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Selenocompounds as Potent Efflux Pump Inhibitors on Gram-positive Bacteria

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In recent years, selenocompounds have gained increasing attention as potential anticancer and antibacterial agents. Several selenoderivatives have been confirmed to act as MDR efflux pump inhibitors, based on their *in vitro* results against the bacterial AcrAB-TolC system and the cancer MDR efflux pump P-glycoprotein. Efflux pumps can contribute directly or indirectly to the virulence of bacteria, as they can reduce the intracellular concentration of antibacterial substances by expelling them out of the cell. The present work aims to study the antibacterial and efflux pump inhibiting properties of four families of selenoesters, namely aspirin-selenoesters, phenone-selenoesters, hydroxy-selenoesters, and benzyl-selenoesters. The real-time ethidium bromide accumulation assay confirmed that these derivatives inhibited the efflux systems of methicillin-resistant *Staphylococcus aureus* (MRSA) without exerting any antibacterial effect. The relative expression of efflux pump gene of NorA transporter was also monitored in the presence of the most potent derivatives on reference *S. aureus*, finding that these derivatives could change the expression of the tested efflux pump gene. Regarding the anti-biofilm activity, aspirin-selenoesters, benzyl-selenoesters, and hydroxy-selenoesters could efficiently inhibit the biofilm production of the MRSA strain. It can be concluded that selenocompounds could act as efflux pump inhibitors, thus reducing the virulence of biofilm-producing bacteria.

Introduction

The emergence of superbugs, which are bacterial strains resistant to the majority of the currently commercially available antibiotics, is a worrying concern in public health, as infections caused by these superbugs kill thousands of patients yearly worldwide.[1,2] Therefore, the discovery of new antibacterial agents is an urgent need for our society.

Selenium is an important trace element, which plays a role in the prevention of cancer. In this context, selenocompounds (Se-compounds) have been successfully employed against multidrug resistant cancers by many studies.[3–7] Selenium has recently gained attention, particularly for its potential in the development of new antibacterial compounds[7–10] and nanoparticles,[11,12] especially against multidrug resistant bacteria.[10] Recently, many nanoparticles have been reported to show antibacterial properties,[11,13–15] and their antibacterial applications are being studied in many different fields. For instance, selenium nanoparticles can be used as food preservatives to prevent the bacterial growth in food,[13] to maintain a disinfected surface on chitosan films for wound healing applications,[14] for the photocatalytic removal of bacteria,[15] or to encapsulate antibiotics for more efficient and targeted delivery.[11] The mechanisms of action of these selenium nanoparticles are diverse and include: the release of metal cations, the disruption of bacterial biofilm, the damage of the bacterial cell wall and the affection of the gene expression.[16,17]

Similarly, specific Se-compounds have been reported to have antibacterial activities. One of these antibacterial Se-compounds is ebselen[8,18,19] (2-phenyl-1,2-benzisoselenazol-3-(2*H*)-one), a well-known antioxidant[20] compound with anti-inflammatory[21] properties that can also affect resistant bacteria through different mechanisms, such as the inhibition of the enzyme thioredoxin reductase, the sensitization to known antimicrobials, the inhibition of biofilm formation, and the quorum sensing.[8] Besides, its combination with silver is effective against different bacteria,[18,19] such as *Escherichia coli*[18] and *Acinetobacter baumannii*.[19] Other selenium-containing derivatives with antibacterial, antiparasitic or antifungal activities include substituted 1,2,3-thiaselenazoles,[9] selenocyanates,[22,23] diselenides,[23–25] organoselenium-palladium complexes,[26] sodium selenite in combination with silver nitrate,[27] polyselenonium salts,[28] selenoflavones,[29]

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phenoselenazines,[30] 1,2,3-selenadiazoles[31] and selenazolinium salts.[10]

In our previous studies, the antibacterial activity of selenoesters has been reported,[32–34] showing that compounds containing ketone functional groups in the alkyl moiety bound to the selenium atom of the selenoester exhibited noteworthy activity.[32–34] Symmetrical compounds were also of interest, as they enabled that compounds having other functional groups (e.g. nitriles), also showed potent antibacterial activities.[33] In addition, previously reported selenoesters had the ability of preventing the formation of bacterial and fungal biofilms, as well as the capacity to disrupt biofilms.[33,35,36] Furthermore, combining the same structure with active moieties such as aspirins and selenoesters have rendered active anticancer and antioxidant derivatives,[37–39] although their potential application in the search of antimicrobial compounds is still unknown.

The previously studied selenoesters have a substituted phenyl ring directly bound to the carbonyl of the selenoester and an alkyl moiety bound to the selenium atom. These previous studies evaluated compounds containing methylketones[32–36] and *tert*-butyl ketones[33–35] as the above-mentioned ketone-containing functional group present in the alkyl moiety. To explore different ketone-containing moieties, a series of seven selenoesters that contain a phenone (phenyl ketone) moiety has been designed, varying the substituents bound at the phenone ring, which were: 2,4-dihydroxy [compounds MB-20, MB-25, MB-27, and MB-31); 2-hydroxy (compound MB-44) and 4-chloro (compounds MB-43 and MB-49); Figure 1]. One of them, the MB-43 derivative, contains a 2-acetylphenyl moiety bound to the carbonyl, conforming an aspirin-selenoester. Four additional aspirin-selenoesters were obtained (MB-2, MB-3, MB-4, and MB-18; Figure 1).

Finally, to determine whether the ketone is important for the activity of the ketone-containing selenoesters, two additional structural variations were considered. Firstly, the ketone (methylketone, in the form of a 2-oxopropryl moiety) was substituted by an alcohol (2-hydroxypropyl moiety) in the compounds MB-68 and MB-70. Finally, the ketone was removed, to obtain compounds without this functional group or any equivalent to it. As the synthesis of the respective propyl selenoesters was not successful, a series of benzyl-selenoesters was designed, leading to the synthesis and isolation of MB-77, MB-80, and MB-81: these compounds could be considered as phenones in which the carbonyl of the ketone has been removed.

In summary, herein we report the design of selenoester derivatives and the assessment of their antibacterial activity. These selenoesters have different functionalities (phenones, hydroxyl group, benzyls) or bioactive scaffolds (aspirin), to determine which enhance the antibacterial activity.

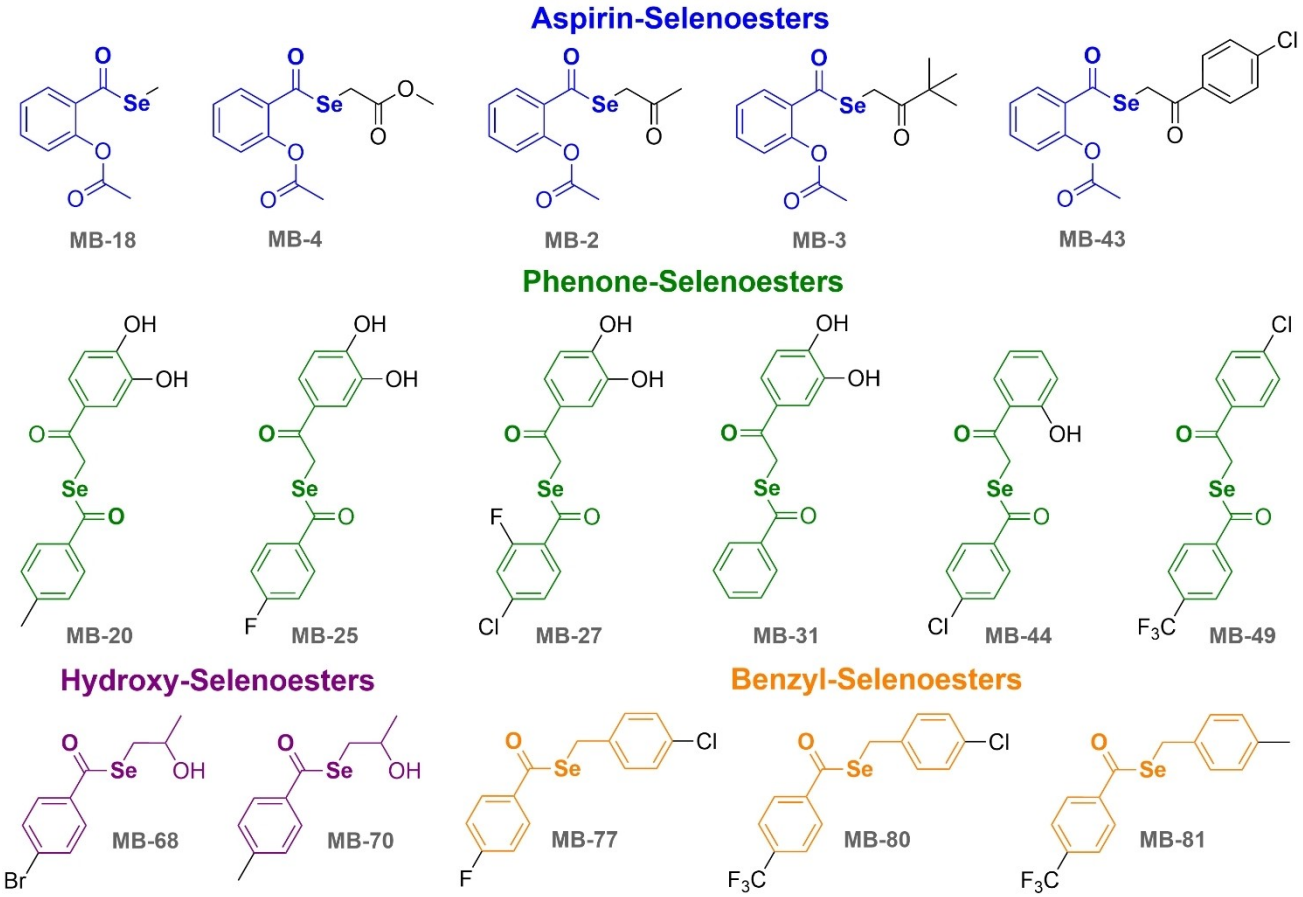


Figure 1. Structure of the compounds evaluated in this work.

The evaluated activities included assessing the antibacterial effects of the compounds and their resistance modifying activities in combination with ciprofloxacin and tetracycline. Additionally, the study examined their capacity to inhibit bacterial efflux pumps, the influence of the most active compounds on the gene expression of these pumps, their ability to inhibit bacterial biofilm formation, and the membrane disrupting effects of Se-compounds.

Results and Discussion

Synthesis of the (2-hydroxypropyl) Benzoselenoates

Selenoesters are usually synthesized following a reaction route that consists of three steps: the formation of the selenating agent (the sodium hydrogen selenide) by reduction of elemental selenium with sodium borohydride, the attack of this agent to an aroyl chloride to form a benzoselenoate reactive intermediate salt benzoselenoate, and the nucleophilic attack of this salt to an alkyl halide to form the selenoester. The aspirin-selenoesters, the phenone-selenoesters and the benzyl-selenoesters were obtained following this synthetic procedure, reported in a patent of our group.[40]

However, the synthesis of the hydroxy-selenoesters did not follow this path. Instead of performing the third reaction with an alkyl halide, an epoxide (propylene oxide) was added, and the selenium intermediate could open the epoxide ring, leading to the formation of the desired alcohol, as shown in Scheme 1. To the best of our knowledge, this is the first time that selenium intermediates can open epoxide rings. Other Se-compounds, like diselenides or phenylselenolates (as PhSeZnCl), have been previously used to open epoxides.[41,42]

The full reaction to synthesize the hydroxy-selenoesters had a reasonable yield: 39% for MB-68 and 43% for MB-70. The yield of the last step (the ring opening) is unknown because the selenium salt was prepared *in situ* and used quickly in the reaction without isolation or purification, to avoid its oxidation or degradation.

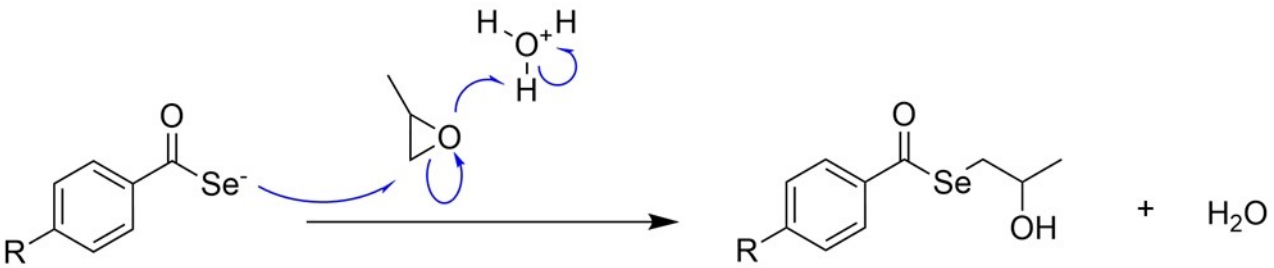
Antibacterial Activity: Determination of the Minimum Inhibitory Concentration (MIC)

The Se-compounds had no antibacterial activity on the tested Gram-negative bacterial strains; however, they were very active towards Gram-positive bacteria (Table 1). The solvent dimethyl sulfoxide (DMSO) had no antibacterial effect.

The selenoaspirins MB-2 and MB-43 exerted a potent activity on *Staphylococcus epidermidis* ATCC 12228, with MICs of 0.78 μM and <0.19 μM, respectively, Furthermore, the derivatives of hydroxy-phenone MB-20, MB-27, and MB-31 showed an MIC of 0.78 μM. In contrast, the compounds containing the hydroxyl phenone moiety, MB-25 and MB-44, displayed MIC values of 0.39 μM and less than 0.19 μM, respectively. In addition, the *p*-chloro-phenone derivative MB-49 showed also an MIC below 0.19 μM. Interestingly, all tested compounds with a phenone moiety (including the aspirin-selenoester MB-43, which also has a phenone moiety) showed submicromolar MIC values against *S. epidermidis*, regardless of substituents (–OH, –Cl) and the substitution patterns of the phenone ring considered herein. This fact is interesting and may point to the possibility that this moiety can be an active pharmacophore toward this bacterial strain and can open an interesting approach to the search for new active Se-compounds against *S. epidermidis*. In contrast, the hydroxy-selenoesters and the benzyl-selenoesters did not show antibacterial activity as their MIC values were higher than 100 μM, with the exception of the benzyl-selenoester MB-80 towards *S. epidermidis* that showed an MIC value of 12.5 μM.

In the case of *S. aureus* ATCC 25923, the selenoaspirin MB-43 had an MIC below 1 μM (MIC: 0.39 μM), the other derivative MB-2 showed an MIC of 1.56 μM. The *p*-chloro-phenone MB-49 was similarly effective with an MIC of 1.56 μM. Among the hydroxyl-phenone derivatives, MB-25, MB-27, and MB-31 were potent compounds that showed an MIC of 6.25 μM, in addition MB-20 and MB-44 had an MIC of 12.5 μM.

As expected, the derivatives were generally less active on the methicillin-resistant *S. aureus* 272123 (MRSA) strain. Selenoaspirins MB-43 and MB-2 had MIC above 1 μM (MIC: 1.56 and 6.25 μM, respectively). Among the hydroxyl-phenone derivatives, the most potent was MB-44 having the MIC at 3.125 μM, whereas the remaining hydroxyl-phenone derivatives MB-20, MB-25, MB-27 and MB-31 had the MIC at 6.25 μM. Out of the Se-compounds, the most potent compound was the *p*-chloro-phenone derivative MB-48 with an MIC of 0.78 μM.



Scheme 1. Epoxide opening that leads to the formation hydroxyl selenoesters. R = –Br, $-CH_3$ (MB-68 and MB-70, respectively).

Table 1. Antibacterial activity of the aspirin-, phenone-, hydroxy-, and benzyl-selenoesters. MIC values in bold are those equal to or lower than 25 μM.

Compounds	MIC (μM)					
	Gram-positive strains				Gram-negative strains	
	S. aureus ATCC 25923	*S. aureus* 272123 (MRSA)	*E. faecalis* ATCC 29212	*S. epidermidis* ATCC 12228	*E. coli* AG100	*E. coli* AG100 A
Aspirin-selenoesters						
MB-18	>100	>100	>100	>100	>100	>100
MB-2	**1.56**	**6.25**	100	**0.78**	>100	>100
MB-3	50	**100**	100	**25**	>100	>100
MB-4	**25**	100	>100	**12.5**	>100	>100
MB-43	**0.39**	**1.56**	**6.25**	<**0.19**	>100	>100
Phenone-selenoesters						
MB-20	**12.5**	**6.25**	**25**	**0.78**	>100	100
MB-25	**6.25**	**6.25**	**25**	**0.39**	>100	>100
MB-27	**6.25**	**6.25**	**25**	**0.78**	>100	>100
MB-31	**6.25**	**6.25**	**25**	**0.78**	>100	100
MB-44	**12.5**	**3.125**	>100	<**0.19**	>100	>100
MB-49	**1.56**	**0.78**	**25**	<**0.19**	>100	>100
Hydroxy-selenoesters						
MB-68	>100	>100	>100	>100	>100	>100
MB-70	>100	>100	>100	>100	>100	>100
Benzyl-selenoesters						
MB-77	>100	>100	>100	>100	>100	>100
MB-80	>100	>100	>100	**12.5**	>100	>100
MB-81	>100	>100	>100	>100	>100	>100
DMSO	>1%	>1%	>1%	>1%	>1%	>1%

In general, the derivatives were less potent on *Enterococcus faecalis* ATCC 29212. The most active derivative was selenoaspirin MB-42 (MIC: 6.25 μM). The hydroxy-phenone derivatives MB-20, MB-25, MB-27, MB-31, and the *p*-chloro-phenone derivative MB-49 all showed the MIC at 25 μM.

Inhibition of Bacterial Efflux Pumps

Bacterial efflux pump inhibitors (EPIs) are a class of compounds that have gained significant attention in the field of antimicrobial research. The efflux pumps are specialized transport proteins found in bacterial cell membranes, which play a crucial role in the intrinsic or acquired bacterial resistance to various antibiotics and other toxic compounds e.g. biocides. Due to the efflux pumps, these substances can be actively expelled out of the cell, thereby reducing their intracellular concentration, consequently, their effectiveness toward multidrug resistant bacteria.

Regarding the efflux pump inhibition, one derivative was an active EPI on the sensitive ATCC 25923 strain and the resistant 272123 clinical isolate of *S. aureus*. The benzyl-selenoester MB-77 was a potent EPI on *S. aureus* strains: the relative fluorescence indexes (RFIs) on the reference strain were 0.60 and 0.317 on the resistant strain (Figure 2). MB-68 and MB-70 hydroxy-selenoesters acted as EPI compounds on the reference *S. aureus* strain: MB-68 had an RFI of 0.51 and the RFI was 0.70 in the presence of MB-70. There was another promising compound: MB-3 (RFI was 0.262) could inhibit the efflux pump to the same extent as the positive control verapamil (VER). The RFI values obtained for the reference VER were 0.70 and 0.26 in sensitive and resistant strains, respectively.

On the Gram-negative *Escherichia coli* AG100 strain expressing the AcrAB-TolC system, only the hydroxy-phenone derivative MB-20 showed notable activity (RFI was 2.74) compared to the positive control promethazine (PMZ; RFI was 2.85).

Relative Gene Expression of *norA*

As mentioned above, three derivatives (the hydroxy-selenoesters MB-68 and MB-70, and the benzyl-selenoester MB-77) were potent EPIs on *S. aureus* ATCC 25923. Thus, to widen the knowledge of the mechanisms underlying this activity, the effect of these Se-compounds was investigated on the relative gene expression of the *norA* efflux pump gene.

NorA is a multidrug efflux pump that is part of the core genome of *S. aureus* strains.[43,44] Under certain conditions, such

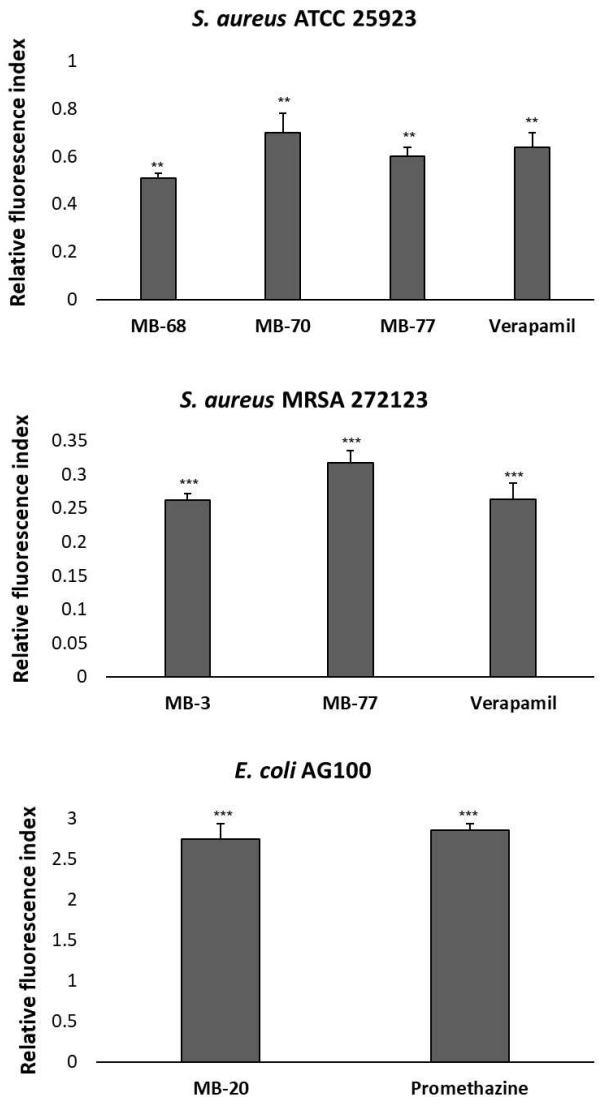


Figure 2. Relative fluorescence index (RFI) values determined for the most effective selenoesters in selected sensitive and resistant bacterial strains. The levels of significance were $^{**}p < 0.01$, and $^{***}p < 0.0001$.

as exposure to subinhibitory concentrations of antibiotics, the expression of the *norA* gene can be upregulated, leading to increased production of the NorA pump production and the subsequent development of antibiotic resistance.[45] This upregulation occurs through a complex regulatory network that involves multiple genes and regulatory factors. We wanted to monitor the events that occur in the real-time ethidium bromide (EB) accumulation assay, and for this reason we used a short incubation time of 30 min. Furthermore, the selected compounds (MB-68, MB-70, and MB-77) were used at the same concentrations (50 μM) in the EB accumulation and relative gene expression assays (Figure 3).

After 0 and 30 min of incubation and culture in the presence of the tested derivatives, the changes in the expression rate of the *norA* gene were monitored. In the presence of MB-68, a 2.71-fold increase in *norA* expression was detected, and in case of MB-77, 9.85-fold increase in *norA* expression was measured after 30 min. This increased expression is evidence of the bacterial adaptation to the toxic substance (here Se-compounds) present in the cellular environment. Interestingly, *norA* expression was still downregulated (1.05-fold reduction) in the presence of MB-70. This fact could be explained by taking into account that the development of stress response of the bacteria may require a longer period of time.[46]

Enhancement of Antibiotic Activity in the Presence of Se-compounds

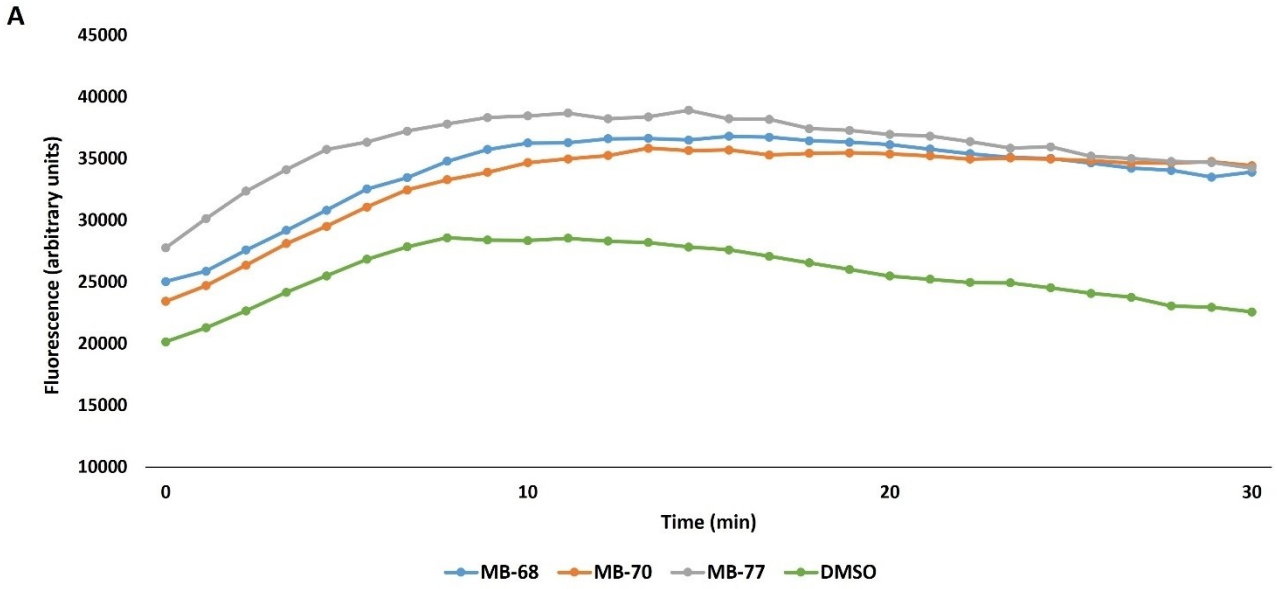
In the absence of the compounds, the MIC value of ciprofloxacin (CIP) was 2 μM against *S. aureus* ATCC 25923 and tetracycline (TET) was 6.25 μM against *S. aureus* MRSA 272123. MB-68 and MB-70 showed synergism with CIP: these compounds applied at 50 μM reduced the MIC value of CIP by two-fold (1 μM) against sensitive *S. aureus* strain. The MIC of TET, in combination with MB-3, was decreased three-fold against resistant *S. aureus* (data not shown).

Inhibition of Biofilm Formation

The tested Se-compounds were added at $\frac{1}{3}$ MIC or 50 μM (where the MIC was >100 μM) to the bacterial suspension in the biofilm inhibition assay. The selenoaspirines MB-2 and MB-3 showed anti-biofilm activity against *S. aureus* ATCC 25923 (Figure 4). The hydroxy-phenone derivative MB-20 and the hydroxy-selenoester MB-70 could also inhibit the biofilm formation of this *S. aureus* strain; furthermore, the most potent inhibition was achieved in the presence of the hydroxy-selenoester MB-68.

Surprisingly, the Se-compounds were highly active anti-biofilm agents against the MRSA strain. The selenoaspirins MB-3 and MB-18 were effective compounds that could inhibit biofilm formation, MB-3 being the most active with 92.14% inhibition (Figure 5). The hydroxy-selenoesters MB-68 and MB-70 inhibited the biofilm formation with a determined efficacy of 67.21 and 65.41%, respectively. Among the benzyl-selenoesters, MB-77 was the most active, with a measured inhibition% of 85.75%. Furthermore, the benzyl-derivatives MB-80 and MB-81 were less powerful than MB-77, as their inhibition rates determined were 70.18 and 66.34%, respectively.

Three selenoaspirins MB-3, MB-4, and MB-43 could inhibit the biofilm production of *E. coli* AG100. Of these three derivatives, MB-4 was the most effective (Figure 6). The hydroxy-phenone derivatives MB-20, MB-25, and MB-31 could also decrease the mass of the biofilm. A hydroxy-selenoester (MB-68) and a benzyl-selenoester (MB-80) were also anti-biofilm agents. MB-68 was the most potent anti-biofilm derivative of these two.



B

Compound	Relative gene expression level of *norA*	
	0 min	30 min
MB-68	1.95	2.71
MB-70	-3.51	-1.05
MB-77	-6.28	9.85

Figure 3. (A) Efflux pump inhibition in *S. aureus* ATCC 25923: EB accumulation curves of selected Se-compounds and negative control DMSO. (B) Relative gene expression levels of the *norA* gene in the presence of Se-compounds after 0 and 30 min.

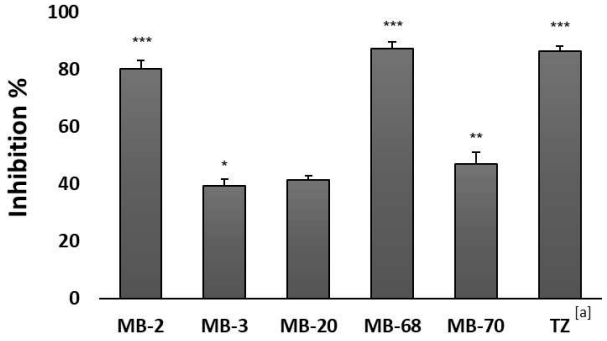


Figure 4. Biofilm inhibition exerted by the selenoesters on the *S. aureus* ATCC 25923 bacterial strain. The levels of significance were *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0001$.
[a] TZ: thioridazine; positive control.

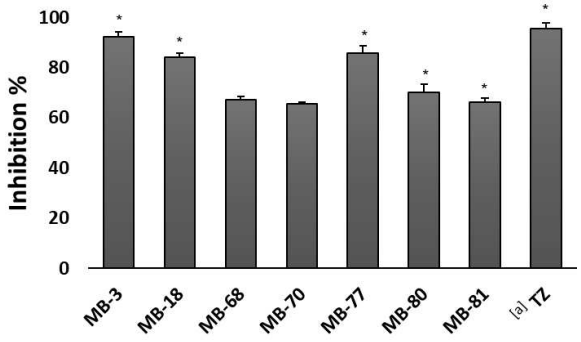


Figure 5. Biofilm inhibition in the presence of selenoesters on *S. aureus* MRSA 272123 strain. The level of significance was *$p < 0.05$.
[a] TZ: thioridazine; positive control.

Membrane-damaging Effect of Se-compounds

MB-3 (17.57%) and MB-77 (14.65%) were less active against *S. aureus* MRSA 272123, in addition, there was nearly 16% damage in *S. aureus* ATCC 25923 in the presence of MB-77. The phenone-selenoester MB-20 was able to disrupt 82.06% of the *E. coli* AG100 membrane compared to the untreated bacteria control (data not shown). The other Se-compounds had no membrane-damaging effect on the tested bacterial strains.

Discussion

Firstly, the synthesis of the hydroxy-selenoesters opens a new tool to explore the chemistry of the selenoesters: the inclusion of alcohols through the opening of an epoxide. This is a much safer method attempting to reduce a ketone selenoester, as the reduction conditions can lead to the breakage of the carbon-selenium bond of the selenoester. In addition, it is neither easy nor economical to find commercially available alkyl halides that also have hydroxyl groups. The yield was acceptable (39–43%), considering that it was the combined yield of three reactions in

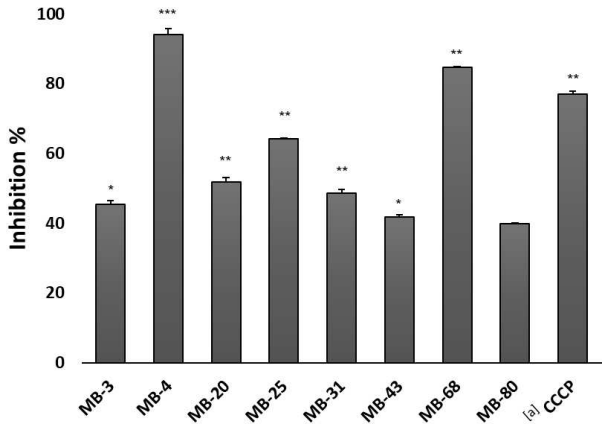


Figure 6. Biofilm inhibition in the presence of selenoesters on *E. coli* K-12 AG100 strain. The levels of significance were *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0001$.
[a] CCCP: Carbonyl cyanide 3-chlorophenylhydrazone; positive control.

a pot, without isolating or purifying the intermediates to minimize their losses due to oxidation or other side reactions.

As mentioned in the Introduction, selenoesters with antibacterial and/or antifungal activity have been reported in former works.[32–36] These studies indicated that compounds containing a methyl ketone or a *tert*-butylketone in the alkyl moiety bound to the selenium atom of the selenoester showed a noteworthy activity.[32–36] Herein, as a continuation of previous works, novel selenoesters with different moieties have been explored: (i) a series of aspirin-selenoesters that insert this interesting pharmacophore in the molecule, (ii) a series of phenones, in which the alkylketones are replaced by a phenone (phenylketone); (iii) a series of compounds in which the methyl ketone has been replaced by its reduced hydroxyl equivalent; and (iv) a series with a benzyl group, thus removing the ketone moiety.

It is interesting to highlight that, except for MB-4 (which contains an oxygen methyl ester at the alkyl moiety of the molecule) the compounds containing a ketone in the alkyl moiety bound to the selenium atom had an antibacterial MIC value below 100 μM against the tested Gram-positive strains (Table 1). According to this table, all tested phenones showed antibacterial activity, together with the two selenoaspirins MB-2 and MB-43, in which the alkyl moiety bound to the selenium of the selenoester has a methyl ketone and a *p*-chloro-phenone, respectively. None of the hydroxy- or benzyl-selenoesters showed an MIC value below 100 μM in the tested strains. These experimental results highlight that the presence of the ketone moiety is crucial for intrinsic antibacterial activity. Among the different substitutions of the evaluated phenones, *p*-chloro-phenones are the ones with the most potent antibacterial activity: compound MB-43 (aspirin, *p*-chloro-phenone) showed the lowest MIC against *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212 and *S. epidermidis* ATCC 12228; while MB-49 had the lowest MIC against *S. aureus* ATCC 272123 (MRSA) and *S. epidermidis* ATCC 12228, this last one similarly to compounds MB-43 and MB-44. MB-4 containing a methyl ester instead of a methyl ketone, showed MIC values below 100 μM, but clearly much less potent than the equivalent methyl ketone MB-2. For instance, the MIC of MB-4 was 16-fold higher than that of MB-2 in the two *S. aureus* strains and in the *S. epidermidis* strain evaluated. In addition, it can be concluded based on previous studies[32–35] and the present results that the compounds are much less effective against Gram-negative strains.

Regarding the inhibition of the bacterial efflux pumps, it is noteworthy that the non-active hydroxy-selenoesters and benzyl-selenoesters in the MIC determination were the ones with the ability to inhibit the efflux pumps in Gram-positive bacteria. This effect is quite interesting, as these compounds could be used as adjuvants to enhance the antibacterial activity of antibiotics currently in clinical use affected by the emergence of multidrug resistance. The MB-20 phenone-selenoester increased EB retention in the *E. coli* AG100 strain being an active EPI without antibacterial activity on *E. coli* AG100. In the combination assays, both hydroxy-selenoesters (MB-68 and MB-70) reduced the MIC value of CIP by two-fold on susceptible *S. aureus* ATCC 25923. The MB-3 aspirin-selenoester increased the susceptibility of resistant *S. aureus* ATCC 272123 to TET; this compound reduced the MIC value of TET by three-fold.

Concerning the gene expression assay, the results show that the active EPIs against *S. aureus* (hydroxy-selenoesters MB-68 and MB-70; benzyl-selenoester MB-77) can alter the expression of efflux pump genes, such as *norA*, which can be explained by the bacterial stress response. The relative gene expression level of EP genes (*acrA* and *acrB*) was already studied in the *E. coli* AG100 strain. It was observed that cyclic selenoanhydride and meta-substituted benzene selenodiester up-regulated the Resistance Nodulation Division (RND) transporter subunit *acrB* gene after 4 h of exposure.[35]

The hydroxy-selenoesters, benzyl-selenoesters, and two aspirin-selenoesters (MB-18 and MB-3) can inhibit the formation of *S. aureus* MRSA 272123 biofilm, with an inhibition of up to 92.14% in the case of MB-3. It is difficult to extract reliable SAR (structure-activity relationships) due to the reduced number of compounds, but apparently biofilm inhibition in *S. aureus* MRSA 272123 is affected by the substituent present in the ring. In *S. aureus* ATCC 25923 the compounds that exerted inhibition were two aspirin-selenoesters MB-2 and MB-3 (which have an acetyl moiety at the phenyl ring to conform the aspirin scaffold), and the compounds MB-20 and MB-68, which are hydroxy-selenoesters. These two derivatives have in common a *p*-methylbenzoyl aromatic moiety bound to the selenium of the selenoester. Among the derivatives with halogens in this aromatic moiety, only the hydroxy-selenoester MB-70 (with a *p*-bromobenzoyl aromatic moiety) showed the ability to inhibit biofilm production of *S. aureus* ATCC strain and with less potency than the equivalent *p*-methyl derivative. None of the phenones or benzyl-selenoesters unsubstituted or substituted with fluoro, chloro or trifluoromethyl groups showed activity. This fact could indicate that halogen atoms, apart from bulky bromine, remove the activity, and that electron-donating groups such as methyl or acetyl, enhance it. This effect is not observed in *S. aureus* MRSA, in which the benzyl-selenoesters were anti-biofilm compounds, in spite of the halogens present in their structures. In *E. coli* AG100 the most potent inhibitors of

the biofilm formation were an aspirin-selenoester (MB-4) and the hydroxy-selenoester MB-68.

Finally, the most potent membrane disrupting Se-compound was the hydroxy-phenone MB-20 at 50 μM concentration, which showed 82.06% damage to *E. coli* AG100 strain. The compounds tested had less effect on the membrane integrity of the *S. aureus* strains. The difference was observed because the structure of the cell membrane is different in Gram-negative and Gram-positive bacteria.[47]

Conclusions

The most interesting compounds among the ones tested would be the aspirin-selenoester MB-43 and the phenone-selenoester MB-49: both showed potent antibacterial activity against Gram-positive bacteria; together with the hydroxy-selenoesters MB-68 and MB-70; and the benzyl-selenoester MB-77: these three latest had an interesting ability to inhibit bacterial efflux pumps, alter the gene expression of the NorA pump, and inhibit the formation of bacterial biofilm. Results indicated that the aspirin-selenoester MB-3 and hydroxy-selenoesters MB-68 and MB-70 exhibited synergism with antibiotics against *S. aureus* strains. These results suggested that the active EPI properties of compounds in Gram-positive strains can make them promising adjuvants of the antibiotics that are used in medical practice to combat bacterial infections.

Experimental Section

Compounds

The synthesis of the aspirin-, phenone-, and benzyl-selenoesters was performed according to the protocol described in the patent application EP3628659 A1.[40] These compounds were appropriately characterized using analytical techniques typical in Organic Chemistry, as nuclear magnetic resonance (NMR), mass spectrometry (MS) and infrared (IR) spectrometry; whereas their purity was determined by means of elemental analysis. All compounds showed a purity appropriate for them to be tested in biological assays (> 95%), according to elemental analysis. Briefly, in a first step the selenating agent (NaHSe, sodium hydrogen selenide) was generated by the slow addition of sodium borohydride ($NaBH_4$) over a suspension of grey selenium powder in water. The addition of the adequate substituted benzoyl chloride generated a selenium reactive intermediate salt that attacks as nucleophile the adequate alkyl halide to form the final desired compound.

Hydroxy-selenoesters were obtained following a variation of this procedure that will be discussed below in section Synthetic procedure for the hydroxyl-selenoesters. In brief, procedure is similar to the one used for the remaining compounds, but in the last step propylene oxide is added instead of an alkyl halide. The benzoselenoate intermediate salt has sufficient nucleophilic ability to trigger the opening of the epoxide, and to the best of our knowledge, this is the first time that a selenoate salt has been used to attack an epoxide. Nevertheless, in a work found in bibliography,[41,42] diselenides and phenylselenolates have been used to open epoxides and form the adequate hydroxyl compounds.

Chemical Reagents and Chemical Characterization

The chemical reagents, solvents and materials used to synthesize the compounds presented in this work were purchased at the following vendors: Acros Organics and Alfa Aesar (both brands of Thermo Fisher Scientific, Geel, Belgium), Fluorochem (Hadfield, Derbyshire, United Kingdom), Honeywell Riedel de Haën (Seelze, Germany), Panreac Química S.L.U (Castellar del Vallés, Barcelona, Spain), Scharlab S.L. Spain (Sentmenat, Barcelona, Spain) and Sigma-Aldrich Merck S.L.U. Spain (Madrid, Spain).

NMR spectra were taken at a Varian Inova-300 spectrometer (Agilent Technologies, Santa Clara, California, USA) to monitor whether the reactions took place adequately. The purity of the compounds with a clean spectrum at the 300 MHz spectrometer was assessed by means of elemental analysis, at a LECO CHNS-932 microanalyser (LECO Europe B.V., Geleen, Netherlands), at a temperature of 990 °C and using He as carrier gas and silver capsules to introduce the sample in the analyser. To be considered pure each derivative synthesized need to have a deviation minor than 0.40% in each element analysed (C, H, N, S). The NMR spectra included at Supporting Information (NMR-^{1}H, NMR-^{13}C, COSY, HMBC, HSQC), were acquired at a Bruker Avance III HD-400 (Billerica, Massachusetts, USA) spectrometer using TMS as internal standard. Mass spectra were obtained in a quadrupole HP 5973 MSD spectrometer (Hewlett Packard, currently Agilent Technologies, Santa Clara, California, USA) with direct insertion probe and electronic impact (EI) in positive mode as ionization source, at a 70 eV ionization energy and with an m/z precision of ±0.05. Finally, melting points were measured in a Reichert-Kofler heating system coupled with a microscope; and were provided as obtained by visual inspection, without correction. Recorded spectra have been included in the Supporting Information (Figures S1A–S2F).

Synthetic Procedure for the Hydroxy-selenoesters

A procedure that consists of three reactions performed consecutively in the same reaction flask was followed to prepare the 2-hydroxypropyl selenoesters MB-68 and MB-70.

The reaction route started by the suspension of an equivalent of selenium grey powder in 20 mL of water. Afterwards, 2 equivalents of $NaBH_4$ were added slowly, keeping all the time a gentle stirring, till the finalization of the release of hydrogen, a gas by-product of this first reaction. This enables the formation of sodium hydrogen selenide, which in the next step attacks as a nucleophile the carbonyl group of the corresponding acyl chloride, added then over the reaction solution; which is then kept 90 min at 50–70 °C; to ensure the formation of the corresponding acyl selenide. Prior to the last step, the crude reaction mixture was filtered to eliminate the formed boron salts. In this moment, an equivalent of propylene oxide was added over the filtrate. The acyl selenide attacks the epoxide, leading to its ring opening and to the formation of the corresponding 2-hydroxypropyl benzoselenoester. The mixture was kept 1 h at 50 °C and then 1 h at room temperature to ensure the completion of the reaction. The obtained hydroxy-selenoester was isolated and purified by application of the most adequate techniques in each case, as precipitation if the compound is solid and extraction if it is liquid.

Chemical Description of the Hydroxy-selenoesters

Se-(2-hydroxypropyl) 4-bromobenzoselenoate (MB-68)

From sodium borohydride (0.160 g, 4.23 mmol), grey selenium (0.158 g, 2.00 mmol), *p*-bromobenzoyl chloride (0.447 g, 2.04 mmol)

and propylene oxide (0.116 g, 0.14 mL, 2.00 mmol). Final compound precipitated as a yellow solid powder that was isolated by filtration and washed with water, rendering 252 mg (39%). MW: 322.07. DIP-MS m/z (abundance %): 76 (16), 154.95/156.95 (28/27), 182.95/184.95 (100/98, Br). ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.78 (d, $J_{2\text{-}3,6\text{-}5}$ = 8.6 Hz, 2H, H_2+H_6), 7.60 (d, $J_{3\text{-}2,5\text{-}6}$ = 8.6 Hz, 2H, H_3+H_5), 4.07 (double sextet, $J_{CH\text{-}CH3rCH\text{-}CH2}$ = 6.1 Hz, 1H, –CH), 3.15 and 3.33 (3.15: dd, J = 12.9 Hz, J = 7.0 Hz; 3.33: dd, J = 12.9 Hz, J = 4.2 Hz; together 2H, $-SeCH_2$), 2.00 (bs, 1H, –OH), 1.33 (d, $J_{CH3\text{-}CH}$ = 6.1 Hz, 3H, $-CH_3$). ^{13}C-NMR (101 MHz, $CDCl_3$, δ: 194.0 (COSe), 137.3 (C_1), 132.3 (C_3+C_5), 129.1 (C_4), 128.8 (C_2+C6), 67.4 (CHOH), 35.2 ($SeCH_2$), 23.1 ($-CH_3$). Elemental analysis for $C_{10}H_{11}BrO_2Se$, calculated/found (%): C: 37.29/37.27; H: 3.44/3.476.

Se-(2-hydroxypropyl) 4-methylbenzoselenoate (MB-70)

From sodium borohydride (0.158 g, 4.18 mmol), grey selenium (0.161 g, 2.04 mmol), *p*-toluoyl chloride (0.303 g, 0.26 mL, 1.96 mmol) and propylene oxide (0.116 g, 0.14 mL, 2.00 mmol). Final compound was obtained as a yellow liquid by extraction with methylene chloride and subsequent drying with sodium sulphate, filtering, and evaporation in rotary evaporator, rendering 219 mg (43%). MW: 257.20. DIP-MS m/z (abundance %): 65.15 (24), 91.15 (82), 119.15 (100), 257.95 (0, M+). ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.82 (d, $J_{2\text{-}3,6\text{-}5}$ = 8.2 Hz, 2H, H_2+H_6), 7.25 (d, $J_{3\text{-}2,5\text{-}6}$ = 8.2 Hz, 2H, H_3+H_5), 4.07 (dsx, $J_{CH\text{-}CH3,CH\text{-}CH2}$ = 6.1 k Hz, J = 2.0 Hz, 1H 1H, –CH), 3.12 and 3.31 (3.12: dd, J = 12.9 Hz, J = 7.0 Hz; 3.31: dd, J = 12.9 Hz, J = 4.2 Hz; together 2H, $-SeCH_2$); 3.40 (s, 3H, $-CH_3$ Ar.) 2.04 (s, 1H, –OH), 1.32 (d, $J_{CH3\text{-}CH}$ = 6.2 k Hz, 3H, $-CH_3$ Alk.). ^{13}C-NMR (101 MHz, $CDCl_3$, δ: 194.4 (COSe) 145.0 (C_4), 136.4 (C_1), 129.6 (C_3+C_5), 128.0 (C_2+C_6), 67.5 (CHOH), 34.9 ($SeCH_2$), 23.1 ($-CH_3$ Alk.), 21.9 ($-CH_3$ Ar.). Elemental analysis for $C_{11}H_{14}O_2Se$, calculated/found (%): C: 51.37/51.50; H: 5.49/5.413.

Bacterial Strains

Compounds were evaluated against two Gram-negative strains. Firstly, the wild-type *Escherichia coli* K-12 AG100 strain [argE3 thi-1 rpsL xyl mtl Δ(gal-uvrB) supE44], expressing the AcrAB–TolC efflux pump system 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 four Gram-positive strains. *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923 was 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). In addition, *Staphylococcus epidermidis* ATCC 12228 and *Enterococcus faecalis* ATCC 29212 strains were used in the MIC determination assay.

Determination of MIC by Microdilution Method

The determination of MICs of the tested Se-compounds followed the guidelines of the Clinical and Laboratory Standard Institute (CLSI).[48] The results were visually inspected to determine the MIC values of the compounds. DMSO was used as a negative control to rule out any potential antibacterial effects.

Real-time Ethidium Bromide Accumulation Assay

Real-time fluorimetry was used based on the intracellular accumulation of the efflux pump substrate EB, to assess the efflux pump inhibiting activity of Se-compounds on *S. aureus* ATCC 25923, *S. aureus* MRSA 272123, *E. coli* AG100 and *E. coli* AG100 A strains. CLARIOstar Plus plate reader (BMG Labtech, UK) was used to monitor the accumulation of EB. VER was applied at 50 μg/mL and PMZ at 25 μg/mL as positive controls. Additionally, DMSO was applied as a negative control at a 1 v/v% concentration. The bacterial cultures were incubated at 37 °C in a shaking incubator until they reached an optical density (OD) of 0.6 at 600 nm. Afterwards, they were washed with phosphate buffered saline (PBS; pH 7.4), centrifuged, and resuspended in PBS. The Se-PBS compounds were added at $\frac{1}{3}$ MIC or 50 μM (where the MIC was >100 μM) concentration to PBS containing a non-toxic concentration of EB (2 μg/mL). The solutions were then pipetted into a 96-well black microtiter plate (Greiner Bio-One Hungary Kft, Hungary), and 50 μL of bacterial suspension (OD_{600} 0.6) were added to each well. The plates were placed into the CLARIOstar plate reader, and the fluorescence was monitored every minute for one hour at the excitation and emission wavelengths of 530 nm and 600 nm, respectively. Based on the real-time data, the RFI of the last time point (minute 60) of the EB accumulation assay was calculated according to the following equation:

$$RFI = (RF_{treated} - RF_{untreated})/RF_{untreated}$$

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).[49] Data analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA; www.graphpad.com), applying the two-tailed *t*-test.

Resistance Modulation Assay

The resistance modulating effect of compounds with ciprofloxacin (CIP) and tetracycline (TET) antibiotics were evaluated by the MIC reduction assay on *S. aureus* ATCC 25923, *S. aureus* MRSA 272123 and *E. coli* AG100 strains. In this assay, only the compounds with efflux pump inhibitory effects were tested. Briefly, CIP or TET was diluted in a 96-well microtiter plate by two-fold serial dilution in Mueller Hinton (MH) broth and then the compounds were added at subinhibitory concentrations ($\frac{1}{3}$ MIC or 50 μM). Finally, 10^{-4} dilution of the overnight bacterial culture in MH was added to each well. The microtiter plates were incubated at 37 °C for 18 h. MIC values in the presence of the antibiotics alone and in combination with Se-compounds were determined by visual inspection.[34]

Inhibition of Biofilm Formation

The strains used were the Gram-negative *E. coli* AG100 and the Gram-positive *S. aureus* ATCC 25923 and *S. aureus* MRSA 272123. The detection of the biofilm formation was possible with the use of the dye crystal violet [CV; 0.1% (v/v)]. The initial inoculum was incubated in Luria-Bertani broth (LB) (for *E. coli*) or in tryptic soy broth (TSB, for *S. aureus*) overnight, and then diluted to an OD_{600} of 0.1. Then, the bacterial suspension was added to 96-well microtiter plates and the compounds were added at $\frac{1}{3}$ MIC or 50 μM (where the MIC was >100 μM). The final volume in each well was 200 μL. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for *E. coli* AG100 and thioridazine (TZ) for *S. aureus* strains were used as positive controls. The plates were incubated at 30 °C for 48 h, with gentle

stirring (100 rpm). After the incubation, the medium was discarded, and unattached cells were removed by washing the plates with tap water. Then, 200 μL CV was added to the wells and incubated for 15 min at room temperature (~24 °C). In the next step, CV was eliminated from the wells, the plates were washed again with tap water, and 200 μL of 70% ethanol was added to the wells. The biofilm formation was established by measuring the OD_{600} using a Multiscan EX ELISA plate reader (Thermo Labsystems, Cheshire, WA, USA). The anti-biofilm effect of the samples was expressed in the percentage (%) of the decrease of biofilm formation in respect to the control. Data analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA; www.graphpad.com), applying the two-tailed *t*-test.

Measuring Bacterial Membrane Damage

To explore how the structure of bacterial membrane changes in the presence of compounds, membrane integrity was measured using LIVE/DEAD Baclight™ Bacterial Viability Kit (Thermo Fisher Scientific, Waltham USA). The exponential phase bacterial culture was washed twice with PBS, centrifuged, and resuspended in PBS (OD_{600} 0.2). After resuspending, 1 mL of the culture was heat-treated at 80 °C for 15 min to obtain the dead culture, whereas the treated cultures with $\frac{1}{3}$ of the MIC or 50 μM of selected Se-compounds were incubated at 37 °C for 15 min. A mixture of dyes–SYTO 9™ and propidium iodide (PI)–was added to 2 mL filtered (0.2 μm pore size filter) distilled water. 100 μL of this dye mixture was added to 100 μL *S. aureus* ATCC 25923, *S. aureus* MRSA 272123 and *E. coli* AG100 cultures that were treated with Se-compounds. The dye mixture was also added to the control samples (untreated and heat-treated controls). To measure the membrane disruption of the live cells, the kinetics of the intracellular penetration of PI and SYTO 9 were followed by measuring the relative fluorescence units in 60 s intervals over 1 h at 485/530 nm (excitation/emission) using a CLARIOstar Plus microplate reader (BMG Labtech, UK). The experiments were carried out in triplicates in 96-well black microtiter plates. The membrane disruption (%) was calculated from the average of the data obtained from the last 10 min measurement, and then average values of the Se-compounds treated cultures were compared with the untreated ones (represents 100%).[50]

Expression Analyses of Genes by Real-time Reverse Transcriptase Quantitative Polymerase Chain Reaction

S. aureus ATCC 25923 strain was cultured in TSB broth and was incubated overnight at 37 °C with shaking. On the day of RNA isolation, the bacterial suspension (OD of 0.6 at 600 nm) was transferred to 10 mL tubes in 3 mL aliquots, and 50 μM of MB-68, MB-70 and MB-77 were added to the respective tubes, which were incubated at 37 °C. At the 0 and 30 min 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. The purified RNA was stored in RNase-free water in nuclease-free collection tubes and was maintained at −20 °C until the quantification was performed. The concentration of the extracted RNA templates was assessed by spectrophotometry (NanoDrop Lite, Thermo Scientific) at 260 nm. The expression of the efflux pump gene *norA* was studied by reverse transcription of the total RNA. The data obtained for the gene targets were normalized against the *S. aureus* 16S ribosomal RNA measured in the same sample. The primers[51] used in the assay were the following ones:

1. Sequence (5′-3′) of *norA* (246 bp)

TCGTCTTAGCGTTCGG TTTA (Fw)

TCCAGTAACCATCGGC AATA (Rv)

2. Sequence (5′-3′) of *16S rRNA* (492 bp)

AGAGTTTGATCMTGGC TCAG (Fw)

GWATTACCGCG GCKGCTG (Rv)

The real-time quantification of the RNA templates by real-time one-step reverse transcriptase quantitative polymerase chain reaction (PCR) 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.[52]

Supporting Information Summary

The Supporting Information contains the following data: Figure S1A: DIP-MS spectrum of MB-68, Figure S1B: 1H-NMR spectrum ($CDCl_3$, 400 MHz) of MB-68, Figure S1C: 13 C-NMR spectrum ($CDCl_3$, 101 MHz) of MB-68, Figure S2A: DIP-MS spectrum of MB-70, Figure S2B: 1H-NMR spectrum ($CDCl_3$, 400 MHz) of MB-70, Figure S2C: 13 C-NMR spectrum ($CDCl_3$, 101 MHz) of MB-70, Figure S2D: 1H-1H COSY NMR spectrum ($CDCl_3$) of MB-70 (aromatics), Figure S2E: 1H-13 C HSQC NMR spectrum ($CDCl_3$) of MB-70, Figure S2F: 1H-13 C HMBC NMR spectrum ($CDCl_3$) of MB-70.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Keywords: Biofilm · Biological activity · Efflux pump · Multidrug resistance · Selenium

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