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First Identification of Boronic Species as Novel Potential Inhibitors of the *Staphylococcus aureus* NorA Efflux Pump

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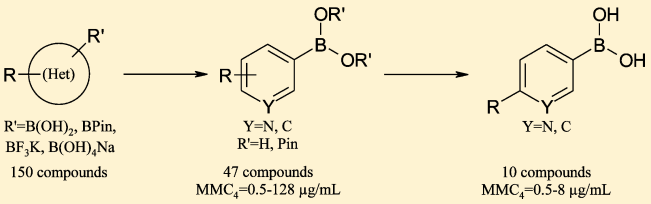
ABSTRACT: Overexpression of efflux pumps is an important mechanism of bacterial resistance that results in the extrusion of antimicrobial agents outside the bacterial cell. Inhibition of such pumps appears to be a promising strategy that could restore the potency of existing antibiotics. The NorA efflux pump of *Staphylococcus aureus* confers resistance to a wide range of unrelated substrates, such as hydrophilic fluoroquinolones, leading to a multidrug-resistance phenotype. In this work, approximately 150 heterocyclic boronic species were evaluated for their activity against susceptible and resistant strains of *S. aureus*. Twenty-four hit compounds, although inactive when tested alone, were found to potentiate ciprofloxacin activity by a 4-fold increase at concentrations ranging from 0.5 to 8 μg/mL against *S. aureus* 1199B, which overexpresses NorA. Boron-free analogues showed no biological activity, thus revealing that the boron atom is crucial for biological activity. This work describes the first reported efflux pump inhibitory activity of boronic acid derivatives.



INTRODUCTION

From the first therapeutic use of penicillin in 1941 to the advent of antibacterial drugs, each newly marketed antibiotic has invariably resulted in the emergence of resistant bacterial pathogens.[1,2] At first limited to one or few structurally related antibiotics, bacterial resistance then progressed toward cross-resistance between antibiotics that have unrelated scaffolds and different mechanisms of action, leading to multidrug-resistant (MDR) microorganisms.[3,4] The spread of those superbugs, which are resistant to almost all available antibiotics, is thus becoming a worldwide healthcare problem.[5−7] Bacteria have developed three main mechanisms of resistance: (i) enzymatic degradation and modification of antibiotics,[8] (ii) alteration or modification of the antibiotic target(s),[9] and (iii) reduction of intracellular antibiotic concentrations by reduced uptake and/or activation of the efflux.[10] These mechanisms can be simultaneously expressed within one single bacterial strain and are responsible for enhanced resistance.

Efflux-mediated drug resistance was first described in 1980 following the identification of tetracycline resistance in *Escherichia coli*.[11] This phenomenon results in the release of different substances outside the bacterial cell mediated by membrane transporters called efflux pumps[12,13] and is found in both Gram-positive[14] and Gram-negative bacteria.[15] The original physiological role of such pumps is to protect bacteria from environmental toxins as well as allowing them to secrete endogenous products.[16] Most efflux pumps are thus able to remove a broad spectrum of structurally unrelated substrates. As a collateral consequence of this self-defense mechanism, antibiotics that may be incidental substrates of efflux pumps are actively extruded from the bacterial cell. Intrinsic resistance to various drugs by active efflux is widespread among bacteria: transporters are expressed at a basal level, resulting in a low level of resistance.[17,18] However, the intensive use of antibiotics results in overexpression of efflux pumps for which antimicrobial agents are good substrates. In addition, while allowing bacteria to survive in the presence of sublethal drug concentrations, efflux pumps may in turn predispose the microorganisms to develop mutations in drug target genes.[19,20] Efflux-mediated resistance is thus a growing cause of concern because of its key role in the selection of high-level target-based resistance.[21]

The quest for new potent antibiotics remains the main strategy against drug resistance. In particular, research focuses on the structural pharmacomodulation of existing antibacterial classes to overcome microbial-resistance mechanisms[22] and on the development of antibiotics with novel mechanisms of action. Nonetheless, over the past 30 years, only two completely new

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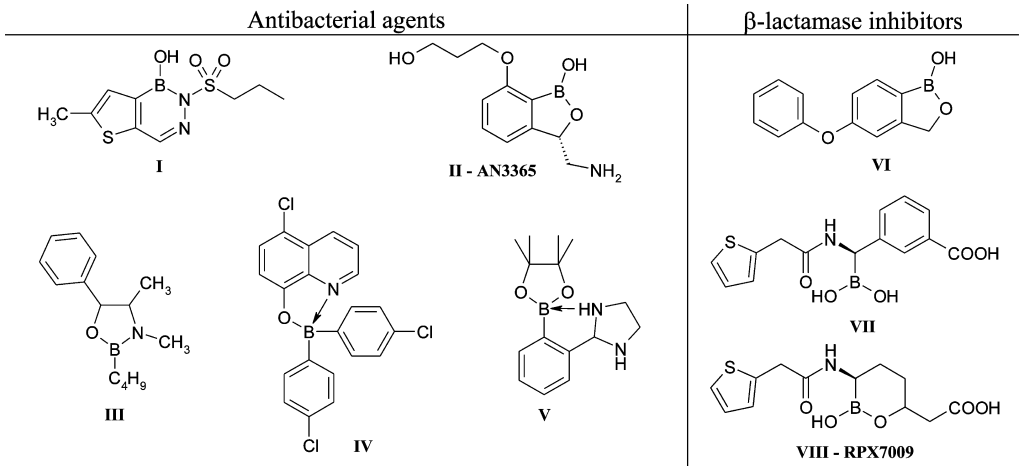

Figure 1. Antibacterial properties of organoboron compounds.

classes of compounds have been launched on the market: the oxazolidinone linezolid in 2000 and the lipopeptide daptomycin in 2003,[23] and resistance to these has already emerged.[24] New strategies are thus needed to fight resistant pathogens.[25] Recently, research has been moving toward another path: the fight against resistance mechanisms, particularly the inhibition of efflux pumps, which appears to be a promising approach that could restore the potency of existing antibiotics. This strategy is based on the combination between an efflux pump inhibitor (EPI) and the active antibiotic agent, preventing its release from bacteria and therefore increasing its intrabacterial concentration.[26] This approach is similar to the association between a *β*-lactam antibiotic and a *β*-lactamase inhibitor for which examples are already used in the clinic or are undergoing clinical development.[27] Therefore, EPIs are expected to increase the potency of antibiotics and to expand their spectrum of activity. Moreover, as efflux pump are involved in the selection of high-level resistant strains, such inhibitors may have enhanced clinical benefits in reducing the rate of resistance development.[28]

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are of particular concern among resistant microorganisms because they are responsible for numerous community- and hospital-acquired infections.[29] Although MRSA strains are characterized by the presence of the *mecA* gene that confers resistance to *β*-lactam antibiotics, these organisms also have the ability to acquire resistance to several antimicrobial agents such as fluoroquinolones, macrolides, aminoglycosides, and tetracyclines.[30] These cross resistances result from different mechanisms, of which the MsrA pump accounts for a significant part. The MsrA pump is an ABC transporter that uses energy derived from ATP hydrolysis to efflux antibacterial agents. A recent study revealed that the increased expression of at least one MDR efflux pump gene is frequently observed in *S. aureus* isolates and that MRSA predominates among those strains.

NorA-overexpressing strains have been shown to be the most common among MRSA strains.[31] NorA, a transporter of the major facilitator superfamily, catalyzes the antiport-coupled transport of the antibiotic with a proton gradient. Its overexpression confers resistance to a wide range of unrelated substrates such as hydrophilic fluoroquinolones (ciprofloxacin and norfloxacin but not lipophilic ones), various biocides (acriflavine, cetrimide, and benzalkonium chloride), and dyes (ethidium bromide).[32,33] In recent years, several NorA inhibitors have been identified.[34] The alkaloid reserpine, the first natural identified NorA EPI, is commonly used as a control in pump inhibitory assays, but it is not clinically relevant because of neurotoxicity at concentrations required for activity.[35] Other natural-based EPIs include flavonolignan and flavone compounds,[36,37] *N*-caffeoylphenalkylamide derivatives,[38] piperine and piperine analogues,[39] and polyacylated oligosaccharides.[40] Synthetic inhibitors are mainly based on heterocyclic scaffolds, such as indoles,[41] phenothiazines and thioxanthenes,[42,43] quinolines and quinolones,[44,45] and tricyclic compounds.[46] Considering the structural diversity of these compounds, we were hopeful to discover new classes of NorA EPIs.

For several years, our laboratory has focused on the synthesis and physicochemical study of various heterocyclic compounds bearing boronic acid or boronic ester functions.[47] Besides their key role in cross-coupling reactions, some boronic species have been described for their antimicrobial,[48] antineoplastic,[49] and enzyme-inhibitory activities.[50] Boronic acids are well-known as serine-protease inhibitors that act as transition-state analogues through nucleophilic attack of the active serine on the boron open shell. Of particular interest are the antibacterial properties of various organoboron compounds (Figure 1) such as diazaborines (**I**, enoyl acyl carrier protein reductase inhibitors),[51] benzoxaboroles (**II** (AN3365), leucyl-tRNA synthase inhibitors,[52] and **VI**, *β*-lactamases inhibitors),[53] oxazaborolidines (**III**),[54] borinic (**IV**)[55] and boronic (**V**) esters,[56] and boronic acids (**VII** and **VIII** (RPX7009), *β*-lactamases inhibitors).[57,58] This prompted us to investigate the potential biological properties of a series of boronic species prepared in our laboratory over the past decade.

In this article, we describe the first systematic antibacterial evaluation of heterocyclic boronic acids against *S. aureus* strains susceptible or resistant to quinolone or macrolide antibacterials by active efflux processes. In particular, these compounds were found to be a novel class of potential NorA efflux inhibitors that restores the antibacterial activity of ciprofloxacin against SA-1199B.

RESULTS AND DISCUSSION

Microbiological Assays. Compounds were investigated for their activity against the susceptible *S. aureus* ATCC25923 strain and two resistant *S. aureus* strains that express active efflux pumps. Between the latter, the *S. aureus* 1199B strain shows reduced susceptibility to fluoroquinolones through overexpression of the NorA efflux pump and also possesses a mutation in

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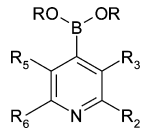
Table 1. Antibacterial Activity of Pyridine-4-boronic Compounds



				Activity (μg/mL) against Se[a] and Rt[b] *S. aureus*							
				R = H				R = pinacol			
R_2	R_3	R_5	R_6	Id.	Se MIC	Rt NorA MIC	Rt NorA MMC_4	Id.	Se MIC	Rt NorA MIC	Rt NorA MMC_4
H	H	H	H		N.D.			**2a**	-	-	>128
Cl	H	H	H	**1a**	-	-	>128	**2b**	-	-	128
F	H	H	H	**1b**	-	-	>128	**2c**	-	-	2
Me	H	H	H		N.D.			**2d**	-	-	>128
H	Br	H	H		N.D.			**2e**	-	-	>128
H	Cl	H	H		N.D.			**2f**	-	-	>128
H	F	H	H		N.D.			**2g**	-	-	>128
H	CN	H	H	**1c**	-	-	>128	**2h**	-	-	>128
Cl	Cl	H	H	**1d**	-	-	32	**2i**	-	-	32
F	Cl	H	H	**1e**	-	-	16	**2j**	-	-	16
Cl	CN	H	H	**1f**	-	-	>128	**2k**	-	-	>128
OMe	Cl	H	H	**1g**	-	-	32	**2l**	-	-	32
Cl	H	Cl	H	**1h**	-	-	64	**2m**	-	-	32-64
Ampicillin					0.25				0.25		
Ciprofloxacin						16				16	
Reserpine							4-8				4-8

[a]Se, susceptible *S. aureus* ATCC25923. [b]Rt, resistant *S. aureus* 1199B (NorA). –, inhibition of growth <90% at a concentration of 100 μg/mL. N.D., no data.

the gyrase subunit A. Ciprofloxacin has a MIC of 0.5 μg/mL against the parent susceptible *S. aureus* 1199 strain and of 16 μg/mL against the resistant clone. The second resistant strain was *S. aureus* RN4220, harboring the multicopy plasmid pUL 5054 that includes the *msr*(A) gene, conferring resistance to 14- and 15-membered macrolides through overexpression of the MsrA efflux pump. Erythromycin has a MIC of 0.5 and 128 μg/mL against the susceptible and resistant strains, respectively.

In each microbiological assay, all compounds were initially evaluated at a concentration of 100 μg/mL, and for those that inhibit more than 90% of the bacterial growth, the MIC was then determined. In a first screening assay, compounds were evaluated for their intrinsic antibacterial activity against both susceptible and resistant strains. Compounds that were inactive alone were then investigated for their ability to restore the potency of ciprofloxacin or erythromycin against the resistant SA-1199B and SA-RN4220 strains to identify potential NorA or MsrA efflux pump inhibitors. Therefore, compounds were evaluated in combination with a subinhibitory concentration of the antimicrobial agent: (i) MIC/4 (i.e., 4 μg/mL of ciprofloxacin) and (ii) MIC/8 (i.e., 16 μg/mL of erythromycin). Potentiating activities of inhibitors are reported as minimum modulatory concentration MMC_4 or MMC_8 values, which are the lowest concentration of the inhibitor required to achieve antibacterial activity in combination with corresponding subinhibitory concentrations of ciprofloxacin or erythromycin. Reserpine, which is a known inhibitor of the NorA efflux pump, was used as an internal standard and displayed a MMC_4 of 4–8 μg/mL.

Structure–Activity Relationships. A laboratory-made chemical library composed of approximately 150 various organoboron compounds, mainly heterocyclic boronic species as well as trifluoroborate salts, was tested. Because both boronic acids and esters display biological activity (Figure 1), we decided to evaluate both protected and unprotected boronic species. All compounds from the screening were originally prepared in our laboratory[47] or were a gift from BoroChem S.A.S., even if some of these are currently commercially available. The most significant biological results are reported in Tables 1–5. None of these compounds were significantly active when used alone against the susceptible ATCC25923 straing, with MICs ≥ 64 μg/mL. Except for **14d** and **14o–p**, all compounds were inactive when used alone against the fluoroquinolone-resistant 1199B strain. Phenylboronic acids **14d** and **14o–p** showed moderate intrinsic antibacterial activity against the 1199B strain, with MICs of 32 μg/mL. In contrast, all compounds, in combination with 4 μg/mL of ciprofloxacin, were found to inhibit more than 90% of the bacterial growth of the 1199B strain at the initial 100 μg/mL concentration. On the contrary, only compounds **14d** and **14o–p**, showing a moderate intrinsic antibacterial activity, were found to be active to the same extent (MMC_8 = 32 μg/mL, data not shown) against the macrolide-resistant RN4220 strain in combination with erythromycin. Differences in activity of the compounds between both efflux pumps may be due to their structural specificity: the NorA pump belongs to the MFS family and catalyzes the antiport-coupled transport of the antibiotic with a proton gradient,[59]

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and the MsrA pump is an ABC transporter which uses energy derived from ATP hydrolysis.[60]

Determination of the activity of compounds in combination with ciprofloxacin revealed the presence of 24 promising molecules with MMC_4 ranging from 0.5 to 8 μg/mL, half of which were pyridine-3-boronic derivatives (**3d**, **5a**−**b**, **5d**−**g**, **5i**−**j**, **7b**, **7d**, and **11a**) and seven were benzene boronic compounds (**14f**−**g**, **14i**, **14n**, and **14r**−**t**). Other active compounds included the pyridine-4-boronic acid pinacol ester **2c**, the thiophene-3-boronic acid **15b**, the pyrazole-5-boronic acid pinacol ester **15f**, and 2 indazole boronic derivatives **16b** and **16g**. The following discussion will refer to the MMC_4 of compounds against the SA-1199B strain with 4 μg/mL of ciprofloxacin.

Results are shown in Table 1 for pyridine-4-boronic compounds and in Table 2 for pyridine-3-boronic ones. The first structure−activity relationship (SAR) studies revealed that (i) pyridine-3-boronic acids displayed the best potentiating activity of ciprofloxacin with 11 compounds (**3d**, **5a**−**b**, **5d**−**g**, **5i**−**j**, **7b**, and **7d**) having MMC_4 ranging from 1 to 8 μg/mL; (ii) except for compounds **5a** and **11a** that had the same MMC_4, pyridine-3-boronic esters (**9a**−**b**, **11b**−**f**, **12a**−**b**, and **13a**) showed lower activities than their corresponding boronic acids (**3d**−**e**, **5b**−**c**, **5e**−**g**, **6a**−**b**, and **7b**), and inactive acid derivatives (**3h**−**i**, **6c**, and **7c**) led to inactive ester analogues (**9c**−**d**, **12c**, and **13b**); (iii) pyridine-4-boronic derivatives were mostly inactive except the fluorinated ester **2c**, which showed a surprisingly low MMC_4 of 2 μg/mL, and pyridine-4-boronic acids (**1d**−**e** and **1g**−**h**) and pyridine-4-boronic esters (**2i**−**j** and **2l**−**m**) displayed equivalent moderate activity; (iv) for active boronic acids (**5b**−**c** and **6a**), the shift of the boronic moiety from the C-3 to the C-4 position (**1a**−**b** and **1h**) resulted in a loss of activity; and (v) the trigonal boronic acid function of compound **3a** gave better results than the negatively charged tetrahedric potassium trifluoroborate (**8a**) or sodium boronate (**8b**) salts.

Considering the pyridine-3-boronic acids, the 6-substituted and 5,6-disubstituted derivatives appeared to be the most promising compounds except for compounds **5h** and **6c**, which were inactive. 6-Benzyloxypyridine-3-boronic acid **5f** displayed the highest activity and was able to potentiate ciprofloxacin by a 4-fold increase at a concentration as low as 1 μg/mL. SAR studies of the monosubstituted pyridine-3-boronic acids revealed that (i) apart from compound **3d**, 2-substituted-pyridine-3-boronic acids (**3b**−**c** and **3e**−**h**) showed a significative loss of activity compared to their corresponding 6-substituted derivatives (**5a**−**b** and **5d**−**g**); (ii) the 5-substituted **4b** was slightly less potent that its 6-substituted analogue **5j** but still exhibited interesting activity; and (iii) cyclisation at the C-5/C-6 positions (**7d**) or introduction of a methyl group at the C-5 position (**7a**−**b**) was well-tolerated.

The data reported in Table 3 reveal similar SAR for benzene boronic derivatives. Except for **14h**, which is inactive, benzene analogues of the active pyridine-3-boronic acids (compounds **14f**−**g**, **14i**, **14n**, and **14s** vs **5a**−**b**, **5f**, **7b**, and **7d**) displayed the best activities with MMC_4 ranging from 0.5 to 8 μg/mL. Along with **14r**, this revealed the importance of a substituent in the para position with respect to the boronic moiety, as was observed for pyridine-3-boronic derivatives. Cyclisation at the C-3/C-4 positions (**14s**−**t**) or introduction of a methyl group in the meta position of the benzene ring (**14n**) was also well-tolerated.

Biological results for five-membered heterocyclic and indazole boronic derivatives (Tables 4 and 5) did not allow for SAR to be clearly established. One thiophene (**15b**), one pyrrole (**15f**), and two indazole (**16b** and **16g**) compounds showed promising results, with MMC_4 of 8−16 μg/mL.

Chemistry. Once we identified pyridine-3- and benzene-boronic acids as promising NorA inhibitors, we sought to confirm the importance of the boronic acid moiety for biological activity on the resistant 1199B strain by testing analogues devoid of the boron atom. Biological activity of boronic acids relies on their ability to shift from a neutral and trigonal planar sp^2 configuration to an anionic tetrahedral sp^3 one through nucleophilic attack on the empty p-type orbital of boron. In this case, electrophilic functions, such as carboxylic acids and esters, aldehydes, silicate, phosphate, and α-keto acid derivatives are also able to act as protease inhibitors.[61] We chose to limit our study to the carboxylic acid analogues **19a**−**k** that are bioisosters of boronic acids and to their aldehyde counterparts, **20a**−**k**. Because some bacteria can perform hydroxydeboronation reactions that transform boronic acids into corresponding alcohols,[62] we were also interested in hydroxylated analogues **21a**−**k** and **22**. Chosen compounds for this investigation were pyridine-3-boronic acid (**3a**,), 6-bromo- (**5a**), 6-chloro- (**5b**), 6-benzyloxy- (**5f**), and 6-chloro-5-methylpyridine-3-boronic acids (**7b**), quinoline-3-boronic acid (**7d**), and their benzenic counterparts (**14a**, **14f**−**g**, **14n**, and **14s**) that gave the best results from the primary screening (Tables 2 and 3) and whose desired analogues were easily available either commercially or synthetically. In particular, all benzene derivatives were purchased from commercial sources.

We prepared carboxylic acids **19d**−**e** and aldehyde compounds **20d**−**e** from halogeno-pyridines **18d**−**e** using the general halogen−metal-exchange procedure developed in our laboratory for the pyridine series.[47a] The halogen−lithium exchange was carried out with *n*-BuLi in anhydrous ether at −78 °C, and the lithiated intermediate species were trapped either by carbon dioxide or dimethylformamide followed by appropriate hydrolysis. Specific attention must be given to the carboxylic acid workup because they tend to adopt a zwitterionic form because of the concomitant presence of the carboxylic acid and the pyridine functions. Thus, desired product with protonated carboxylic acid and free amine is only predominant in the aqueous phase in a narrow pH range that could be theoretically determined using the MarvinSketch protonation calculator. Optimized workup involved slow addition of a 4% aqueous NaOH solution to the reaction mixture, which allowed solubilization of the carboxylate salt in the aqueous layer while retaining organic byproducts in the ethereal phase. The resulting aqueous phase was then carefully acidified by 3 N HCl until complete precipitation of expected carboxylic acids **19d**−**e** were obtained in good yields (58−87%). Such a procedure was not necessary for aldehyde derivatives that are predominant at neutral pH, and hydrolysis followed by classical workup led to compounds **20d**−**e** in 55−78% yields (Scheme 1).

Then, we obtained hydroxypyridines **21d**−**f** in good yields (71−93%) from corresponding boronic acids using the hydrogen peroxide-mediated hydroxydeboronation described in our laboratory (Scheme 2).[63]

Finally, we prepared 6-benzyloxypyridine-3-methanol **22** in moderate yield (45%) through reduction of carboxylic acid **19d** in the presence of lithium aluminum hydride (LAH) in anhydrous THF (Scheme 3).

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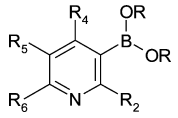
Table 2. Antibacterial Activity of Pyridine-3-boronic Compounds



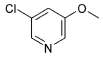
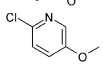
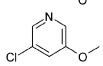
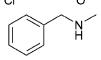
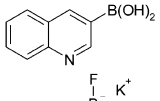
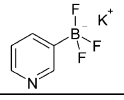
				Activity (μg/mL) against Se[a] and Rt[b] *S. aureus*							
				R = H				R = pinacol			
R_2	R_4	R_5	R_6	Id.	Se MIC	Rt NorA MIC	Rt NorA MMC_4	Id.	Se MIC	Rt NorA MIC	Rt NorA MMC_4
H	H	H	H	**3a**	-	-	32			N.D.	
Br	H	H	H	**3b**	-	-	>128			N.D.	
Cl	H	H	H	**3c**	-	-	>128			N.D.	
F	H	H	H	**3d**	-	-	8-16	**9a**	-	-	>128
OMe	H	H	H	**3e**	-	-	16	**9b**	-	-	>128
OEt	H	H	H	**3f**	-	-	>128			N.D.	
OBn	H	H	H	**3g**	-	-	32			N.D.	
SMe	H	H	H	**3h**	-	-	128->128	**9c**	-	-	>128
CN	H	H	H	**3i**	-	-	>128	**9d**	-	-	>128
H	H	Br	H			N.D.		**10a**	-	-	>128
H	H	Cl	H			N.D.		**10b**	-	128	>128
H	H		H	**4a**	64	>128	16-32			N.D.	
H	H		H	**4b**	-	-	16			N.D.	
H	H	H	Br	**5a**	-	-	8	**11a**	-	-	8
H	H	H	Cl	**5b**	-	-	8	**11b**	-	-	128
H	H	H	F	**5c**	-	-	32	**11c**	-	-	128
H	H	H	OMe	**5d**	-	-	4			N.D.	
H	H	H	OEt	**5e**	-	-	4-8	**11d**	-	-	128
H	H	H	OBn	**5f**	-	-	1	**11e**	-	-	64-128
H	H	H	SMe	**5g**	-	-	8	**11f**	-	-	>128
H	H	H		**5h**	-	-	>128			N.D.	
H	H	H		**5i**	-	-	4-8			N.D.	
H	H	H		**5j**	-	-	8			N.D.	
H	H	H				N.D.		**11g**	-	-	128
Cl	H	Cl	H	**6a**	-	>128	16	**12a**	-	128	>128
Cl	H	Br	H	**6b**	-	-	32-64	**12b**	-	-	128
F	H	Br	H	**6c**	-	>128	128	**12c**	-	128	64
H	H	Me	Br	**7a**	-	-	32			N.D.	
H	H	Me	Cl	**7b**	-	>128	4-8	**13a**	-	-	32
H	H	Me		**7c**	-	-	64-128	**13b**	-	-	32-64
				**7d**	-	-	4			N.D.	
				**8a**	-	-	128->128				

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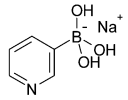
Table 2. continued

				Activity (μg/mL) against Se[a] and Rt[b] *S. aureus*							
				R = H				R = pinacol			
R_2	R_4	R_5	R_6	Id.	Se MIC	Rt NorA MIC	Rt NorA MMC_4	Id.	Se MIC	Rt NorA MIC	Rt NorA MMC_4
				**8b**	-	-	128				
		Ampicillin			0.25				0.25		
		Ciprofloxacin				16				16	
		Reserpine					4-8				4-8

[a]Se, susceptible *S. aureus* ATCC25923. [b]Rt, resistant *S. aureus* 1199B (NorA). −, inhibition of growth <90% at a concentration of 100 μg/mL. N.D., no data.

Biological evaluation of these compounds is reported in Table 6. The MIC of compound **22** is >128 μg/mL against SA-1199B, and its MMC_4 is of 64−128 μg/mL with ciprofloxacin. No derivatives showed any intrinsic antibacterial activity against the SA-1199B strain, with MICs ≥ 128 μg/mL, as well as in combination with 4 μg/mL of the fluoroquinolone (MMC_4 ≥ 32 μg/mL). These values are significantly higher than those displayed by the corresponding boronic acid derivatives (MMC_4 ≤ 8 μg/mL). These results highlight the requirement of the boron atom for biological activity, which cannot be simply replaced by carbon.

A survey of the required features for biological activity of pyridine-3- and benzene boronic acids is summarized in Figure 2.

Cytotoxic Assays. After the evaluation of the active molecules as antibacterial agents, their cytotoxicity was ascertained on KB cells at a concentration of 10 μM. KB cells are epithelial cells originated from an epidermal carcinoma of the mouth sensitive to a variety of structurally different agents and lacking the efflux pump P-gp (ABC B1). Docetaxel was used as positive control at its IC_{50} concentration (0.15 nM). The results are shown in Table 7 as the percent of cellular growth inhibition. Most molecules, including compounds **5f**, **14i**, and **14d**, inhibited less than 80% of the growth of KB cells and were thus considered as noncytotoxic. 6-Benzyloxypyridine-3-boronic acid **5f** and 4-benzyloxybenzene boronic acid **14i** were thus confirmed as hit compounds for their activity as potential inhibitors of the NorA efflux pump, and 2-phenoxybenzene boronic acid **14d**, for its antibacterial activity against the SA-1199B strain. 4-Formylbenzene boronic acids **14o** and **14p** were the most cytotoxic at 10 μM, and this may be correlated with the presence of the aldehyde moiety.

CONCLUSIONS

In this study, we conducted the first investigation on the antibacterial properties of a series of boronic derivatives against susceptible and resistant *S. aureus* strains. Starting from a chemical library of approximately 150 compounds, we identified 24 hit compounds for their ability to restore the activity of ciprofloxacin by a 4-fold increase at concentrations ranging from 0.5 to 8 μg/mL against the NorA-overexpressing SA-1199B strain. The first structure−activity relationship studies pointed out the necessity of the boron atom for activity as well as the presence of a substituent in the para position with respect to the boronic moiety. 6-Benzyloxypyridine-3-boronic acid **5f** and 4-benzyloxybenzene boronic acid **14i** were found to be the most promising NorA inhibitors: they potentiate the activity of ciprofloxacin with respective MMC_4 values of 1 and 0.5 μg/mL, they have no significant intrinsic antibacterial activity, and they show reduced cytotoxicity against human cell lines. Chemical pharmacomodulation of compound **5f** has been undertaken to design NorA inhibitors with improved potency and will be the subject of a future publication.

EXPERIMENTAL SECTION

Synthesis. All chemical products, reagents, and solvents were purchased from commercial sources and used without further purification except THF, which was distilled from Na/benzophenone. Chromatography was carried out on a column using flash silica gel 60 Merck (0.063−0.200 mm) as the stationary phase. The eluting solvent indicated for each purification was determined by thin-layer chromatography (TLC) performed on 0.2 mm precoated plates of silica gel $60F_{254}$ (Merck), and spots were visualized using a UV lamp. Melting points were determined on a Kofler melting-point apparatus. IR spectra were recorded on KBr disks using a PerkinElmer BX FT-IR spectrophotometer. The band positions are given in reciprocal centimeters (cm^{-1}). ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a JEOL Lambda 400 spectrometer. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a Bruker 500 Avance III spectrometer. Chemical shifts (δ) are expressed in parts per million downfield from tetramethylsilane as an internal standard, and coupling constants (J) are in hertz. The abbreviations used are as follows: s, singlet; bs, broad singlet; d, doublet; dd, double of doublets; t, triplet; and m, multiplet. The purities of all tested compounds were analyzed by LC−MS, with the purity of all compounds being higher than 95%. Analyses were performed using a Waters alliance 2695, and MS detection was performed with a SQDetector.

All compounds from the screening were originally prepared in our laboratory[47] or were a gift from BoroChem S.A.S., even if some of these are currently commercially available. All carbonylated and hydroxylated benzene analogues and non described pyridinyl ones (Table 6) were purchased from commercial sources and used as provided by the manufacturers without additional verification.

6-Benzyloxypyridine-3-carboxylic Acid (19d). To a slurry of 2.5 M of *n*-BuLi (1.89 mL, 4.73 mmol) in anhydrous diethyl ether (80 mL) cooled at −78 °C was added a solution of 2-benzyloxy-5-bromopyridine (1.0 g, 3.79 mmol) in anhydrous THF (10 mL). The mixture was allowed to react at this temperature over 60 min. A CO_2(g)-inflated balloon was bubbled in the reaction medium and

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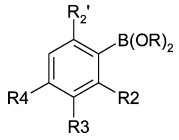
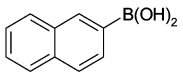
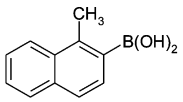
Table 3. Antibacterial Activity of Benzene Boronic Acid Compounds



						Activity (μg/mL) against Se[a] and Rt[b] *S. aureus*		
R	R_2	R_3	R_4	$R_{2'}$	Id.	Se MIC	Rt NorA MIC	Rt NorA MMC_4
H	H	H	H	H	**14a**	-	-	32
H	F	H	H	H	**14b**	-	-	16
H	Ph	H	H	H	**14c**	-	-	>128
H	OPh	H	H	H	**14d**	>128	32	32
H	COOEt	H	H	H	**14e**	-	-	>128
H	H	H	Br	H	**14f**	N.D.	>128	2-4
H	H	H	Cl	H	**14g**	N.D.	64	1-2
H	H	H	OMe	H	**14h**	N.D.	>128	64-128
H	H	H	OBn	H	**14i**	N.D.	>128	0.5
H	H	H	COOH	H	**14j**	-	-	>128
H	H	H	$NHCH(CH_3)_2$	H	**14k**	-	-	64
H	Me	H	H	Me	**14l**	-	-	>128
H	OMe	OMe	H	H	**14m**	-	-	>128
H	H	Me	Cl	H	**14n**	N.D.	>128	2
H	H	CF_3	CHO	H	**14o**	>128	32	16
H	H	Cl	CHO	H	**14p**	>128	32	4
Pinacol	H	H	Et	H	**14q**	-	-	32
Pinacol	H	H	CH_2OCOMe	H	**14r**	-	-	4-8
					**14s**	N.D.	>128	4-8
					**14t**	-	-	8-16
		Ampicillin				0.25		
		Ciprofloxacin					16	
		Reserpine						4-8

[a]Se, susceptible *S. aureus* ATCC25923. [b]Rt, resistant *S. aureus* 1199B (NorA).–, inhibition of growth <90% at a concentration of 100 μg/mL. N.D., no data.

left to react for 60 min. The mixture was allowed to warm to room temperature and was quenched by slow addition of a 4% aqueous NaOH solution (50 mL). The resulting aqueous layer was collected and acidified to pH 2.5 by dropwise addition of 3 N HCl. The resulting precipitate was filtered on a sintered-glass filter, washed with ether, and dried to give **19d** as a white solid. Yield 87%. mp 174 °C. IR (KBr): ν (cm^{-1}) 3028, 1701, 1606, 1289, 1132, 725. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.47 (2H, s, OCH_2), 6.86 (1H, d, J = 8.8 Hz, H-5), 7.32–7.48 (5H, m, H-Ph), 8.23 (1H, dd, J = 8.8 and 1.9 Hz, H-4), 8.94 (1H, d, J = 1.9 Hz, H-2). ^{13}C NMR (125 MHz, DMSO-d_6): δ 166.1, 165.5, 149.3, 140.1, 136.6, 128.4, 128.0, 127.9, 120.7, 110.8, 67.6. LC–MS (ESI): t_R = 6.39 min; $[M + H]^+$ 230.13.

6-Chloro-5-methylpyridin-3-ylcarboxylic Acid (19e). Following the procedure for carboxylic acid **19d** synthesis, 2-chloro-5-iodo-3-methylpyridine (1.0 g, 3.95 mmol) was reacted with *n*-BuLi (1.97 mL, 4.93 mmol). The mixture was then treated as described in the representative procedure (pH 2) to give **19e** as a white solid. Yield 68%. mp 171 °C. IR (KBr): ν (cm^{-1}) 3054, 1688, 1592, 1435, 1392, 1071. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.38 (3H, s, CH_3), 8.24 (1H, d, J = 2.4 Hz, H-4), 8.70 (1H, d, J = 2.4 Hz, H-2), 13.55 (1H, sl, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 165.6, 154.3, 147.9, 140.4, 132.5, 126.2, 18.9. LC-MS (ESI): t_R = 5.02 min; $[M + H]^+$ 172.37, 174.38.

6-Benzyloxypyridine-3-carboxaldehyde (20d). To a slurry of 2.5 M of *n*-BuLi (1.89 mL, 4.73 mmol) in anhydrous diethyl ether

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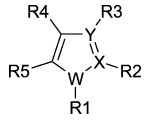
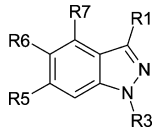
Table 4. Antibacterial Activity of Other Heterocyclic Compounds



									Activity (μg/mL) against Se[a] and Rt[b] *S. aureus*		
W	X	Y	R_1	R_2	R_3	R_4	R_5	Id.	Se MIC	Rt NorA MIC	Rt NorA MMC_4
N	C	C	Boc	$B(OH)_2$	H	H	H	**15a**	-	-	>128
S	C	C		H	$B(OH)_2$	H	H	**15b**	-	-	8-16
S	C	C		Me	$B(OH)_2$	H	Me	**15c**	-	-	16-32
N	N	C	H		$B(OH)_2$	Me	H	**15d**	-	-	64
N	N	C	Bz		H	BPin	H	**15e**	-	-	128
N	N	C	Me		H	H	BPin	**15f**	-	-	8-16
N	N	C	THP		H	H	BPin	**15g**	-	-	>128
N	N	C	THP		H	Me	BPin	**15h**	-	-	>128
N	C	N	THP	H		H	BPin	**15i**	-	-	>128
N	C	N	Me	Cl		H	BPin	**15j**	-	-	128
N	C	N	Bz	Ph		H	BPin	**15k**	-	-	32
S	C	N		H		H	BPin	**15l**	-	-	>128
S	C	N		NHCOOtBu		H	BPin	**15m**	-	-	>128
S	C	N		NHCOOEt		H	BPin	**15n**	-	-	>128
O	C	N		O-TIPS		H	BPin	**15o**	-	-	>128
				Ampicillin					0.25		
				Ciprofloxacin						16	
				Reserpine							4-8

[a]Se, susceptible *S. aureus* ATCC25923. [b]Rt, resistant *S. aureus* 1199B (NorA). −, inhibition of growth <90% at a concentration of 100 μg/mL.

Table 5. Antibacterial Activity of Indazole Boronic Compounds



						Activity (μg/mL) against Se[a] and Rt[b] *S. aureus*		
R_1	R_3	R_5	R_6	R_7	Id.	Se MIC	Rt NorA MIC	Rt NorA MMC_4
H	H	H	$B(OH)_2$	H	**16a**	-	-	32
H	THP	H	$B(OH)_2$	H	**16b**	-	-	8
H	THP	H	BPin	H	**16c**	-	-	128
H	Boc	H	BPin	H	**16d**	-	-	>128
H	SEM	H	BPin	H	**16e**	-	-	16
Me	COMe	H	BPin	H	**16f**	-	-	128
H	COMe	H	H	BPin	**16g**	-	-	8
H	THP	BPin	H	H	**16h**	-	-	128
		Ampicillin				0.25		
		Ciprofloxacin					16	
		Reserpine						4-8

[a]Se, susceptible *S. aureus* ATCC25923. [b]Rt, resistant *S. aureus* 1199B (NorA). −, inhibition of growth <90% at a concentration of 100 μg/mL.

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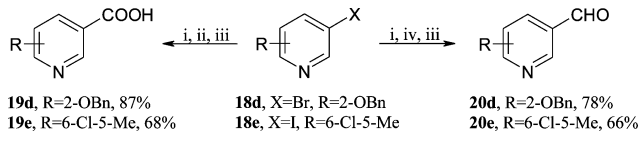
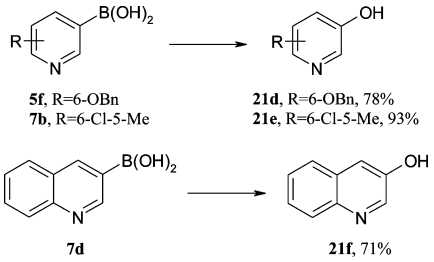
Scheme 1. Synthesis of Carbocylic Acids 19d−e and Aldehydes 20d−e[a]



19d, R=2-OBn, 87%
19e, R=6-Cl-5-Me, 68%
18d, X=Br, R=2-OBn
18e, X=I, R=6-Cl-5-Me
20d, R=2-OBn, 78%
20e, R=6-Cl-5-Me, 66%

[a]Reagents and conditions: (i) *n*-BuLi (1.25 equiv), anhyd. Ether, −78 °C, 1 h; (ii) CO_2(g), −78 °C to rt, 1 h; (iii) hydrolysis; (iv) DMF (1.25 equiv), −78 °C to rt, 1 h. Isolated yields.

Scheme 2. Synthesis of Hydroxypyridines 21d−f[a]



5f, R=6-OBn
7b, R=6-Cl-5-Me
21d, R=6-OBn, 78%
21e, R=6-Cl-5-Me, 93%

7d
21f, 71%

[a]Reagents and conditions: H_2O_2 (35%, 3 equiv), CH_2Cl_2, rt, 12 h. Isolated yields.

Scheme 3. Synthesis of 6-Benzyloxypyridine-3-methanol 22[a]

19d
22, 45%

[a]Reagents and conditions: LAH (2.5 equiv), anhyd. THF, 0 °C to rt, 4 h. Isolated yield.

(80 mL) cooled at −78 °C was added a solution of 2-benzyloxy-5-bromopyridine (1.0 g, 3.79 mmol) in anhydrous THF (10 mL). The mixture was allowed to react at this temperature over 60 min. A solution of anhydrous DMF (0.37 mL, 4.73 mmol) was then added and left to react for 60 min. The mixture was allowed to warm to room temperature, quenched with water, and extracted with ethyl acetate. The organic phase was washed with brine, dried on $MgSO_4$, filtered, and evaporated. The crude product was purified by silica gel chromatography (cyclohexane/EtOAc 9:1), affording **20d** as a beige solid. Yield 78%. mp < 50 °C. IR (KBr): ν (cm^{-1}) 3033, 2956, 2886, 1686, 1603, 1568, 1491, 1349, 1262, 1222, 991, 841, 744, 696. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.48 (2H, s, OCH_2), 6.90 (1H, d, J = 8.8 Hz, H-5), 7.33−7.48 (5H, m, H-Ph), 8.08 (1H, dd, J = 8.8 and 2.4 Hz, H-4), 8.65 (1H, d, J = 2.4 Hz, H-2), 9.96 (1H, s, CHO). ^{13}C NMR (100 MHz, $CDCl_3$): δ 189.5, 167.1, 152.8, 137.6, 136.3, 128.6, 128.2, 128.1, 126.8, 112.3, 68.6. LC−MS (ESI): t_R = 6.81 min; $[M + H]^+$ 214.48.

6-Chloro-5-methylpyridine-3-carboxaldehyde (20e). Following the procedure for carboxaldehyde **20d** synthesis, 2-chloro-5-iodo-3-methylpyridine (1.0 g, 3.95 mmol) was reacted with *n*-BuLi (1.97 mL, 4.93 mmol) and DMF (0.38 mL, 4.93 mmol). The crude product was purified by silica gel chromatography (cyclohexane/EtOAc 9:1), affording **20e** as a white solid. Yield 66%. mp 59 °C. IR (KBr): ν (cm^{-1}) 3034, 2965, 2879, 1685, 1589, 1388, 1067, 732. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.49 (3H, s, CH_3), 8.03 (1H, d, J = 2.2 Hz, H-4), 8.70 (1H, d, J = 2.2 Hz, H-2), 10.08 (1H, s, CHO). ^{13}C NMR (125 MHz, DMSO-d_6): δ 191.5, 155.4, 138.9, 133.3, 130.9, 19.0. LC−MS (ESI): t_R = 5.16 min; $[M + H]^+$ 156.39, 158.40.

6-Benzyloxy-3-hydroxypyridine (21d). To a stirred solution of 6-benzyloxypyridine-3-boronic acid **5f** (0.3 g, 1.31 mmol) in dichloromethane (20 mL) was slowly added a solution of 35% hydrogen peroxide (0.34 mL, 3.93 mmol). The reaction was continued at room temperature for 12 h and then concentrated to dryness. The residue was diluted with water (50 mL) and extracted with ethyl acetate. The organic phase was washed with brine, dried on $MgSO_4$, filtered, and evaporated. The crude product was purified by silica gel chromatography (cyclohexane/EtOAc 8:2), affording **21d** as a white solid. Yield 78%. mp 91 °C. IR (KBr): ν (cm^{-1}) 3060, 2870, 1496, 1265, 1043, 755. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.27 (2H, s, OCH_2), 5.55 (1H, sl, OH), 6.71 (1H, d, J = 8.8 Hz, H-5), 7.18 (1H, dd, J = 8.8 and 3.2 Hz, H-4), 7.27−7.43 (5H, m, H-Ph), 7.76 (1H, d, J = 3.2 Hz, H-2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 158.1, 147.4, 137.3, 132.5, 128.4, 127.84, 127.8, 127.78, 111.6, 68.0. LC−MS (ESI): t_R = 5.95 min; $[M + H]^+$ 202.45.

6-Chloro-3-hydroxy-5-methylpyridine (21e). Following the procedure for hydroxypyridine **21d** synthesis, 6-chloro-5-methylpyridine-3-boronic acid **7b** (0.3 g, 1.75 mmol) was reacted with a solution of 35% hydrogen peroxide (0.45 mL, 5.25 mmol). The crude product was purified by silica gel chromatography (cyclohexane/EtOAc 8:2), affording **21e** as a white solid. Yield 93%. mp 125 °C. IR (KBr): ν (cm^{-1}) 3012, 2884, 2634, 1581, 1452, 1320, 1236, 743, 644. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.23 (3H, s, CH_3), 7.17 (1H, d, J = 2.9 Hz, H-4), 7.75 (1H, d, J = 2.9 Hz, H-2), 10.09 (1H, s, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 153.4, 139.8, 134.6, 132.5, 126.5, 19.1. LC−MS (ESI): t_R = 4.85 min; $[M + H]^+$ 144.37, 146.39.

3-Hydroxyquinoline (21f). Following the procedure for hydroxypyridine **21d** synthesis, quinoline-3-boronic acid **7d** (0.4 g, 2.31 mmol) was reacted with a solution of 35% hydrogen peroxide (0.60 mL, 6.94 mmol). The crude product was purified by silica gel chromatography (cyclohexane/EtOAc 7:3), affording **21e** as a beige solid. Yield 71%. mp 190 °C. IR (KBr): ν (cm^{-1}) 2580, 1602, 1349, 1213, 747. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.47−7.50 (3H, m, H-4, H-6, H-7), 7.77−7.79 (1H, m, H-5), 7.87−7.89 (1H, m, H-8), 8.56 (1H, d, J = 2.9 Hz, H-2), 10.30 (1H, s, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 150.9, 143.9, 142.4, 129.0, 128.6, 126.7, 126.5, 125.9, 115.3. LC−MS (ESI): t_R = 1.63 min; $[M + H]^+$ 146.42.

6-Benzyloxypyridin-3-ylmethanol (22). To a solution of 6-benzyloxypyridine-3-carboxylic acid **19d** (1.0 g, 4.36 mmol) in anhydrous THF (50 mL) cooled at 0 °C was slowly added lithium aluminum hydride (0.41 g, 10.91 mmol). The mixture was allowed to warm to room temperature and to react at this temperature for 4 h. Then, it was quenched with water and extracted with ethyl acetate. The organic phase was washed with brine, dried on $MgSO_4$, filtered, and evaporated. The crude product was purified by silica gel chromatography (cyclohexane/EtOAc 6:4), affording **22** as a beige solid. Yield 45%. mp 70 °C. IR (KBr): ν (cm^{-1}) 3327, 1609, 1489, 1289, 1261, 1003, 830, 699. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.87 (1H, t, J = 5.1 Hz, OH), 4.62 (2H, d, J = 5.1 Hz, CH_2OH), 5.37 (2H, s, OCH_2), 6.81 (1H, d, J = 8.8 Hz, H-5), 7.30−7.46 (5H, m, H-Ph), 7.63 (1H, dd, J = 8.8 and 2.9 Hz, H-4), 8.12 (1H, d, J = 2.9 Hz, H-2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.4, 145.7, 138.5, 137.1, 129.1, 128.4, 127.9, 127.8, 111.3, 67.7, 62.5. LC−MS (ESI): t_R = 5.63 min; $[M + H]^+$ 217,13.

Microbiological Assays. *Bacterial Strains.* *S. aureus* ATCC 25923 was purchased from the Institut Pasteur (CRBIP, Paris, France). The sensitive SA-1199 strain and the overproducing NorA mutant SA-1199B were obtained from G.W. Kaatz (University of Michigan, USA).[64] *S. aureus* RN4220 pUL5054 possessing the multicopy plasmid pUL5054 including the *msr*(A) gene, thus overproducing MsrA, was a gift from C. Bebear (University of Bordeaux, France).[65] All strains were grown at 37 °C in Mueller−Hinton broth (Bio-Rad, Mitry Mory, France) or spread on Mueller−Hinton agar plates for counting. Colony forming unit (CFU) monitoring was carried out by counting colonies present in 2 × 10 μL of serial log dilutions of bacteria inoculum spotted on MH agar plates. Plates were examined for growth after overnight incubation at 37 °C.

Media, Antibiotics, and Culture Conditions. Mueller−Hinton broth (MH, Bio Rad) was used for all bacteria overnight cultures and susceptibility testing. Ciprofloxacin was obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Reserpine was purchased from Alfa Aesar (Schiltigheim, France). Stock solutions (ciprofloxacin) were prepared in sterile water except for reserpine, which was dissolved in dimethyl sulfoxide (DMSO).

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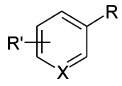
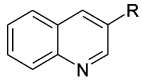
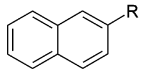
Table 6. Evaluation of Antibacterial Activity of Carbonylated and Hydroxylated Analogues[a]



			Activity (μg/mL) against SA-1199B								
R			COOH			CHO			OH		
R'	X	Id.	Id.	MIC	MMC_4	Id.	MIC	MMC_4	Id.	MIC	MMC_4
H	N	**3a**	**19a**	>128	>128	**20a**	>128	>128	**21a**	>128	>128
6-Br	N	**5a**	**19b**	>128	>128		N.D.		**21b**	>128	>128
6-Cl	N	**5b**	**19c**	>128	>128		N.D.		**21c**	>128	>128
6-OBn	N	**5f**		N.D.		**20d**	>128	32		N.D.	
6-Cl-5-Me	N	**7b**	**19e**	>128	>128		N.D.		**21e**	>128	>128
		**7d**	**19f**	>128	32	**20f**	>128	>128	**21f**	>128	>128
H	C	**14a**	**19g**	>128	>128	**20g**	>128	>128	**21g**	>128	>128
6-Br	C	**14f**	**19h**	>128	>128	**20h**	>128	>128	**21h**	>128	128
6-Cl	C	**14g**	**19i**	128	64	**20i**	>128	>128	**21i**	>128	128
6-Cl-5-Me	C	**14n**	**19j**	>128	>128		N.D.		**21j**	>128	64
		**14s**	**19k**	>128	>128	**20k**	>128	64	**21k**	>128	64

[a]N.D., no data.

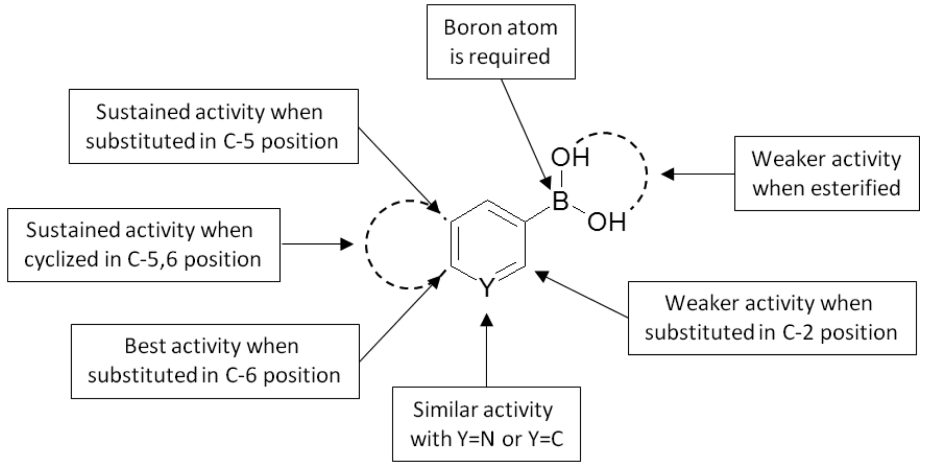


Figure 2. SAR studies for pyridine and benzene boronic species.

MIC Determination. The inhibitory potential of the boronic derivatives was tested through the determination of the minimum inhibitory concentration (MIC) of these complexes against *S. aureus* ATCC25923 and *S. aureus* 1199B overexpressing the efflux pump. The bacterial inoculum was prepared from a frozen culture incubated in 5 mL of MH broth medium at 37 °C for 18 h. Cells were inoculated into MH broth and dispensed at 200 μL/well in 96-well microtiter plates (bacterial concentration in each well = 10^6 CFU/mL).

Tested compounds, solubilized at 10 mg/mL in DMSO, were then added using a Biomek 2000 handling robot using 2-fold serial dilutions, with the highest being 128 mg/L for fully soluble compounds. The MIC was defined as the concentration that completely inhibited cell growth during a 24 h incubation at 37 °C. Growth was assayed with a microplate reader by monitoring absorption at 620 nm. In addition, the plates were read visually. All experiments were performed in duplicate.

The highest final DMSO concentration used (2.56% v/v) induced no significant bacterial growth inhibition. Controls were performed on each microplate with antibiotics known to inhibit the strain's growth: ampicillin for *S. aureus* ATCC25923 (0.25 mg/L), ciprofloxacin (MIC 16 mg/L) for *S. aureus* 1199B (4 and 32 mg/L), and erythromycin (MIC 128 mg/L) for *S. aureus* RN4220 pUL5054 (16 and 256 mg/L). The plates were incubated at 37 °C, and optical densities were read over a 24 h period. The accepted variance of the MIC value is estimated to be a 2-fold difference because of the microdilution method used.

Cytotoxic Assays. KB cells were provided by the NCI and grown in DMEM supplemented with 10% FCS, penicillin-streptomycin, fungizone, and glutamine at 37 °C under 5% CO_2. Cells were plated in 96-well tissue culture plates in 200 μL of medium and treated 24 h later with 2 μL stock solution of compounds dissolved in DMSO using

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Table 7. Evaluation of the Cytotoxicity

Id.	cytotoxicity[a]	Id.	cytotoxicity[a]	Id.	cytotoxicity[a]
2c	0	**5j**	0	**14o**	100
3d	4	**7b**	0	**14p**	100
5a	1	**7d**	0	**14r**	6
5b	1	**11a**	0	**14s**	3
5d	0	**14d**	10	**14t**	0
5e	0	**14f**	0	**15b**	0
5f	2	**14g**	0	**15f**	0
5g	0	**14i**	6	**16b**	0
5i	0	**14n**	0	**16g**	0

[a]Cytotoxicity is given as the percent inhibition of cellular proliferation of KB cells.

a Biomek 3000 (Beckman-Coulter). Controls received the same volume of DMSO (1% final volume). After a 72 h exposure, MTS reagent (Promega) was added, and plates were incubated for 3 h at 37 °C; the absorbance was monitored at 490 nm, and results are expressed as the inhibition of cell proliferation calculated as the ratio (1 − (OD490 treated/OD490 control))100 in triplicate experiments. For IC_{50} determination (50% inhibition of cell proliferation), cells were incubated for 72 h following the same protocol with compound concentrations ranging from 5 nM to 100 μM in separate duplicate experiments.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

EPI, efflux pump inhibitor; MMC, minimum modulatory concentration; MFS, major facilitator superfamily; ABC, ATP binding cassette; *n*-BuLi, *n*-butyllithium

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