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# 3-Aryl-4-methyl-2-quinolones Targeting Multiresistant *Staphylococcus aureus* Bacteria

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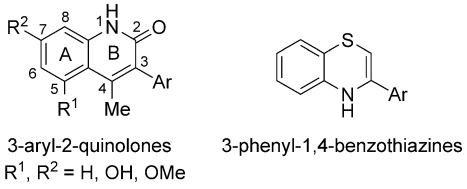
The NorA efflux pump lowers intracellular fluoroquinolone concentrations by expelling antibiotics through the membrane of *Staphylococcus aureus*. We identified 3-aryl-4-methyl-2-quinolin-2-ones as compounds able to restore the activity of the NorA substrate, ciprofloxacin, against resistant *S. aureus* strains, and acting as efflux pump inhibitors (EPI). In particular, 5-hydroxy-7-methoxy-4-methyl-3-phenylquinolin-2-one (**6c**) presents both an EPI and an antimicrobial effect. Its efficacy and safety make it a potential candidate for further investigations.

The membrane-based bacterial efflux pumps contribute to the emergence of resistance against antibiotics, inducing failures during chemotherapies. This phenomenon, which was first discovered in 1980 with the transport of tetracyclines out of the enterobacteria, is due to numerous efflux systems described both in Gram-negative and Gram-positive bacteria.[1–6] In the *Staphylococcus aureus* genome (2.8 Mb), approximately 253 open reading frames (ORFs) have been described as encoding putative transport pumps. One of these pumps (NorA) is a drug/proton antiporter, belonging to the major facilitator superfamily (MFS) that effluxes a broad spectrum of anti-infectious molecules, such as fluoroquinolones, ethidium bromide, rhodamine and acridines, conferring a multidrug resistance (MDR) phenotype to the bacteria.[7–9] Therefore, the search for new molecules to reinforce the antibiotic arsenal against more and more virulent bacteria is needed. The circumvention or reversion of MDR to antibiotics could be achieved through two strategies. The first is based on the search for novel antibiotics that are not substrates of the efflux pumps, and the second strategy relies on the inhibition of the efflux system by using efflux pump inhibitors (EPIs) in association with antibiotics.[9] The latter strategy could lead to an extension of the clinical utility of existing antibiotics and a decrease of the administered antibiotic doses.[10]

In the last decade, many EPIs were reported, but so far none was brought to the clinic, mainly because of high toxicity and/or low in vivo efficacy.[11–19] In this regard, the naturally occurring alkaloid, reserpine, is frequently used as a positive control of EPI;[20] unfortunately, it is too toxic at clinically relevant doses.

In this study, we targeted novel molecules that have intrinsic antibacterial activity and act as EPIs with the aim to get synergy gains, thus, allowing us to investigate phenyl quinolones. While phenyl quinolones share structural similarities with fluoroquinolones, conferring them potential antibiotic activity, they are also structurally close to 3-phenyl-1,4-benzothiazines (Figure 1), known as inhibitors of *S. aureus* NorA multidrug efflux pump.[16] After screening different subclasses of aryl quinolones, we focused our investigations on 3-aryl-4-methyl-2-quinolones, shown in Figure 1. The emphasized structural modifications mainly concerned the methoxylation and hydroxylation at C-5 and C-7 positions. This substitution pattern was adopted because it showed its usefulness among flavo-




3-aryl-2-quinolones
$R^1$, $R^2$ = H, OH, OMe

3-phenyl-1,4-benzothiazines

**Figure 1.** The general structures of studied 3-aryl-2-quinolones and the structure of 3-phenyl-1,4-benzothiazines.[16]

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noids, structurally close compounds that were found as promising inhibitors of several efflux pumps.[21]

## Chemistry

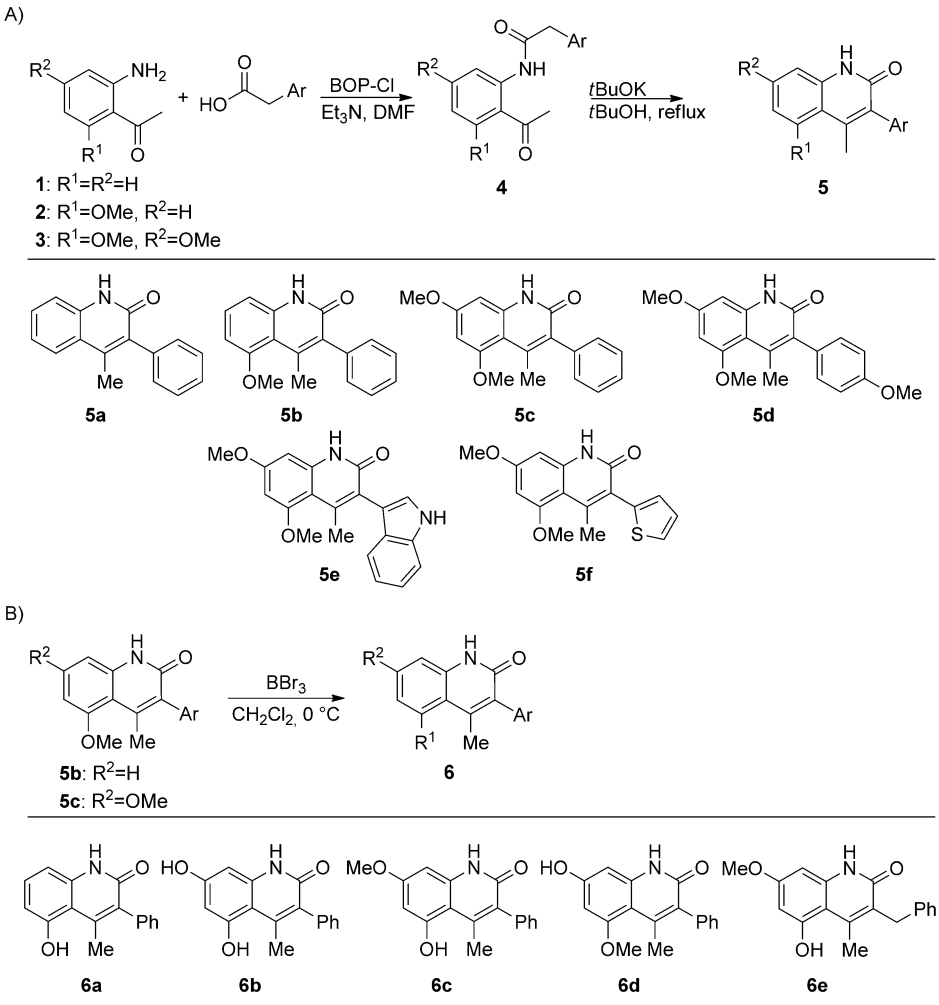
Access to the investigated compounds shown in Figure 1 was accomplished starting from the key blocks, 2-aminoacetophenones **1**–**3** (Scheme 1). Compound **1** is commercially available, and derivatives **2** and **3** were prepared according to a previously reported method from our laboratory.[22] The next step was the conversion of key acetophenones **1**–**3** to 3-aryl-4-methylquinolin-2-ones (**5**). Thus, treatment of **1**–**3** with arylacetic acids in the presence of bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl) as a coupling agent and triethylamine afforded the corresponding amides **4**. The cyclization of obtained amides **4** to quinolones **5** was conducted with *t*BuOK in *t*BuOH. The synthesis of A-ring hydroxylated analogs **6** was achieved by treatment of the methoxylated derivatives **5** with boron tribromide ($BBr_3$) in dichloromethane. During the O-demethylation step, we found that treatment of 5,7-dimethoxyquinolone (**5c**) with $BBr_3$ provided the fully O-demethylated compound **6b** as well as the two partially O-demethylated derivatives **6c** and **6d**. In this case, a chromatographic separation was necessary to isolate the three pure compounds.

## Results and Discussion

The ability of the synthesized compounds to restore the activity of ciprofloxacin was assessed by the microdilution method, through the determination of the minimum inhibitory concentrations (MICs) of ciprofloxacin on *S. aureus* ATCC 29213.[23,24]

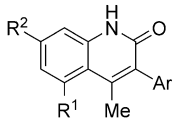
The results are shown in Table 1. To determine the structural features of 2-quinolones responsible for the activity, we used the simplest compound (**5a**) as the starting hit. Derivative **5a** has a 2-fold reduced MIC compared with ciprofloxacin at 98.7 μM. Using compound **5a**, we evaluated the impact of methoxylation at the C-5 and C-7 position. The introduction of a methoxy group at C-5 (**5b**) led to a sensitive increase of ciprofloxacin activity. The methoxylation at both C-5 and C-7 positions (**5c**) induced a positive impact on the activity. Next, we decided to investigate the role of the 3-aryl ring by varying its structure. In order to do this, we used derivative **5c** as a model by keeping all positions unmodified and introduced different aryl rings chosen among substituted phenyl rings and heterocycles. As shown in Table 1, only substitution with a *para*-methoxyphenyl (**5d**) provided a higher-activity derivative, whereas substitution with heterocycles (**5e** and **5f**) was not fruitful. It should be highlighted that in the case of a halogenated phenyl ring, the activity was not measured due to poor solubility. Next, we examined the A-ring hydroxylation effect. The introduction of a hydroxyl group at C-5 provided derivative **6a**, which was less active than the methoxy analogue **5b**. The O-demethylation at one of the two methoxy groups of compound **5c** led to compounds **6c** and **6d**. As shown in Table 1, compound **6c** showed the highest activity in the series. The hydroxylation at both C-5 and C-7 positions (**6b**) had a similar effect as the dimethoxylated analogue. Finally, the evaluation of analogue **6e** having a $CH_2$-group as a spacer between the 3-phenyl group and C-3, led to a decrease of activity compared with compound **6c**.

Having identified the most active compound (**6c**), we searched for a short, straightforward and a scale-up compatible




**Scheme 1.** A) Synthesis and B) O-demethylation of 3-aryl-4-methyl-2-quinolones.

**Table 1.** Effect of compounds **5a–f** and **6a–e** on reduction of ciprofloxacin in *S. aureus*.



| Compd | $R^1$ | $R^2$ | Ar | Concentration [μM] | MIC reduction[a] |
|---|---|---|---|---|---|
| **5a** | H | H | Ph | 98.7 | 2 |
| **5b** | OMe | H | Ph | 36 | 2 |
| **5c** | OMe | OMe | Ph | 12.8 | 1 |
| **5d** | OMe | OMe | 4-OMe-Ph | 13.2 | 2 |
| **5e** | OMe | OMe | 3-indolyl | 15.5 | 2 |
| **5f** | OMe | OMe | 2-thiophenyl | 22 | 2 |
| **6a** | OH | H | Ph | 75 | 1 |
| **6b** | OH | OH | Ph | 27.7 | 2 |
| **6c**[b] | OH | OMe | Ph | 31.25 | 4 |
| | | | | 3.9 | 2 |
| **6d** | OMe | OH | Ph | 46 | 4 |
| **6e** | OH | OMe | Benzyl | 31 | 2 |
| Reserpine | | | | 20 | 4 |

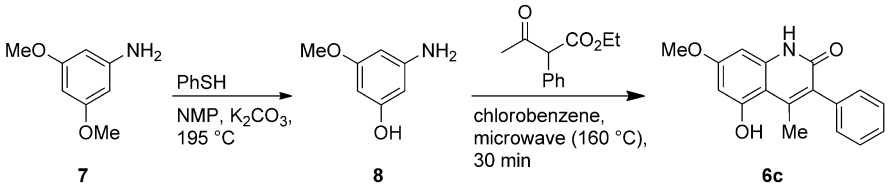
[a] Fold reduction of ciprofloxacin at the given concentration. [b] For comparison purposes, the most active compound **6c** was evaluated at two different concentrations.

curve of ciprofloxacin alone and the curve of ciprofloxacin in association with compound **6c** (Figure 2).

Interestingly, we also observed a bactericidal activity on *Enterococcus* strains (*E. faecalis* and *E. faecium*), and even some vancomycin-resistant strains (VRE), suggesting that other Gram-positive *cocci* could be targeted by this association.

In order to investigate the role of NorA efflux pump in the reversal effect of compound **6c** on ciprofloxacin, we performed checkerboard assays using *S. aureus* 1199B, which overexpress NorA derived from a MSSA bloodstream isolate and *S. aureus* K1712, a *norA* knock-out strain derived from the *S. aureus* 8325.4 strain.[18] First, the lack of NorA did not influence on the intrinsic bactericidal activity of **6c**, since the MIC value of **6c** alone on *S. aureus* K1712 was not different from the MIC of the mother strain 8325.4 (62.5–125 μM or 17.6–35.1 μg mL$^{-1}$). Second, we observed a synergy between ciprofloxacin and compound **6c** on strains 1199, 1199B and 8325.4 with an increased effect on strain 1199B, and no synergy between these two molecules on strain K1712. Furthermore, compound **6c** did not show any potentiation effect on ciprofloxacin on *S. aureus* K1712 being deleted in NorA, suggesting that NorA is involved in this activity. In addition, no potentiation by **6c** was observed on the bactericidal activity of several non-NorA sub-

method for its preparation (Scheme 2). Hence, we developed a two-step method starting from the commercially available 3,5-dimethoxyaniline (**7**). This compound was partially O-demethylated according to a known procedure,[25] providing aminophenol **8**. The latter was condensed with the commercially available ethyl 3-oxo-2-phenylbutanoate by using microwave irradiation to provide the desired compound (**6c**) in one step with high yield (90%) and purity. This method allows the quick preparation of multigram quantities of **6c** in a short period of time.




**Scheme 2.** Rapid, alternative two-step synthesis of compound **6c**.

Because of its potent effect in restoring the antibacterial activity of ciprofloxacin, we focused our further investigation on derivative **6c**. First, we investigated the intrinsic antibacterial activity, and we found that **6c** presented a bactericidal activity (MIC = 62.5–125 μM or 17.6–35.1 μg mL$^{-1}$). The latter activity was effective against a variety of bacteria, including methicillin-sensitive *S. aureus* (MSSA) strains, methicillin-resistant *S. aureus* (MRSA) strains and glycopeptide-intermediate *S. aureus* (GISA) strains. A checkerboard assay was conducted to measure the synergy between **6c** and some classic antibiotics on three types of *S. aureus* strains (MSSA, MRSA and GISA). On all of them, we observed a synergy (FIC ≤ 0.5) between compound **6c** and ciprofloxacin. This synergy was confirmed using a time–kill curve, in which 2 $\log_{10}$ reduction was observed between the

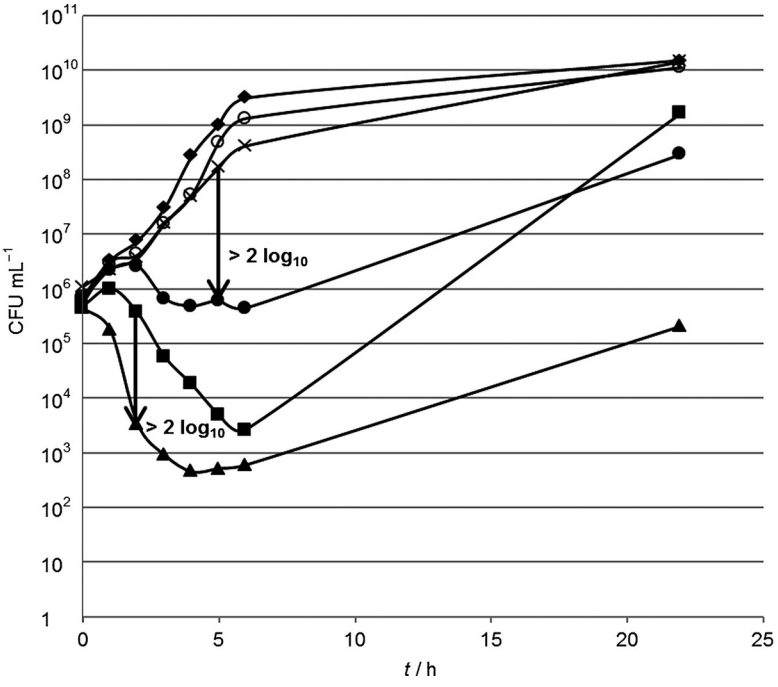



**Figure 2.** Time–kill curves of *S. aureus* obtained by association of ciprofloxacin and **6c**: alone (♦); 0.5 μg mL$^{-1}$ ciprofloxacin (■); 0.125 μg mL$^{-1}$ ciprofloxacin (○); 31.25 μM **6c** alone (×); 0.125 μg mL$^{-1}$ ciprofloxacin and 31.25 μM **6c** (●); 0.5 μg mL$^{-1}$ ciprofloxacin and 31.25 μM **6c** (▲).

strates (oxacillin, erythromycin, tetracycline and vancomycin), strengthening the idea that **6c** is a NorA inhibitor. We further performed an efflux inhibition assay using *S. aureus* 1199B strain previously loaded with ethidium bromide (EBr), which is a substrate of NorA and whose efflux can be measured by loss of fluorescence. EtBr efflux was inhibited by **6c** with a potency that is comparable to reserpine, the reference used as NorA inhibitor, confirming that this pump was targeted by **6c** for its EPI activity (**6c**: $IC_{50} = 18$ μM versus reserpine: $IC_{50} = 14$ μM). Because NorA substrates and inhibitors could induce similar effects on P-glycoprotein (P-gp), in our hand, no effect on P-gp was observed even at high concentrations (results not shown).

Toxicity evaluation of compound **6c** showed that no toxicity was observed with concentrations up to 100 μM (20.07 μg mL$^{-1}$) on HME-1 cells in vitro. For in vivo experiments, no weight loss or death was observed with BALB/cByJ mice treated with **6c**, indicating that the compound was not toxic at the tested doses. However, additional studies with various administration schemes would be necessary before concluding on this point.

In conclusion, by using a simple design strategy and very simple chemistry, we accessed a novel class of antibacterial agents that target resistant *S. aureus* strains through an EPI activity. The most promising derivative (**6c**) can be prepared in two steps with high yield. The lack of toxicity as evidenced by cellular and in vivo investigations make the lead compound (**6c**) a serious candidate for preclinical trials as a novel antibacterial agent.

## Experimental Section

### *Chemistry*

**General**: NMR spectra were recorded on a Bruker AC-400 instrument (400 MHz). Electrospray ionization (ESI) mass spectra were acquired by the Analytical Department of Grenoble University on an Esquire 300 Plus Bruker Daltonis instrument with a nanospray inlet. Combustion analyses were performed at the Analytical Department of Grenoble University. IR spectra were collected on a Bruker Vector 22 spectrometer. Thin-layer chromatography (TLC) used Merck silica gel F-254 plates (thickness 0.25 mm). Flash chromatography used Merck silica gel 60, 200–400 mesh. Unless otherwise stated, reagents were obtained from commercial sources (Sigma–Aldrich and Acros Organics) and were used without further purification. Derivative **3** was prepared according to a previously reported method from our laboratory.[22]

***N*-(2-Acetyl-5-methoxy-3methoxyethoxymethoxyphenyl)arylamide (4)**: Acetophenone derivative **1**, **2** or **3** was dissolved in dimethylformamide (DMF; 7 mL mmol$^{-1}$) under argon atmosphere and treated with $Et_3N$ (5 equiv). Bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl; 2 equiv) and arylacetic acid (2 equiv) were added. The reaction was stirred at RT for 48 h, then a solution of $NaHCO_3$ (5%, 10 mL) was added. The solution was partially evaporated in vacuo, and the crude was extracted with AcOEt (2×20 mL), washed with brine (2×10 mL), dried over $MgSO_4$ and concentrated. Purification over column chromatography (silica gel, $CH_2Cl_2$) afforded the pure product.

**3-Aryl-4-methylquinolin-2-one (5)**: Amides **4** obtained from the previous step were dissolved in *t*BuOH (6 mL mmol$^{-1}$), and *t*BuOK (2.1 equiv) was added. The solution was stirred overnight at RT. *t*BuOH was evaporated in vacuo and a saturated solution of $NH_4Cl$ (20 mL) was added. After extraction in AcOEt (2×20 mL), the organic layer was washed with water (10 mL), brine (10 mL), dried over $MgSO_4$ and concentrated. Finally, the crude material was purified by column chromatography (silica gel, $CH_2Cl_2$).

**4-Methyl-3-phenylquinolin-2-one (5a)**: White powder (167 mg starting from 1 mmol of the corresponding amide **4**, 71%); $R_f$ = 0.42 ($CH_2Cl_2$/MeOH, 99:1); $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 11.03$ (s, 1H); 7.59 (dd, $J_1 = 7.0$ Hz, $J_2 = 2.9$ Hz, 1H), 7.43 (dd, $J_1 = 7.2$ Hz, $J_2 = 3.2$ Hz, 2H), 7.38 (dd, $J_1 = 7.3$ Hz, $J_2 = 3.0$ Hz, 1H), 7.22 (dd, $J_1 = 7.2$ Hz, $J_2 = 7.0$ Hz, 2H), 7.10–7.00 (m, 3H), 2.05 ppm (s, 3H); MS (ESI$^+$): *m/z*: 236 $[M+H]^+$; Anal. calcd for $C_{16}H_{13}NO$: C 81.68, H 5.57, N 5.95, found: C 81.64, H 5.53, N 5.94.

**5-Methoxy-4-methyl-3-phenylquinolin-2-one (5b)**: White powder (344 mg starting from 2 mmol of the corresponding amide **4**, 65%); $R_f$ = 0.23 ($CH_2Cl_2$/MeOH, 99:1); mp: 320 °C (decomposition); $^1$H NMR (400 MHz, $CD_3OD$): $\delta = 7.77$ (d, $J = 8$ Hz, 1H), 7.43–7.26 (m, 3H), 7.26 (d, $J = 8$ Hz, 2H), 6.92–6.86 (m, 2H), 3.89 (s, 3H), 2.29 ppm (s, 3H); MS (ESI$^+$): *m/z*: 266 $[M+H]^+$; Anal. calcd for $C_{17}H_{15}NO_2$: C 76.96, H 5.70, N 5.28, found: C 76.93, H 5.66, N 5.26.

**5,7-Dimethoxy-4-methyl-3-phenylquinolin-2-one (5c)**: White powder (841 mg starting from 3 mmol of the corresponding amide **4**, 98%); $R_f$ = 0.2 ($CH_2Cl_2$/MeOH, 99:1); mp: 266–268 °C; $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta = 11.57$ (s, 1H), 7.37–7.29 (m, 2H), 7.29–7.26 (m, 1H), 7.14–7.12 (m, 2H), 6.44 (ls, 1H), 6.30 (ls, 1H), 3.78 (s, 3H), 3.76 (s, 3H), 2.28 ppm (s, 3H); MS (ESI$^+$): *m/z*: 296 $[M+H]^+$, 318 $[M+Na]^+$; Anal. calcd for $C_{18}H_{17}NO_3$: C 73.20, H 5.80, N 4.74, found: C 73.15, H 5.79, N 4.72.

**5,7-Dimethoxy-3-(4-methoxyphenyl)-4-methylquinolin-2-one (5d)**: White powder (390 mg starting from 1.5 mmol of the corresponding amide **4**, 80%); $R_f$ = 0.19 ($CH_2Cl_2$/MeOH, 99:1); mp: 282–284 °C; $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta = 12.12$ (s, 1H), 7.24 (d, $J = 8.8$ Hz, 2H), 6.95 (d, $J = 8.8$ Hz, 2H), 6.37 (d, $J = 2.4$ Hz, 1H), 6.21 (d, $J = 2.4$ Hz, 1H), 3.85 (s, 3H), 3.83 (s, 3H), 3.78 (s, 3H), 2.46 ppm (s, 3H); MS (ESI$^+$): *m/z*: 326 $[M+H]^+$, 348 $[M+Na]^+$; Anal. calcd for $C_{19}H_{19}NO_4$: C 70.14, H 5.89, N 4.31, found: C 70.08, H 5.84, N 4.28.

**5,7-Dimethoxy-3-(indol-3-yl)-4-methylquinolin-2-one (5e)**: White powder (300 mg starting from 2 mmol of the corresponding amide **4**, 45%); $R_f$ = 0.27 ($CH_2Cl_2$/MeOH, 95:5); mp: 335 °C (decomposition); $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta = 11.52$ (s, 1H), 11.19 (s, 1H), 7.41 (m, 1H), 7.30 (s, 1H), 7.14 (m, 1H), 7.08 (m, 1H), 6.97 (m, 1H), 6.49 (ls, 1H), 6.34 (ls, 1H), 3.84 (s, 3H), 3,81 (s, 3H), 2.41 ppm (s, 3H); MS (ESI$^+$): *m/z*: 335 $[M+H]^+$, 357 $[M+Na]^+$; Anal. calcd for $C_{20}H_{18}N_2O_3$: C 71.84, H 5.43, N 8.38, found: C 71.81, H 5.40, N 8.34.

**5,7-Dimethoxy-4-methyl-3-(thiophen-2-yl)quinolin-2-one (5f)**: White powder (284 mg starting from 1.5 mmol of the corresponding amide **4**, 63%); $R_f$ = 0.24 ($CH_2Cl_2$/MeOH, 99:1); mp: 310 °C (decomposition); $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 12.57$ (s, 1H), 7.42 (m, 1H), 7.11 (m, 1H), 7.02 (m, 1H), 6.48 (ls, 1H), 6.23 (ls, 1H), 3.84 (s, 6H), 2.62 ppm (s, 3H); MS (ESI$^+$): *m/z*: 302 $[M+H]^+$; Anal. calcd for $C_{16}H_{15}NO_3S$: C 63.77, H 5.02, N 4.65, found: C 63.74, H 4.98, N 4.61.

**Synthesis of derivatives 6a–d**: Compound **5b** or **5c** (450 mg, 1.52 mmol) was dissolved in $CH_2Cl_2$ (15 mL mmol$^{-1}$). $BBr_3$ (1 equiv) was slowly added, and after 3 h, $H_2O$ (5 mL) was added. The solution was filtered and washed with $H_2O$ (10 mL). Column chromatography (silica gel) using a gradient of $CH_2Cl_2$/MeOH (100:0–95:5)

allowed to purify **6a** and separate the different hydroxylated isomers **6b–d**.

**5-Hydroxy-4-methyl-3-phenylquinolin-2-one (6a)**: White powder (188 mg, 50%); $R_f = 0.30$ ($CH_2Cl_2$/MeOH, 97:3); mp: 318 °C (decomposition); $^1$H NMR (400 MHz, $CD_3OD$): $\delta = 7.70$ (d, $J = 8$ Hz, 1H), 7.44–7.35 (m, 3H), 7.25 (d, $J = 8$ Hz, 2H), 6.81–6.75 (m, 2H), 2.27 ppm (s, 3H); MS (ESI$^+$): *m/z*: 252 $[M+H]^+$; Anal. calcd for $C_{16}H_{13}NO_2$: C 76.48, H 5.21, N 5.57, found: C 76.45, H 5.18, N 5.55.

**5,7-Dihydroxy-4-methyl-3-phenylquinolin-2-one (6b)**: Beige powder (344 mg starting from 1.5 mmol of **5b** and using 5 equiv of $BBr_3$, 86%); $R_f = 0.14$ ($CH_2Cl_2$/MeOH, 95:5); mp: 320 °C (decomposition); $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta = 11.34$ (s, 1H), 9.82 (s, 1H), 7.38–7.30 (m, 3H), 7.15 (m, 2H), 6.20 (ls, 1H), 6.12 (ls, 1H), 3.34 (s, 3H), 2.33 ppm (s, 3H); MS (ESI$^+$): *m/z*: 268 $[M+H]^+$, 290 $[M+Na]^+$; Anal. calcd for $C_{16}H_{13}NO_3$: C 71.90, H 4.90, N, 5.24, found: C 71.87, H 4.86, N, 5.22.

**5-Hydroxy-7-methoxy-4-methyl-3-phenylquinolin-2-one (6c)**: White powder (375 mg, 89%); $R_f = 0.33$ ($CH_2Cl_2$/MeOH, 95:5); mp: 159–160 °C; $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta = 11.46$ (s, 1H), 10.26 (s, 1H), 7.39–7.37 (m, 2H), 7.30 (m, 1H), 7.16 (m, 2H), 6.34 (ls, 1H), 6.22 (ls, 1H), 3.74 (s, 3H), 2.36 ppm (s, 3H); MS (ESI$^+$): *m/z*: 282 $[M+H]^+$; Anal. calcd for $C_{17}H_{15}NO_3$: C 72.58, H 5.37, N 4.98, found: C 72.54, H 5.33, N 4.95.

**7-Hydroxy-5-methoxy-4-methyl-3-phenylquinolin-2-one (6d)**: Beige powder (17 mg starting from 1.5 mmol of **5b** and using 1 equiv of $BBr_3$, 4%); $R_f = 0.18$ ($CH_2Cl_2$/MeOH, 95:5); mp: 290–292 °C (decomposition); $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta = 11.50$ (s, 1H), 10.08 (s, 1H), 7.40–7.36 (m, 2H), 7.31 (m, 1H), 7.15 (m, 2H), 6.35 (ls, 1H), 6.20 (ls, 1H), 3.79 (s, 3H), 2.30 ppm (s, 3H); MS (ESI$^+$): *m/z*: 282 $[M+H]^+$; Anal. calcd for $C_{17}H_{15}NO_3$: C 72.58, H 5.37, N 4.98, found: C 72.51, H 5.31, N 4.96.

**3-Benzyl-5-hydroxy-7-methoxy-4-methylquinolin-2(1*H*)-one (6e)**: Compound **6e** was prepared according to two different methods: a) the same method applied for the synthesis of **6a–d** starting from crude 3-benzyl-5,7-dimethoxy-4-methylquinolin-2-one, b) according to scheme 2. Compound **6e** was obtained as white powder to give **6e** as a white powder (259 mg according to scheme 2 and starting from 1 mmol of **8**), 88%): $R_f = 0.36$ ($CH_2Cl_2$/MeOH, 95:5); mp: 278 °C (decomposition); $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta = 11.45$ (s, 1H), 10.23 (s, 1H), 7.12–7.23 (m, 5H), 6.34 (d, $J = 2.45$ Hz, 1H), 6.2 (d, $J = 2.45$ Hz, 1H), 3.96 (s, 2H), 3.72 (s, 3H), 2.53 ppm (s, 3H); MS (ESI$^+$): *m/z*: 296 $[M+H]^+$, 318 $[M+Na]^+$; Anal. calcd for $C_{18}H_{17}NO_3$: C 73.20, H 5.80, N 4.74, found: C 73.14, H 5.77, N 4.72.

### *Biology*

*Bacterial strains*: The strains of *S. aureus* used in this study were ATCC 29213, the two mutated strains SA-1199B (over-expressing *norA* and also possesses an A116E Grl A substitution) and SA-K1712 (*norA*-deleted) and their isogenic parents, respectively SA-1199 and SA 8325-4[7] kindly provided by Dr. G. W. Kaatz (Detroit, USA). To evaluate the spectrum of activity of **6c**, ten methicillin-resistant *S. aureus* (MRSA) and five vancomycin-intermediate *S. aureus* (VISA) isolates were also employed as well as 16 *Enterococcus* isolates (seven *E. faecalis* and nine *E. faecium*) kindly provided by Dr. S. Boisset and Dr. O. Dumitrescu (Lyon, France).

*Susceptibility studies and MIC evaluation*: Bacterial susceptibilities to different molecules were assessed by the determination of antimicrobial minimal inhibitory concentration (MIC) values using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI; Wayne, PA, USA) methodology and the Comité de l'Antibiogramme de la Société Française de Microbiologie (CASFM; Paris, France), using Mueller–Hinton II (MH II).[23,24] The effect of combining test compound on the MICs of ciprofloxacin was also investigated.

*Toxicity*: In vitro toxicity was evaluated by cell-survival experiments using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and HME-1 cells as previously described.[26] In vivo toxicity was evaluated on female BALB/cByJ mice (Charles River, L'Arbresle, France) with intraperitoneal injections of compound **6c** (15 mg kg$^{-1}$ per day) for 5 days and daily weighing of animals.

### *Evaluation of synergy*

*Checkerboard combination studies*: The MICs of ciprofloxacin in combination with compound **6c** were determined on *S. aureus* strains by the broth microdilution technique using a two-dimensional checkerboard with twofold dilutions of each drug. Growth control wells containing medium were included in each plate. Microplates were incubated for 24 h at 37 °C. The fractional inhibitory concentration index (FICi) was the sum of the fractional inhibitory concentration (FIC) of ciprofloxacin ($FIC_{cip}$) and the FIC of compound **6c** ($FIC_{6c}$) calculated as follows: $FIC_{cip}$ = (MIC of ciprofloxacin in combination)/(MIC of ciprofloxacin alone) and $FIC_{6c}$ = (MIC of **6c** in combination)/(MIC of compound **6c** alone). The synergy was determined as follows using the lowest FICi value of the checkerboard: FICi $\leq 0.5$: synergy, FICi = 0.5–1.0: additive activity, FICi > 1.0–2.0: no interaction, and FICi > 2.0: antagonism.[27]

*Ethidium bromide efflux*: The loss of ethidium bromide (EtBr) cation from *S. aureus* strain 1199B loaded with EtBr was determined fluorometrically.[7,28] Cells were grown overnight on MH agar and bacterial suspensions were realized ($OD_{660}$ of 0.8 to 1) in buffer (110 mM NaCl, 7 mM KCl, 50 mM $NH_4Cl$, 0.4 mM $Na_2HPO_4$, 52 mM Tris base and 0.2% glucose, adjusted pH 7.5 with HCl). Ethidium loading of cells was accomplished by the addition of ethidium and CCCP (final concentrations, 10 μg mL$^{-1}$ and 100 μM, respectively). After 20 min of incubation at 37 °C, cells were pelleted and then resuspended in fresh buffer with or without various concentrations of compound **6c** or reserpine, and the fluorescence of the suspension was monitored continuously ($\lambda_{ex} = 530$ nm, $\lambda_{em} = 600$ nm).

*Time–kill studies*: Colony-forming units (CFU) of *S. aureus* strain ATCC 29213 were determined in six-well plates using MH broth inoculated with exponentially growing bacteria to a final concentration of $10^6$ CFU mL$^{-1}$. Each assay included a growth control well with no antibiotic, two wells with ciprofloxacin alone (0.125 μg mL$^{-1}$ or to 0.5 μg mL$^{-1}$ final concentration), one well with **6c** alone (31.25 μM = 7.87 μg mL$^{-1}$) and two wells with a combination of ciprofloxacin (0.125 or 0.5 μg mL$^{-1}$) and **6c** (31.25 μM). Cultures were incubated at 37 °C, and samples were recovered hourly between 1–5 h and after 22 h. Samples were serially diluted and plated onto MH II agar media for colony counts.[29] Synergy and antagonism were defined respectively as $\geq 2 \log_{10}$ CFU mL$^{-1}$ fold decrease and $\geq 2 \log_{10}$ CFU mL$^{-1}$ fold increase by the combination compared with the most active single molecule.[30]

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**Keywords:** antibiotics · efflux pumps · inhibitors · resistance

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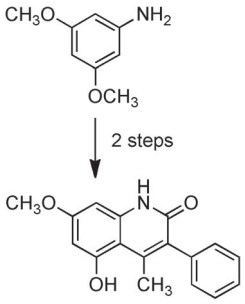


$IC_{50}$ (NorA) = 18 μM
$IC_{50}$ (reserpine) = 14 μM

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**3-Aryl-4-methyl-2-quinolones Targeting Multiresistant *Staphylococcus aureus* Bacteria**