylidene)hexane-1,3-dione (**PY8**), which had little or no effect on the intracellular EB accumulation in both strains. Among the P-ylide series, compounds 1,1,1-trifluoro-3-oxo-1-methoxy-3-(triphenylphosphoranylidene)propane-2-one (**PY2**), 4,4,4-trifluoro-3-oxo-2-(triphenylphosphoranylidene)butanal (**PY4**) and 1,1,1-trifluoro-3-(triphenylphosphoranylidene)pentane-2,4-dione (**PY5**) exhibited strong AcrAB-TolC pump-inhibiting properties compared to the AcrAB-TolC pump-deficient mutant strain. The most potent derivative was **PY4** and its effect was more pronounced on the multidrug resistant *E. coli* strain compared to the pump deleted *E. coli* strain (Figure 12).

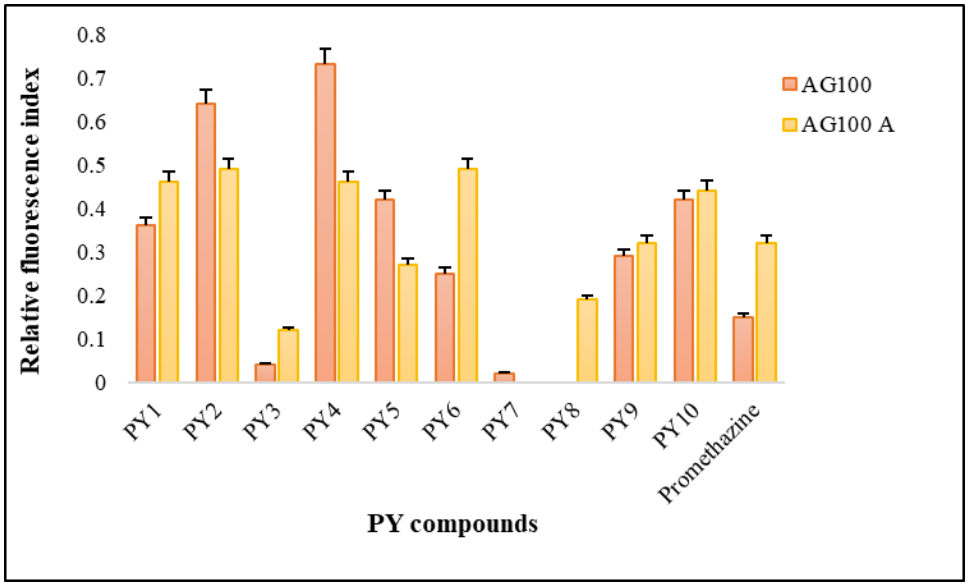


Figure 12 Relative final fluorescence index (RFI) of P-ylides (**PY1**-**10**) on the AcrAB-TolC expressing *E. coli* AG100 and pump deleted *E. coli* AG100 A strains at 50 μg/mL (except PY6 (25 μg/mL) on AG100 A strain)

2.3. Selenocompounds

The ability of the selenocompounds to inhibit the efflux of AcrAB-TolC transporter was determined on *E. coli* AG100 and AG100 A strains. **EDA1** and meta-substituted benzene selenodiester (**EDA4**), strongly inhibited the efflux mediated by AcrAB-TolC in *E. coli* AG100 compared to the positive control PMZ (RFI: 0.15). Methoxycarbonylmethyl selenoester (**EDA7**) and **EDA9–11** caused moderate inhibitory action, whereas thiophene selenodiester (**EDA2**), pyridine selenodiester (**EDA3**), para-substituted benzene selenodiester (**EDA5**), carbamoylmethyl selenoester (**EDA6**) and phenoxycarbonyl selenoester (**EDA8**) showed weak or no activity on the intracellular EB accumulation in *E. coli* AG100. Nevertheless, no EP inhibitory action was found in the *E. coli* AG100 A strain in case of selenocompounds (**EDA1–11**) (Figure 13).

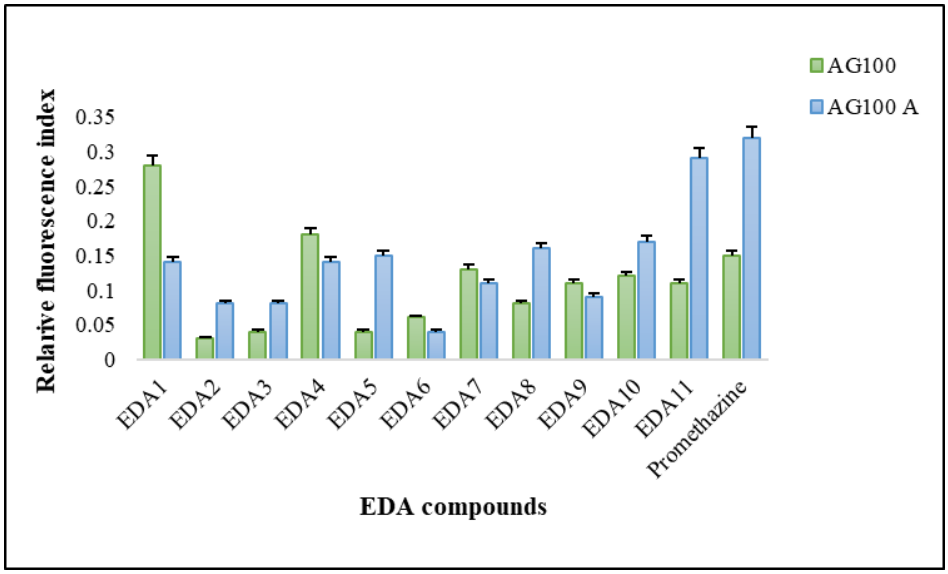


Figure 13 Relative final fluorescence index (RFI) of selenocompounds (**EDA1-11**) on the AcrAB-TolC expressing *E. coli* AG100 and pump deleted *E. coli* AG100 A strains at 50 μM

3. Efflux pump inhibiting activity (efflux assay)

In the EB efflux assay, after loading the wild-type *S.* Typhimurium SL1344 and *acrB* mutant L644 strains with EB the active efflux of the dye was measured as the time taken for fluorescence drops by 25% and 50% compared to the starting fluorescence level. Each selenocompound showed a 25% and 50% decrease in fluorescence at an earlier time point (25%: between 4.5th and 17.8th min; 50%: between 7.9th and 79.2nd min), compared with positive control CCCP (25%: in 52.1st min; 50%: in 122.6th min) in *S.* Typhimurium SL1344 (Table 3). The EB efflux was most effectively inhibited in the presence of **EDA9** as fluorescence intensity of EB was reduced by half in the 79.2nd minutes. For the L644 strain only **EDA7** was able to prevent the efflux of EB more effectively than carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Table 3).

Samples	*Time taken (min) for percentage*			
	S. Typhimurium SL1344		*S. Typhimurium L644*	
	-25%	**-50%**	**-25%**	**-50%**
Untreated control = Bacterial control	5.4	9.6	8	14.1
CCCP	52.1	122.6	42.1	117.6
EDA1	9.7	55.3	28	78.4
EDA2	17.8	56.8	31.5	88.6
EDA3	4.5	7.9	9.9	21
EDA4	17.2	56.1	21.6	83.2
EDA5	5.3	11.0	10.1	26.3
EDA6	5.4	11.0	11.8	30.5
EDA7	12.2	58.9	**55.2**	**119.5**
EDA8	5.5	9.5	9	16.8
EDA9	15.6	79.2	37.8	86.1
EDA10	17.1	65.6	18.8	80.8
EDA11	19.8	30.7	15	32

Table 3 Time taken for fluorescence to drop by 25% and 50% of starting value in wild-type *S.* Typhimurium SL1344 and its *acrB* mutant L644 strains in the presence of selenocompounds at 50 μM

4. Anti-biofilm activity of selenocompounds

The anti-biofilm effect of selenocompounds (**EDA1-11**) was evaluated by microdilution method using crystal violet (CV) on *S.* Typhimurium 14028s strain. Except compounds **EDA6** and dimethoxy-substituted *tert*-butylketone selenoester (**EDA11**) all derivatives showed significant (>45%; $p<0.05$) or higher biofilm inhibition at 50 μM on *S.* Typhimurium. The most potent selenocompounds with anti-biofilm effect were **EDA4** and -**5** at 50 μM showing 75% and 73% of inhibition, respectively (Figure 14).

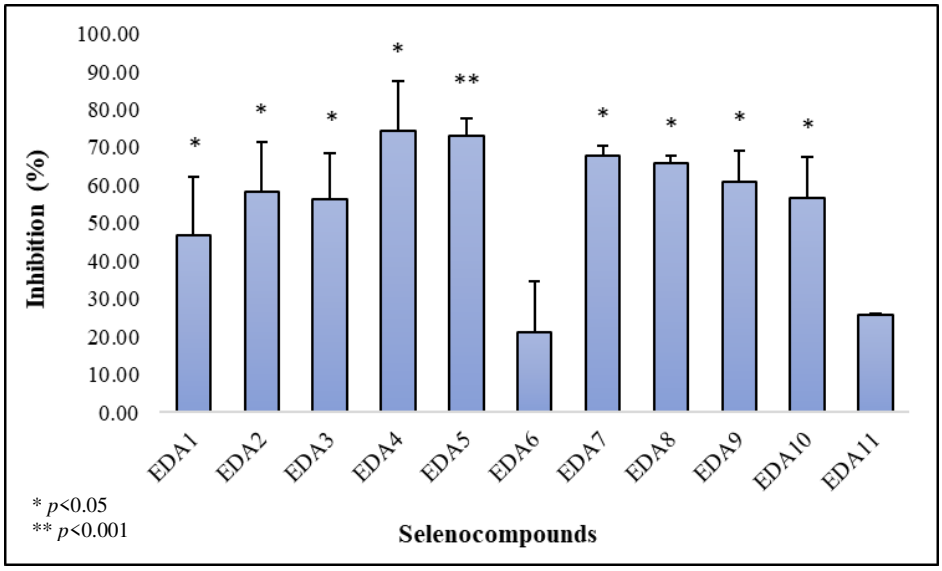


Figure 14 Anti-biofilm effect of selenocompounds on *S.* Typhimurium 14028s at 50 μM

5. Combined effects of chamanetin (CK2) and dichamanetin (CK4) with antibiotics

The type of interaction between the antibacterial compounds **CK2, CK4** and tetracycline (TET) and the fluoroquinolone antibiotic ciprofloxacin (CIP) was evaluated on reference (ATCC 25923) and methicillin resistant (MRSA 272123) *S. aureus* strains by checkerboard assay. The results are presented in Tables 5 and 6 as combination index values[157]. As it can be observed the combined effect of TET and compounds **CK2** or **CK4** on *S. aureus* ATCC 25923 resulted in synergism. The most effective ratio of antibiotic and compound was 1:20 and 1:1, respectively. Similarly, CIP also acted synergistically with compounds **CK2** and **CK4** and the most active ratio of antibiotic and compound was 1.3:12.5 and 1.3:1, respectively (Table 4).

***Staphylococcus aureus* ATCC 25923**				
Combination	**Best Ratio**	**CI at ED_{90}**	**SD (+/-)**	**Interaction**
TET + **CK2**	1:20	0.64	0.13	Synergism
TET + **CK4**	1:1	0.42	0.1	Synergism
CIP + **CK2**	1.3:12.5	0.82	0.24	Slight synergism
CIP + **CK4**	1.3:1	0.69	0.28	Synergism

Ratio: antibiotic and tested compound (μM). CI: combination index. CI < 1, CI = 1 and CI > 1 represent synergism, additive effect (or no interaction), and antagonism, respectively.

Table 4 Combined effect of **CK2** and **CK4** with antibiotics on *S. aureus* ATCC 25923 strain

On the methicillin resistant *S. aureus* strain **CK2** showed antagonistic effect with TET and slight synergism with CIP. The interactions of **CK4** with TET and CIP on the resistant *S. aureus* strain were synergism and moderate antagonism respectively (Table 5).

***Staphylococcus aureus* 272123**				
Combination	**Best Ratio**	**CI at ED_{90}**	**SD (+/-)**	**Interaction**
TET + **CK2**	6:25	1.5	0.29	Antagonism
TET + **CK4**	100:1	0.59	0.35	Synergism
CIP + **CK2**	13:8	0.85	0.22	Slight synergism
CIP + **CK4**	32:1	1.35	0.25	Moderate antagonism

Ratio: antibiotic and tested compound (μM). CI: combination index. CI < 1, CI = 1 and CI > 1 represent synergism, additive effect (or no interaction), and antagonism, respectively.

Table 5 Combined effect of **CK2** and **CK4** with antibiotics on *S. aureus* 272123 strain

6. Enhancement of the activity of antibiotics in the presence of selenocompounds

The combination effect of selenocompounds with TET and CIP was defined by MIC reduction assay on AcrAB-TolC expressing *E. coli* AG100 strain. In the absence of selenocompounds, TET showed MIC value of 4.2 μM and CIP exhibited MIC of 0.02 μM. **EDA9** potentiated the effect of TET and CIP, furthermore **EDA10** reduced the MIC value of CIP by two-fold in *E. coli* strain (Table 6).

Compounds	***Escherichia coli* AG100**	
	Reduction of TET MIC	***Reduction of CIP MIC***
EDA1-8; EDA11	no effect	no effect
EDA9	**2-fold**	**2-fold**
EDA10	no effect	**2-fold**

MIC values of TET and CIP alone: 4.2 μM and 0.02 μM, respectively.

Table 6 Fold change in reduction of the MICs of selected antibiotics on *E. coli* AG100 in the presence of selenocompounds

7. Anti-quorum sensing activity

7.1. Bioactive compounds from *C. kirkii*

The QS inhibiting activity of compounds was defined as measuring the colorless zone around the disc on *C. violaceum* as described previously[153]. Compounds **CK1**, -**2**, -**4**, and **-5**

were able to inhibit effectively the QS between *C. violaceum* and *E. cloacae* compared to the positive control PMZ (Table 7).

Compounds	*QS inhibition zone in mm*
CK1	**51**
CK2	**50**
CK3	-
CK4	**53**
CK5	**52**
Promethazine (PMZ)	46

10 µL of 10 mM stock solution was added onto the filter paper discs (10 µM/disc) and the colorless zone around the disc was determined on *C. violaceum.* The inhibition was measured after incubation for 24–48 h at room temperature

Table 7 Inhibitory effects of compounds **CK1–5** on quorum sensing (QS) signal transmission.

7.2. Fluorinated β-diketo phosphorus ylides

P-ylides were not able to inhibit the QS (inhibition zone: 0 mm) in the applied systems compared to the positive control PMZ.

8. Relative expressions of genes related to antibiotic resistance, quorum sensing and efflux pumps

8.1. Bioactive compounds from *C. kirkii*

In order to evaluate the effect of compounds on the relative expression of EP genes in both *S. aureus* strains, the most effective compounds **CK2** and **CK4** were selected for gene expression studies. In the reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) assay the genes of NorA and MepA transporters were investigated. As shown in Figure 15/A **CK2** at 5 µM significantly up-regulated the expression of *norA* and *mepA* genes after 4 h of exposure in methicillin resistant *S. aureus* strain. Compound **CK4** at 0.5 µM also significantly up-regulated both EP genes after 4 h of exposure in *S. aureus* 272123 as presented in Figure 15/B. In *S. aureus* ATCC 25923 strain, the expression level of the *mepA* gene was not influenced. Nevertheless, the *norA* gene was significantly up-regulated by compounds **CK2** (19.84-fold increase) at 5 µM and **CK4** (2.39-fold increase) at 0.5 µM (data not shown).

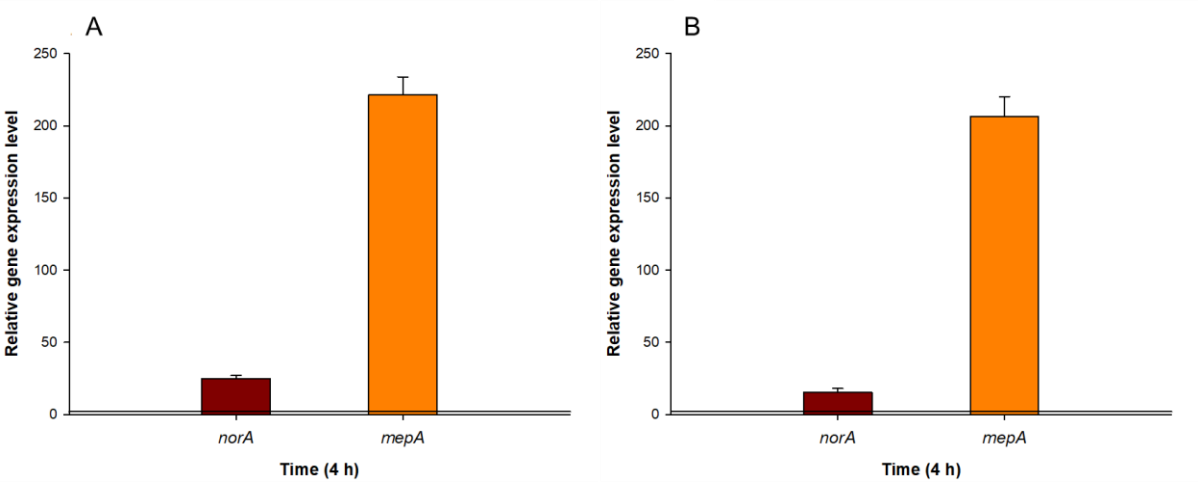


Figure 15 Relative gene expression levels of *norA* and *mepA* genes in the presence of **CK2** (A) and **CK4** (B) at the concentration of 5 μM (**CK2**) and 0.5 μM (**CK4**), respectively in *S. aureus* 272123, after 4 h exposure. The line denotes the threshold value, which was set at a two-fold increase in transcripts

8.2. Fluorinated β-diketo phosphorus ylides

Regarding the effect of P-ylides on the relative expression of EP and QS genes in *E. coli* AG100 the most effective **PY2**, -**4**, and -**5** compounds were selected for gene expression studies. In the assay the gene of the multidrug EP subunit AcrB, the periplasmic AcrA subunit, the component of the *E. coli mar* locus and the gene of the LuxR homologue SdiA were investigated. As shown in Figure 16/A **PY2** at 50 μg/mL up-regulated all the genes studied after 4 h of exposure, however, after 18 h the gene expression returned to basal levels. **PY4** also significantly up-regulated the secondary resistance-nodulation-division family (RND) transporter gene *acrB* (approximately 2-fold increase) after 4 h and 18 h exposures as well. Surprisingly, there was an up-regulation in the expression of *sdiA* after 18 h compared to the expression level after 4 h implicating the ability of **PY2** to influence *sdiA*, however, this increase was not significant (Figure 16/B). **PY5** up-regulated the expression levels of *acrA* and *acrB* after 4 h, although after 18 h the up-regulation of these genes was not significant (less than 2-fold increase) as presented by Figure 16/C.

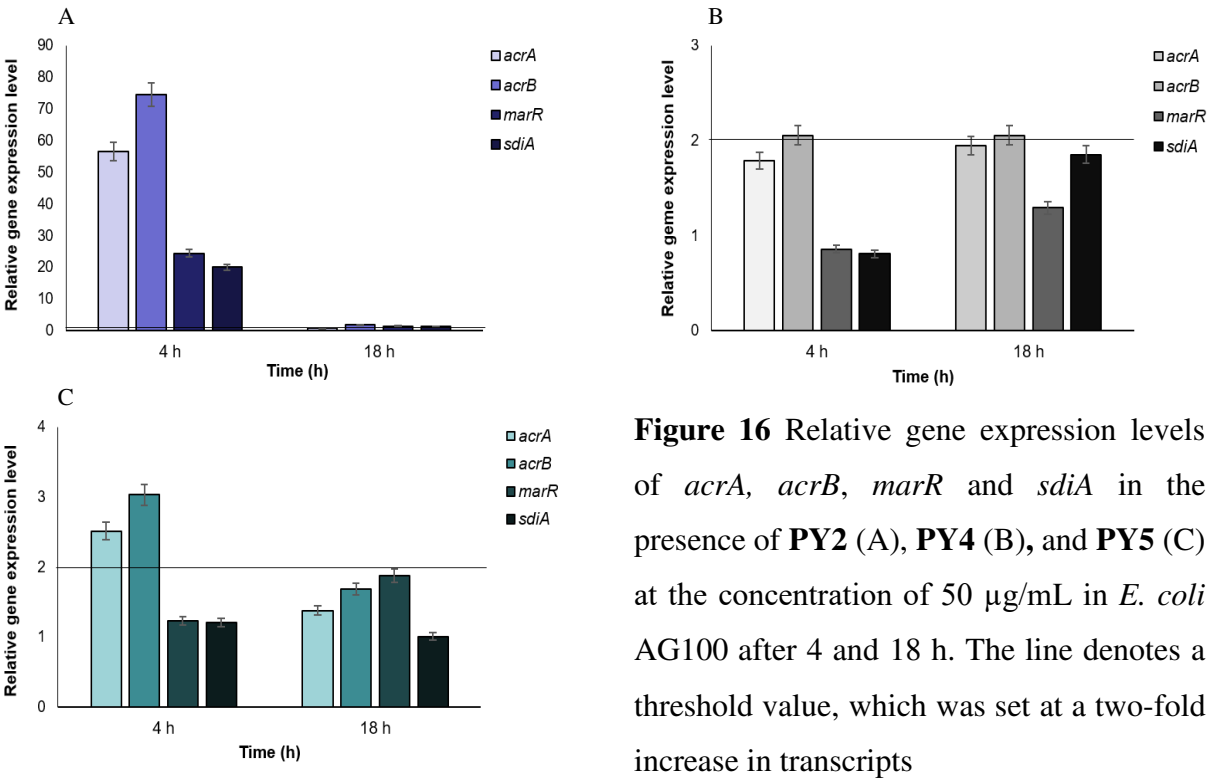


Figure 16 Relative gene expression levels of *acrA, acrB*, *marR* and *sdiA* in the presence of **PY2** (A), **PY4** (B)**,** and **PY5** (C) at the concentration of 50 µg/mL in *E. coli* AG100 after 4 and 18 h. The line denotes a threshold value, which was set at a two-fold increase in transcripts

8.3. Selenocompounds

Regarding the effect of selenocompounds on the relative expression of EP, antibiotic resistance and QS genes in *E. coli* AG100, the most effective **EDA** compounds -**1**, -**4** and -**7** were examined by gene expression analysis. In the assay, the gene of AcrB, AcrA, SdiA and the component of the *E. coli mar* locus were investigated. As shown in Figure 17/A, **EDA1** at 50 µM significantly up-regulated *acrB*, *marR* and *sdiA* genes studied after 4 h of exposure, however, after 18 h, the expression of *acrB* gene returned to basal level and the *marR* and *sdiA* genes increased significantly. **EDA4** up-regulated the expression of *acrB*, *marR* and *sdiA* after 4 h although after 18 h the expression levels of *acrB* and *marR* genes decreased. The QS gene *sdiA* was significantly up-regulated after 18 h (Figure 17/B). **EDA7** also significantly up-regulated *marR* after 4 h and 18 h exposures. After 18 h the RND transporter subunit genes (*acrA*, *acrB*) were significantly up-regulated in the presence of **EDA7** (Figure 17/B).

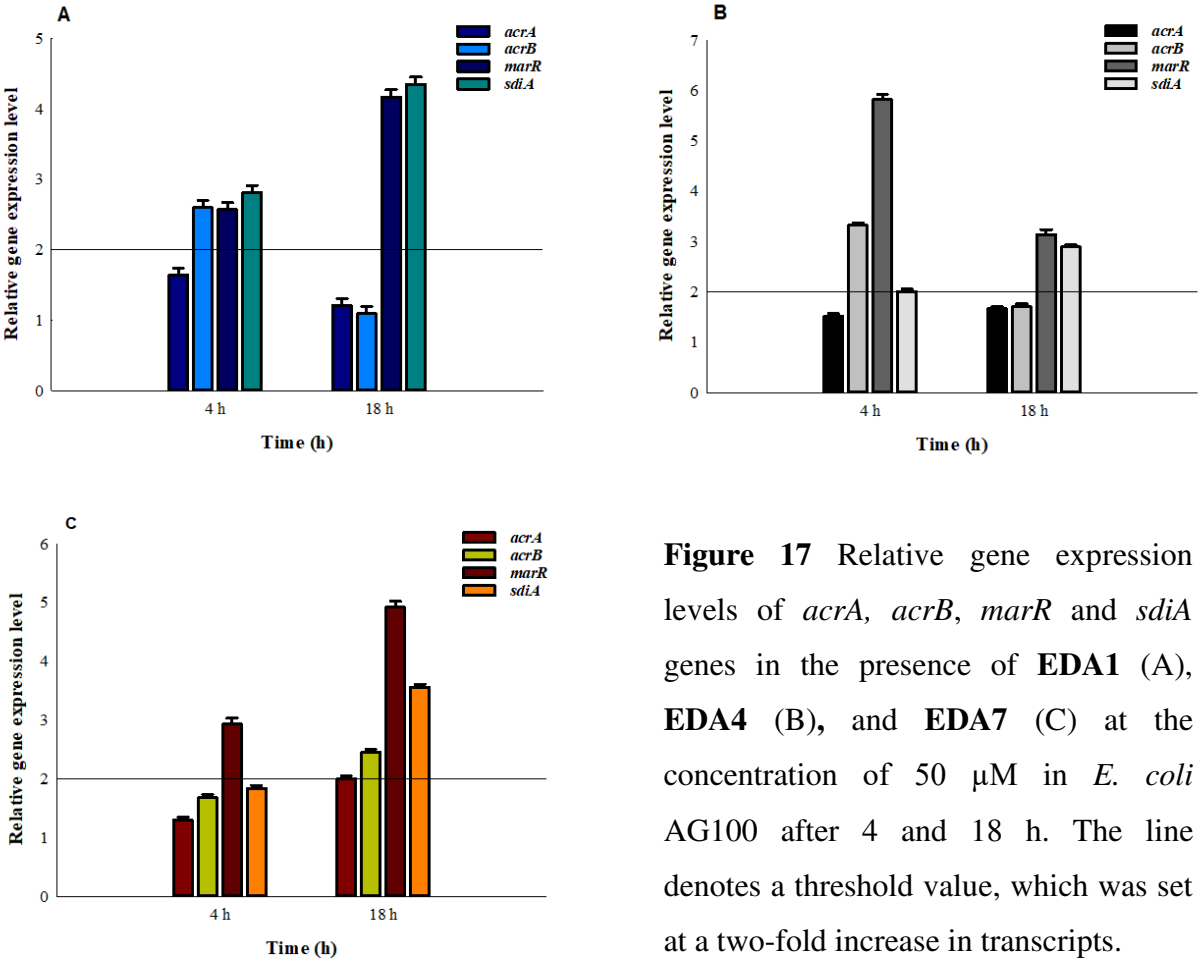


Figure 17 Relative gene expression levels of *acrA*, *acrB*, *marR* and *sdiA* genes in the presence of **EDA1** (A), **EDA4** (B)**,** and **EDA7** (C) at the concentration of 50 μM in *E. coli* AG100 after 4 and 18 h. The line denotes a threshold value, which was set at a two-fold increase in transcripts.

DISCUSSION

Multidrug resistance to antibiotics has become a serious problem in the treatment of infectious diseases. One of the most important mechanisms causing multidrug resistance is the over-expression of efflux pumps (EPs), whereby cells pump out toxic substances to the exterior of the cells. Infections caused by multidrug resistant bacteria lead to increased treatment costs and may result in fatal outcomes; consequently, it is a major challenge for drug development in order to discover new efflux pump inhibitors (EPIs).

The natural, plant-derived or synthetic compounds may represent a valuable source of new antibacterial agents because they can inhibit the growth of bacteria and the activity of bacterial efflux systems which indirectly prevent the formation of biofilm and the bacterial cell-to-cell communication system, furthermore, they can potentiate the efficacy of antibiotics as well.

The main goal of our study was to evaluate the antibacterial and multidrug resistance reversing effects of bioactive compounds from *Chleistochlamys kirkii* (**CK1-5**), fluorinated β-diketo phosphorus ylides (**PY1-10**) and selenocompounds (**EDA1-11**) in different bacterial models. The following methods were used in the studies: minimum inhibitory concentration (MIC) determination, ethidium bromide (EB) accumulation and efflux assay, checkerboard combination method, MIC reduction assay, biofilm, quorum sensing (QS) inhibitory test and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR).

1. Bioactive compounds from *C. kirkii* (CK1-5)

According to this study chamanetin (**CK2**) and dichamanetin (**CK4**) had the most pronounced antibacterial activity in case of methicillin sensitive and resistant *Staphylococcus aureus* strains. These results are supported by previous studies[139]. Dichamanetin showed stronger effect than chamanetin, due to its higher lipophilic character resulting from the extra benzyl group at C-6. In contrast to chamanetin, isochamanetin (**CK3**), differing only in the position of benzyl substituents, was inactive at the concentration tested. Thus, besides the importance of lipophilicity, the presence of a benzyl moiety at C-8 appears to play a decisive role in the antibacterial activity of this type of compounds. In the combination assays, chamanetin and dichamanetin showed synergism with tetracycline (TET) and ciprofloxacin (CIP) on the *S. aureus* reference strain. Furthermore, on the methicillin resistant *S. aureus* (MRSA) strain, chamanetin and dichamanetin, combined with TET and CIP, respectively, also showed synergism, which indicates that these compounds could be potential adjuvants in

the therapy. Besides having an antibacterial effect, both chamanetin and dichamanetin could inhibit the activity of EPs compared to the positive control. Furthermore, these compounds inhibited the QS between *Chromobacterium violaceum* 026 and *Enterobacter cloacae* 31298, and they caused over-expression of EP genes (*norA* and *mepA*) after 4 h of exposure in the MRSA strain. The change in gene expression could be due to the stress response against chamanetin and dichamanetin because these compounds as potential noxious agents for *S. aureus* had to be extruded from the cytoplasm of the bacterium as soon as possible. This stress response can be the explanation for the up-regulation of the EP genes after 4 h. Chamanetin and dichamanetin influenced the expression of *mepA* gene in the MRSA strain, however, these compounds did not have any effect on the expression level of *mepA* in the reference *S. aureus* strain. The difference between methicillin resistant *S. aureus* and methicillin susceptible reference *S. aureus* strains is due to the over-expression of the *mepA* gene in the resistant strain and it has low expression level in the reference strain. It has been demonstrated by other studies that the over-expression of EPs confers a fitness cost for the organism, for example, a resistant isolate over-expressing EPs shows reduced production of virulence determinants. Usually the over-expression of pump genes is often related to global effects on bacterial physiology influencing virulence[161]. In Gram-negatives, compounds **CK1**–**5** did not show antibacterial activity. The highly lipophilic polycarpol (**CK1**) inhibited the resistance-nodulation-division (RND) transporter AcrAB-TolC transport system in *Escherichia coli* AG100 strain because it could increase the membrane permeability of bacteria[139]. In contrast polycarpol had no EPI effect on the pump mutant *E. coli* AG100 A strain confirming that it may have a direct EPI effect on the AcrAB-TolC EP. In addition, apart from chamanetin and dichamanetin, polycarpol and acetylmelodorinol (**CK5**) were also able to inhibit effectively the bacterial communication, suggesting that they could be applied as anti-QS agents.

2. Fluorinated β-diketo phosphorus ylides (PY1-10)

In our previous study trifluoro-1-phenyl-2-(triphenylphosphoranylidene)butane-1,3-dione (**PY3**), 4,4,5,5,5-pentafluoro-1-phenyl-2-(triphenylphosphoranylidene)pentane-1,3-dione (**PY7**) and 4,4,5,5,6,6,6-heptafluoro-1-phenyl-2-(triphenylphosphoranylidene)hexane-1,3-dione (**PY8**) have been shown to have activity against the EPs of cancer cells[141], but did not show any activity against the EPs of *E. coli* strains. The aim of the previous work was to examine the primary ATP-binding cassette subfamily B member (ABCB1) EP inhibitory effect of **PY** compounds in cancer cells, in the present study the compounds have been tested

against the secondary AcrAB-TolC system in bacteria. It is important to note that ATP-binding cassette (ABC) transporters derive their energy from the hydrolysis of ATP, however, the AcrAB-TolC system is a proton motive force (PMF)-dependent multidrug efflux system. The most effective compounds in *E. coli* AG100 were 1,1,1-trifluoro-3-oxo-1-methoxy-3-(triphenylphosphoranylidene)propane-2-one (**PY2**), 4,4,4-trifluoro-3-oxo-2-(triphenylphosphoranylidene)butanal (**PY4**), and 1,1,1-trifluoro-3-(triphenylphosphoranylidene)pentane-2,4-dione (**PY5**), which inhibited the AcrAB-TolC system and influenced the expression of the transporter genes *acrA* and *acrB*. In addition, although the compounds are not QS inhibitors, **PY4** increased the expression of *sdiA* after 18 h exposure. The compounds in the R^1 chain contain a trifluoromethyl ketone ($COCF_3$) and the R^2 chain is a methoxy (OMe; **PY2**), formyl (CHO; **PY4**), and acetyl (COMe; **PY5**) substituent. Thus, some structurally related fluorinated β-diketo phosphorus ylides differ in their multidrug resistance reversal activities between cancer cells and bacterial strains, indicating that the compounds act differently as inhibitors of ABCB1 and AcrB EPs because these pumps differ in their structure and energy source driving the pump (ATP and PMF, respectively)[74].

3. Selenocompounds (EDA1-11)

These novel selenocompounds were studied previously for anticancer and ABCB1 EP inhibitory activity in different cancer cells[149–151,162]. According to the results on bacteria it was found that the ketone-containing selenoesters **EDA9-11** showed antibacterial activity against *S. aureus* reference strain, furthermore the methylketone selenoester (**EDA9)** was effective on *Enterococcus faecalis* ATCC 29212. In the EB accumulation assay the cyclic selenoanhydride (**EDA1)** and the meta-substituted benzene selenodiester (**EDA4)** could inhibit the activity of the AcrAB-TolC EP system and they were more potent than the positive control promethazine (PMZ) in *E. coli* AG100. Methylketone selenoester significantly inhibited the efflux mechanism of wild-type *Salmonella enterica* serovar Typhimurium SL1344 strain. EP inhibiting activity has been found for methoxycarbonylmethyl selenoester (**EDA7)** in the *acrB* mutant *S.* Typhimurium strain. From the results obtained in the EB efflux assay, it can be concluded that the same compounds had EP inhibitory activity in the wild-type and mutant *S.* Typhimurium strains as well. The similarities observed in the efflux assay of compounds mean that the derivatives do not directly inhibit the AcrAB-TolC system of *S.* Typhimurium.
Results showed that the meta-substituted benzene selenodiester (**EDA4**) and para-substituted benzene selenodiester (**EDA5**), which contain a phenyl ring, were the strongest inhibitors of

biofilm formation in *S.* Typhimurium 14028s, indicating the promising potential of these two difunctionalized derivatives against this Gram-negative strain assayed. **EDA7**-**10** and the thiophene selenodiester (**EDA2**) and pyridine selenodiester (**EDA3**) also exerted a biofilm inhibiting activity higher than 50% and the inclusion of an amide group (carbamoylmethyl selenoester; **EDA6**) or of a 3,5-dimethoxyphenyl moiety (dimethoxy-substituted *tert*-butylketone selenoester; **EDA11**) significantly reduced the biofilm formation in *S.* Typhimurium 14028s. Methylketone selenoester reduced the MIC value of TET by 2-fold, furthermore this compound and chloro-substituted *tert*-butylketone selenoester potentiated the activity of TET and CIP on *E. coli* AG100. In the gene expression analysis, we observed that cyclic selenoanhydride, meta-substituted benzene selenodiester, and methoxycarbonylmethyl selenoester influenced the gene expression of resistance (and transporter) genes because these derivatives significantly up-regulated the *marR* gene after 4 and 18 h exposure, respectively. Furthermore, the QS gene *sdiA* was significantly up-regulated in the presence of cyclic selenoanhydride, after exposures of 4 h and 18 h.

According to the results these studies indicated that natural and synthetic compounds could be used as potential antibacterial agents alone or combination with antibiotics for the treatment of infectious diseases. In addition, our studies are suggesting the importance of the substituent's topology and moiety for the biological potency. In future studies, it will be necessary to design and analyze systematically more compounds and understand the structure-activity relationships in order to develop potent resistance modifiers.

NEW FINDINGS

1. **Bioactive compounds from *Cleistochlamys kirkii***
   - Chamanetin (**CK2**) and dichamanetin (**CK4**) with a benzyl moiety at C-8 showed potent antibacterial effect against methicillin susceptible *Staphylococcus aureus* ATCC 25923 and methicillin and ofloxacin resistant *S. aureus* 272123 strains. Moreover, dichamanetin proved to be the most active compound isolated from *C. kirkii* on these strains and has higher lipophilic character compared to chamanetin due to its extra benzyl group at C-6.
   - Dichamanetin had synergistic activity with tetracycline and ciprofloxacin on *S. aureus* reference strain. Furthermore, dichamanetin showed synergism in combination with tetracycline on the methicillin and ofloxacin resistant *S. aureus* strain.
   - Chamanetin and acetylmelodorinol (**CK5**) had potent efflux pump modulatory effects in *S. aureus* reference strain. Polycarpol (**CK1**) was the most effective efflux pump inhibitor on *Escherichia coli* AG100 inhibiting the AcrAB-TolC transporter system.
   - All bioactive compounds of *C. kirkii* except isochamanetin (CK3) were able to inhibit effectively the bacterial quorum sensing system.
2. **Fluorinated β-diketo phosphorus ylides**
   - 1,1,1-trifluoro-3-oxo-1-methoxy-3-(triphenylphosphoranylidene)propane-2-one (**PY2**), 4,4,4-trifluoro-3-oxo-2-(triphenylphosphoranylidene)butanal (**PY4**), and 1,1,1-trifluoro-3-(triphenylphosphoranylidene)pentane-2,4-dione (**PY5**) inhibited directly the AcrAB-TolC efflux transporter in *E. coli* AG100 strain compared to the AcrAB-TolC pump mutant AG100 A strain.
3. **Selenocompounds**
   - Methylketone selenoester (**EDA9**) had antibacterial activity against the reference *S. aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 strains.
   - Methylketone selenoester potentiated the effect of tetracycline and ciprofloxacin on the AcrAB-TolC expressing *E. coli* AG100 strain.
   - Almost all selenocompounds showed potent anti-biofilm activity against *Salmonella enterica* serovar Typhimurium 14028s strain.
   - Cyclic selenoanhydride (**EDA1**), meta-substituted benzene selenodiester (**EDA4**) and methoxycarbonylmethyl selenoester (**EDA7**) were the most active inhibitors of the AcrAB-TolC system in *E. coli* AG100 strain.

SUMMARY

Multidrug resistance is a major concern in the treatment of bacterial infections due to the reduced or missing response of microorganisms to the applied antimicrobial agents. One of the most important mechanisms of multidrug resistance is the increased expression of efflux pumps (EPs) that can extrude distinct classes of antibiotics from bacteria to the environment. In order to overcome this process, efflux pump inhibitors (EPIs) could be designed and applied alone or in combination with antibiotics as adjuvants. For this reason, the objective of this study was to investigate the antimicrobial and resistance modifying activity of natural compounds isolated from *Cleistochlamys kirkii*, fluorinated β-diketo phosphorus ylides and selenocompounds on different Gram-positive and Gram-negative strains. The effects of compounds on bacterial growth were determined by microdilution method. The EP inhibiting activity of compounds on the accumulation and/or efflux of the general EP substrate ethidium bromide was assessed by real-time fluorimetry on *Escherichia coli*, *Salmonella* Typhimurium and *Staphylococcus aureus* strains. The anti-biofilm effect of selenocompounds was investigated by crystal violet assay using *S.* Typhimurium 14028s strain. The combined effects of antibiotics (tetracycline and ciprofloxacin) and compounds were examined by minimum inhibitory concentration reduction assay or checkerboard microdilution method. The quorum sensing inhibition of natural compounds and phosphorus ylides was studied by agar diffusion method, furthermore the relative gene expression level of EP and/or quorum sensing genes were determined by reverse transcriptase quantitative polymerase chain reaction. Results showed that chamanetin (**CK2**) and dichamanetin (**CK4**) from *C. kirkii* showed synergism with antibiotics on methicillin sensitive *S. aureus*, all the natural compounds except isochamanetin (CK3) were able to inhibit the quorum sensing. The most potent representative of phosphorus ylides was 4,4,4-trifluoro-3-oxo-2-(triphenylphosphoranylidene)butanal (**PY4**) and its effect was more pronounced on the AcrAB-TolC efflux system expressing *E. coli* strain. According to the results the methylketone selenoester (**EDA9**) had remarkable antibacterial activity against Gram-positive strains. The most selenocompounds were able to inhibit the biofilm formation of *S.* Typhimurium. The selenoanhydride (**EDA1**) and the meta-substituted benzene selenodiester (**EDA4**) were active inhibitors of the AcrAB-TolC system in *E. coli* AG100. These results suggested that natural compounds could be effective adjuvants in the antibiotic treatment of infections. Phosphorus ylides might be valuable EPI compounds to reverse efflux related multidrug resistance in bacteria and selenocompounds can be effective antibacterials and EPIs.

ÖSSZEFOGLALÁS

A multidrog rezisztencia során az előzetesen alkalmazott antibakteriális szer hatástalanná válik, mely súlyos problémát okoz a bakteriális fertőzések terápiájában. A multidrog rezisztencia kialakulásának egyik legjelentősebb mechanizmusa az efflux pumpák (EPs) túltermelése, amelyek a különböző hatásmechanizmusú antibiotikumokat képesek a baktériumsejtből a környezetbe juttatni. Az efflux mechanizmusokhoz köthető rezisztencia leküzdéséhez fontos lenne olyan efflux pumpa gátló (EPI) vegyületek tervezése, melyek önmagukban vagy antibiotikummal kombinációban alkalmazhatóak a fertőzések kezelésében. A jelen disszertáció célja három vegyületcsoport (*Cleistochlamys kirkii*-ből izolált természetes vegyületek, foszfor-ilidek, illetve szelénvegyületek) antimikrobiális és rezisztencia visszafordító hatásának vizsgálata *in vitro* Gram-pozitív, illetve Gram-negatív baktérium modellek felhasználásával. A vegyületek antibakteriális hatását mikrodilúciós módszerrel határoztuk meg, az EP gátlást etídium-bromid akkumulációs, illetve efflux vizsgálattal tanulmányoztuk valós idejű fluoreszcencia méréssel *Escherichia coli*, *Salmonella* Typhimurium, illetve *Staphylococcus aureus* törzsek felhasználásával. A szelénvegyületek biofilm gátló aktivitását kristályibolyás festéssel határoztuk meg *S.* Typhimurium 14028s törzsön. A természetes vegyületeket és a szelénvegyületeket kombinációban vizsgáltuk antibiotikumokkal (ciprofloxacinnal és tetraciklinnel), valamint a foszfor-ilidek és a *C. kirkii* származékok quorum-sensing (QS) gátló hatását agardiffúziós módszerrel határoztuk meg. Mind a három vegyületcsoportból a leghatásosabb EP gátló aktivitással rendelkező vegyületek génexpresszióra gyakorolt hatását RT-qPCR reakcióval teszteltük. Eredményeink alapján elmondható, hogy a *C. kirkii*-ből izolált kamanetin (**CK2**) és dikamanetin (**CK4**) a kísérletben alkalmazott antibiotikumokkal szinergizmust mutatott a methicillin érzékeny *S. aureus* törzsön, továbbá az izokamanetint (CK3) kivéve a vegyületek hatékonyan gátolták a QS-et. A foszfor-ilidek közül a 4,4,4-trifluoro-3-oxo-2-(trifenilfoszforanilidin)butanal (**PY4**) gátolta a leghatékonyabban az *E. coli* AcrAB-TolC efflux rendszerét. A metilketon szelenoészter (**EDA9**) a vizsgált Gram-pozitív törzseken jelentős antibakteriális hatással rendelkezett. A szelénvegyületek hatékonyan gátolták a *S.* Typhimurium 14028s biofilm képzését, illetve a ciklikus szelenoanhidrid (**EDA1**) és a meta-szubsztituált benzol szelenodiészter (**EDA4**) erősen gátolta az *E. coli* AG100 AcrAB-TolC rendszerét. A mérési eredményeink alapján megállapítható, hogy a *C. kirkii*-ből izolált vegyületek hatásosak lehetnek adjuvánsként, a foszfor-ilidek EPI vegyületekként, a szelénszármazékok pedig antibakteriális, illetve EPI vegyületként alkalmazva a bakteriális fertőzések kezelésében.

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APPENDIX

Appendix 1 IUPAC names of the P-ylides compounds

PY1: 4,4,4-trifluoro-3-oxo-2-(triphenylphosphoranylidene)butanenitrile

PY2: 1,1,1-trifluoro-3-oxo-1-methoxy-3-(triphenylphosphoranylidene)propane-2-one

PY3: trifluoro-1-phenyl-2-(triphenylphosphoranylidene)butane-1,3-dione

PY4: 4,4,4-trifluoro-3-oxo-2-(triphenylphosphoranylidene)butanal

PY5: 1,1,1-trifluoro-3-(triphenylphosphoranylidene)pentane-2,4-dione

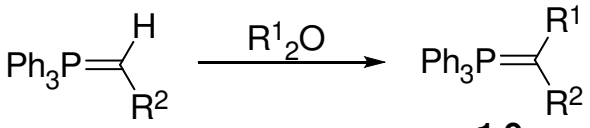
PY6: ethyl-4,4,4-trifluoro-3-oxo-2-(triphenylphosphoranylidene)butanoate

PY7: 4,4,5,5,5-pentafluoro-1-phenyl-2-(triphenylphosphoranylidene)pentane-1,3-dione

PY8: 4,4,5,5,6,6,6-heptafluoro-1-phenyl-2-(triphenylphosphoranylidene)hexane-1,3-dione

PY9: 3-oxo-2-(triphenylphosphoranylidene)butanenitrile

P10: 2-(triphenylphosphoranylidene)butanenitrile



1-9

Compound	R^1	R^2
PY1	$COCF_3$	CN
PY2	$COCF_3$	OMe
PY3	$COCF_3$	COPh
PY4	$COCF_3$	CHO
PY5	$COCF_3$	COMe
PY6	$COCF_3$	CO_2Et
PY7	COC_2F_5	COPh
PY8	COC_3F_7	COPh
PY9	$COCH_3$	CN
PY10	H	CN

Appendix 2 Structures of P-ylides (**PY1-10**)

Appendix 3 IUPAC names of the selenocompounds

EDA1: Benzo[c]selenophen-1,3-dione (cyclic selenoanhidride)

EDA2: Dimethyl thiophene-2,5-dicarboselenoate (thiophene selenodiester)

EDA3: Dimethyl pyridine-2,6-dicarboselenoate (pyridine selenodiester)

EDA4: Dimethyl benzene-1,3-dicarboselenoate (meta-substituted benzene selenodiester)

EDA5: Dimethyl benzene-1,4-dicarboselenoate (para-substituted benzene selenodiester)

EDA6: Carbamoylmethyl benzoselenoate (carbamoylmethyl selenoester)

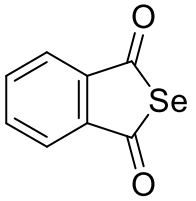
EDA7: Methoxycarbonylmethyl p-chlorobenzoselenoate (methoxycarbonylmethyl selenoester)

EDA8: Phenoxycarbonylmethyl benzoselenoate (phenoxycarbonyl selenoester)

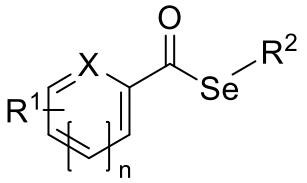
EDA9: 2-Oxopropyl 4-chlorobenzoselenoate (methylketone selenoester)

EDA10: 3,3-Dimethyl-2-oxobutyl 4-chlorobenzoselenoate (chloro-substituted *tert*-butylketone selenoester)

EDA11: 3,3-Dimethyl-2-oxobutyl 3,5-dimethoxybenzoselenoate (dimethoxy-substituted *tert*-butylketone selenoester)



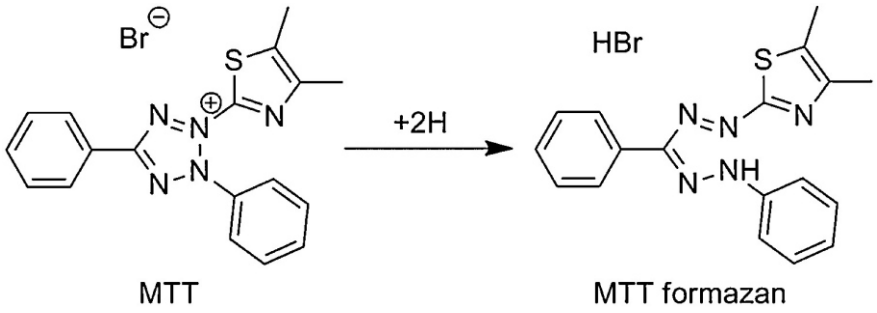
I. Cyclic selenoanhydride (**EDA1**)



II. Selenoesters (**EDA2-11**)

Compound	R^1	X	n	R^2
EDA1	-	-	-	-
EDA2	5-$COSeCH_3$	S	0	-CH_3
EDA3	6-$COSeCH_3$	N	1	-CH_3
EDA4	3-$COSeCH_3$	C	1	-CH_3
EDA5	4-$COSeCH_3$	C	1	-CH_3
EDA6	-H	C	1	-CH_2CONH_2
EDA7	4−Cl	C	1	-CH_2COOCH_3
EDA8	-H	C	1	-CH_2COOPh
EDA9	4−Cl	C	1	-CH_2COCH_3
EDA10	4−Cl	C	1	-$CH_2COC(CH_3)_3$
EDA11	3,5-diOCH_3	C	1	-$CH_2COC(CH_3)_3$

Appendix 4 Structures of the selenoanhydride (**EDA1**) and selenoesters (**EDA2-11**)



Appendix 5 MTT assay: reduction of MTT bromide to MTT formazan[163]

Gene	***Primer sequence (5'-3')***	***Amplicon size (bp)***
acrA	CTTAGCCCTAACAGGATGTG	189
	TTGAAATTACGCTTCAGGAT	
acrB	CGTACACAGAAAGTGCTCAA	183
	CGCTTCAACTTTGTTTTCTT	
marR	AGCGATCTGTTCAATGAAAT	170
	TTCAGTTCAACCGGAGTAAT	
sdiA	CTGATGGCTCTGATGCGTTTA	163
	TCTGGTGGAAATTGACCGTATT	
gapdh	ACTTACGAGCAGATCAAAGC	170
	AGTTTCACGAAGTTGTCGTT	
norA	TCGTCTTAGCGTTCGGTTTA	246
	TCCAGTAACCATCGGCAATA	
mepA	TGCTGCTGCTCTGTTCTTTA	198
	GCGAAGTTTCCATAATGTGC	
16S rRNA	AGAGTTTGATCMTGGCTCAG	492
	GWATTACCGCGGCKGCTG	

Appendix 6 Forward and reverse primers used in RT-qPCR reaction

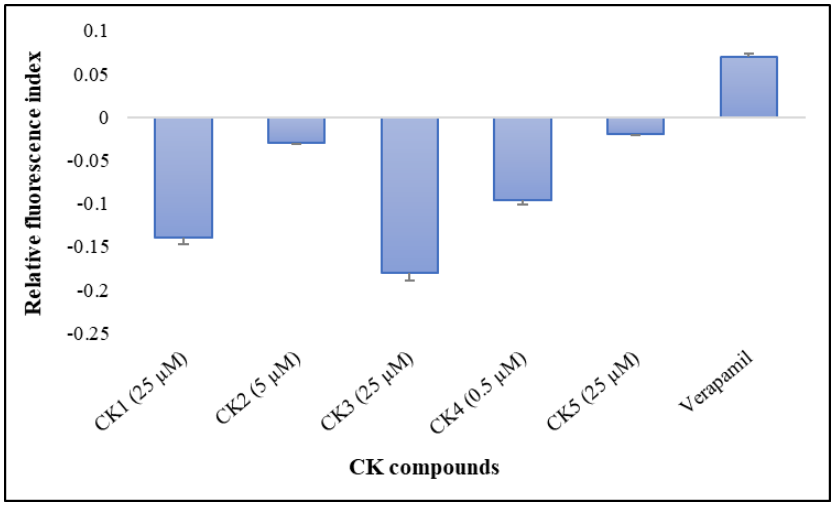
Compounds	*MIC (µg/mL)*			
	***E. coli* AG100**	***E. coli* AG100 A**	***C. violaceum* 026**	***E. cloacae* 31298**
PY1	>100	>100	>100	>100
PY2	>100	>100	>100	>100
PY3	>100	>100	>100	>100
PY4	>100	>100	>100	>100
PY5	>100	>100	>100	>100
PY6	>100	**50**	>100	>100
PY7	>100	>100	>100	>100
PY8	>100	>100	>100	>100
PY9	>100	>100	>100	>100
PY10	>100	>100	>100	>100

Appendix 7 Minimum inhibitory concentrations (MICs) of fluorinated P-ylides (**PY1-10**) on the Gram-negative strains

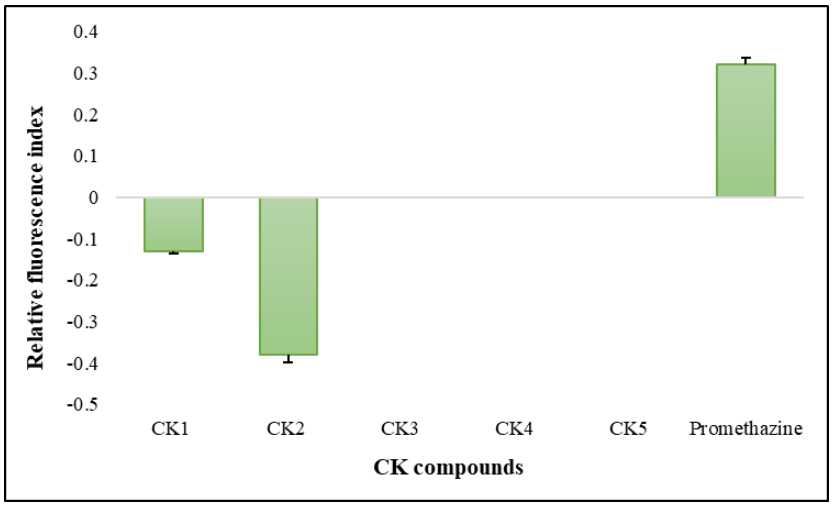
Compounds	*MIC (μM)*						
	25923	**29212**	**AG100**	**AG100 A**	**14028s**	**SL1344**	**L644**
EDA1	>100	>100	>100	>100	>100	>100	>100
EDA2	100	>100	>100	>100	>100	>100	>100
EDA3	100	>100	>100	>100	>100	>100	>100
EDA4	100	>100	>100	>100	>100	>100	>100
EDA5	>100	>100	>100	>100	>100	>100	>100
EDA6	>100	>100	>100	>100	>100	>100	>100
EDA7	100	>100	>100	>100	>100	>100	>100
EDA8	100	>100	>100	>100	>100	>100	>100
EDA9	**3.12**	**12.5**	>100	>100	>100	>100	>100
EDA10	**25**	>100	>100	>100	>100	>100	>100
EDA11	**50**	>100	>100	>100	>100	>100	>100

25923: *S. aureus* ATCC 25923; **29212**: *E. faecalis* ATCC 29212; **AG100**: *E. coli* AG100; **AG100 A**: *E. coli* AG100 A; **14028s**: *S. Typhimurium* 14028s; **SL1344**: *S. Typhimurium* SL1344; **L644**: *S. Typhimurium* L644 (ΔacrB)

Appendix 8 Minimum inhibitory concentrations (MICs) of selenocompounds (**EDA1**-**11**) on Gram-positive and Gram-negative strains



Appendix 9 Relative final fluorescence index (RFI) of **CK** compounds (**CK1**-**5**) on methicillin and ofloxacin resistant *S. aureus* 272123 at different concentrations



Appendix 10 Relative final fluorescence index (RFI) **CK** compounds (**CK1**-**5**) on *E. coli* AG100 A at 50 µM

TÁRSSZERZŐI LEMONDÓ NYILATKOZAT

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....

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szerző

.....

Dr. Spengler Gabriella

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Fluorinated Beta-diketo Phosphorus Ylides Are Novel Efflux Pump Inhibitors in Bacteria

ANNAMÁRIA KINCSES[1], ÁGNES MÍRA SZABÓ[1], RYOSUKE SAIJO[2], GENKI WATANABE[2], MASAMI KAWASE[2], JOSEPH MOLNÁR[1] and GABRIELLA SPENGLER[1]

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Abstract. *Background: One of the most important resistance mechanisms in bacteria is the increased expression of multidrug efflux pumps. To combat efflux-related resistance, the development of new efflux pump inhibitors is essential. Materials and Methods: Ten phosphorus ylides were compared based on their MDR-reverting activity in multidrug efflux pump system consisting of the subunits acridine-resistance proteins A and B (AcrA and AcrB) and the multidrug efflux pump outer membrane factor TolC (TolC) of Escherichia coli K-12 AG100 strain and its AcrAB-TolC-deleted strain. Efflux inhibition was assessed by real-time fluorimetry and the inhibition of quorum sensing (QS) was also investigated. The relative gene expression of efflux QS genes was determined by real-time reverse transcriptase quantitative polymerase chain reaction. Results: The most potent derivative was* $Ph_3P=C(COC_2F_5)CHO$ *and its effect was more pronounced on the AcrAB-TolC-expressing E. coli strain, furthermore the most active compounds,* $Ph_3P=C(COCF_3)OMe$, $Ph_3P=C(COC_2F_5)CHO$ *and* $Ph_3P=C(COCF_3)COMe$, *reduced the expression of efflux pump and QS genes. Conclusion: Phosphorus ylides might be valuable EPI compounds to reverse efflux related MDR in bacteria.*

Multidrug resistance (MDR) is a serious problem for the treatment of various diseases, such as bacterial and fungal infections and cancer, due to reduction or deficit response of microorganisms as well as cancer cells to applied chemotherapeutic agents (1, 2).

One of the major mechanisms of MDR is the overexpression of efflux pumps (EPs), which reduces the accumulation of toxic agents. In bacteria the resistance nodulation division (RND) transporters have a great impact on MDR phenotype. The major cause for the MDR phenotype is due to overexpression of efflux pumps that are part of the RND family of transporters, for example the acridine-resistance proteins A and B (AcrA and AcrB) and the multidrug efflux pump outer membrane factor TolC (TolC) AcrAB-TolC system (1). These efflux pumps have the ability to recognize and extrude a large variety of unrelated antibiotics from the periplasmic space of the cell envelope, or from the cytoplasm. The energy required for the operation of the efflux pump is provided by the proton motive force created by the proton gradient resulting from electron transport (3).

The quorum-sensing system (QS) in bacteria is a regulatory system that controls gene expression depending on the density of the bacterial cell population. A transcriptional regulator (LuxR homolog), signal synthase (LuxI homolog) and autoinducer (acyl homoserine lactone) are crucial for the QS in most Gram-negative bacteria. SdiA, an *E. coli* LuxR homolog, has a great impact on the colonization of *E. coli* (4, 5). SdiA is a homolog of QS regulators that detects *N*-acylhomoserine lactone (AHL) signals from other bacteria. SdiA represses the expression of virulence factors by interacting with unknown stationary-phase signals in *E. coli* O157:H7, and enhances multidrug resistance by activating MDR efflux pumps in *E. coli* (6).

Organic compounds of phosphorus ylides (P-ylides) are a fascinating class of compounds (7). The biological activity of P-ylides related to their ATP-binding cassette subfamily B member 1 (ABCB1)-modulating activity in mouse lymphoma cells has already been described (8), however, additional information is needed to describe their valuable biological activities in other aspects.

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Key Words: Phosphorus ylides, multidrug resistance, bacterial AcrAB-TolC, efflux pump, quorum sensing.

In this article, we report the MDR-modulating activities of P-ylides in bacteria related to the inhibition of efflux activity and QS.

Materials and Methods

Compounds. The synthesis of P-ylides was described previously (8) and the structures of the P-ylides (compounds **1-10**) screened for their MDR-modulating activities are shown in Table I. The compounds were dissolved in dimethyl sulfoxide (DMSO) for the experiments.

Acridine orange (AO), ethidium bromide (EB), and LB broth were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Modified LB agar (LB*) contained 5 g yeast extract, 10 g trypton, 10 g NaCl, 1 g K_2HPO_4, 0.3 g $MgSO_4.7H_2O$ and 36 mg FeNaEDTA in 1.0 l of distilled water. Mueller Hinton broth was purchased from Scharlau Chemie S.A. (Barcelona, Spain).

Bacterial strains. Wild-type *E. coli* K-12 AG100 strain [argE3 thi-1 rpsL xyl mtl Δ(gal-uvrB) supE44] expressing the AcrAB-TolC EP at its basal level and its AcrAB-TolC-deleted mutant strain (*E. coli* K-12 AG100A). The strains were kindly provided by Professor Dr. Hiroshi Nikaido (Department of Molecular and Cell Biology and Chemistry, University of California, Berkeley, CA, USA).

Strains used for QS tests: *Chromobacterium violaceum* 026 (CV026) as sensor strain; *Sphingomonas* spp. EZF 10-17 (*Sphingomonadaceae*) isolated from a grapevine crown gall tumor (used as AHL producer), this strain induced pigment production by CV026 and proved to be efficient for the study of QS interactions; *Enterobacter cloaceae* 31298 (clinical wound isolate, used as AHL producer).

Determination of minimum inhibitory concentrations. The minimum inhibitory concentrations (MICs) of P-ylides were tested according to Clinical and Laboratory Standard Institute guidelines (9).

Real-time accumulation assay by Roche LightCycler real-time thermocycler. The effect of the studied compounds on the real-time accumulation of EB was assessed by an automated EB method (10), using a LightCycler real-time thermocycler (LightCycler 1.5; Roche, Indianapolis, IN, USA). Briefly, an aliquot of an overnight culture of the strain in LB medium was transferred to fresh LB medium and incubated until it reached an optical density (OD) of 0.6 at 600 nm. It was then washed with phosphate-buffered saline (PBS; pH 7.4) and centrifuged at 13,000 × *g* for 3 min, the pellets re-suspended in PBS (pH 7.4) and the OD adjusted to 0.6 at 600 nm. The compounds were added individually at different concentrations (in double concentrated form) in 5 μl volumes of their stock solutions to 45 μl of EB solution of 2 mg/l in PBS. Then, 10 μl of the EB solution containing the compound were transferred into standard glass capillary tubes of 20 μl maximum volume (Roche) and 10 μl of bacterial suspension (OD of 0.6 at 600 nm) was added to the capillaries. The capillaries containing the samples were placed into a carousel (Roche) and the fluorescence was monitored at the FL-2 channel every minute on a real-time basis.

From the real-time data, the activity of the compound, namely the relative fluorescence index (RFI) of the last time point (minute 30) of the EB accumulation assay was calculated according to the formula:

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

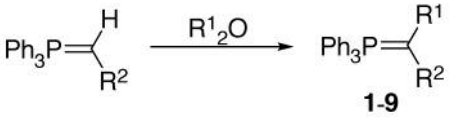
where $RF_{treated}$ is the relative fluorescence at the last time point of the EB retention curve in the presence of an inhibitor, and $RF_{untreated}$ is the relative fluorescence at the last time point of the EB retention curve of the untreated solvent control (DMSO).

Assay for QS inhibition. LB* was used for these experiments. The sensor strain *C. violaceum* 026 (CV026) and the AHL producer strains EZF 10-17 *Sphingomonas* spp. (*Sphingomonadaceae*) or *E. cloaceae* 31298 were inoculated as parallel lines and incubated at room temperature (20˚C) for 24-48 h. QS inhibition was monitored by agar diffusion method. Filter paper discs (7.0 mm in diameter) were impregnated with 10 μl of stock solutions of the compounds in DMSO (10 mg/ml in DMSO). The discs were placed between the parallel lines of sensor and AHL producer strains on the surface of the nutrient agar. The plates were incubated at room temperature for a further 24-48 h and the interactions between the strains and compounds were evaluated for the reduction in size of the zone of pigment production and the zone of growth inhibition of the affected strains, in millimeters. AO was applied as positive control (11).

Expression analyses of genes by real-time reverse transcriptase quantitative polymerase chain (RT-qPCR) reaction. Bacteria were cultured in LB broth and were incubated overnight at 37˚C with shaking. On the day of RNA isolation, the bacterial suspensions (OD of 0.6 at 600 nm) were transferred to 10 ml tubes in 3 ml aliquots and 50 μg/ml of compounds were added to the tubes which were the incubated at 37˚C. At intervals of 4 and 18 h of culture, the tubes were centrifuged at 12,000 × *g* for 2 min. Pellets were suspended in 100 μl TE buffer containing 1 mg/ml lysozyme by vigorous vortexing and were incubated at 37˚C for 10 min. The total RNA was isolated in an RNase-free environment using NucleoSpin RNA kit (Macherey Nagel, Germany) according to the manufacturer's instructions. Purified RNA was stored in RNase-free water in nuclease-free collection tubes and was maintained at −20˚C until quantification was performed. The concentration of the extracted RNA templates was assessed by spectrophotometry at 260 nm.

Expression of the *acrA*, *acrB*, multiple antibiotic resistance protein R (*marR*) and quorum-sensing transcriptional activator (*sdiA*) genes was studied by reverse transcription of total RNA. The data obtained for gene targets were normalized against the *E. coli* house-keeping gene glyceraldehyde-3-phosphate-dehydrogenase (*gapdh*) measured in the same sample. Real-time quantification of the RNA templates by real-time one-step RT-qPCR was performed in a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA) strictly adhering to the manufacturer recommendations of the SensiFAST™ SYBR No-ROX One-Step Kit (Bioline GmbH, Luckenwalde, Germany). Briefly, each well of 96-well microtiter plates contained, in a final volume of 20 μl, 10 μl of the 2x SensiFAST™ SYBR No-ROX One-Step Mix, 0.2 μl Reverse Transcriptase, 0.4 μl RiboSafe RNase Inhibitor, 5.4 μl diethylpyrocarbonate-treated water, 500 nM of each primer and approximately 20 ng of total RNA in RNAase-free water. Thermal cycling was initiated with a denaturation step of 5 min

Table I. *Structures of P-ylides (compounds **1-10**).*



1-9

Compound	R^1	R^2
1	$COCF_3$	CN
2	$COCF_3$	OMe
3	$COCF_3$	COPh
4	$COCF_3$	CHO
5	$COCF_3$	COMe
6	$COCF_3$	CO2Et
7	COC_2F_5	COPh
8	COC_3F_7	COPh
9	$COCH_3$	CN
10	H	CN

at 95˚C, followed by 40 cycles each of 10 s at 95˚C, 30 s at 57˚C and 20 s at 72˚C.

The forward and reverse primers used for assessment of the activity of the transporter genes *acrA*, *acrB*, the regulator *marR* and the QS regulator *sdiA* are shown in Table II.

Results

Compounds **1-10** did not have any antibacterial effect on the AcrAB-TolC-expressing *E. coli* AG100 (MDR) strain nor its AcrAB-TolC-deleted progeny *E. coli* AG100A (EP-deleted strain) (MIC above 100 μg/ml), except for **6**, which had a mild, non-significant effect on the EP-deleted strain (MIC=50 μg/ml).

The activity of the compounds was compared based on the relative final fluorescence index (RFI) of the real-time accumulation curves (Table III).

In the case of real-time EB accumulation by Light Cycler thermocycler, the amount of EB accumulated by cells is higher if the difference between $RF_{treated}$ and $RF_{untreated}$ is greater, therefore, the degree of inhibition of the EP system by the compound becomes greater.

The majority of the P-ylides were found to inhibit the AcrAB-TolC system of *E. coli* except $Ph_3P=C(COCF_3)COPh$ (**3**), $Ph_3P=C(COC_2F_5)COPh$ (**7**) and $Ph_3P=C(COC_3F_7)COPh$ (**8**), which had little or no effect on the intracellular EB accumulation in the *E. coli* AG100 and the AG100A strains. Among the P-ylide series, $Ph_3P=C(COCF_3)OMe$ (**2**), $Ph_3P=C(COCF_3)CHO$ (4) and $Ph_3P=C(COCF_3)COMe$ (**5**) exhibited strong AcrAB-TolC pump-inhibiting properties compared to the AcrAB-TolC pump-deficient mutant strain. The most potent derivative was **4** and its effect was more

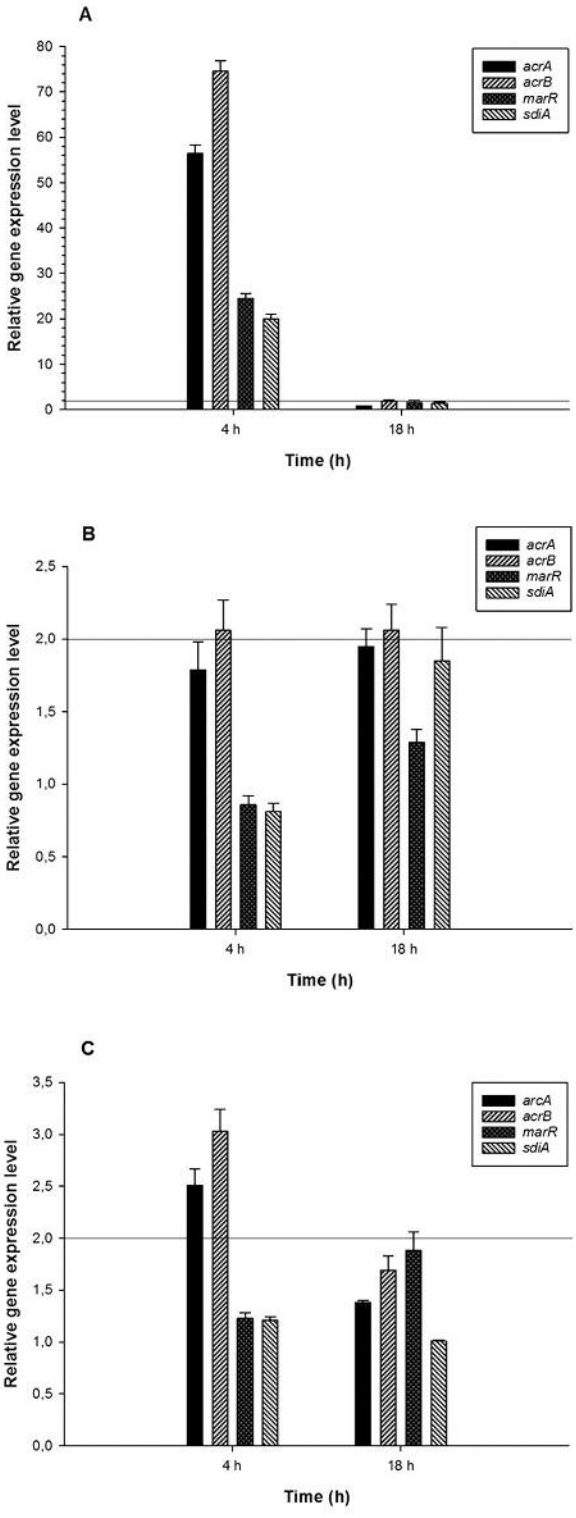


Figure 1. *Relative gene expression levels of the genes of the acridine resistance protein A (acrA), acridine resistance protein B (acrB), multiple antibiotic resistance protein R (marR) and quorum-sensing transcriptional activator (sdiA) in the presence of compounds **2** (A), **4** (B), and **5** (C) after 4 and 18 h exposure in LB. The line denotes the threshold value, which was set at a two-fold increase in transcripts.*

Table II. *Forward and reverse primers used for assessment of the activity of the transporter genes acrA and acrB, the regulator marR and the quorum-sensing regulator sdiA of Escherichia coli AG100.*

Gene	Full name	Primer sequence (5'-3')	Amplicon size (bp)	Reference
acrA	Acridine-resistance protein A	CTTAGCCCTAACAGGATGTGTTGAAATTACGCTTCAGGAT	189	(12)
acrB	Acridine-resistance protein B	CGTACACAGAAAGTGCTCAACGCTTCAACTTTGTTTTCTT	183	(12)
marR	Multiple antibiotic-resistance protein R	AGCGATCTGTTCAATGAAATTTCAGTTCAACCGGAGTAAT	170	(12)
sdiA	Quorum-sensing transcriptional activator	CTGATGGCTCTGATGCGTTTATCTGGTGGAAATTGACCGTATT	163	This study
gapdh	Glyceraldehyde-3-phospate dehydrogenase	ACTTACGAGCAGATCAAAGCAGTTTCACGAAGTTGTCGTT	170	(12)

pronounced on the MDR *E. coli* strain compared to the pump-deleted *E. coli* strain. This activity suggests that the compound may interfere with the proton motive force because AcrB utilizes proton motive force as energy for its transport function.

The P-ylides were not able to inhibit the QS in the applied systems compared to the positive control AO (data not shown).

Regarding the effect of P-ylides on the relative expression of efflux pump and QS genes in *E. coli* AG100, the most effective compounds **2**, **4**, and **5** were selected for gene-expression studies. In the assay, the gene of the multidrug efflux pump subunit AcrB, the gene of the periplasmic AcrA subunit, the component of the *E. coli mar* locus (multiple antibiotic resistance), and the gene of the LuxR homolog SdiA were investigated. As shown in Figure 1A, compound **2** at 50 μg/ml up-regulated all the genes studied after 4 h of exposure, however, after 18 h, the gene expression returned to basal levels. Compound **4** also significantly up-regulated the secondary RND transporter gene *acrB* (approximately 2-fold increase) after 4 h and 18 h exposures as well. Surprisingly, there was up-regulation of *sdiA* expression after 18 h compared to the expression level after 4 h implicating the ability of compound **2** to influence the QS gene *sdiA*, however, this increase was not significant (Figure 1B). Compound **5** up-regulated the expression levels of *acrA* and *acrB* after 4 h, although after 18 h, the up-regulation of these genes was not significant (less than 2-fold increase) as presented by Figure 1C.

Table III. *Relative final fluorescence index (RFI) for the effect of P-ylides (compounds **1-10**) on Escherichia coli AG100 expresing acridine-resistance proteins A and B and the multidrug efflux pump outer membrane factor TolC, and the pump-deleted E. coli AG100A strain at 50 μg/ml.*

Compound	RFI[a]	
	AG100	AG100A
1	0.36	0.46
2	0.64	0.49
3	0.04	0.12
4	0.73	0.46
5	0.42	0.27
6	0.25	0.49
7	0.02	0
8	0	0.19
9	0.29	0.32
10	0.42	0.44

[a]RFI was calculated according to the formula:

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

where $RF_{treated}$ is the relative fluorescence at the last point (30 min) of the ethidium bromide (EB) retention curve in the presence an P-ylide and $RF_{untreated}$ is the relative fluorescence at the last point (30 min) of the EB retention curve of the untreated solvent control dimethyl sulfoxide.

Discussion

Some phenothiazines and hydantoins are known to be EPIs against both bacteria and cancer cells (13). However, P-ylides **3**, **7** and **8** have been shown to have activity against the EPs of cancer cells (8), but not to have activity against the EP of bacteria. It is important to note that ABC transporters are primary efflux pumps deriving their energy from the hydrolysis of ATP, however, the AcrAB-TolC system is a three-component proton motive force-dependent multidrug efflux system. The most effective compounds in the present bacterial system were compounds **2**, **4**, and **5**, which inhibited the AcrAB-TolC system and influenced the expression of the genes *acrA* and *acrB*, which are constituents of the AcrAB-TolC system. In addition, although the compounds are not QS inhibitors, compound **4** did increase the expression of *sdiA* after 18 h exposure.

It can be concluded that in the $COCF_3$ series (compounds **1-6**), the MDR-reverting activity in the MDR *E. coli* strain was intensified in the following order: CHO (**4**) > OMe (**2**), COMe (**5**) >> CO_2Et (**6**), COPh (**3**), CN (**1**).

Thus, some structurally related trifluoroacetylated P-ylides differ in their MDR-reversal activities between cancer cells and bacterial strains, indicating that the compounds differently act as inhibitors of primary (ABCB1) and secondary (AcrB) efflux pumps because these pumps differ in their energy source for driving the pump (ATP and PMF, respectively).

The present study demonstrated that trifluoroacetylated P-ylides may be attractive lead EPIs for further development as a MDR-reversing agents, however, their mode of action should be elucidated by structure–activity relationship studies.

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RESEARCH ARTICLE

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Bioactive compounds from the African medicinal plant *Cleistochlamys kirkii* as resistance modifiers in bacteria

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Cleistochlamys kirkii (Benth) Oliv. (Annonaceae) is a medicinal plant traditionally used in Mozambique to treat infectious diseases. The aim of this study was to find resistance modifiers in *C. kirkii* for Gram-positive and Gram-negative model bacterial strains. One of the most important resistance mechanisms in bacteria is the efflux pump-related multidrug resistance. Therefore, polycarpol (**1**), three *C*-benzylated flavanones (**2**–**4**), and acetylmelodorinol (**5**) were evaluated for their multidrug resistance-reverting activity on methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* and *Escherichia coli* AG100 and AG100 A strains overexpressing and lacking the AcrAB–TolC efflux pump system. The combined effects of antibiotics and compounds (**2** and **4**) were also assessed by using the checkerboard microdilution method in both *S. aureus* strains. The relative gene expression of the efflux pump genes was determined by real-time reverse transcriptase quantitative polymerase chain reaction. The inhibition of quorum sensing was also investigated. The combined effect of the antibiotics and compound **2** or **4** on the methicillin-sensitive *S. aureus* resulted in synergism. The most active compounds **2** and **4** increased the expression of the efflux pump genes. These results suggested that *C. kirkii* constituents could be effective adjuvants in the antibiotic treatment of infections.

KEYWORDS

C-benzylated flavanones, *Cleistochlamys kirkii*, efflux pump, *Escherichia coli* AG100, methicillin-resistant *Staphylococcus aureus*, quorum sensing

1 | INTRODUCTION

The increasing resistance of bacterial isolates is a high concern in the therapy of infectious diseases. The widespread and inappropriate use of antibiotics has contributed to the selection of resistant bacteria. Multidrug-resistant (MDR) bacteria show resistance against a broad range of antimicrobials, and one of the most important resistance mechanisms is the presence of multidrug-resistant efflux pumps (EPs). These membrane proteins have physiological functions, and they are involved in the extrusion of toxic substances into the environment (Aparna, Dineshkumar, Mohanalakshmi, Velmurugan, & Hopper, 2014; Webber & Piddock, 2003).

In *Staphylococcus aureus*, the most studied MDR pump is the chromosomally encoded NorA, which is a member of the major facilitator superfamily. NorA can transport hydrophilic compounds, quaternary ammonium compounds, and dyes. The MepA chromosomally encoded efflux transporter described in *S. aureus* belongs to the multidrug and toxic compound extrusion family. MepA can recognize fluoroquinolones,

glycylcyclines, dyes, and quaternary ammonium compounds (Costa, Viveiros, Amaral, & Couto, 2013).

The discovery of plant-derived antimicrobials has drawn particular attention (Balogh et al., 2014; Hintz, Matthews, & Di, 2015). Numerous phytochemicals have minimal toxic activity, and they could be used in order to overcome drug resistance in bacteria by blocking multidrug EPs (Stavri, Piddock, & Gibbons, 2007). It is important to note that EP inhibitors (EPIs) from plant sources can inhibit the activity of bacterial efflux systems; furthermore, they can potentiate the efficacy of antibiotics as well. It has been described that reserpine, isolated from the roots of *Rauwolfia vomitoria*, inhibits the Bmr EP of *Bacillus subtilis* (Klyachko, Schuldiner, & Neyfakh, 1997). Several *Berberis* species producing berberine were also found to synthesize 5′-methoxyhydnocarpin, an inhibitor of the NorA MDR pump of *S. aureus* (Stermitz, Lorenz, Tawara, Zenewicz, & Lewis, 2000). Cucurbitane-type triterpenoids, isolated from the aerial parts of the African medicinal plant *Momordica balsamina*, have shown activity against the EP systems of Gram-positive bacteria (Ramalhete, Da Cruz, et al., 2011a; Ramalhete, Lopes, et al., 2011b; Ramalhete, Spengler, et al., 2011c).

The bacterial quorum sensing (QS) is a cell-to-cell communication system that is based on chemical signals, namely, autoinducers (AIs). In Gram-negative bacteria, the most common AI is the *N*-acyl homoserine lactone (AHL; Varga et al., 2011). EPs and QS signals play an important role in the development of bacterial virulence. The QS system and the AIs are able to influence the expression of transporter genes. These EPs have the ability to transport AI molecules to the external environment of bacteria, thus facilitating cell-to-cell communication (Spengler, Kincses, Gajdács, & Amaral, 2017).

Cleistochlamys kirkii (Benth) Oliv. (Annonaceae) is an African medicinal plant traditionally used in Mozambique for the treatment of wound infections, tuberculosis, and rheumatism (Verzár & Petri, 1987).

Previously, in a research for antimicrobial compounds from African medicinal plants (Cabral et al., 2015; Kolaczkowski et al., 2010; Madureira, Ramalhete, Mulhovo, Duarte, & Ferreira, 2012; Pereira et al., 2016; Ramalhete et al., 2010), bioassay-guided fractionation of the methanol extract of the root barks of *C. kirkii* led to the isolation of several compounds with different scaffolds. Among them, *C*-benzylated flavanones, namely, dichamanetin and the α,β-unsaturated lactone (−)-cleistenolide, have shown relevant antibacterial activity against Gram-positive bacteria, including drug-resistant strains (Pereira et al., 2016). Moreover, in the combination with β-lactam antibiotics and vancomycin, polycarpol bearing a triterpenic scaffold exhibited a strong synergistic effect against Gram-positive resistant strains. In this study, polycarpol (**1**) and *C*-benzylated flavanones **2**–**4** and a heptane derivative (**5**) were further investigated for their ability as both EP and QS inhibitors of the efflux systems of Gram-negative and Gram-positive bacteria. Furthermore, the ability of compounds **2** and **4** to potentiate the effect of tetracycline and the fluoroquinolone antibiotic ciprofloxacin on methicillin-susceptible and methicillin-resistant *S. aureus* (MRSA) strains was also described.

2 | MATERIALS AND METHODS

2.1 | Compounds tested

Triterpene polycarpol (**1**), *C*-benzylated flavanones chamanetin (**2**), isochamanetin (**3**), dichamanetin (**4**), and the heptane derivative acetylmelodorinol (**5**; Figure 1) were isolated from the methanol extract of the root barks of *C. kirkii*, as it has previously been described (Pereira et al., 2016). The purity of all the compounds was higher than 95% based on high-performance liquid chromatography analysis and nuclear magnetic resonance spectroscopy. The compounds were dissolved in dimethyl sulfoxide (DMSO).

2.2 | Reagents and media

Promethazine (PMZ; EGIS), ethidium bromide (EB), verapamil, tetracycline-hydrochloride (TET), ciprofloxacin-hydrochloride (CIP), and Luria–Bertani (LB) broth, and LB agar were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The modified LB medium (LB*) was prepared from yeast extract 5 g/L, tryptone 10 g/L, NaCl 10 g/L, K_2HPO_4 1 g/L, $MgSO_4 \times 7H_2O$ 0.3 g/L, and FeNaEDTA

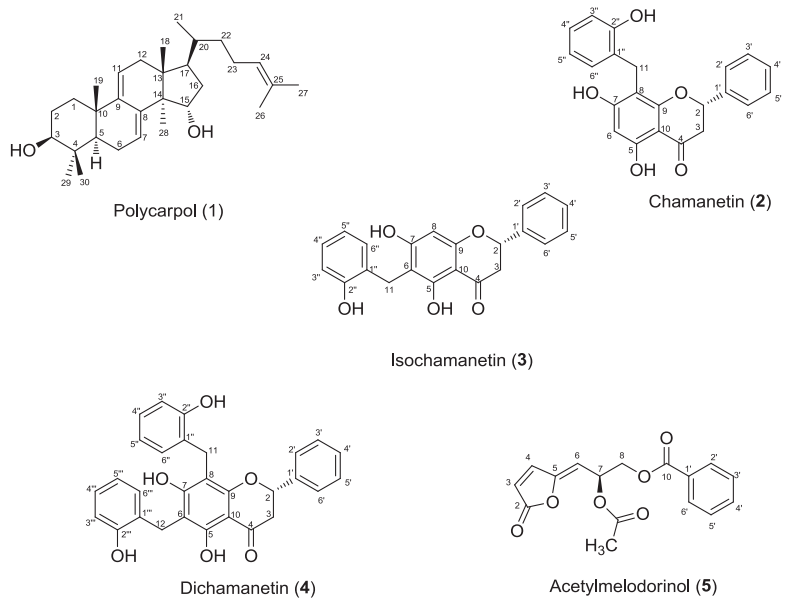

FIGURE 1 Chemical structures of compounds **1**–**5**

36 mg/L. In case of modified LB* agar, the LB* medium was supplemented with agar 20 g/L (Difco). pH was adjusted to 7.2. Tryptic soy broth (TSB), tryptic soy agar, and Mueller–Hinton (MH) broth were purchased from Scharlau Chemie S. A. (Barcelona, Spain).

2.3 | Bacterial strains

Compounds were evaluated against the Gram-negative wild-type *Escherichia coli* K-12 AG100 strain [argE3 thi-1 rpsL xyl mtl Δ(gal-uvrB) supE44], expressing the AcrAB–TolC EP at its basal level and its AcrAB–TolC-deleted mutant *E. coli* AG100 A strain. These strains were kindly provided by Prof. Dr. Hiroshi Nikaido (Department of Molecular and Cell Biology and Chemistry, University of California, Berkeley, CA, USA).

The compounds were further evaluated against two Gram-positive strains, *S. aureus* American Type Culture Collection (ATCC) 25923, used as the methicillin-susceptible reference strain, and the methicillin and ofloxacin-resistant *S. aureus* 272123 clinical isolate, which was kindly provided by Prof. Dr. Leonard Amaral (Institute of Hygiene and Tropical Medicine, Lisbon, Portugal).

For QS tests, the following strains were used: *Chromobacterium violaceum* 026 (CV026) as sensor strain and *Enterobacter cloaceae* 31298 as AHL producer strain (a clinical isolate from a wound). When *C. violaceum* reaches a high cell density, it produces a purple pigment, namely, violacein (Ballantine, Beer, Crutchley, Dodd, & Palmer, 1958).

2.4 | Determination of minimum inhibitory concentrations by microdilution method

The minimum inhibitory concentrations (MICs) of compounds were determined according to the Clinical and Laboratory Standard Institute guidelines (2017) in three independent assays. The solvent DMSO had no antibacterial effect.

2.5 | Interaction between antibiotics and compounds

The combined effect of compounds and antibiotics on the growth inhibition of *S. aureus* was evaluated by the checkerboard method. Twofold serial dilutions of antibiotics were prepared in MH broth on the horizontal rows of microtiter plate and then cross-diluted vertically by twofold serial dilutions of the compounds (Wolfart et al., 2006). For this assay, only the compounds with well-defined MIC values could be used. Consequently, the combination assays were carried out on methicillin-susceptible and MRSA strains. The dilutions of the antibiotics (TET or CIP) were made in a horizontal direction in 100 µl, and the dilutions of compounds were made vertically in the microtiter plate in 50 µl. After the dilution of an overnight culture, bacterial cells were resuspended in MH medium containing 1×10^4 cells and distributed into each well. The plates were incubated for 18 hr at 37 °C. The cell growth rate was determined after MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) staining, as described elsewhere (Wolfart et al., 2006). The combination index (CI) values at 90% growth inhibition (ED_{90}) were determined by using CompuSyn software to plot 4 or 5 data points for each ratio (www.combosyn.com, ComboSyn, Inc., Paramus, NJ, USA). CI values were calculated by means of the median-effect equation, where CI < 1, CI = 1, and CI > 1 represent synergism, an additive effect (or no interaction), and antagonism, respectively (Chou & Martin, 2005).

2.6 | Real-time accumulation assay by Roche LightCycler real-time thermocycler

The activity of compounds on the real-time accumulation of EB was assessed by the automated EB method (Viveiros et al., 2008) using a LightCycler real-time thermocycler (LightCycler 1.5, Roche, Indianapolis, IN, USA). Briefly, an aliquot of an overnight culture of the *S. aureus* strain in TSB medium was transferred to fresh TSB medium, and it was incubated until it reached an optical density (OD) of 0.6 at 600 nm. In case of *E. coli*, the medium used in the assay was LB broth; the preparation of the inoculum was similar to the one of *S. aureus*. The cells were washed with phosphate-buffered saline (PBS; pH 7.4) and centrifuged at 13,000 × *g* for 3 min, the pellets were resuspended in PBS (pH 7.4), and the OD was adjusted to 0.6 at 600 nm. The compounds were added individually at different concentrations at MIC/2, MIC/3, MIC/4, or MIC/5 (in double concentrated form) to the EB solution in PBS. The final concentration of EB was based on the MIC and the fluorescent signal produced by this amount of EB. In case of *S. aureus* strains, the concentration of EB was 0.5 µg/ml, for *E. coli* AG100 1 µg/ml, and in case of *E. coli* AG100 A, it was 0.25 µg/ml. Then, 10 µl of the EB solution containing the compound was transferred into standard glass capillary tubes of 20 µl maximum volume (Roche), and 10 µl of bacterial suspension (OD of 0.6 at 600 nm) was added to the capillaries. The capillaries containing the samples were placed into the carousel (Roche), and the fluorescence was monitored at the FL-2 channel in every minute on a real-time basis.

From the real-time data, the activity of the compound, namely, the relative final fluorescence index (RFI) of the last time point (minute 30) of the EB accumulation assay, was calculated according to the following formula:

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

where RF_{treated} is the relative fluorescence at the last time point of EB retention curve in the presence of an inhibitor and $RF_{\text{untreated}}$ is the relative fluorescence at the last time point of the EB retention curve of the untreated control having the solvent control (DMSO). Verapamil was applied as positive control on Gram-positive strains, and PMZ was used on Gram-negative strains.

2.7 | Assay for quorum sensing inhibition

LB* was used for these experiments. The sensor strain *C. violaceum* 026 and the AHL producer strains *E. cloaceae* 31298 were inoculated as parallel lines and incubated at room temperature (20 °C) for 24–48 hr. QS inhibition was monitored by the agar diffusion method. Filter paper discs (7.0 mm in diameter) were impregnated with 10 µl of stock solutions (10 mM) of the compounds in DMSO. The discs were placed between the parallel lines of the sensor and the AHL producer strains on the surface of the nutrient agar. The plates were incubated at room temperature for another 24–48 hr, and the interactions between the strains and compounds were evaluated for the

reduction in the size of the zone of pigment production and the zone of growth inhibition of the affected strains, in millimeters. PMZ was applied as positive control (Varga et al., 2011).

2.8 | Expression analyses of genes by real-time reverse transcriptase quantitative polymerase chain reaction

S. aureus ATCC 25923 and *S. aureus* 272123 strains were cultured in TSB broth and were incubated overnight at 37 °C with shaking. On the day of RNA isolation, the bacterial suspensions (OD of 0.6 at 600 nm) were transferred to 10-ml tubes in 3-ml aliquots, and 5 μM of compound **2** or 0.5 μM of compound **4** was added to the tubes, which were incubated at 37 °C. After 4 hr of culturing, the tubes were centrifuged at 12,000 × *g* for 2 min. Pellets were suspended in 100 μl Tris–EDTA buffer containing 1 mg/ml lysozyme by vigorous vortexing, and they were incubated at 37 °C for 10 min. The total RNA was isolated in an RNase-free environment using the NucleoSpin RNA kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. Purified RNA was stored in RNase-free water in nuclease-free collection tubes and was maintained at −20 °C until quantification was performed. The concentration of the extracted RNA templates was assessed by spectrophotometry at 260 nm. Expression of the EP genes *norA* and *mepA* was studied by reverse transcription of the total RNA. The data obtained for gene targets were normalized against the *S. aureus* 16S ribosomal RNA measured in the same sample. The primers (Couto, Costa, Viveiros, Martins, & Amaral, 2008) used in the assay were the following:

1. Sequence (5′-3′) of *norA* (246 bp)	TCGTCTTAGCGTTCGG TTTA (Fw)
	TCCAGTAACCATCGGC AATA (Rv)
2. Sequence (5′-3′) of *mepA* (198 bp)	TGCTGCTGCTCTGTTC TTTA (Fw)
	GCGAAGTTTCCATAAT GTGC (Rv)
3. Sequence (5′-3′) of *16S rRNS* (492 bp)	AGAGTTTGATCMTGGC TCAG (Fw)
	GWATTACCGCG GCKGCTG (Rv)

Real-time quantification of the RNA templates by real-time one-step reverse transcriptase quantitative polymerase chain reaction was performed in a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA), and the manufacturer's recommendations of the SensiFAST™ SYBR No-ROX One-Step Kit (Bioline GmbH, Luckenwalde, Germany) were strictly adhered to. Briefly, each well of the 96-well microtiter plates in a final volume of 20 μl contained 10 μl of the 2× SensiFAST™ SYBR No-ROX One-Step Mix, 0.2 μl reverse transcriptase, 0.4 μl RiboSafe RNase Inhibitor, 5.4 μl diethyl pyrocarbonate-treated water, 500 nM of each primer, and approximately 20 ng of the total RNA in RNAase-free water. Thermal cycling was initiated with a denaturation step of 5 min at 95 °C, followed by 40 cycles, each of 10 s at 95 °C, 30 s at 57 °C, and 20 s at 72 °C.

3 | RESULTS

3.1 | In vitro antibacterial activity of compounds

Compounds (**1**–**5**) were assessed for their antibacterial activity against methicillin-susceptible *S. aureus* ATCC 25923, and the methicillin- and ofloxacin-resistant *S. aureus* 272123 clinical isolate. Wild-type *E. coli* K-12 AG100 strain, and *E. coli* AG100 A strain, overexpressing and lacking the AcrAB–TolC EP system, respectively, were used as Gram-negative models. In addition, the antibacterial activity of the compounds was tested on QS strains *C. violaceum* and *E. cloaceae*.

Concerning the antibacterial effect of the compounds, chamanetin (**2**) and dichamanetin (**4**) had a potent antibacterial effect on the *S. aureus* strains. MIC value of compound **2** was 12.5 μM on reference *S. aureus*; however, the MIC of the methicillin- and ofloxacin-resistant strain was 25 μM. Compound **4** was the most effective flavanone because its MIC value on *S. aureus* ATCC 25923 was 0.8 μM; furthermore, on the methicillin-resistant strain, it was 1.56 μM.

The compounds had no antibacterial effect on the Gram-negative *E. coli* AG100, AG100 A, *C. violaceum*, and *E. cloaceae* strains.

3.2 | Combination effects of chamanetin (2) and dichamanetin (4) with antibiotics

The type of interaction between the antibacterial *C*-benzylated flavanones **2** and **4** and tetracycline and the fluoroquinolone antibiotic ciprofloxacin was evaluated on methicillin-susceptible (ATCC 25923) and MRSA strains by the checkerboard assay. The results are presented in Tables 1a and 1b as CI values. CI values <1 indicate a synergistic interaction between the compound and the antibiotic (Chou & Martin, 2005). As it can be observed, the combined effect of TET and compound **2** or **4** on *S. aureus* ATCC 25923 resulted in synergism. The most effective ratio of antibiotic and compound was 1:20

TABLE 1a Combination assays on *Staphylococcus aureus* ATCC 25923 strain

Staphylococcus aureus ATCC 25923				
Combination	**Best ratio**	**CI at ED_{90}**	**SD (+/−)**	**Interaction**
Tetracycline + chamanetin (**2**)	1:20	0.63786	0.13419	Synergism
Tetracycline + dichamanetin (**4**)	1:1	0.42093	0.10354	Synergism
Ciprofloxacin + chamanetin (**2**)	1.3:12.5	0.81577	0.23974	Slight synergism
Ciprofloxacin + dichamanetin (**4**)	1.3:1	0.68615	0.27953	Synergism

Note. Starting concentration of tetracycline: 5.2 μM; ciprofloxacin: 2.6 μM; and compound **2**: 25 μM and compound **4**: 2 μM. ATCC = American Type Culture Collection; CI = combination index.

TABLE 1b Combination assays on MRSA 272123 strain

Staphylococcus aureus 272123				
Combination	**Best ratio**	**CI at ED_{90}**	**SD (+/−)**	**Interaction**
Tetracycline + chamanetin (**2**)	6:25	1.4595	0.29390	Antagonism
Tetracycline + dichamanetin (**4**)	100:1	0.59402	0.34521	Synergism
Ciprofloxacin + chamanetin (**2**)	13:8	0.85030	0.21648	Slight synergism
Ciprofloxacin + dichamanetin (**4**)	32:1	1.35064	0.24946	Moderate antagonism

Note. Starting concentration of tetracycline: 100 μM; ciprofloxacin: 64 μM; and compound **2**: 50 μM and compound **4**: 3 μM. Ratio: antibiotic and tested compound (μM). CI < 1, CI = 1, and CI > 1 represent synergism, an additive effect (or no interaction), and antagonism, respectively. CI = combination index; MRSA = methicillin-resistant *Staphylococcus aureus*.

and 1:1, respectively. Similarly, CIP also acted synergistically with compounds **2** and **4**, being the most active ratio of antibiotic and compound 1.3:12.5 and 1.3:1, respectively (Table 1a).

Against the MRSA strain, compound **2** showed antagonistic effect with TET and slight synergism with CIP. The interactions of compound **4** with TET and CIP on the MRSA strain were synergism and moderate antagonism, respectively (Table 1b).

3.3 | Efflux pump-inhibiting activity

The EB accumulation assay provides information about the intracellular accumulation of the general EP substrate EB. A potential EPI increases the fluorescence level of EB because of its accumulation within the bacterial cell. The EP-inhibiting activity of the compounds was compared on the basis of the RFI of the real-time accumulation curves in Gram-positive and Gram-negative strains (Figure 2a). In case of real-time EB accumulation by the LightCycler thermocycler, the amount of EB accumulated by cells is higher if the difference between $RF_{treated}$ and $RF_{untreated}$ is greater; therefore, the degree of inhibition of the EP system by the compound becomes greater.

As shown in Figure 2a, compounds **2**, **3**, and **5** had EP-inhibiting activity compared with verapamil (RFI: 0.29) on the *S. aureus* ATCC 25923 strain, and the most active compound was compound **2**. However, compounds **1–5** had no EPI activity on the MRSA strain at the concentrations applied in the assay.

Concerning the inhibitory activity on Gram-negatives, triterpene polycarpol (**1**) and acetylmelodorinol (**5**) compared with the PMZ (RFI: 0.15) could inhibit the AcrAB–TolC system of *E. coli* AG100. Compound **1** proved to be the most effective EPI (Figure 2a). On the basis of the real-time accumulation data, compounds **1–5** had no effect on the *E. coli* AG100 A strain lacking the AcrAB–TolC pump.

3.4 | Anti-quorum sensing activity

The QS inhibition activity of compounds was defined measuring the colorless zone around the disc on *C. violaceum* as described previously (Varga et al., 2011). Compounds **1**, **2**, **4**, and **5** were able to inhibit effectively the QS between CV026 and *E. cloaceae* compared with the positive control PMZ (Table 2).

3.5 | Relative expression of efflux pump genes

In order to evaluate the effect of compounds on the relative expression of EP genes in both *S. aureus* strains, the most effective compounds **2** and **4** were selected for gene expression studies. In the real-time quantitative RT-PCR assay, the genes of NorA and MepA transporters were investigated. As shown in Figure 2bA, compound **2** at 5 μM significantly up-regulated the expression of *norA* and *mepA* genes after 4 hr of exposure in the MRSA strain. Compound **4** at 0.5 μM also significantly up-regulated both EP genes after 4 hr of exposure in the MRSA as presented in Figure 2bB. In the *S. aureus* ATCC strain, the expression level of the *mepA* gene was not influenced; nevertheless, the *norA* gene was significantly up-regulated by compounds **2** (19.84-fold increase) at 5 μM and **4** (2.39-fold increase) at 0.5 μM (data not shown).

4 | DISCUSSION

Natural compounds isolated from plants represent a valuable source of new antibacterial agents. When analyzing the results, the potent antibacterial activity found for dichamanetin (**4**) and also the significant activity of chamanetin (**2**) corroborated our previous studies on this set of rare *C*-benzylated flavanones (Pereira et al., 2016). The strongest activity of dichamanetin (**4**), when compared with chamanetin (**2**), seems to be due to its higher lipophilic character resulting from the extra benzyl group at C-6. In contrast to chamanetin (**2**), isochamanetin (**3**), differing only in the position of the benzyl group, was inactive at the concentration tested. Thus, besides the importance of lipophilicity, the presence of a benzyl moiety at C-8 appears to play a decisive role in the antibacterial activity of this type of compounds.

In the combination assays, chamanetin (**2**) and dichamanetin (**4**) had synergistic activity with tetracycline and ciprofloxacin on the *S. aureus* ATCC 25923 strain. Furthermore, on the MRSA strain, dichamanetin (**4**) and chamanetin (**2**), combined with tetracycline and ciprofloxacin, respectively, also showed synergism, which indicates that these compounds could be potential adjuvants in the therapy.

Besides having an antibacterial effect, both compounds (**2** and **4**) could inhibit the activity of EPs, they were QS inhibitors, and they significantly increased the expression of EP genes *norA* and *mepA* after 4 hr of exposure in the MRSA strain. Furthermore, the NorA and MepA pumps of the MRSA strain could not be inhibited because of the overexpression of these pumps. The change in gene expression could be due to the stress response against compounds **2** and **4** because these compounds as potential noxious agents for *S. aureus* had to be extruded from the cytoplasm as soon as possible. This stress response can be the explanation for the up-regulation of the EP genes after 4 hr. The difference between MRSA and ATCC is due to the overexpression

(a)

Compound	*E. coli* AG100			*S. aureus* ATCC 25923		
	MIC (μM)	Concentration (μM)	RFI	MIC (μM)	Concentration (μM)	RFI
1	>100	50	0.484	>100	25	-0.035
2	>100	50	-0.414	12.5	5	-0.267
3	>100	50	-0.032	>100	25	0.174
4	>100	50	-0.643	0.8	0.5	-0.151
5	>100	50	0.161	>100	25	0.139

(b)

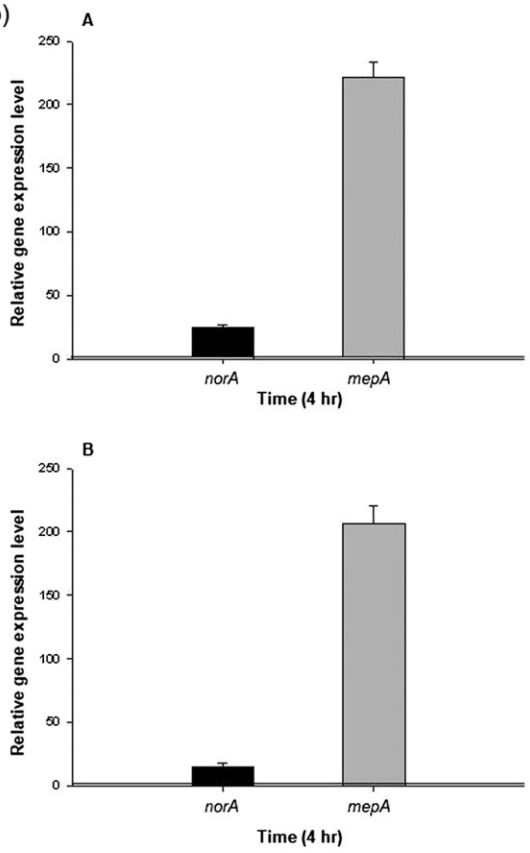

FIGURE 2 The minimum inhibitory concentration (MIC) and efflux pump modulating effect of compounds **1–5**. (a) Relative final fluorescence index (RFI) for the effect of compounds **1–5** on the AcrAB–TolC-expressing *Escherichia coli* AG100 and *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923 strains. (b) Relative gene expression levels of *norA* and *mepA* genes in the presence of (A) chamanetin (**2**) and (B) dichamanetin (**4**) in methicillin resistant *Staphylococcus aureus* (MRSA) 272123, after 4-hr exposure. The line denotes the threshold value, which was set at a twofold increase in transcripts

TABLE 2 Inhibitory effects of compounds **1–5** on QS signal transmission

Compound	QS inhibition zone in mm
1	51
2	50
3	–
4	53
5	52
Promethazine	46

Note. Ten microliters of 10 mM stock solution was added into the filter paper discs (10 μM per disc), and the colorless zone around the disc measured on *Chromobacterium violaceum* was measured after incubation for 24–48 hr at room temperature. QS = quorum sensing.

of the *mepA* gene in the resistant strain and it has low expression level in the ATCC strain. Compounds **2** and **4** increased the expression of *mepA* gene in the MRSA, but in the ATCC strain, these compounds did not influence the expression level of *mepA*. It has been demonstrated by other studies that the overexpression of EPs confers a fitness cost for the organism, for example, a resistant isolate overexpressing EPs shows reduced production of virulence determinants. Usually the overexpression of pump genes is often related to global effects on bacterial physiology influencing virulence (Ledda et al., 2017).

In Gram-negatives, compounds **1–5** had no antibacterial activity. Polycarpol (**1**) was the most effective EPI, inhibiting the AcrAB–TolC transport system in the *E. coli* AG100 strain. The AcrAB–TolC system belongs to the resistance nodulation division transporters, utilizing the proton motive force to extrude toxic agents out of the bacterial

cell. Since compound **1** had no EPI effect on the AcrAB–TolC pump-deleted *E. coli* strain, it may have a direct EPI effect on the AcrAB–TolC transporter system. The effect of polycarpol could be due to its high lipophilicity (Pereira et al., 2016), which can increase the membrane permeability of bacteria.

In addition to flavanones **2** and **4**, polycarpol (**1**) and acetylmelodorinol (**5**) were also able to inhibit effectively the bacterial communication, suggesting that they could be applied as anti-QS agents.

5 | CONCLUSIONS

To summarize our findings, *C. kirkii* constituents, chamanetin (**2**) and dichamanetin (**4**), inhibited the growth of both *S. aureus* strains. The three flavanones tested (**2**–**4**) increased the accumulation of the EP substrate EB on sensitive *S. aureus* strain, and compounds **1**, **2**, **4**, and **5** prevented the cell-to-cell communication. Polycarpol (**1**) was an effective inhibitor of the AcrAB–TolC system of *E. coli*, which might be explained by its interference with membrane permeability and modulation of the function of the pump. Dichamanetin (**4**) and chamanetin (**2**) showed synergistic effect on MRSA and sensitive *S. aureus* strains implying that they could restore and increase the efficacy of antibiotics, especially in MDR infections. It can be assumed that the advantageous properties of *C. kirkii* constituents can influence the QS system and operation of EPs decreasing virulence and antibiotic resistance (Varga et al., 2011).

According to the results, the most active compounds, chamanetin (**2**) and dichamanetin (**4**), could be possible resistance modifiers because they possess antibacterial, EPI, and anti-QS properties; moreover, these compounds might be used as adjuvants in the antibiotic therapy because they may be able to reduce the bacterial virulence. Furthermore, they can potentiate the activity of antibiotics.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Article

Selenocompounds as Novel Antibacterial Agents and Bacterial Efflux Pump Inhibitors

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Abstract: Bacterial multidrug resistance is becoming a growing problem for public health, due to the development and spreading of bacterial strains resistant to antimicrobials. In this study, the antibacterial and multidrug resistance reversing activity of a series of seleno-carbonyl compounds has been evaluated. The effects of eleven selenocompounds on bacterial growth were evaluated in *Staphylococcus aureus*, methicillin resistant *S. aureus* (MRSA), *Enterococcus faecalis, Escherichia coli*, and *Chlamydia trachomatis* D. The combination effect of compounds with antibiotics was examined by the minimum inhibitory concentration reduction assay. Their efflux pump (EP) inhibitory properties were assessed using real-time fluorimetry. Relative expressions of EP and quorum-sensing genes were studied by quantitative PCR. Results showed that a methylketone selenoester had remarkable antibacterial activity against Gram-positive bacteria and potentiated the activity of oxacillin in MRSA. Most of the selenocompounds showed significant anti-chlamydial effects. The selenoanhydride and the diselenodiester were active inhibitors of the AcrAB-TolC system. Based on these results it can be concluded that this group of selenocompounds can be attractive potential antibacterials and EP inhibitors. The discovery of new derivatives with a significant antibacterial activity as novel selenocompounds, is of high impact in the fight against resistant pathogens.

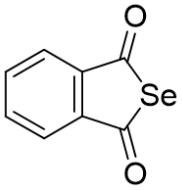
Keywords: selenocompounds; selenoesters; AcrAB-TolC efflux pump; *Chlamydia trachomatis* D; *Escherichia coli* K-12 AG100; *Staphylococcus aureus*

1. Introduction

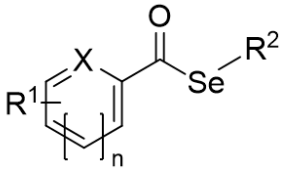
Multidrug resistance is becoming a serious problem in the treatment of resistant bacterial infections. The discovery of novel antibacterial or multidrug resistance reversing agents is extremely urgent as soon we may lack effective drugs to treat bacterial infections caused by the arising superbugs resistant to the majority of the clinically available antibiotics [1]. Selenium (Se)-containing molecules could be possible alternatives in the development of a new approach to combat infections caused by multidrug resistant (MDR) pathogens. Se is an important element in biological molecules in archea, bacteria, and eukaryotes [2]. In humans, Se is an essential trace element and also has chemopreventive effects [3].

In this context, a few studies have reported that certain selenocompounds have shown an interesting antibacterial activity. First, a series of selenides-bearing benzenesulfonamide moieties has been found to strongly inhibit the carbonic anhydrases VchCAα and VchCAβ of *Vibrio cholerae*, thus, exerting an inhibition on the growth and pathogenicity of this bacterium [4]. In addition, a degraded selenide polysaccharide, extracted from *Enteromorpha prolifera*, has been found to show antibacterial activity against *Escherichia coli* [5]. Additionally, a series of fused selenazolinium salts have been shown to have a potent activity against **ESKAPE** pathogens, which are: vancomycin-resistant **E**nterococci, methicillin-resistant ***S**taphylococcus aureus* (MRSA), ***K**lebsiella pneumoniae, **A**cinetobacter baumannii, **P**seudomonas aeruginosa*, and carbapenem-resistant ***E**nterobacteriaceae*. The majority of these compounds have minimum inhibitory concentration (MIC) values below 1 μg/mL, in resistant bacterial strains of MRSA, *K. pneumoniae*, *A. baumanii*, and *P. aeruginosa* [6]. This last work highlights the potential applications of selenocompounds, in the treatment of infections caused by the MDR bacterial strains. Our previous studies have demonstrated that the selenoanhydride **1** and selected selenoesters **2–11** (Table 1) have shown potent anticancer activity against ATP-Binding cassette sub-family B member 1 (ABCB1)-overexpressing MDR mouse T-lymphoma cells and MDR colon adenocarcinoma cells [7,8]. The ABC family of protein transporters also plays an important role in bacterial multidrug resistance [9]. Several members of the ABC family, e.g., MsrA in staphylococci [10,11] or Msr(D) in *Streptococcus pneumoniae* [12], significantly contribute to the efflux of antibiotics, and are considered as attractive protein targets in experimental chemotherapy. A major factor of bacterial and cancer drug resistance is assigned to the MDR efflux transporter proteins, expelling toxic compounds and drugs out of the cells. Based on the energy source of these pumps, the primary transporter derives their energy from the hydrolysis of ATP (ABC-transporters) and secondary transporters use proton or ion gradients to drive the extrusion of toxic compounds. Using selenocompounds, such as chemosensitizers, these compounds have been shown to inhibit the ABCB1 in cancer cells [7,8], and based on these results, our aim was to investigate the efflux pump inhibitory properties of these selenocompounds on the representative bacterial efflux system AcrAB-TolC. RND (Resistance–Nodulation–Division) family transporters are widespread, especially among Gram-negative bacteria, and catalyse the efflux of antibiotics and biocides. This tripartite efflux system consists of an outer membrane channel and periplasmic adaptor proteins, and the inner membrane transporter AcrB [13]. The MarR transcription factor regulates resistance to diverse antibiotics, organic solvents and oxidative stress agents by controlling the expression of efflux pumps (including AcrAB-TolC) through the repression of the operon that encodes the transcriptional activator MarA. The antibiotic resistance arises when the MarR protein is inactivated or the expression of *marR* genes is inhibited [14]. Although the expression of AcrAB-TolC efflux pump is regulated at several levels, the MarR the AcrR also regulates it negatively, meanwhile, the MarA, SoxS, and Rob are activators of this efflux pump [15]. In addition, the quorum sensing (QS) regulators, such as SdiA could also affect the expression of AcrAB-TolC efflux pump in *E. coli*, since AcrAB-TolC has been proposed to pump out QS signals [16].

Table 1. Selenocompounds evaluated as antibacterial and as multidrug resistance reversing agents—selenoanhydride (**1**) and selenoesters (**2**–**11**).



I. Cyclic selenoanhydride (**1**)



II. Selenoesters (**2**–**11**)

Compound	Group	R^1	X	n	R^2
1	**I**	-	-	-	-
2	**II**	5-$COSeCH_3$	S	0	-CH_3
3	**II**	6-$COSeCH_3$	N	1	-CH_3
4	**II**	3-$COSeCH_3$	C	1	-CH_3
5	**II**	4-$COSeCH_3$	C	1	-CH_3
6	**II**	-H	C	1	-CH_2CONH_2
7	**II**	4–Cl	C	1	-CH_2COOCH_3
8	**II**	-H	C	1	-CH_2COOPh
9	**II**	4–Cl	C	1	-CH_2COCH_3
10	**II**	4–Cl	C	1	-$CH_2COC(CH_3)_3$
11	**II**	3,5-diOCH_3	C	1	-$CH_2COC(CH_3)_3$

Furthermore, coating surfaces with Se could reduce the bacterial attachment to prosthetic devices [17], whereas sodium selenite exhibited ulcer healing and antibacterial activity against *Helicobacter pylori* [18]. Various studies have highlighted the antimicrobial properties of elemental Se, in the form of nanoparticles (SeNP) against *S. aureus* [19–22], *Staphylococcus epidermidis*, *K. pneumoniae*, *Bacillus subtilis* [23], *P. aeruginosa*, *E. coli*, and *A. baumannii* [24]. Additionally, biogenic SeNPs, synthesized by different non-pathogenic bacterial strains and stabilized with bacterial proteins, have shown activity against pathogenic bacteria [25,26].

In addition, there is an emerging evidence that *Chlamydia trachomatis* is developing resistance to antibiotics, as certain clinical isolates have shown single- or multidrug resistance [27,28]. Consequently, the development of new antibacterials and multidrug resistance reversing compounds is required to overcome this emerging problem. Although there are numerous studies that have investigated the antibacterial activity of Se-containing (in)organic compounds and SeNPs, according to our knowledge, no report has been described regarding anti-chlamydial activity of selenocompounds. Furthermore, the selenocompounds found as anticancer agents and cancer efflux pump inhibitors have not yet been tested on any bacterial strains.

Herein, we report the antibacterial effects of selenocompounds **1**–**11** on Gram-negative and Gram-positive bacteria, such as *E. coli*, *C. trachomatis* D, *Enterococcus faecalis*, and *S. aureus* (including methicillin resistant strain, MRSA).

2. Results

2.1. Antibacterial Activity: Determination of the MIC

The ketone-containing selenoesters **9**–**11** showed a potent antibacterial activity against the Gram-positive *S. aureus* ATCC 25923 and MRSA HEMSA 5. The methylketone selenoester **9** was the most active agent with noteworthy MIC values in the low micromolar range (3.12 and 3.91 μM). The *tert*-butylketone selenoesters **10** and **11** showed lower antibacterial activity than methylketone selenoester **9**, but was still significant (25 and 50 μM). The selenoanhydride **1** and the remaining selenoesters **2**–**8** evaluated were inactive as their MIC were equal or above 100 μM. The selenoester **9** showed also significant antibacterial activity towards *E. faecalis*, but this Gram-positive strain was less

sensitive to **9** than *S. aureus* and MRSA (MIC = 12.5 μM), and also was not sensitive to the rest of the selenocompounds tested (MIC > 100 μM), (Table 2).

In contrast, none of the eleven Se derivatives demonstrated antibacterial effects against the two Gram-negative strains evaluated in this study, which are the AcrAB-TolC-expressing *E. coli* AG100 and the AcrAB-TolC-deleted mutant *E. coli* AG100A. In these two strains, all compounds showed MIC values above 100 μM (data not shown).

Table 2. Minimum inhibitory concentration (MICs) of the selenocompounds on the Gram-positive bacteria. In bold—MIC values <10 μM.

Compounds	MIC (μM)		
	Staphylococcus aureus **ATCC 25923**	***Staphylococcus aureus*** **HEMSA 5**	***Enterococcus Faecalis*** **ATCC 29212**
1	>100	>125	>100
2	100	>125	>100
3	100	>125	>100
4	100	>125	>100
5	>100	>125	>100
6	>100	>125	>100
7	100	125	>100
8	100	>125	>100
9	**3.12**	**3.91**	12.5
10	25	>125	>100
11	50	>125	>100

2.2. Enhancement of the Activity of Antibiotics

In order to determine if selenocompounds **1**–**11** enhance the activity of antibiotics, they were tested in combination with antibiotics commonly used in clinical therapy, which are substrates of the AcrB pump—tetracycline [29] and ciprofloxacin [30]. The combined effects of selenocompounds and these antibiotics were tested on the AcrAB-TolC expressing Gram-negative *E. coli* AG100 strain. In addition, the chemosensitizing effects of selenocompounds on the Gram-positive MRSA HEMSA 5 strain were studied in combination with oxacillin. These antibiotics have been selected among the ones that are more widely used in clinical practice, in an attempt to cover different mechanisms of action, to see which ones are more affected by the selenocompounds. Gram-negative efflux pumps of the RND superfamily in Gram-negative bacteria are crucial to the cellular defence mechanisms, but the overexpression of these pumps can lead to multidrug resistance, which is an alarming problem for health care. The AcrAB-TolC system containing the RND type pump AcrB has been studied extensively, due to its importance in bacterial resistance. As an in vitro model system we used the AcrAB-TolC overexpressing *E. coli* AG100 strain and its pump-deleted mutant strain *E. coli* AG100A, in order to find effective efflux pump inhibitor (EPI) compounds. EPIs as chemosenzitizers could reverse the resistant phenotype, and in combination with antibiotics, they could enhance the activity of these conventional antibiotics. Moreover, the Gram-positive methicillin resistant *Staphylococcus aureus* (MRSA) is a major concern in healthcare facilities, for this reason our aim was to test the selenocompounds on reference ATCC and resistant MRSA strains. The enhancement of the activity of oxacillin was studied as the ability of compounds **1**–**11**, to reduce MIC of oxacillin against MRSA, whereas the enhancement of tetracycline or ciprofloxacin was studied analogously for AcrAB-TolC-expressing *E. coli* strain (Table 3).

In the absence of the selenocompounds, oxacillin showed MIC value of 374 μM (150 μg/mL) against MRSA, since this strain was highly resistant to this β-lactam antibiotic. The methylketone selenoester **9**, at a low concentration of 1.95 μM (0.537 μg/mL), exerted a noteworthy 64-fold reduction of the MIC value of oxacillin to 5.84 μM (2.34 μg/mL). Hence, this compound can be useful as a potent agent to reverse the resistance of MRSA to oxacillin. Interestingly, the rest of the compounds **1**–**8**, **10**, and **11** were not active, even at the concentration of 62.5 μM (MIC reduction of oxacillin ≤ 2).

However, none of the tested selenocompounds were able to improve the efficacy of antibiotics against the AcrAB-TolC-overexpressing strain of *E. coli* with the above-mentioned excellent 64-fold factor. The highest reductions observed in *E. coli* were a 2-fold reduction of the MIC values of tetracycline (compound **9**) and of ciprofloxacin (compounds **9, 10**).

Table 3. Numerical value of the reduction of the MICs of selected antibiotics in methicillin resistant *S. aureus* (MRSA) or in *E. coli* AG100 exerted by selenocompounds when administered in combination with antibiotics.

Cpd[1]	MRSA HEMSA 5		*Escherichia coli* AG100		
	Concentration of Compound [μM][2]	Reduction of Oxacillin MIC	Concentration of Compound [μM]	Reduction of Tetracycline mic	Reduction of Ciprofloxacin mic
1	62.5	no effect	50	no effect	no effect
2	ND	ND [3]	50	no effect	no effect
3	62.5	2-fold	50	no effect	no effect
4	62.5	no effect	50	no effect	no effect
5	62.5	no effect	50	no effect	no effect
6	62.5	no effect	50	no effect	no effect
7	62.5	≥ 2-fold	50	no effect	no effect
8	62.5	2-fold	50	no effect	no effect
9	1.95	64-fold	25	2-fold	2-fold
10	62.5	no effect	50	no effect	2-fold
11	62.5	no effect	50	no effect	no effect

[1] Cpd: Compound. [2] Starting concentration of tetracycline: 8.4 μM; ciprofloxacin: 1.4 μM; and oxacillin: 747 μM. [3] ND: Not determined.

2.3. Anti-Chlamydial Activity

Before the assessment of the anti-chlamydial activity of the selenocompounds, a cytotoxicity assay was performed on HeLa cells to determine the ranges of concentrations at which the selenocompounds can be evaluated without showing direct toxic effects to HeLa cells. Selenocompounds **2**, **3**, **5**, **7**, and **9**–**11**, significantly inhibited the formation of chlamydial inclusions at selected concentrations (Figure 1).

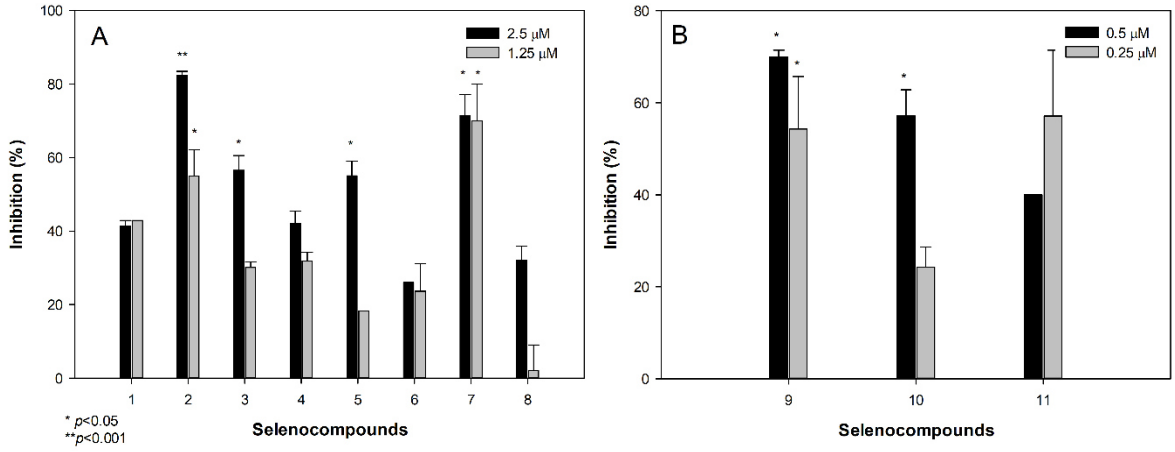


Figure 1. Anti-chlamydial effect of selenocompounds at 1.25 and 2.5 μM (**A**), and at 0.25 and 0.5 μM (**B**).

Compounds **2** and **7** at 2.5 μM, showed 82% and 71% inhibition, compared to the control, respectively. In addition, **2** and **7** were effective at 1.25 μM, whereas **9** and **10** inhibited the formation of inclusions at low submicromolar concentrations of 0.5 μM. The most potent anti-chlamydial selenocompounds were **9** and **11**, as they inhibited more than 50% of the growth of *C. trachomatis* D, at a concentration of 0.25 μM (0.0689 and 0.0858 μg/mL, respectively).

2.4. Real-Time Accumulation Assay

Since ethidium bromide (EB) is a substrate of the AcrB efflux pump, the intracellular accumulation of EB provides information about the inhibition of the AcrAB-TolC system, in the presence of selenocompounds, in a time-dependent manner. The assay records the real-time accumulation of EB, using a real-time thermocycler, by monitoring the fluorescence of EB inside the cells [31]. The activities of compounds **1**–**11** in the real-time EB accumulation assay, were given in terms of the relative fluorescence index (RFI) of the real-time accumulation curves (Table 4). In case of the real-time EB accumulation, the amount of EB accumulated by cells was higher if the difference between $RF_{treated}$ and $RF_{untreated}$ was greater, therefore, the degree of inhibition of the efflux pump system by the compound became greater. Compounds **9** and **10** possessed EPI activity and decreased the MIC of ciprofloxacin on *E. coli* AG100. However, the selenoanhydride **1** and the selenoester **4**, compared with the positive control promethazine (PMZ, RFI: 0.15), strongly inhibited the efflux of AcrAB-TolC in *E. coli* AG100; they had no effect in combination with the antibiotics, suggesting that other cellular mechanisms might also be involved in the mode of action, such as interaction with cell wall components, formation of reactive oxygen species (ROS), or membrane destabilizing effects. Without investigating the possible metabolites of the selenocompounds, no further conclusions can be drawn, for this reason we are planning to study the metabolites of these compounds in future works. Derivatives **7** and **9**–**11** caused moderate inhibitory action, whereas **2**, **3**, **5**, **6** and **8** showed weak or no activity on the intracellular EB accumulation in *E. coli* AG100. Among derivatives **7** and **9**–**11**, compound **7**, which contained a methyl oxoester in the alkyl moiety bound to Se, was the most active agent (RFI = 0.13).

Table 4. Relative fluorescence index (RFI) for the effect of selenocompounds and positive control promethazine (PMZ) on the AcrAB-TolC-expressing *Escherichia coli* AG100 strain.

Compound	RFI[a]	Compound	RFI[a]	Compound	RFI[a]
	***Escherichia coli* AG100**		***Escherichia coli* AG100**		***Escherichia coli* AG100**
1	0.28	**5**	0.04	**9**	0.11
2	0.03	**6**	0.06	**10**	0.12
3	0.04	**7**	0.13	**11**	0.11
4	0.18	**8**	0.08	**PMZ**	0.15

Nevertheless, no efflux pump inhibitory action of selenocompounds (**1**–**11**) was found in the *E. coli* AG100A strain.

2.5. Gene Expression Analysis by Quantitative PCR

For the effect of the selenocompounds on the relative expression of the efflux pump, antibiotic resistance and QS genes in *E. coli* AG100—the most effective compounds in the EB real-time accumulation assay—were examined (compounds **1**, **4**, **7**; Figure 2). In this assay, the genes of the multidrug efflux pump (AcrAB), the component of the *E. coli mar* locus (multiple antibiotic resistance), and the gene of SdiA were investigated. The changes in gene expression from reverse transcription quantitative PCR experiments were normalized to the expression of *gapdh* (internal control), in the same sample, and compared to the expression of the examined genes obtained from the untreated, control samples.

As shown in Figure 2A, compound **1** at 50 μM concentration significantly up-regulated the *acrB*, *marR*, and *sdiA* genes, after 4 h of exposure. However, after 18 h, the expression of the *acrB* gene returned to the basal levels and the expression of the *marR* and *sdiA* genes, significantly increased. Compound **4** up-regulated the expression levels of *acrB*, *marR*, and *sdiA*, after 4 h, although after 18 h, the expression levels of the *acrB* and *marR* genes decreased. The QS gene *sdiA* was significantly up-regulated after 18 h (Figure 2B).

Compound **7** also significantly up-regulated *marR*, after exposures of 4 h and of 18 h. After 18 h, the expression level of the RND transporters subunit genes (*acrA*, *acrB*) was significantly increased (Figure 2C).

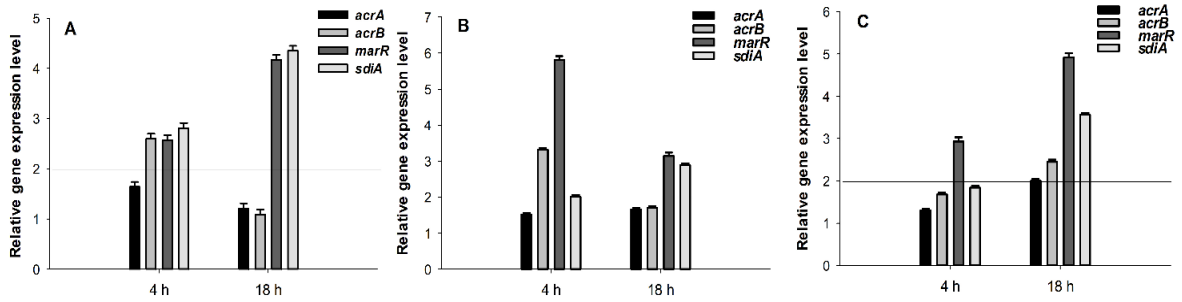


Figure 2. Relative gene expression levels of the genes of the *acrA*, *acrB*, *marR*, and *sdiA* in the presence of compounds **1** (**A**), **4** (**B**), and **7** (**C**), after 4 and 18 h exposure. The line denotes a threshold value, which was set at a two-fold increase in transcripts.

3. Discussion

Results of these studies indicate that selenoesters and selenoanhydrides, previously found as active anticancer or ABCB1 efflux pump inhibitors in cancer cells [7,8,32–35], also displayed a promising antimicrobial potential against the MDR bacterial strains.

3.1. Antibacterial Activity

The evaluation of the compounds proved that the ketone-containing selenoesters **9**–**11** showed an antibacterial activity against the Gram-positive reference *S. aureus* strain, whereas, the methylketone selenoester **9** was also active against the MRSA HEMSA 5 and *E. faecalis*. However, none of the compounds were active against the Gram-negative *E. coli* ag100. The background of the different antibacterial activity of **9** against the tested Gram-positives and Gram-negatives was unknown; further experiments are required to clarify whether the mechanism of action of the methylketone selenoester could be related with any kind of interaction between this compound and the bacterial cell wall that is typical for gram-positive bacteria. In contrast, the remaining alkyl groups (–CH_3) or alkyl-functionalized moieties (–CH_2CONH_2, –CH_2COOCH_3 and –CH_2COOPh, Table 1) bound to the Se atom rendered selenoesters that were ineffective against the tested strains. Interestingly, the compounds **9**–**11** were also the most potent anticancer agents in previous works [7,8,33], and they also showed a good selectivity towards cancer cells, with respect to non-tumour cell lines, as they showed selectivity indexes ranging from 8.4 to 14.4 [8].

In previous works, it was hypothesized that the possible mechanism of action of these compounds could be the hydrolysis of the compound and the subsequent liberation of the ionic species of Se, which could be responsible for the activity of the compounds [33]. In this case, this phenomenon enables us to hypothesize that the CH_3COCH_2SeH, or its anionic form, are the chemical forms of Se that could be behind the observed activities. The lack of activity of the non-ketone selenoesters, directs a special attention to this –$SeCH_2COCH_3$ ketone-containing moiety.

3.2. Enhancement of the Activity of Antibiotics

The activity of **9** on the MDR clinical isolate (MRSA) was very promising, because compound **9** reduced the MIC of oxacillin in 64-fold (from 374 μM to 5.84 μM). These results supported the potential applications of the methylketone selenoesters, such as antimicrobials, and the multidrug resistance reversing agents. These results were in accordance with the activity shown by these compounds as enhancers of the anticancer activity of chemotherapy drugs [35], suggesting that these selenium derivatives have the ability to effectively interact with the resistance mechanisms developed by the resistant bacterial strains and by the resistant cancer cells. This work intends to carry out a screening of the potential applications of the selenocompounds, and in future works we will attempt to ascertain the possible mechanisms of actions of the activities described herein, as this observed potential enhancement of the activity of oxacillin exerted by compound **9** in an MRSA clinical isolate.

3.3. Anti-Chlamydial Activity

Previous studies have reported that selected selenocompounds, such as certain selenocyanates, selenoureas, and diselenides, showed antiproliferative activities against the intracellular forms of *Leishmania spp.* [36,37]. Taking those results into account, this study provided a new line of evidence for the action of selenoanhydride/selenoesters on an obligate intracellular chlamydial strain. In particular, different selenoesters, such as **2**, **3**, **5**, **7**, and **9**–**11**, have exerted a noteworthy activity against *C. trachomatis* D. Furthermore, the activities of the methyl (**9**) and the *tert*-butyl (**11**) derivatives were very promising, as they inhibited the formation of more than 50% of the chlamydial inclusions, at a very low concentration (0.25 μM). However, their mode of action has not been ascertained in this study.

Regarding the observed structure activity relationships of the anti-chlamydial assays, the ketone selenoesters **9**–**11** showed noteworthy activity at lower concentrations (0.25 μM, 0.5 μM), compared to the rest of the series (1.25 μM, 2.5 μM). Among the remaining selenoesters, the symmetric dimethyl selenodiester, which contains a thiophene ring **2**, and the methyl oxoester derivative **7** showed a better activity, and the activities of the symmetric dimethyl selenodiesters **3** and **5** were also remarkable. These fact highlights the importance of the symmetry for the activity against intracellular pathogens [36].

3.4. Interaction of the Compounds with Bacterial Efflux Pumps

The resistance to the current antibacterial drugs is one of the major therapeutic challenges in the treatment of bacterial infections, and knowing the potential of these derivatives as multidrug resistance reversing agents (proved both by the capacity to enhance the activity of antibiotics described above and by the enhancement of anticancer drugs reported in previous works), we have studied here the procedure through which selenocompounds interact with the bacterial AcrAB-TolC system in the *E. coli* AG100 strain.

The results obtained revealed that the cyclic selenoanhydride **1** significantly inhibited this bacterial AcrAB-TolC efflux pump in the *E. coli* AG100 strain. Similarly, EP inhibiting activity has been found for compounds **4** and **7**. The second most potent inhibitor was the symmetrical benzene derivative 1,3-disubstituted with methylselenoester moieties (**4**). Interestingly, its 1,4-disubstituted analogue (**5**) showed an EP-inhibitory activity, 4.5-fold lower, suggesting the importance of the substituents' topology for the expected biological effect. Taking into account the distinct difference in electron density properties between *m*- and *p*-substituted phenyl rings, this factor seems to have affected the mechanisms of EP inhibition.

The well-characterized RND-type transporter, AcrB is associated with TolC and AcrA and is the major efflux pump of *E. coli* [38]. These efflux pumps recognize and extrude a large variety of antibiotics from the cytoplasm. The energy required for the operation of the efflux pump is provided by the proton motive force, created by the proton gradient resulting from electron transport [39]. This fact suggests that those selenocompounds, which possessed EP inhibitor activity, might interfere with the proton motive force. Surprisingly, compounds **1** and **4**, which inhibited the AcrAB-TolC system, influenced the expression of the gene *acrB*, which is a constituent of the AcrAB-TolC system. In addition, the compounds increased the expression of the QS gene *sdiA*, after 18 h of exposure, which suggests their roles in QS, although their QS inhibitory activities were not investigated in this study.

4. Materials and Methods

4.1. Chemistry

Eleven pure selenocompounds obtained as described earlier [33], were examined (**1**–**11**, Table 1). All compounds were stable in air and their purity was assessed by elemental analysis and ^{1}H and ^{13}C NMR, as reported in a previous work [35]. Before their use in biological assays, they were dissolved in dimethyl sulfoxide (DMSO; Merck KGaA, Darmstadt, Germany), to obtain stock solutions. Working solutions were prepared by dilutions in the culture medium.

4.2. Bacterial Strains

Wild-type *E. coli* K-12 AG100 strain [argE3 thi-1 rpsL xyl mtl Δ(gal-uvrB) supE44] and its AcrAB-TolC-deleted mutant strain *E. coli* AG100A (a kind gift from Hiroshi Nikaido, Department of Molecular and Cell Biology and Chemistry, University of California, Berkeley, USA) were used for the evaluation of the EPI activity of the tested selenocompounds.

S. aureus ATCC 25923 and *E. faecalis* ATCC 29212 strains were used to determine the MIC. A methicillin-resistant *S. aureus* strain (MRSA HEMSA 5, a clinical isolate) was used in the combination assay, with oxacillin, to determine the capacity of compounds to enhance the antibacterial effect of this antibiotic. *C. trachomatis* reference strain (serovar D, UW-3/Cx, ATCC, VR-885D) was used in the anti-chlamydial assay.

4.3. Propagation of C. trachomatis D

C. trachomatis D was propagated on the HeLa 229 cells (ATCC, CCL-2.1), as described earlier [40]. The titre of the infectious elementary bodies was determined by an indirect immunofluorescence assay. Serial dilutions of the elementary bodies' preparation were inoculated onto the HeLa monolayers and, after a 48-h culture, the cells were fixed with acetone, and stained with monoclonal anti-*Chlamydia* LPS antibody (AbD Serotec, Oxford, UK) and FITC-labelled anti-mouse IgG (Merck KGaA, Darmstadt, Germany). The inclusions of *C. trachomatis* D were enumerated under a UV microscope.

4.4. Determination of MIC

The effects exerted by different concentrations of the compounds on the bacterial growth in *S. aureus*, *E. faecalis*, and *E. coli* AG100 were tested in 96-well plates. The MICs of selenocompounds were determined, according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [41]. The DMSO exerted no antibacterial effect. Alternatively, the MIC of the oxacillin in MRSA HEMSA 5 was determined by the broth microdilution method, in a cation-adjusted Mueller–Hinton Broth (MHB II), according to the recommendations of the CLSI. Results were recorded after a 20- or 24-hour incubation at 37 °C.

4.5. Enhancement of the Activity of Antibiotics

The chemosensitizing effect of the tested selenocompounds was evaluated through the determination of the MIC values of the antibiotics, in the presence of sub-inhibitory concentrations of the compounds (MIC/2 or MIC/4), in both Gram-negative (*E. coli* AG100) and Gram-positive (MRSA) strains. The MICs were evaluated in the *E. coli* strain, by a two-fold broth microdilution method in the 96-well plates, using serial dilutions of tetracycline (from 8.4 to 0.16 μM) and ciprofloxacin (from 1.4 to 2.7×10^{-3} μM). The first four rows contained two-fold dilutions of antibiotics, and the combinations of the antibiotics and tested compounds were added into the last four rows. 10^{-4} dilution of an overnight bacterial culture in 50 μL of MHB was then added to each well, with the exception of the medium control wells. The plates were then incubated at 37 °C for 18 h. MIC values of the antibiotics and their combination with the tested compounds were determined by naked eyes. In the assay with oxacillin in the MRSA HEMSA 5 bacterial strain, a microdilution method in MHB II was used.

4.6. Anti-Chlamydial Assay

Elementary bodies of *C. trachomatis* D (4×10^3 IFU/mL) were incubated with compounds at selected concentrations (0.25, 0.5, 1.25, 2.5 μM) in sucrose-phosphate-glutamic acid buffer (SPG) for 2 h at 37 °C. As a control, *C. trachomatis* D was also incubated alone in the SPG. To quantify the anti-chlamydial effects of the compounds, HeLa cells were seeded in 24-well plates with 13-mm cover glasses. The confluent cells were infected with compound-treated *C. trachomatis* D or with the non-treated controls. After 48 h, the cells were fixed with acetone at −20 °C for 10 min. The titre of the infectious elementary bodies was determined by the indirect immunofluorescence assay, as described earlier [42].

4.7. Real-Time Accumulation Assay

The effect of the studied selenocompounds on the real-time accumulation of ethidium bromide (EB) was assessed by an automated EB method [43], using a LightCycler real-time thermocycler (LightCycler 1.5; Roche). The compounds were added individually at different concentrations at MIC/2 to the EB solution in PBS. The final concentration of EB was 1 and 0.25 μg/mL for *E. coli* AG100 and AG100A, respectively. The method for the calculation of the relative fluorescence index (RFI) of the last time point (minute 30) was described earlier by Kincses et al. [44]. Promethazine (PMZ; EGIS) was applied as a positive control.

4.8. Expression Analyses of Genes by Quantitative PCR

Total RNA was isolated from *E. coli* AG100 (OD of 0.6 at 600 nm) using the NucleoSpin RNA kit (Macherey Nagel) according to the manufacturer's instructions. The concentration of the extracted RNA templates was assessed by spectrophotometry at 260 nm.

The expression of the *acrA*, the *acrB*, the multiple antibiotic resistance protein R (*marR*), and the quorum-sensing transcriptional activator (*sdiA*) genes was studied by reverse transcription quantitative PCR (RT-qPCR), as described earlier [44]. The real-time one-step PCR was performed in a CFX96 Touch real-time PCR detection system (Bio-Rad), strictly adhering to the manufacturer recommendations of the SensiFAST™ SYBR No-ROX One-Step Kit (Bioline GmbH, Luckenwalde, Germany). The forward and reverse primers used in the experiment are shown in Table 5 [44,45]. The cycle threshold (Ct) values were determined with the Bio-Rad CFX Manager Software version 3.1. Relative quantification analysis was carried out using the Livak method [46]. The expression of *gapdh* was used as the internal control and the untreated *E. coli* AG 100 served as the external control. We have defined a threshold value—increases greater than 2-fold in the amount of transcripts relative to the control samples were considered significant.

Table 5. Primers used in the RT-qPCR.

Gene	Full Name	Primer Sequence (5′–3′)	Amplicon size (bp)	Ref.
acrA	Acridine resistance protein A	CTTAGCCCTAACAGGATGTG TTGAAATTACGCTTCAGGAT	189	[45]
acrB	Acridine resistance protein B	CGTACACAGAAAGTGCTCAA CGCTTCAACTTTGTTTTCTT	183	[45]
marR	Multiple antibiotic resistance protein R	AGCGATCTGTTCAATGAAAT TTCAGTTCAACCGGAGTAAT	170	[45]
sdiA	Quorum-sensing transcriptional activator	CTGATGGCTCTGATGCGTTTA TCTGGTGGAAATTGACCGTATT	163	[44]
gapdh	Glyceraldehyde-3-phospate dehydrogenase	ACTTACGAGCAGATCAAAGC AGTTTCACGAAGTTGTCGTT	170	[45]

5. Conclusions

Herein, we have reported the evaluation of the antibacterial and multidrug resistance reversing activity of 11 novel selenocompounds. The most active compound in the antibacterial assay, the methylketone selenoester **9**, showed potential antibacterial activity against the different strains of *S. aureus, E. faecalis*, and *C. trachomatis* D, even at very low concentrations (0.25 μM for *C. trachomatis* D). This selenocompound also enhanced the efficacy of antibiotics, namely it multiplied by 64-fold the antibacterial action of oxacillin, against the MDR clinical isolate of *S. aureus*. Alternatively, three compounds (the selenoanhydride **1** and the selenoesters **4** and **7**) inhibited the tripartite multidrug resistance efflux pump AcrAB-TolC in *E. coli*, and affected the expression of the different genes related to these resistance processes.

Based on these results, it can be concluded that this group of selenocompounds can be attractive potential EP inhibitors and antibacterial lead scaffolds, for further development of new chemical tools, to overcome bacterial multidrug resistance.

Author Contributions: E.D.-Á. and C.S. synthesized the compounds evaluated. T.M., G.S., J.H. and K.K.-K. conceived the experiments; T.M., A.K., A.C., Á.S.T., K.W., M.A.M. and G.S. performed the biological experiments; T.M., G.S. and E.D.-Á. wrote the paper; T.M., C.S., J.H., K.K.K., G.S. and E.D.-Á. revised the manuscript; T.M. and G.S. provided the funding for the biological evaluation; and E.D.-Á and M.A.M. provided the funding of the article publication.

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Sample Availability: Not available.

IV.

SZELÉNVEGYÜLETEK EFFLUX PUMPA ÉS BIOFILM GÁTLÓ HATÁSÁNAK VIZSGÁLATA SALMONELLA TYPHIMURIUM TÖRZSEKEN

Kincses Annamária
Szegedi Tudományegyetem ⋄ Általános Orvostudományi Kar
Orvosi Mikrobiológiai és Immunbiológiai Intézet

Szerzőtárs, témavezető: Spengler Gabriella

A baktériumokban az egyik legfontosabb rezisztencia mechanizmus a multidrog efflux pumpák megnövekedett expressziója, melyek jelentős szerepet játszanak a bakteriális biofilm képzésben is. Ezen kutatásunk során 11 újonnan szintetizált szelénvegyület AcrAB-TolC efflux pumpa, valamint biofilm gátló aktivitását vizsgáltuk meg Salmonella enterica serovar Typhimurium törzsek felhasználásával. Az AcrAB-TolC-t expresszáló vad típusú törzsben a leghatásosabb efflux pumpa gátlást a 9-es vegyület mutatta. A S. Typhimurium 14028s biofilm képzését pedig a 4-es és az 5-ös szelénvegyület gátolta a legnagyobb mértékben. Eredményeink alapján elmondható, hogy a szelénvegyületek adjuvánsként alkalmazva a biofilm termelő S. Typhimurium által okozott fertőzés antibiotikumos kezelése során javíthatják a terápia hatékonyságát.

A bakteriális multidrog rezisztencia (MDR) jelensége évről évre súlyos problémákat okoz világszerte. A jelenség az antibiotikumok és a mikróbák elleni szerek kiterjedt használatának következménye. A rezisztens baktériumok száma folyamatosan nő, megnehezítve ezzel a fertőző betegségek terápiájának hatékonyságát. Az MDR kialakulása többféle mechanizmushoz is köthető, melyek közül az egyik típus az efflux transzporterek megemelkedett expressziója *(Blair et al., 2015; Piddock, 2006)*. Ezek a pumpák fiziológiásan is megtalálhatók a baktériumokban, feladatuk a toxikus vegyületek eltávolítása a sejtből. A káros anyagok mellett a pumpák az antibakteriális kemoterápiás szereket is kipumpálhatják a sejtből, ez pedig a terápia sikertelenségéhez vezethet *(Blair et al., 2015)*. Az efflux pumpák további jellemzője, hogy több tanulmány szerint jelentős szerepet játszanak a baktériumok biofilm képzésében. Néhány kutató szerint az efflux pumpák közvetett módon képesek befolyásolni a biofilm képzésben szerepet játszó géneket, továbbá a bakteriális kommunikációban (quorum sensing) szerepet játszó molekulákat, melyek hozzájárulnak a biofilm képzéshez *(Alav et al., 2018)*. **A bakteriális biofilm olyan mikrobiális közösség, melyben a szesszilis baktériumok egymáshoz, illetve a felszínhez tapadnak egy önmaguk által termelt extracelluláris polimer mátrix segítségével** *(Moraes et al., 2018)*. A biofilm további jellemzője, hogy a planktonikus sejtekhez képest alkalmazkodni tudnak a környezeti feltételekhez, továbbá a bakteriális biofilmet felépítő baktériumoknak lassabb a metabolizmusuk és a növekedésük is. **A biofilmben a baktériumok megnövekedett rezisztenciát mutatnak az antimikrobiális anyagokkal szemben** *(Singh et al., 2017)*. **Emiatt az efflux mechanizmusokhoz és a biofilm képzéshez köthető rezisztencia visszafordításához fontos lenne olyan új antimikrobiális vegyületek vagy adjuvánsok fejlesztése, melyek hatékonyan gátolnák ezeket a rezisztencia mechanizmusokat.**

A szelén egy fontos nyomelem, mely jótékony hatással bír a daganatok vagy a gyulladásos megbetegedések megelőzésében. A szakirodalomban számos példát találunk a szeléntartalmú vegyületek antibakteriális hatására is: a kutatások leginkább olyan baktériumok felhasználásával történnek, melyek nozokómiális fertőzéseket okoznak. A kórházi kezelés során szerzett infekcióért gyakran az *Escherichia coli*, illetve a *Staphylococcus aureus* a felelős. Az ezen baktériumok által okozott fertőzések a biofilm képző képességük révén nehezen gyógyíthatók. Az egyik kutatócsoport által használt perihidroszelenoxantin vegyület antibakteriális hatással rendelkezett *S. aureus* törzsön *(Sonkusre–Singh, 2015)*. Egy másik kutatócsoport *E. coli*, illetve *S. aureus* biofilm képző törzseket szeléntartalmú nanorészecskékkel kezelt. 24 óra elteltével az optikai denzitás (OD) mérése után azt tapasztalták, hogy a szelénnel kezelt baktériumok növekedése csökkent a kontrollhoz képest *(Guisbiers et al., 2016)*.

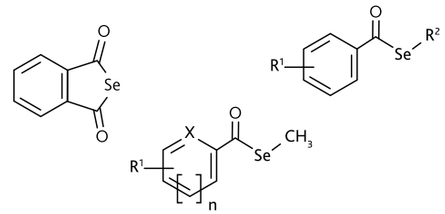
A *Salmonella enterica* Gram-negatív, élelmiszerek révén terjedő humán kórokozó. Az általa okozott kórkép a szalmonellózis. Az élelmiszergyártás során biofilm képző tulajdonsága miatt perzisztálhat, ezért a baktérium teljes elpusztítása nehéz feladat *(Moraes et al., 2018)*.

Kísérleteink célja 11 új szelénvegyület anti-biofilm, illetve efflux pumpa gátló aktivitásának meghatározása volt *Salmonella Typhimurium* törzsek felhasználásával.

ANYAGOK ÉS MÓDSZEREK

Vegyületek

Kutatásaink során 10 szelenoészter (**2-11**), illetve egy ciklikus szelenoanhidrid (**1**) vegyületet vizsgáltunk meg (1. ábra). A származékokat dimetilszulfoxidban (DMSO) oldottuk, a törzsoldatok koncentrációja 10 mM volt. A vegyületeket Dr. Enrique Domínguez-Álvarez (Consejo Superior de Investigaciones Científicas, Instituto de Química Orgánica General, Spanyolország) és volt témavezetője, Prof. Dr. Carmen Sanmartín (University of Navarra, Department of Organic and Pharmaceutical Chemistry, Spanyolország; Instituto de Investigación Sanitaria de Navarra, Spanyolország) bocsátotta a rendelkezésünkre *(Domínguez-Álvarez et al., 2016)*.



A. Ciklikus szelenoanhidrid (**1**) **B.** Heteroaril szelenoészterek (**2-3**) **C.** Aril szelenoészterek (**4-11**)

1. ábra A vizsgált szelénvegyületek szerkezete

Baktériumtörzsek

Vizsgálataink során az alábbi baktériumtörzseket használtuk fel: *Salmonella enterica* serovar Typhimurium SL1344 vad típusú törzset, mely expresszálja az AcrAB-TolC efflux pumpát, valamint ennek az acrB mutáns törzsét (L644) *(Smith-Blair, 2014)*. A vegyületek anti-biofilm hatását a biofilm képző *S. enterica* serovar Typhimurium 14028s törzsön vizsgáltuk. A törzseket Dr. Jessica M. Blair (University of Birmingham, Institute of Microbiology and Infection, Egyesült Királyság) bocsátotta a rendelkezésünkre.

A legkisebb gátló koncentráció meghatározása mikrodilúciós módszerrel

A MIC vagy legkisebb gátló koncentráció az antibiotikum azon legkisebb koncentrációja (mg/L), amely a baktériumok látható növekedését már gátolni tudja. Kísérleteink során a vegyületek antibakteriális hatását felező hígításos módszerrel, 96-lyukas mikrotitráló lemezben végeztük el. A vegyületekből kettes léptékű hígítási sort készítettünk 100 µl Müller-Hinton táplevesben.

A származékok kiindulási koncentrációja 100 µM volt. A baktériumsejteket 100 µl táplevesben adtuk a lyukakhoz 104 sejt/ml koncentrációban a médium kontroll lyukak kivételével. A lemezeket 37 °C-on, 18 órán át inkubáltuk. Az inkubációs idő végén a vizsgált vegyületek MIC értékeit szabad szemmel olvastuk le (CLSI irányelvek szerint).

Etídium-bromid efflux vizsgálat

Az etídium-bromid (EB) efflux vizsgálathoz az SL1344 *S. Typhimurium,* valamint az L644 acrB mutáns törzset Luria-Bertani (LB) táptalajban tenyésztettük 37 °C-on rázatva, amíg a tenyészet optikai denzitása (OD) elérte a 0,6-ot 600 nm-en.

A baktériumsejteket ezután 1 mM $MgCl_2$-t tartalmazó 20 mM-os kálium-foszfát oldatban mostuk, majd felszuszpendáltuk a sejteket és beállítottuk az OD-t 0,2-re. A baktériumkultúrához hozzámértük az EB-t 50 µg/ml-es koncentrációban, valamint az efflux pumpa gátló karbonil cianid m-klorofenil hidrazont (carbonyl cyanide m-chlorophenyl hydrazone, CCCP; Sigma-Aldrich) 100 µM-os végkoncentrációban.

A kultúrát tovább inkubáltuk 23 °C-on, rázatva (150 rpm), 60 percen keresztül. Az inkubációs idő letelte után a mintákat 3500 rpm sebességgel 10 percig, 21 °C-on centrifugáltuk. A felülúszót leöntöttük és a pelletet 1 mM-os $MgCl_2$-vel, valamint 5%-os glükózzal (energiaforrás) kiegészített 20 mM-os kálium-foszfát oldatban szuszpendáltuk, majd a kultúrából 200 µl-t mértünk fekete, 96-lyukas mikrotitráló lemez (Corning, Amsterdam) lyukaiba. A lemezbe előzetesen belemértük a tesztelendő anyagok 50 µM-os koncentrációját. Az EB effluxot FLUOstar Optima lemez leolvasóval (BMG Labtech) mértük 530 és 600 nm-en 2 órán keresztül.

A kísérleti eredmények három egymástól független vizsgálati eredmény átlagából származnak. A mérések során azokat az időpillanatokat határoztuk meg, amikor a fluoreszcencia szintje 25, valamint 50%-kal csökkent. Pozitív kontrollként CCCP-t alkalmaztunk, negatív kontrollként DMSO-t *(Smith–Blair, 2014).*

Biofilm gátló vizsgálat

A vegyületek biofilm gátló hatását a biofilm képző *S. Typhimurium* 14028s törzsön vizsgáltuk, 96-lyukas U-alakú mikrotitráló lemezben. A vizsgálat során a NaCl-mentes LB-ben növesztett overnight baktériumkultúrát kihígítottunk 0,1-es OD-ig (600 nm). A baktériumsejteket 200 µl NaCl-mentes LB táplevesben adtuk a lyukakhoz, a médium kontroll lyukak kivételével.

A vegyületeket ezután 50 µM-os koncentrációban adtuk a baktériumkultúrához. A lemezeket 30 °C-on rázatva (100 rpm), 48 órán át inkubáltuk. Az inkubációs idő végén a tápfolyadékot leszívtuk és a lemezeket csapvízzel mostuk. Ezután 200 µl kristályibolyát (0,1 % [v/v]) mértünk a lemezekre és szobahőmérsékleten inkubáltuk 15 percen keresztül. Az inkubációs idő letelte után a kristályibolyát leszívtuk és a lemezeket újra csapvízzel mostuk. 200 µl 70%-os etanolt mértünk a mintákhoz, majd a baktérium biofilm képzését FLUOstar Optima lemez leolvasóval határoztuk meg 600 nm-en.

A kísérleti eredmények négy egymástól független vizsgálati eredmény átlagából származnak. A szelénvegyületek jelenlétében bekövetkező biofilm tömeg csökkenését %-ban fejeztük ki a kontrollhoz viszonyítva. Az eredményeket t-teszttel analizáltuk, a statisztikai analízisnél a $p<0,05$ értéket vettük szignifikánsnak.

EREDMÉNYEK

A vizsgált anyagok nem mutattak antibakteriális hatást a *Salmonella* törzseken. Az EB efflux vizsgálat során az SL1344 vad típusú törzs esetében azt tapasztaltuk, hogy a 25%-os fluoreszcencia csökkenés mindegyik vegyületnél korábbi időpillanatban (a 4,5-17,8. perc között) történt a pozitív kontrollhoz (52,1. perc) képest. **Az eredményeink alapján elmondható, hogy az 50%-os fluoreszcencia csökkenés esetében is ugyanezt tapasztaltuk.** Az **1**-es, **2**-es, **4**-es, **7**-es, illetve a **10**-es vegyület mutatott efflux gátló aktivitást: ezen vegyületek esetében a minták 50%-os fluoreszcencia csökkenése az 55,3. és 65,6. percben következett be. A **leghatásosabb** vegyület a **9**-es volt, ahol ugyanezt a csökkenést a 79,2. percben mértük *(1. táblázat)*.

A mutáns L644 törzsnél a CCCP hatására a fluoreszcencia szint (25%) a 42,1. percben kezdett el csökkenni. Ehhez hasonló eredményt mutatott a **9**-es vegyület (37,8. perc). A leghatásosabb vegyület a **7**-es volt, melynek jelenlétében az EB fluoreszcenciája csak az 55. perc után kezdett el csökkenni. Az 50%-os fluoreszcencia csökkenést a pozitív kontroll esetében 117 perc után tapasztaltuk, míg a **7**-es anyag esetében ez 119 perc volt. Továbbá a mutáns törzsön jelentős efflux pumpa gátlást figyeltünk meg az **1**-es, **2**-es, **4**-es, **9**-es és **10**-es anyagok esetében is. Ezeknél a vegyületeknél az 50%-os csökkenést 78 és 88 perc elteltével detektáltuk (*1. táblázat*).

A vegyületek biofilm gátló aktivitását a *S. Typhimurium* 14028s biofilm képző törzsnél vizsgáltuk. Az eredményeink azt mutatták, hogy a **6**-os és a **11**-es anyagok kivételével a vegyületek 50 µM-os koncentrációban szignifikánsan gátolták a *S. Typhimurium* biofilm képzését. A legkiemelkedőbb anti-biofilm hatással rendelkező szelénvegyület a **4**-es, illetve az **5**-ös volt, melyek szintén 50 µM-os koncentrációban 76% és 74%-kal csökkentették a biofilm mennyiségét a kezeletlen kontroll törzshöz képest *(2. ábra; 2. táblázat)*.

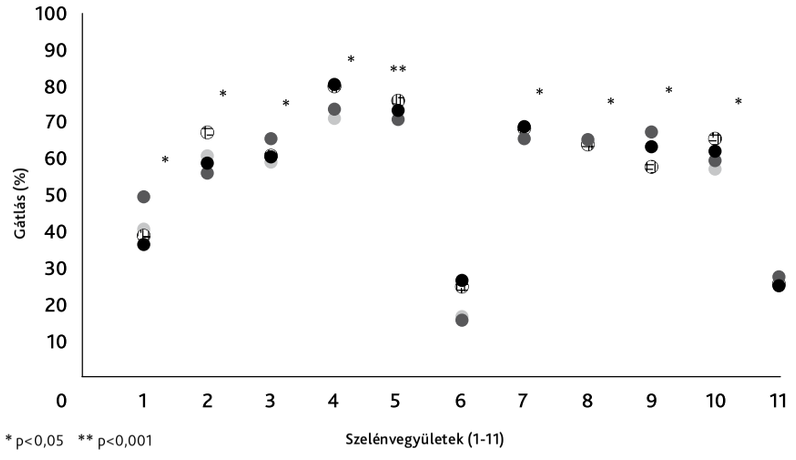
Minták	SL1344		L644	
	-25%	-50%	-25%	-50%
Kontroll	5,4	9,6	8	14,1
CCCP	52,1	122,6	42,1	117,6
1	9,7	55,3	28	78,4
2	17,8	56,8	31,5	88,6
3	4,5	7,9	9,9	21
4	17,2	56,1	21,6	83,2
5	5,3	11,0	10,1	26,3
6	5,4	11,0	11,8	30,5
7	12,2	58,9	55,2	119,5
8	5,5	9,5	9	16,8
9	15,6	79,2	37,8	86,1
10	17,1	65,6	18,8	80,8
11	19,8	30,7	15	32

Kontroll: kezeletlen baktériumkultúra
CCCP: pozitív kontroll

1. tábla Etídium-bromid efflux vizsgálat Salmonella enterica serovar Typhimurium SL1344, illetve az acrB mutáns L644 törzsön. A mérési eredmények azt az időpillanatot (perc) jelölik, amikor a fluoreszcencia 25, valamint 50%-kal csökkent a kiinduláshoz képest.

KÖVETKEZTETÉSEK

Eredményeink alapján megállapítható, hogy a pozitív kontrollként alkalmazott efflux pumpa gátló CCCP mellett a vad típusú SL1344 törzsben a 9-es számú metil-keton szelenoészter jelentős efflux gátlást mutatott. Ellenben a mutáns törzzsel, ahol a szintén metil-ketont tartalmazó 7-es szelenoészter gátolta leginkább az efflux pumpát. Az EB efflux vizsgálat során kapott eredményekből azt is megállapíthatjuk, hogy a vad típusú, illetve a mutáns törzs esetében is ugyanazok a származékok



2. ábra A vegyületek biofilm gátló hatása 50 µM-os koncentrációban *Salmonella enterica* serovar Typhimurium 14028s törzsön

Szelénvegyületek	1	2	3	4	5	6	7	8	9	10	11
Gátlás (%)	41,4	60,9	61,8	76,5	74,1	2,1	67,9	64,5	6,2	61,2	26,2

2. tábla A vegyületek biofilm gátló hatása (4 adat alapján számított átlag) 50 µM-os koncentrációban *Salmonella enterica* serovar Typhimurium 14028s törzsön

rendelkeztek efflux pumpa gátló aktivitással. Ebből arra következtethetünk, hogy a vizsgált vegyületeink nem az AcrAB-TolC efflux transzportert felépítő AcrB alegységet gátolják.

A biofilm gátlás szempontjából a fenil gyűrűt tartalmazó 4-es és 5-ös dimetil szelenodiészter gátolta a leghatásosabban a *S. Typhimurium* törzs biofilm képzését. A -COSeCH2CO- csoportot tartalmazó származékok (7, 8, 9 és 10), a 2-es és a 3-as dimetil heteroaril szelenodiészterek, az amid csoporttal rendelkező 6-os, illetve a 3,5-dimetoxifenilt tartalmazó 11-es származék szignifikánsan gátolták a biofilm kialakulását a kontrollhoz képest. A kísérlet során tesztelt szelénvegyületekhez hasonló anyagokkal még nem végeztek efflux pumpa, illetve biofilm gátló vizsgálatokat *S. Typhimurium* törzsekben.

A 9-es származék az AcrAB-TolC transzporter gátló hatása, valamint a 4-es és az 5-ös számú szelenoészterek a biofilm gátló aktivitásuk miatt kiindulópontjai lehetnek további kísérletek tervezésének, valamint továbbfejlesztve új származékok előállításának alapját képezhetik.

További céljaink között szerepel a vegyületek efflux gátló hatékonyságának vizsgálata acrA, valamint a tolC mutáns *S. Typhimurium* törzsekben is.

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