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Synthesis and Evaluation of 2-Aminothiophene Derivatives as *Staphylococcus aureus* Efflux Pump Inhibitors

Rayssa M. D. da Cruz,[a, b, c, d] Renaud Zelli,[c] Sarah Benshain,[d] Ryldene M. D. da Cruz,[b] José P. Siqueira-Júnior,[e] Jean-Luc Décout,[c] Marie-Paule Mingeot-Leclercq,[d] and Francisco J. B. Mendonça-Junior*[a, b]

2-aminothiophene derivatives (2AT) in which the thiophene ring is fused with a cycloalkyl or a *N*-acylated piperidine ring by positions 5 and 6 and carrying a 3-carbethoxy group were synthesized and their bacterial growth and enzyme inhibitory effects against efflux proteins of *Staphylococcus aureus* leading to resistance to fluoroquinolones and erythromycin (ERY) were investigated. Compounds that most effectively decreases the minimum inhibitory concentrations (MICs) of ciprofloxacin (CIP) were assayed for their dose and time effects on the accumulation and efflux of ethidium bromide (EtBr) in the SA-1 strain. None of the compounds displayed antibacterial activity however, three derivatives carrying 2-amino, 2-aminoacetyl and 2-aminotrifluoroacetyl group enhanced the activity of CIP and ERY by 8- and 16-fold, respectively, and were able to restore the sensitivity of resistant strains, acting as typical efflux pump inhibitors (EPIs). The 2-aminoacetyl and 2-aminotrifluoroacetyl derivatives and two other piperidinyl 2-aminotrifluoroacetyl derivatives increased EtBr accumulation in a dose- and time-dependent manner, and one of them was also able to inhibit the EtBr efflux. Taken together, these results represent an important advance in the development of new EPIs, and demonstrate that 2AT represent a good scaffold for developing new antibiotic adjuvants.

Introduction

The increase in the emergence of multidrug-resistant (MDR) bacteria that do not respond to conventional antibiotic therapy has been one of the main public health problems worldwide.[1,2] *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. constitute a set of bacterial MDR known as "ESKAPE" that are responsible for the nosocomial infections.[3] Some Gram-positive bacteria, including *S. aureus*, are known as "superbugs" because they present resistant to most known antibiotics, including vancomycin,[4] methicillin,[5] linezolid[6] and dalbavancin,[7] which were considered "the last line of defense."

The difficulties associated with treatment of bacterial infections are related to the substantial ability of these microorganisms to acquire resistance mechanisms including the following: enzymatic degradation that allows modification of the chemical structure of antimicrobials; the ability to form biofilms (a cluster of microorganisms surrounded by a glycocalyx); the replacement or modification of bacterial antibiotic targets; and the decrease in intracellular concentration of antibiotics that may be promoted by either decreasing bacterial envelope permeability or by increasing the activity and/or expression of energy-dependent (i.e., active) efflux systems.[8,9]

In *S. aureus*, the main resistance mechanisms to various classes of antibiotics are pump-mediated efflux mechanisms.[10–13] Efflux pumps are integral transmembrane proteins capable of extruding antimicrobials agents and dyes, among other toxic compounds out of the bacterial cell.[14] The efflux proteins are subdivided into five main families.[8,14–18]

Efflux pumps that export various structurally unrelated antimicrobials are known as multidrug resistance pumps (MDR pumps). Among the MDR pumps, NorA and MrsA, belonging to the MFS and RND families, respectively, play important roles in the intrinsic resistance and virulence of *S. aureus*.[19] In Gram-positive bacteria, NorA protein is the most efficient MDR system, affording resistance to a wide range of structurally diverse antibiotics, including quinolones, fluoroquinolones (norfloxacin and ciprofloxacin), pentamidine, berberine and dyes such as ethidium bromide and acridine.[20–23] MrsA protein confers resistance to macrolides such as erythromycin.[24]

Modulators of drug resistance are compounds capable of modulating and even reversing bacterial resistance to certain

[a] *Dr. R. M. D. da Cruz, Prof. F. J. B. Mendonça-Junior*
Department of Biological Sciences
State University of Paraiba, Laboratory of Synthesis and Drug Delivery, João Pessoa, PB, (Brazil)
E-mail: franciscojaime@ccbsa.uepb.edu.br
[b] *Dr. R. M. D. da Cruz, R. M. D. da Cruz, Prof. F. J. B. Mendonça-Junior*
Department of Pharmaceutical Sciences
Federal University of Paraiba, Post-Graduation Program in Natural and Synthetic Bioactive Products, João Pessoa, PB, (Brazil)
[c] *Dr. R. M. D. da Cruz, Dr. R. Zelli, Prof. J.-L. Décout*
Département de Pharmacochimie Moléculaire
University Grenoble Alpes, CNRS, Grenoble, (France)
[d] *Dr. R. M. D. da Cruz, S. Benshain, Prof. M.-P. Mingeot-Leclercq*
Department of Pharmacologie Cellulaire et Moléculaire
Université Catholique de Louvain, Louvain Drug Research Institute, Brussels, (Belgium)
[e] *Prof. J. P. Siqueira-Júnior*
Department of Molecular Biology
Federal University of Paraiba, Laboratory of Microorganism Genetics, João Pessoa/PB, (Brazil)

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antimicrobial agents. Some of these modulators are capable of re-establishing microbial susceptibility and improving the performance of antibiotics, acting as efflux pump inhibitors (EPIs).[25–27]

Because of the constant need for new compounds with antimicrobial properties, associated with little success in the discovery of new classes of antibiotics, researchers have been making significant efforts to synthesize or isolate new molecules capable of reversing bacterial resistance mechanisms. In this scenario, several EPI has been identified by screening of natural[28–30] and synthetic[8,13,19,31,32] molecules.

Among the synthetic compounds, thiophene derivatives have received attention from scientific community due to their ability to behave as EPIs, promoting inhibition of the NorA efflux pump, and re-establishing the activity of ciprofloxacin against *S. aureus* SA-1199B strain that overexpress the NorA efflux pump. In 2007, Chabert et al characterized several thiophenes as inhibitors of the NorA efflux protein in *S. aureus* 1199B. The most promising compounds that showed synergistic action with ciprofloxacin and inhibited the efflux of ethidium bromide (a substrate of the NorA pump) are summarized in Figure 1.[32] More recently, Liger et al. identified two thiophene derivatives that, at concentrations of 0.25–0.5 mg mL^{-1}, were able to decrease the minimum inhibitory concentration (MIC) of ciprofloxacin 16-fold (equivalent to reserpine) against the SA-1199B strain.[33]

In this context, the aim of the present study was to synthesize new 2-amino-thiophene derivatives and to evaluate their antibacterial activity, as well as their ability to decrease drug resistance in *S. aureus* strains by inhibition of NorA and MrsA efflux pumps.

First, the effects of the compounds on the MICs of ciprofloxacin, an efflux substrate of the NorA systems, and erythromycin an efflux substrate of the MrsA systems, were measured against *S. aureus* SA-1 and *S. aureus* RN-4220 strains, respectively. The choice of the SA-1 strain instead of the SA-1199B strain (the most commonly used strain in NorA efflux pump inhibition assays[33–35] was because the former strain overexpresses the NorA efflux pump without having other mutations such as mutations in topoisomerase IV (A116E GrlA) previously reported in SA-1199B.[36]

Then, compounds that resulted in the 4-fold decrease or more in the MIC of ciprofloxacin were assayed for their time-dose effects on the accumulation and on the efflux of ethidium bromide, a substrate of the NorA pump in the *S. aureus* SA-1 strain. Finally, the most active compounds were assayed for their cytotoxicity against macrophages.

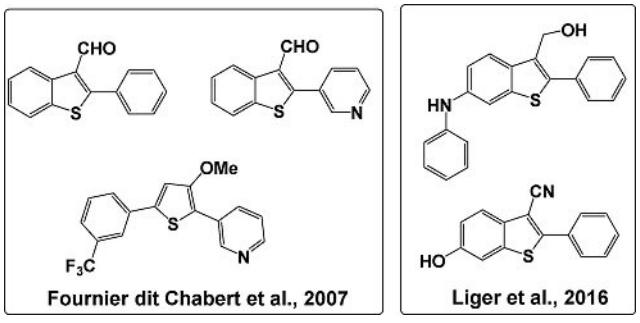


Figure 1. Reported inhibitors of NorA efflux pump of *S. aureus* 1199B strain.

Results and Discussion

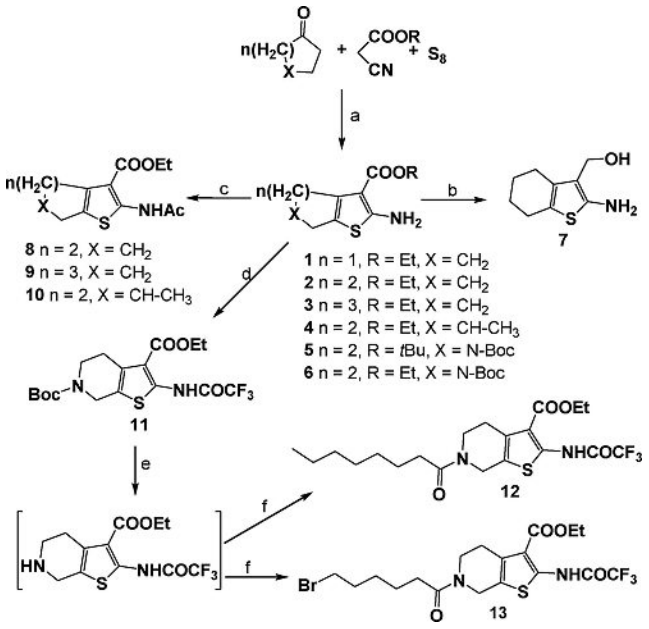
Chemistry

2-Amino-thiophene derivatives (**1–6**) were prepared using the Gewald-type reaction according to Scheme 1.[37,38]

Reduction of the ester of **2** with Na-metal furnished the alcohol **7** with 49% yield.

Compounds **2–4** were subjected to acetylation using acetic anhydride, resulting in compounds **8–10** in yields of 82 to 88%.

To obtain compounds **12** and **13**, initially the 2-amino group of the Gewald adduct was protected in reaction with trifluoroacetic anhydride, yielding compound **11** at 89.3%. Boc deprotection, followed by the reaction of the piperidine with octanoyl chloride (for **12**) and 6-bromohexanoyl chloride (for **13**), yielded compounds containing a lipophilic chain with 8.2% and 59.2% yields, respectively.



Scheme 1. Synthetic route for obtaining 2-aminothiophene derivatives (**1-13**): (a) Morpholine, ethanol, 50–60 °C, 2–3 h; (b) Na, sodium methoxide, methanol, 2 h; (c) acetic anhydride 1,4-dioxane, 80–90 °C, 3–4 h; (d) trifluoroacetic anhydride, Et_3N, CH_2Cl_2, 0–5 °C, 2 h; (e) CH_2Cl_2:TFA (8:2), 0–5 °C, 1 h; (f) DIPEA, acid chloride, CH_2Cl_2, 0–5 °C 1–2 h.

Biological assays

Determination of minimum inhibitory concentrations (MICs), and identification of the potential S. aureus *efflux proteins inhibitors*

The antimicrobial effects of the synthesized 2-aminothiophene derivatives (**1–13**) against *S. aureus*, were initially evaluated against three *S. aureus* strains: wild type *S. aureus* ATCC 25923, SA-1 (expressing NorA), and RN-4220 (expressing MrsA).

Initially the MICs of the reference antibiotics were determined in the presence and absence of compounds **1–13** and reserpine (used as positive control in ATCC and SA-1 strains)[39,40] (Table 1). Meanwhile, the new 2-aminothiophene derivatives synthesized (**1–13**) did not have any intrinsic antimicrobial activity, but some of them showed decrease of MICs when combined with ciprofloxacin or erythromycin on *S. aureus* expressing NorA and MrsA efflux pumps respectively, suggesting they are potential efflux pump inhibitors.

Among the potential inhibitor of efflux pumps of *S. aureus* SA-1 strains, those promoting 4- or 8- fold decrease in MIC of ciprofloxacin **(9, 11–13)** were selected for further study.

Ciprofloxacin and erythromycin displayed good activity against the ATCC 25923 strain, with the MIC values of <0.125 and 0.25 $\mu g\,mL^{-1}$, respectively. As expected, reserpine had no effect on the MIC of ciprofloxacin, as ATCC 25923 does not overexpress the NorA efflux pump.

For SA-1 and RN-4220 strains, the MICs of antibiotics increased, 32- and 1,024-times, respectively, when compared to the MICs obtained with the ATCC 25923 strain, reaching values at 4.0 and 256 $\mu g\,mL^{-1}$. These values are equivalent to those described in the literature,[41] suggesting that the strains are not sensitive to their respective reference antibiotics.

Table 1. MICs of the reference antibiotics against S. aureus strains ATCC 25923, SA-1 and RN-4220 in the presence and absence of reserpine and 2-aminothiophene derivatives. Values are expressed as $\mu g\,mL^{-1}$. Values in parentheses represent the fold of decrease MICs as compared to ciprofloxacin (S. aureus SA-1) or erythromycin (S. aureus RN-4220)

Compound	*S. aureus* strains				
	ATCC 25923	SA-1 (NorA)[a]	MIC decrease	RN-4220 (MrsA)	MIC decrease
Ciprofloxacin	<0.125	4.0		NT	
Erythromycin	0.25	NT		256	
Ciprofloxacin + Reserpine	<0.125	0.25	(**16**×)	NT	
Antibiotic + **1**	-	2.0	(2×)	256	(0)
Antibiotic + **2**	-	4.0	(0)	128	(2×)
Antibiotic + **3**	-	NT		64	(**4**×)
Antibiotic + **4**	-	NT		16	(**16**×)
Antibiotic + **5**	-	4.0	(0)	256	(0)
Antibiotic + **6**	-	NT		256	(0)
Antibiotic + **7**	-	NT		64	(**4**×)
Antibiotic + **8**	-	NT		256	(0)
Antibiotic + **9**	-	0.5	(**8**×)	256	(0)
Antibiotic + **10**	-	NT		64	(**4**×)
Antibiotic + **11**	-	1.0	(**4**×)	256	(0)
Antibiotic + **12**	-	1.0	(**4**×)	NT	
Antibiotic + **13**	-	0.5	(**8**×)	128	(2×)

[a] NT: not tested.

The inhibitory effect of reserpine on the NorA efflux pump, overexpresses in the *S. aureus* SA-1 strain, was confirmed as the combination of ciprofloxacin + reserpine resulted in a 16-fold reduction in the ciprofloxacin MIC (from 4 to 0.25 $\mu g\,mL^{-1}$). Gibbons and colleagues also observed an 8- to 16-fold reduction in MIC of fluoroquinolones (ciprofloxacin and norfloxacin) when associated with reserpine against the *S. aureus* SA-1199B strain.[40,42]

Regarding 2-aminothiophene derivatives, some of them caused modulation of antibiotic activity, reducing the amount of antibiotic needed to re-establish the sensitivity of the strains. These modulation values ranged from 2 to 16 times depending upon the strain, *S. aureus* RN-4220 or SA-1 (Table 1).

First, for *S. aureus* RN-4220 strain (that overexpresses the MrsA efflux pump), half of the evaluated compounds (six out of twelve) caused modulation and reversed the erythromycin resistance. The modulatory effect ranged from 2- to 16-fold, and compound **4** was the most active compound, reducing the MIC16-fold (from 256 to 16 $\mu g\,mL^{-1}$). Compounds **3**, **7** and **10** form the second group of compounds with best modulatory activity, promoting MIC reduction of 4-fold (from 256 to 64 $\mu g\,mL^{-1}$).

Compounds **2** and **13** were weakly active and decreased the MIC of erythromycin 2-fold (from 256 to 128 $\mu g\,mL^{-1}$).

Finally, compounds **1**, **5**, **6**, **8**, **9** and **11** were unable to reverse the *S. aureus* RN-4220 strain resistance to erythromycin; therefore, they are not potential inhibitors of the MrsA efflux pump.

A preliminary assessment of the relationship between chemical structure and modulatory activity of these compounds against the *S. aureus* RN-4220 strain shows that: the presence of the primary amine in C-2 (in compounds **3**, **4** and **7**) is important for the activity, as observed in 75% (3 out of 4) of the most active compounds (promoting MIC reductions from 4- to 16-fold). The only exception was compound **10**, which carries a *N*-acetylated amine. Compound **7** is an interesting compound because it is the only compound in the series in which the ester group in the C-3 position is reduced to alcohol function. The activity of this compound gives us indications that chemical modifications in C-3 are allowed, resulting in no inactive compounds.

In the series of compounds carrying an ethyl ester group and a free amino group (compounds **1–4**), **4** is the most active suggesting that the activity increases with the lipophilicity of the cycloalkyl moiety that increases from **1** to **3** and **4**. The presence of a methyl substituent in **4** also decreases 4-fold the MIC in comparison to compound **3** having a close lipophilicity. This result shows that not only the lipophilicity of the cycloalkyl moiety is involved in the modulating effect and the presence of a methyl group benefits to the activity.

Finally, the presence of cycloalkyl rings attached at the C-4/C-5 positions of the thiophene core, as observed with the most active compounds (**2**, **3**, **4**, **7** and **10**), appears to contribute positively to the activity, because, among the compounds with the best activity, only compound **13** (which has a C-4/C-5-substituted by a piperidine *N*-substituted) is an exception.

Second, for the *S. aureus* SA-1 strain, five of the seven evaluated compounds decreased the MIC of ciprofloxacin (ranging from 2- to 8-fold) suggesting an inhibitory effect on the NorA efflux pump.

The most active compounds, reducing the MIC 8-fold (from 4.0 to 0.5 $\mu g\,mL^{-1}$) were **9** and **13**. Compounds **11** and **12** also showed significant modulatory activity, reducing the MIC by 4-fold (from 4.0 to 1.0 $\mu g\,mL^{-1}$). The other compounds either showed no activity (**2** and **5**) or reduced without much significance (2-fold; **1**) the MIC of ciprofloxacin.

It is difficult to establish structure-activity relationships in the *S. aureus* SA-1 strain with the small number of compounds tested. For example, it appears that the inhibitory effect of the NorA efflux pump is observed only if the amine in the C-2 is substituted. Compounds **1**, **2** and **5**, with a free amino group at C-2 were inactive, while acylated (compounds **9**, **11**, **12** and **13**) were active. It is not possible to delineate a relationship between the structure of the ring fused to the thiophene ring (cyclohexane or substituted piperidine) and the modulatory activity. However, the main outcome of this study suggest interestingly that acylation of the 2-amino group is key to induce a modulator effect on the SA-1 strain and can be contrary to a modulator effect on the MrsA strain.

Comparing our results with the results obtained by Liger et al.,[33] and Chabert et al.,[32] We observed that all works report that most of the thiophenic derivatives did not exert intrinsic antibiotic activity against sensitive or resistant *S. aureus* strains. Chabert et al., who evaluated thiophene derivatives against *S. aureus* resistant to quinolone or macrolides also found that the active compounds are selective for only one of the strains analyzed, being able to promote a reduction in the MIC of the respective antibiotic at most 8 times (3 of 26 compounds). Liger et al. found that six of the 38 compounds analyzed against *S. aureus* SA-1199B strain displayed promising results as NorA efflux pump inhibitors, being able to reduce the ciprofloxacin MIC by at least 4 times (MIC $\leq 8\ mg\,mL^{-1}$).

Based on these results, compounds **9**, **11**, **12** and **13** were selected to evaluate their dose and time effects on accumulation, and on efflux of ethidium bromide (EtBr) in the *S. aureus* SA-1) strain.

Ethidium bromide accumulation assay

Ethidium bromide, a substrates of the NorA efflux pump, is a DNA intercalating agent that emits fluorescence when exposed to UV.[43] The greater the inhibiting power of a compound for the NorA efflux pump is, more EtBr will accumulate within the bacterial cell, and the greater will be the intensity of fluorescence measured.[32,33,44]

Reserpine, a known NorA efflux pump inhibitor,[39] was used as a positive control to evaluate and compare the inhibitory effect of the four selected new 2-aminothiophene derivatives (**9**, **11–13**) potential inhibitors of the NorA efflux pump expressed in *S. aureus* SA-1 strain.

First, to quantify the percentage of EtBr that remains inside the bacterial cell over time the percentage of EtBr accumulation over time was determined in the presence of increasing reserpine concentrations (Figure 2).

In the absence of reserpine, EtBr accumulation reached 49.9% of the maximum accumulation. With increasing concentrations of reserpine, accumulation occurred in a time- and dose-dependent manner. The maximum fluorescence value (100%) was observed when the bacteria were incubated 30 min with reserpine at 30 and 50 μM.

To evaluate the effects of the selected compounds (**9**, **11**, **12** and **13**) on EtBr accumulation in *S. aureus* SA-1 expressing NorA the following two experiments were performed.

The first compared the effects of the selected 2-aminothiophene derivatives and reserpine at a single concentration of 50 μM and time t = 30 min (Figure 3). The second established the dose and time effects on EtBr accumulation of the four compounds evaluated at increasing concentrations and times up to 30 minutes (Figure 4). The 100% accumulation was defined as the EtBr accumulation of reserpine (30 μM, time = 30 min).

In comparison to the effect induced by reserpine, compound **13** showed similar EtBr accumulation profile (Figure 3) suggesting the ability of **13** to inhibit NorA efflux pump of *S. aureus* SA-1 strain. The three other derivatives (**9**, **11**, **12** were

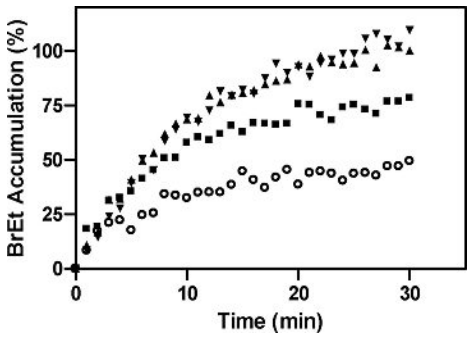


Figure 2. Accumulation of EtBr for 30 min in the presence and absence of increasing reserpine concentrations (○, 0 M; ■,10 M; ▲, 30 M; ▼, 50 M). Experiments were performed in triplicate.

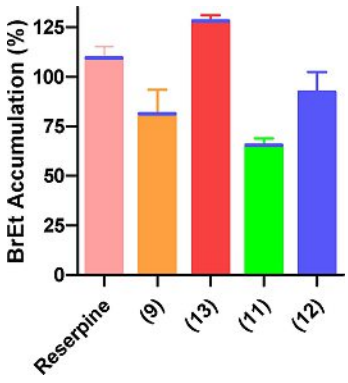


Figure 3. Comparison of EtBr accumulation percentages in the presence of 50 μM of reserpine and selected 2-aminothiophenes after 30 min. Experiments were performed in triplicate. Only **11** was significantly different as compared to reserpine with a certainty of 95% (p-value = 0.066).

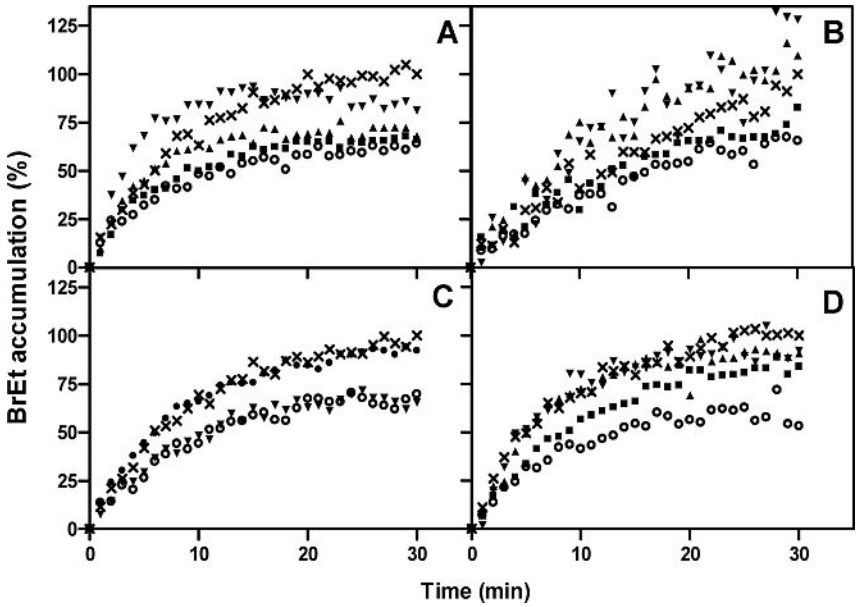


Figure 4. Accumulation of EtBr for 30 min in the presence and absence of increased concentrations of selected 2-amino-thiophene derivatives. A) **9**; B) **13**; C) **11**; D) **12**; (○, 0 M; ■,10 M; ▲, 30 M; ▼, 50 M; ♦, 75 M; ●, 100 M; **x**, reserpine). Experiments were performed in triplicate.

slightly less efficient than reserpine. (**12** > **9** > **11**) since the percentage of EtBr accumulated was slightly lower. Especially, compound **11** showed a significant lower effect, even higher concentrations were used (75 and 100 μM).

In the dose and time effects on EtBr accumulation assessment experiment (Figure 4) we found that, as observed with reserpine, all compounds promoted accumulation of EtBr in a time- and dose-dependent manner. In the absence of an inhibitor (0 μM), the percentage of accumulation of EtBr for the 4 molecules was between 50% and 60% of the maximum accumulation.

Around 15 min, a plateau was reached for all compounds in all concentrations, and at the highest concentration (50 μM) it was possible to classify the compounds according to their EtBr accumulation powers: **13** > **12** > **9** > **11**.

Ethidium bromide efflux assay

After observing the effects of the selected 2-aminothiophene derivatives on EtBr accumulation, we investigated their ability to inhibit EtBr efflux over time (up to 30 minutes), and in increasing concentrations (from 0 to 50 μM for **9**, **12** and **13**, and from 0 to 100 μM for **11**). Reserpine (50 μM) was used as a positive control (Figure 5). If the compounds are capable of inhibiting the NorA efflux pump, they will prevent EtBr efflux. EtBr will remain in the bacteria, and will emit fluorescence whose intensity will be stable over time. On the other hand, if the compounds do not inhibit the efflux pump, the fluorescence intensity will decrease over time, due to EtBr extrusion by the NorA efflux pump.[32,35]

As illustrated in Figure 5, in the absence of an inhibitor (0 μM), the efflux of EtBr gradually increases over time, reaching maximum values around 60% after 30 minutes for all compounds evaluated. The positive control (reserpine at 50 μM) effectively inhibited EtBr efflux and maintained fluorescence values more or less stable and constant over time, with values between 90% and 100%.

As observed in the EtBr accumulation assay (around 15 minutes) an efflux stabilization (plateau) was observed for all molecules at all concentrations. Even with increasing concentrations, compounds **9**, **11** and **13** did maintain fluorescence values associated with EtBr accumulation/efflux.

Among the evaluated compounds, **12** was the only one that inhibited the NorA efflux pump of strain SA-1, and prevented EtBr efflux in a dose-dependent fashion. Fluorescence values observed after 30 minutes were around 75% at concentrations of 10 and 30 μM and around 85% at the highest concentration evaluated (50 μM).

Cytotoxicity

To evaluate the cytotoxicity of the most active compounds against both *S. aureus* strains, murine J774 macrophage cells were subjected to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[45,46]

Compounds were evaluated at concentrations of 10 and 30 μM (exception for compound **11** that was tested at 75 and 100 μM). The results are expressed as percentage of viability (Table 2).

The great majority of the compounds presented moderate cytotoxicity at both evaluated concentrations, presenting a reduction the cell viability between 20% and 40%. Compound **11** showed high cytotoxicity (>90%), but it was evaluated at higher concentrations (75 and 100 μM) because these were the

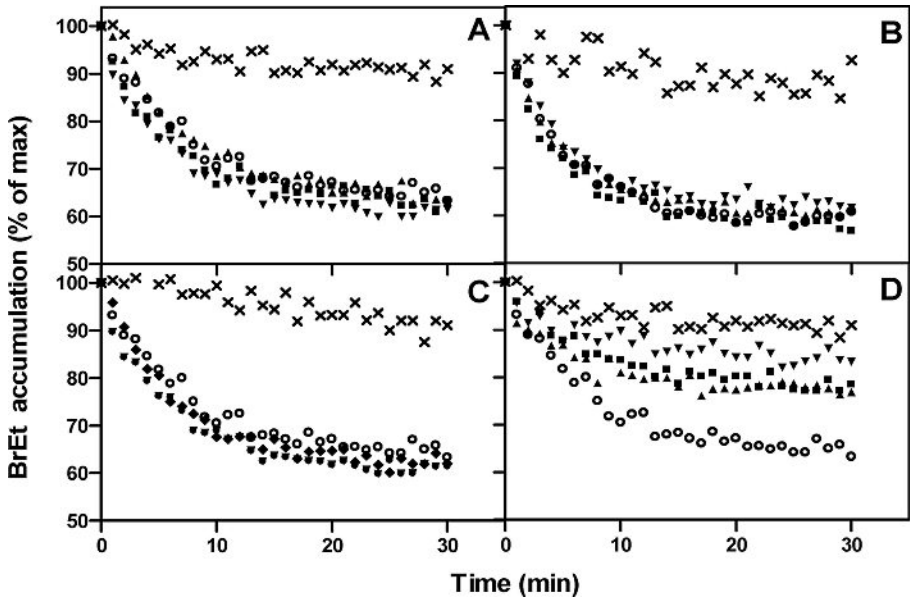


Figure 5. Efflux of EtBr for 30 min in the presence and absence of increased concentrations of selected 2-amino-thiophene derivatives. A) **9**; B) **13**; C) **11**; D) **12**; (○, 0 M; ■,10 M; ▲, 30 M; ▼, 50 M; ♦, 75 M; ●, 100 M; **x**, reserpine). Experiments were performed in triplicate.

Table 2. Cellular viability of the most active compounds against J774 macrophages.

Compound	Cell viability [%] 10 μM	30 μM
1	76	82
2	64	72
3	76	66
4	80	70
7	78	75
8	66	70
9	79	71
10	82	74
11[a]	6[#]	6[##]
12	82	97
13	34	31

[a] For compound **11**, cytotoxicity was evaluated at [#]75 and [##]100 μM.

concentration values at which it has an effect on the accumulation of ethidium bromide. A biochemical oxidation of the *N*-Boc group and/or its removal to lead to the piperidine derivative could induce such a cytotoxicity in comparison to compound **12**. The lack of the bromine atom in **12** in comparison to compound **13** probably explains the observed decrease of the cytotoxicity.

Conclusions

We synthesized new 2-amino-thiophene derivatives (**1**–**13**) and investigated their inhibitory effects on efflux proteins of *S. aureus* strains that exhibit resistance to fluoroquinolones (SA-1) and erythromycin (RN-4220) by overexpressing the efflux pump coding genes. Compounds **9** and **13** enhanced the activity of ciprofloxacin on *S. aureus* SA-1 by 8- fold, and compound **4** the activity of erythromycin on *S. aureus* RN-4220 strain by a 16-fold suggesting their capacity to restore the sensitivity of resistant strains, acting like typical EPIs. Interestingly, compound **13** exhibited inhibition of the two efflux pumps (even the effect on MrsA was low) and would be therefore able to reverse ciprofloxacin and erythromycin resistance. This compound would be very interesting in cases of infections caused by multi-resistant *S. aureus* strains; however, the cost-benefit ratio of using this compound as an antibiotic adjuvant needs to be considered due its potential toxic effects.

Regarding the MrsA, importantly, compounds capable of reversing bacterial resistance to erythromycin by MrsA efflux pump inhibition are not commonly reported. In the literature, the only compounds identified that are capable of modulating erythromycin activity against the RN-4220 strain by reducing MIC by at least 8-fold were three natural products: totarol[47] and carnosic acid[41] that promoted 8-fold potentiation; and the diterpene (4*S*, 9*R*, 14*S*)-4α-acetoxy-9β,14α-dihydroxydolasta-1(15),7-diene isolated from *C. cervicornis*[28] that promoted 16-fold potentiation. Compounds **3**, **4**, **7** and **10** in the present study demonstrated equipotent activity profiles with respect to these natural products. They can be considered as synthetic compounds with the greatest ability to reverse bacterial resistance to erythromycin by MrsA efflux pump inhibition.

Focusing on NorA and results obtained on EtBr accumulation/efflux, one intriguing question concerns the behavior of compounds **9**, **11** and **13** on the dose and time effects with respect to the accumulation and efflux of EtBr against the SA-1 strain. How or why are these compounds capable of increasing EtBr accumulation and not inhibiting its efflux?

Without having a concrete answer, we believe that, for these compounds, the balance of their concentrations and location (intracellular/membranous) and that of EtBr could

affect the accumulation/efflux of EtBr. The lipophilic alkyl part could favor an anchoring in the bacterial membrane and/or an interaction with specific recognition sites in NorA efflux pump. The binding sites involved for EtBr accumulation and efflux are probably different. Meanwhile, the lack of high resolution resolved crystallographic structures of the NorA pump in interaction with EtBr and inhibitors, making it difficult to test this hypothesis.

From a pharmacological point of view, although compound **12** was not the compound with the highest ciprofloxacin modulation (4-fold), it proved to be a very promising antibiotic adjuvant and NorA efflux pump inhibitor. It was the only compound with low toxicity associated with dose and time effects on the accumulation and efflux of EtBr.

Taken together, these results represent an important advance in the development of new EPIs, and demonstrate that 2-amino thiophene derivatives can be good candidates to be used in association with antibiotics against resistant *S. aureus* strains.

Experimental Section

Solvents and reagents: All reagents and solvents were provided by Sigma-Aldrich, Alfa-Aesar, TCI and Fisher Scientific and were used without further purification.

Analytical equipment: NMR spectra were recorded with a Bruker Ultrashield® and Bruker Ultrashield Plus® apparatus (^{1}H NMR: 400 MHz; ^{13}C NMR: 100 MHz; ^{19}F NMR 376 MHz) in $DMSOd_6$ and $CDCl_3$ as solvents. Chemical shifts (δ) are reported in parts per million (ppm) with tetramethyl silane (TMS) as internal standard. The multiplicities of the signals are described as: s-singlet, d-doublet, dd-doublet of doublets, ddd-double double doublet, t-triplet, q-quartet, m-multiplet. HRMS was performed with a LC-QTOF maXIs (Bruker) by using electrospray ionization in positive ionization mode.

Chromatographic analysis: Thin layer chromatography (TLC) plates (Merck® 60 F254 aluminum silica gel plate) were used for reaction monitoring. They were revealed by ultraviolet light (254 or 365 nm) or ninhydrin solution. To separate the compounds, we used the Reveleris® flash chromatography system and classic column chromatography.

Chemical procedures

Gewald's reaction (1-5): Compounds **1–4** were previously synthesized using Gewald-type reaction.[37,38] All compounds were synthesized by reacting ketone (1 eq.), alkyl cyanoacetate (1 eq.) and sulfur (1 eq.) that were dissolved in the reaction solvent (ethanol) in an ice bath. Finally, morpholine (1.3 eq.) was slowly added to the system (drop-wise) in an ice bath. The procedure was performed in a round bottom flask with stirring. After dripping was complete, the reaction proceeded at 50–60 °C for 3–4 h with stirring. After the confirmation of the end of the reaction by TLC (hexane/ethyl acetate (8:2)), the precipitate formed was filtered and washed with ice-cold ethanol. Some compounds were purified by column chromatography through classical systems with various proportions of hexane/ethyl acetate.

Acetylation reaction: Acetylated 2-aminothiophene compounds (**8–10**) were prepared in a round bottom flask with stirring where we added 2-aminothiophene compounds **1–4** (200 mg), 1,4-dioxane (3 mL) with excess of acetic anhydride (2 mL). The mixture was heated in reflux overnight. After the confirmation of the end of the reaction by TLC (hexane/ethyl acetate (8:2)), crushed ice was added to the reaction medium. The precipitate formed was filtered and washed 3 times with water.

Ester's reduction reaction: The reduced 2-aminothiophene compound (**7**) was prepared in a round bottom flask with stirring where we added compound **2** (0.1 g) dissolved in 3 mL of sodium methoxide solution (30% in methanol). A total of 0.3 g of metallic sodium was added and the reactional mixture was stirred at room temperature for 2 h. After the confirmation of the end of the reaction by TLC (hexane/ethyl acetate (8:2)), the reaction was treated with HCl 1 M and water. The aqueous phase was extracted three times with ethyl acetate. The organic phase was dried over sodium sulfate ($NaSO_4$). The solvent was evaporated under reduced pressure and purified by column chromatography (chloroform/methanol (9.5:0.5)).

Trifluoroacetylation: Compound **11** was prepared in inert atmosphere, in a round bottom flask with stirring where was added compound **6** (1 eq, 30.6 mmol) and triethylamine (2 eq, 61.2 mmol) that were dissolved in dichloromethane under an ice bath. To this mixture was had added trifluoro acetic anhydride (1.5 eq, 46 mmol) dropwise. Subsequently, the reaction was maintained at room temperature for 2 h. After the confirmation of the end of the reaction by TLC (hexane/ethyl acetate (8:2)), water was added and the product was extracted 3 times with ethyl acetate. The organic phase was dried over sodium sulfate ($NaSO_4$). The solvent was evaporated under reduced pressure, and the resulting powder was filtered and washed with ice cold ethanol.

Deprotection: The unprotection of the Boc group from compound **11** was performed in a round bottom flask with stirring in an ice bath under an inert atmosphere for 1 h, dissolving (500 mg of **11**) in dichloromethane: trifluoroacetic acid 80:20% solution (5 mL). After the confirmation of the end of the reaction by TLC (dichloromethane/methanol (9.5:0.5)), dichloromethane was added and concentrated on a rotary-evaporator, without drying the solvent completely. The product was used in the subsequent reactions without prior purification.

***N*-Alkylation reaction**: Compounds **12** and **13** were prepared by reacting the unprotected compound (**11**) (1 eq., 15.5 mmol) dissolved in dichloromethane (10 mL) and *N*,*N*-diisopropylethylamine (DIPEA) (2.5 eq., 38.7 mmol) in a round bottom flask with stirring at ice bath under an inert atmosphere. To these mixtures we added (2.5 eq., 38.7 mmol) to 6-bromo hexanoyl chloride (for **13**) or octanoyl chloride (for **12**). After addition of all reagents, the reaction proceeded at room temperature for 17 h. After the confirmation of the end of the reaction by TLC (dichloromethane: methanol (10:0.1), the reaction was concentrated at reduced pressure. The powder formed was filtered off, and washed 3 times with cold ethanol.

Compounds

2-Amino-4,5-dihydrothieno[2,3-*c*]pyridine-3,6(7H)-dicarboxylic ester di-tert-butyl ester (5): $C_{17}H_{26}N_2O_4S$. MM: 354.46. Appearance: light yellow powder. Yield: 76%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.88 (bs, 2H) 4.27 (s, 2H), 3.53 (s, 2H), 2.77 (s, 2H), 1.47 (s, 9H), 1.40 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.3, 161.7, 154.7, 131.6, 113.6, 106.7, 80.5, 80.0, 42.9, 42.3, 40.7, 28.6, 28.5, 27.4. HRMS (ESI), calcd for $[M+H]^+$: 355.1613; found $[M+H]^+$: 355.1687.

2-Amino-4,7-dihydro-5H-thieno[2,3-*c*]pyridine-3,6-dicarboxylic-6-tert-butyl tert-butyl ester (6): $C_{15}H_{22}N_2O_4S$. MM: 326.41. Appearance: light white powder. Yield: 88%. ^{1}H NMR 400 MHz, $CDCl_3$) δ

5.99 (s, 2H), 4.35 (s, 2H), 4.26 (q, J=7.1 Hz, 2H), 3.61 (t, J=5.8 Hz, 2H), 2.80 (s, 2H), 1.48 (s, 9H), 1.34 (t, J=7.1 Hz, 3H). HRMS (ESI), calcd for $[M+H]^+$: 327.1300; found $[M+H]^+$: 327.1375.

2-amino-4,5,6,7-tetrahydrobenzo*[b]*thiophene-3-methanol (7): $C_9H_{13}NOS$. MM: 183.26. Appearance: light brown powder. Yield: 49%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.76 (s, 2H), 2.60–2.69 (m, 2H), 2.41-2.44 (m, 2H), 1.65–1.73 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.5, 161.8, 132.4, 117.7, 105.7, 50.6, 26.9, 24.6, 23.3, 22.8. HRMS (ESI), calcd for $[(M\text{-}4H)+H]^+$: 180.0405; found $[(M\text{-}4H)+H]^+$: 180.0479.

2-Acetamido-4,5,6,7-tetrahydro-4H-benzo*[b]*thiophene-3-carboxylic acid ethyl ester (8): $C_{13}H_{17}NO_3S$. MM: 267.34. Appearance: white powder. Yield: 87%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.32 (q, J=7.1 Hz, 2H), 2.80–2.71 (m, 2H), 2.67–2.61 (m, 2H), 2.25 (s, 3H), 1.84–1.73 (m, 4H), 1.38 (t, J=7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.0, 147.7, 130.8, 126.7, 60.5, 31.0, 26.5, 24.5, 23.8, 23.1, 22.9, 14.5. HRMS (ESI), calcd for $[M+H]^+$: 268.0929; found $[M+H]^+$: 268.1002.

2-Acetamido-5,6,7,8-tetrahydro-4H-cyclohepta*[b]*thiophene-3-carboxylic acid ethyl ester (9): $C_{14}H_{19}NO_3S$. MM: 281.36. Appearance: brown powder. Yield: 82%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.08 (bs, 1H), 4.27 (q, J=7.1 Hz, 2H), 2.94–2.97 (m, 2H), 2.63–2.65 (m, 2H), 2.16 (s, 3H), 1.74–1.78 (m, 2H), 1.51–1.61 (m, 4H), 1.32 (t, J=7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.9, 166.7, 145.6, 136.3, 130.9, 112.7, 60.7, 32.2, 28.6, 28.3, 27.8, 27.0, 23.7, 14.3. HRMS (ESI), calcd for $[M+H]^+$: 282.1086; found $[M+H]^+$: 282.1157.

2-Acetamido-6-methyl-4,5,6,7-tetrahydrobenzo*[b]*thiophene-3-carboxylic acid ethyl ester (10): $C_{14}H_{19}NO_3S$. MM: 281.36. Appearance: light yellow powder. Yield: 88.3%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.17 (bs, 1H), 4.25 (q, J=7.2 Hz, 2H), 2.83–2.88 (m, 1H), 2.54–2.66 (m, 2H), 2.19 (s, 3H), 2.14–2.21 (m, 1H), 1.77–1.82 (m, 2H), 1.55 (bs, 1H), 1.31 (t, J=7.2 Hz, 3H), 1.00 (d, J=6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.9, 166.7, 147.8, 130.4, 126.4, 111.2, 60.5, 32.5, 31.2, 29.3, 26.2, 23.7, 21.4, 14.4. HRMS (ESI), calcd for $[M+H]^+$: 282.1086; found $[M+H]^+$: 282.1157.

2-(2,2,2-Trifluoro-acetylamino)-4,7-dihydro-5H-thieno[2,3-*c*] pyridine-3,6-dicarboxylic-6-tert-butyl ethyl ester (11): $C_{17}H_{21}F_3N_2O_5S$. MM: 422.41. Appearance: yellow powder. Yield: 89.3%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.19 (bs, 1H), 4.47 (s, 2H), 4.32 (q, J=7.2 Hz, 2H), 3.60 (t, J=6.0 Hz, 2H), 2.83 (bs, 2H), 1.41 (s, 9H), 1.33 (t, J=7.2 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.9, 163.8, 1554.5, 144.8, 116.9, 114.4, 144.1, 80.4, 61.5, 42.9, 40.4, 28.5, 26.5, 26.4, 14.2. ^{19}F NMR (376 MHz, $CDCl_3$) δ −75.39 (s). HRMS (ESI), calcd for $[M+Na]^+$: 445.1021; found $[M+Na]^+$: 445.1017.

6-Octanoyl-2-(2,2,2-trifluoroacetamido)-4,5,6,7-tetrahydrothieno [2,3-*c*]pyridine-3-carboxylic ethyl ester (12): $C_{20}H_{27}F_3N_2O_4S$. MM: 448.50. Appearance: yellow powder. Yield: 8.2%. ^{1}H NMR (400 MHz, DMSO) δ 11.87 (bs, 1H), 4.60 (bs, 2H), 4.36 (q, J=7.2 Hz, 2H); 3.74 (t, J=5.6 Hz, 2H); 2.88 bs (2H); 2.33–2.42 (m, 4H); 1.54–1.58 (m, 2H); 1.35 (t, J=7.2 Hz, 3H), 1.28–1.35 (m, 8H); 0.86–0.88 (m, 2H). ^{19}F NMR (376 MHz, $CDCl_3$) δ −75.39 (s). HRMS (ESI), calcd for $[M+H]^+$: 449.1644; found $[M+H]^+$: 449.1717.

6-(6-Bromohexanoyl)-2-(2,2,2-trifluoro-acetylamino)-4,5,6,7-tetrahydro-thieno [2,3-*c*] pyridine-3-carboxylic ethyl ester (13): $C_{18}H_{22}BrF_3N_2O_4S$. MM: 499.34. Appearance: white powder. Yield: 59.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.29 (s, 0.5H), 12.20 (s, 0.5H), 4.65 (s, 1H), 4.52 (s, 1H), 4.25–4.35 (m, 2H), 3.79 (t, J=5.6 Hz, 1H), 3.64 (t, J=5.6 Hz, 1H), 3.32–3.37 (m, 2H), 2.84–2.91 (m, 2H), 2.25–2.39 (m, 2H), 1.80–1.86 (m, 2H), 1.59–1.67 (m, 2H), 1.34 (t, J=7.6 Hz, 3H), 1.19–1.25 (m, 2H). ^{1}H NMR (400 MHz, DMSO) δ 11.88 (s, 1H), 4.67 (s, 1H), 4.36 (q, J=7.1 Hz, 1H), 3.74 (t, J=5.9 Hz, 1H), 3.62 (t, J=6.6 Hz, 1H), 2.89 (s, 1H), 2.43 (t, J=7.2 Hz, 1H), 1.80 (ddd, J=28.0, 14.3, 6.9 Hz, 1H), 1.63–1.55 (m, 1H), 1.46 (dd, J=14.9, 7.9 Hz, 1H), 1.35 (t, J=7.1 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 165.7, 145.0, 129.9, 126.3, 124.6, 114.0, 61.6, 44.2, 42.9, 40.8, 39.3, 33.7, 33.1, 32.6, 28.0, 27.2, 26.2, 24.3, 14.3. ^{19}F NMR (376 MHz, $CDCl_3$) δ −75.38 (s). HRMS (ESI), calcd for $[M+H]^+$: 499.0436; found $[M+H]^+$: 499.0509.

Biological assays

Bacterial strains: *S. aureus* strain used were as follows: wild type *S. aureus* ATCC 25923; *S. aureus* SA-1 (which overexpresses the NorA gene encoding the NorA efflux protein and confers resistance to fluoroquinolones), and *S. aureus* RN-4220 (which overexpresses the MrsA efflux pump and confers resistance to macrolides). *S. aureus* strain SA-1 was obtained in the Louvain Drug Research Institute, UCL-Belgium by culturing the *S. aureus* strain ATCC 25923 with increasing concentrations of EtBr. Prior to use, the cells were grown overnight at 37 °C in tryptone soy agar (TSA) medium. The *S. aureus* RN-4220 strain was provided by professor Simon Gibbons (University of London), and was maintained on blood agar base slants (Laboratory Difco Ltda., Brazil). Prior to use, the cells were grown overnight at 37 °C in brain heart infusion broth (BHI-Laboratory Difco Ltda., Brazil).

Antibiotics, nucleic-acid binding, inhibitors and compounds: All antibiotics were prepared according to Clinical and Laboratory Standards Institute guidelines,[48] and were purchased from Sigma-Aldrich Co. Stock solution of erythromycin at 2,056 μg mL^{-1} (8x concentrated) was prepared in sterile dimethyl sulfoxide (DMSO). Stock solution of ciprofloxacin at 128 μg mL^{-1} (8 times higher than the highest concentration to be evaluated) was prepared in Muller Hinton broth cation adjusted medium. The stock solution of reserpine at 10 mg mL^{-1} was prepared in sterile DMSO. The stock solution of ethidium bromide (EtBr) at 10 μg mL^{-1} was prepared in sterile water. The stock solutions of the 2-amino-thiophenes (compounds **1–13**) were prepared in sterile DMSO solutions where, at its highest final concentration after dilution in broth (4%), no bacterial growth inhibition occurred.[49]

Determination of MICs, and identification of the potential *S. aureus* efflux proteins inhibitors: The MICs of antibiotics and efflux pump inhibitors (reserpine and 2-amino-thiophene derivatives (**1–13**)) were determined by microdilution assay. *S. aureus* SA-1 and *S. aureus* ATCC 25923 strains were inoculated in TSA medium, and the *S. aureus* RN-4220 strain was inoculated in BHI broth, and incubated overnight at 37 °C. For inoculum preparation, a small sample of each bacterium was taken from the medium and placed in a McFarland (McF) tube containing 3 mL of sterile phosphate buffered saline (PBS). The bacterial suspension was adjusted to 0.5 McF turbidity (+/−10^8 bacteria mL^{-1}). This suspension was diluted in MHB CA medium to obtain an inoculum of 10^6 bacteria mL^{-1}. In sterile 96-well plates we added the following: in columns 1 to 11 (positive control) 50 μL of sterile MHB CA medium; and in column 12, (negative control) 200 μL of MHB CA medium.

For MIC determination, 50 μl of antibiotics (ciprofloxacin for SA-1 strain and for ATCC 25923, and erythromycin for RN-4220 strain and for ATCC 25923) stock solution (8x concentrated) were added to the wells of column 1 and two-fold serial dilutions were made in columns 1 to 10. Then 50 μl of a 4x concentrated solution of reserpine (a known NorA efflux pump inhibitor) and/or of a 4x concentrated solution of the potential NorA efflux pump inhibitors (compounds **1–13**) were added to columns 1 to 11. Column 11 served as a positive control to verify that bacterial strains grow normally in the absence of antibiotics but in the presence of compounds **1–13**. Finally, 100 μL of each bacterial inoculum were added to columns 1 to 11. The plates were incubated at 37 °C for 20 h and were read with the naked eye using a mirror. MIC was

defined as the lowest concentration at which no microbial growth was observed. All experiments were carried out at least twice with consistent results.

Ethidium bromide accumulation assays in the presence of a potentially NorA efflux pump inhibitor: To obtain an EtBr/*S. aureus* suspension, *S. aureus* SA-1 strain was grown in MHB CA medium overnight at 37 °C. The bacterial broth was centrifuged for 7 minutes at 4000 rpm in a centrifuge (Eppendorf-Centrifuge 5810R). The supernatant was discarded and the pellet resuspended in the "accumulation/efflux" buffer (NaCl: 110 mM, NH_4Cl: 50 mM, KCl: 7 mM, Na_2HPO_4: 0,4 mM, $C_4H_{11}NO_3$: 52 mM and Glucose: 0.2%) to obtain a final solution with an optical density (O.D.) of 0.2 read at a wavelength of 550 nm in a spectrophotometer (Molecular Devices-SpectraMax M3).[39] EtBr was added to the bacterial suspension to a concentration of 5.07 μM (2 μg mL^{-1}).[39] In a sterile 24-well plate, we added solutions of compounds **9**, **12** and **13** (0, 5, 10, 20, 30, 40 and 50 μM), compound **11** (0, 5, 10, 20, 30, 40 and 50, 75 and 100 μM), and reserpine (30 μM) (positive control) diluted in the "accumulation/efflux" buffer.[33] At least, a volume of the EtBr/*S. aureus* suspension was added and adjusted to each well to obtain a final volume of 500 μL. With a spectrophotometer (Molecular Devices - SpectraMax M3), the fluorescence intensity was measured for 30 minutes. The excitation wavelength was 530 nm and the emission wavelength was 600 nm,[39] 100% corresponds to the fluorescence intensity obtained after incubation with reserpine (30 μM) for 30 minutes. All tests were performed in triplicate. Variance analysis was calculated using the GraphPad Prism program.

Ethidium bromide quantification efflux assays in the presence of a potentially NorA efflux pump inhibitor: The EtBr/*S. aureus* suspension was prepared in the same way as previously described in "EtBr accumulation assay". The differences were as follows: final solution with an O.D. of 0.6, and the suspension was preincubated 30 minutes before adding the solutions of the compounds. In sterile 24-well plates we added solutions of compounds **9**, **12** and **13** (0, 5, 10, 20, 30, 40 and 50 μM), compound **11** (0, 5, 10, 20, 30, 40 and 50, 75 and 100 μM), and reserpine (50 μM) (positive control) diluted in the "accumulation/efflux" buffer.[33] A volume of the EtBr/*S. aureus* preincubated suspension was added and adjusted to each well to obtain a final volume of 500 μL. With a spectrophotometer (Molecular Devices-SpectraMax M3), the fluorescence intensity was measured for 30 minutes. The excitation wavelength was 530 nm and the emission wavelength was 600 nm,[36] 100% corresponds to the fluorescence intensity obtained after incubation with reserpine (50 μM) for 30 minutes. All tests were performed in triplicate.

Cytotoxicity: Murine J774 macrophage cells come from a mouse tumor cell line adhered to sterile 96-well plates at a concentration of 1×10^6 cell mL^{-1}. In RPMI medium, we prepared solutions of compounds **2**, **3**, **4**, **7**, **8**, **9**, **10**, **12** and **13** (10 and 30 μM), compound **11** (75 and 100 μM), and Triton X-100 (2%) (positive control). RPMI was used as the negative control. A total of 100 μL of the test compounds and controls solutions were added to the wells, and the plates were incubated for 24 h at 37 °C under CO_2 (5%). A total of 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution (0.5 mg mL^{-1} in PBS) were added to each well and the plates were incubated for 1 h at 37 °C under CO_2 (5%); 100 μL of sterile DMSO were added to each well and the plates were incubated for additional 10 min at 37 °C under CO_2 (5%). Absorbance was measured with a spectrophotometer (Molecular Devices-SpectraMax M3) at 570 nm. The percentage of cytotoxicity was calculated by comparing the absorbance difference of the negative control (A_{100}) and the sample (A_x) according to the following formula: Toxicity = $(A_{100}\text{-}A_x/A_{100})\times100$.[46] The percentage of cellular viability was calculated according to the following formula: Viability % = (100–toxicity).

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Conflict of Interest

The authors declare no conflict of interest.

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