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Design and synthesis of new polyamine quinoline antibiotic enhancers to fight resistant Gram-negative *P. aeruginosa* bacteria

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Abstract

The lack of novel drugs in development and the combination of increased incidence of drug-resistant strains of bacteria has created the need for the search for new antimicrobials as well as new original strategies to fight bacterial resistance. In this context, a series of polyamine quinoline derivatives were prepared and biologically evaluated, identifying compounds able to sensitize doxycycline activity towards the Gram-negative bacteria *P. aeruginosa*. Of note was the identification of antibiotic enhancing analogues whose cytotoxicity ranged from negligible to significant. The mechanism of action of two of the best compounds was studied against *P. aeruginosa* and *S. aureus* establishing a different behaviour towards integrity or depolarization of bacterial membranes depending on the structure of the considered polyamine quinoline derivatives.

Keywords

Antibiotic enhancers; antimicrobial agents; polyamine quinoline derivatives; Gram-negative bacteria

1. Introduction

Antibiotics are medicines, which have been used for a long time to prevent and treat bacterial infections. However, their misuse and overuse as well as poor infection prevention and control lead to antibiotic resistance occurring when bacteria change in response to the use of these drugs. To date, antibiotic resistance is rising to dangerously high levels in all parts of the world characterized by new resistance mechanisms which are globally spreading, threatening our ability to treat common infectious diseases.¹⁻³ Thus, there is an urgent need of

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new strategies to preserve our health towards emerging multidrug resistant pathogens by using an antibiotic-adjuvants approach to improve antibiotics activities.⁴

In this context, we focused our attention on a strategy to sensitize the drug-resistant phenotype by enhancing or restoring the activity of currently ineffective drugs. In the 80's, it has been reported that simple unsubstituted polyamines, such as spermine and spermidine could synergistically act at 1 mM dose with a range of antibiotics.⁵⁻⁷ Recent studies in our laboratory on polyamine-containing natural or synthetic products identified molecules such as squalamine **1**⁸, motuporamine MOTUN44 **2**⁹, ianthelliformisamine **3**¹⁰, 6-bromoindolglyoxamide **4**¹¹, polyaminoisoprenyl derivative NV716 **5**^{12,13} as being intrinsically antimicrobial or able to enhance the activity of antibiotics potentially through membrane permeability improvement and/or membrane depolarization⁴ (Figure 1).

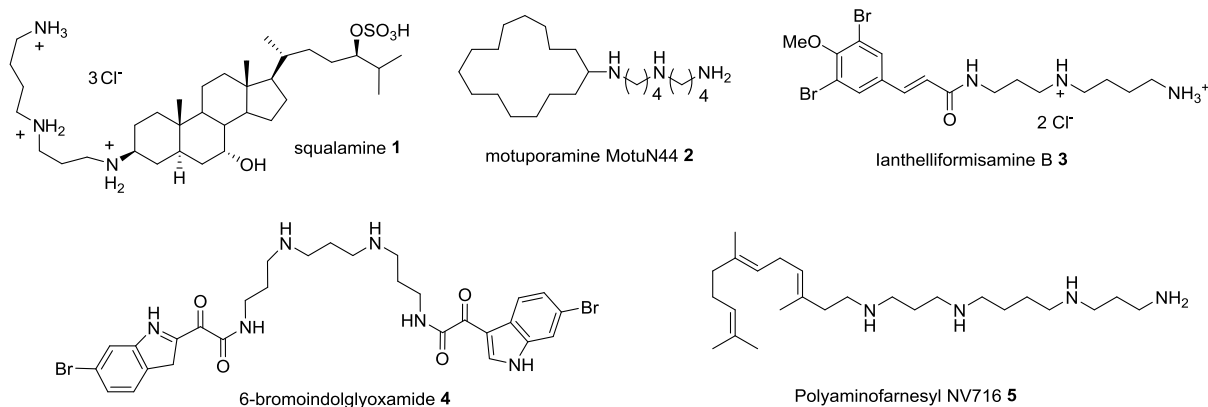


Figure 1. Structure of squalamine **1**, motuporamine MOTUN44 **2**, ianthelliformisamine B **3**, indoleglyoxylpolyamine **4**, polyaminofarnesyl NV716 **5**

In the continuation of our studies, we envisioned to investigate the design, synthesis, and biological evaluation of several polyamine quinoline analogues of chloroquine as antibiotic enhancers. Indeed, the good antimalarial activity of this quinoline analogue linked to its low cytotoxicity let us presume potent good biological activities. Herein, we present a one-step synthesis of the compounds of interest, a structure-activity relationship study related to the nature of the polyamine core, as well as their ability to potentiate the action of doxycycline against resistant Gram-negative bacteria *P. aeruginosa*, and an investigation of the mechanism of action of the best compound towards membranes permeability improvement and their potent depolarization.

2. Results and Discussion

Our initial goal was to investigate a one-step reaction for the synthesis of polyamine quinoline derivatives analogous to well-known antimalarial chloroquine **6** and hydroxychloroquine **7** (Figure 2).

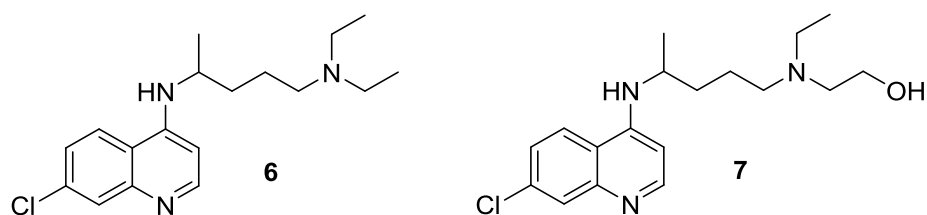
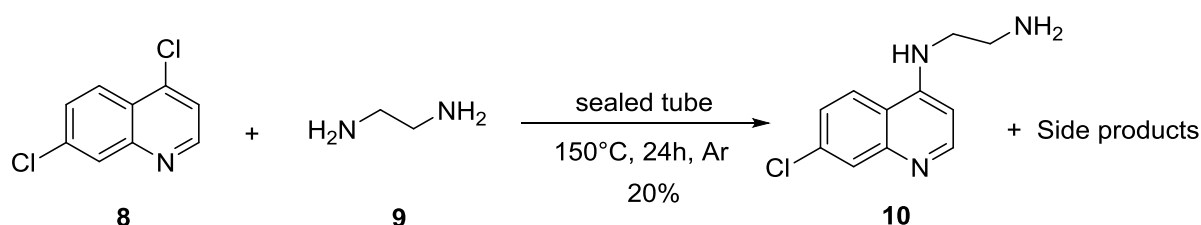


Figure 2. Structure of chloroquine **6** and hydroxychloroquine **7**

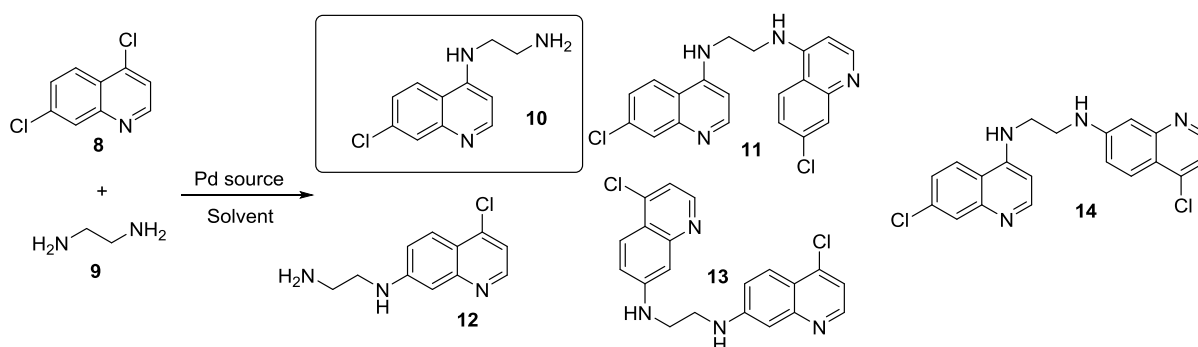
Our first approach consisted in the heating of a mixture of 4,7-dichloroquinoline **8** and ethylenediamine **9** at high temperature (150°C, sealed tube under argon) in the absence or presence of solvent (Scheme 1).



Scheme 1. First envisioned synthetic pathway for the preparation of derivative **10**

Unfortunately, whatever the experimental conditions (data not shown), it was impossible to obtain the expected polyamine quinoline derivative **10** in an acceptable state of purity and good conversion, since the formation of numerous side products was always observed. As an alternative, we considered a strategy involving a palladium catalyzed amination procedure. Palladium has become, over the last 30 years, the most versatile transition metal in metal-catalyzed reactions particularly those involving carbon-nitrogen and carbon-carbon bond formation but, to the best of our knowledge few of these couplings involve a polyamine as a nucleophile.¹⁴

4,7-Dichloroquinoline **8** was chosen as test substrate and the reaction was carried out using ethylenediamine **9** as polyamine nucleophile under various experimental conditions. The formation of potentially different products was monitored by HPLC to investigate the best experimental reaction conditions by varying the nature of the solvent, of the palladium source as well as of the ligand even if numerous works in this field recommend dioxane/tBuONa/Xantphos or RuPhos as best protocols¹⁵ (Scheme 2).



Scheme 2. Pd(0)-catalyzed amination of 4,7-dichloroquinoline **8** with ethylenediamine **9**

In all cases compound **10** appeared to be the major compound formed of the expected ones (Table 1). Diarylated product **11** was also isolated but none of the isomers **12-14** was detected under the applied experimental conditions.

Table 1. Influence of the experimental conditions on the palladium catalyzed amination of 4,7-dichloroquinoline **8** with ethylenediamine **9**

Entry	Experimental conditions	Conversion yield (%) ^[j]				
		8	10	11	12-13-14	By-product
1 ^[a]	EtOH 96%	1	0	0	0	99
2 ^[a]	THF	1	35	20	0	44 ^[k]
3 ^[a]	DMF	58	2	0	0	42
4^[a]	Dioxane	0	87	13	0	0
5 ^[a]	Toluene	95	0	0	0	5 ^[k]
6 ^[a]	CH ₂ Cl ₂	36	0	0	0	64 ^[k]
7 ^[a]	CH ₃ CN	31	33	6	0	30 ^[k]
8 ^[a]	N-methyl pyrrolidone	32	0	0	0	68 ^[k]
9 ^[b]	Na ₂ CO ₃	0	25	7	0	32 ^[k]
10 ^[b]	NaOAc	52	3	0	0	45 ^[k]
11 ^[b]	<i>tert</i> -BuOK	8	70	15	0	7 ^[k]
12 ^[b]	K ₂ CO ₃	47	21	0	0	32 ^[k]
13 ^[b]	NaOMe	64	15	1	0	20 ^[k]
14 ^[b]	K ₃ PO ₄	46	36	1	0	17 ^[k]
15 ^[b]	Cs ₂ CO ₃	15	72	12	0	1 ^[k]
16	Pd(PPh ₃) ₄ (2.4 mol%) ^[d]	99	1	0	0	0
17 ^[c]	L ₁ ^[e]	16	69	2	0	13 ^[k]
18 ^[c]	L ₂ ^[f]	71	2	0	0	27 ^[k]

19 ^[c]	L ₃ ^[g]	64	1	0	0	35 ^[k]
20 ^[c]	L ₄ ^[h]	75	1	0	0	24 ^[k]
21 ^[c]	L ₅ ^[i]	82	5	0	0	13 ^[k]

[a] Pd(dba)₂/BINAP(1/2) (2.4 mol%), *t*-BuONa, solvent, 12 h, reflux. [b] Pd(dba)₂/BINAP(1/2) (2.4 mol%), Base, dioxane, reflux, 12 h. [c] Pd(dba)₂/Ligand(1/2) (2.4 mol%), *t*-BuONa, dioxane, reflux, 12 h. [d] *t*-BuONa, dioxane, reflux, 12 h. [e] L₁ = 4,5-Bis(diphenylphosphino)-9,9-dimethylxanthene. [f] L₂ = 1,1'-Bis(diphenylphosphino)-ferrocene. [g] L₃ = 1,2-Bis(diphenylphosphino)ethane [h] L₄ = 1,1'-Bis[bis(5-methyl-2-furanyl)phosphino]ferrocene. [i] L₅ = (1*R*,2*R*)-*N,N*-Bis[2-diphenylphosphino)benzyl]cyclohexane-1,2-diamine. [j] HPLC conversion based on 4,7-dichloroquinoline. [k] non-identified by-products.

Although compound **10** was formed in 87% conversion yield (74% isolated yield) when the reaction was conducted in dioxane (Table 1, entry 4), it clearly appeared that the nature of the solvent had a great influence on the outcome of the reaction. Thus, classical solvents such as THF and acetonitrile led to the formation of the expected product **10** in low conversion yields (35 and 33% yield, respectively (Table 1, entries 2, 7)) whereas no conversion occurred using toluene (Table 1, entries 5). By using ethanol as the solvent, unexpected compound **15** was obtained in 99% yield and 4-chloroquinolidinone **16** was isolated in 42% yield by performing the reaction in DMF containing traces of water, respectively (Figure 3).

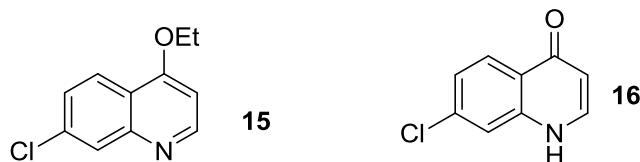


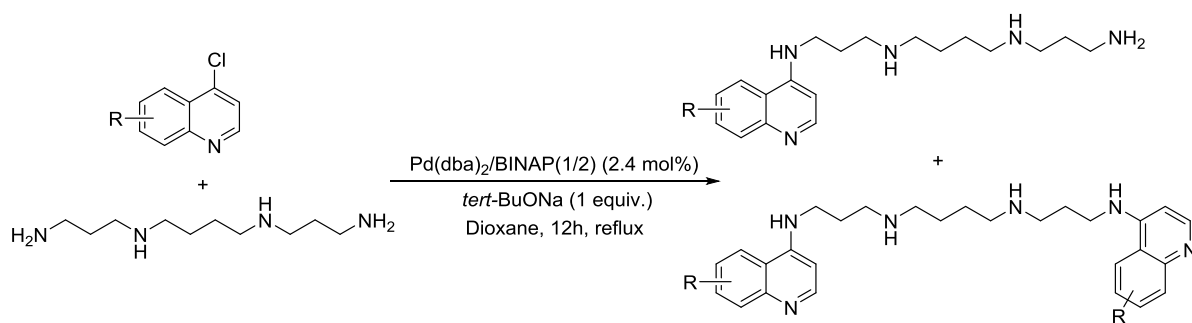
Figure 3. Structure of the isolated by-products **15** and **16**

Reactions performed in THF, CH₃CN, CH₂Cl₂, *N*-methyl pyrrolidinone lead to the formation of numerous non identified by-products, which we have not fully characterized.

As far as ligands are concerned, we have investigated the importance of the nature and structure of different organophosphorus derivatives. Low conversions were observed using classical monodentate PPh₃ or more complex bidentate ligands and BINAP remained the ligand of choice for this reaction.

Under the best experimental conditions (Table 1, entry 4) and by using various substituted chloroquinoline derivatives, we were also able to successfully perform the reaction with spermine as polyamine nucleophile (Table 2). In all experiments, the expected monoarylated derivative was obtained in moderate to good chemical yields.

Table 2. Palladium catalyzed amination of various quinoline substrates with spermine as polyamine nucleophile

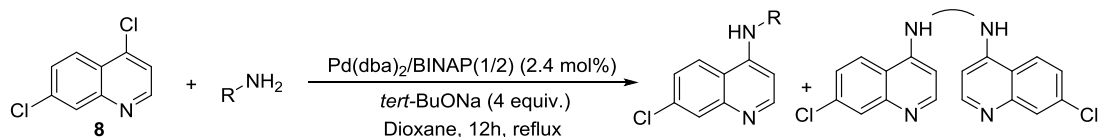


Entry	Substrate	Product (isolated yield (%)) ^[a]
1		64% 17 8% 18
2		63% 20 6% 21
3		32% 23 2% 24
4		30% 26

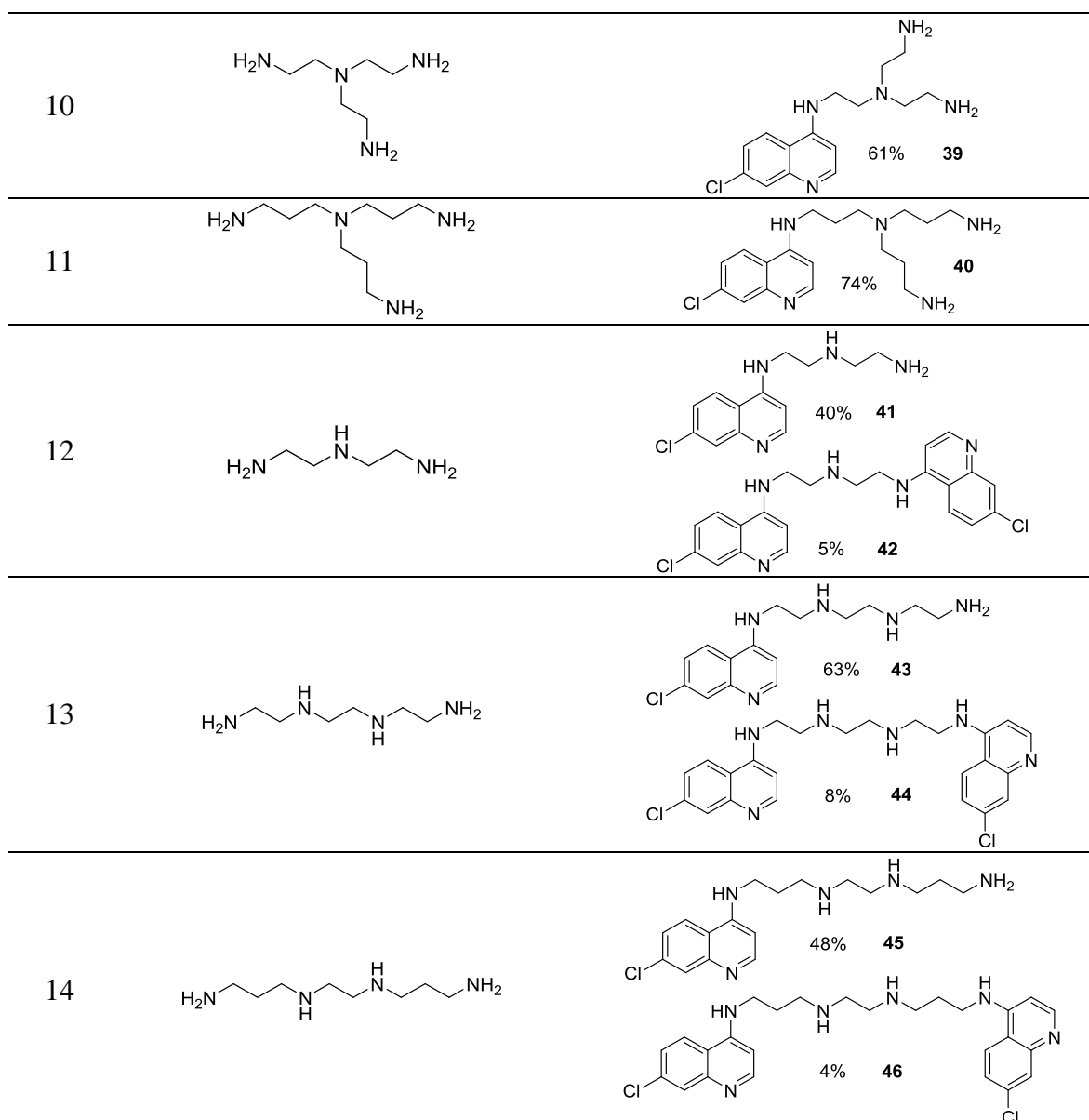
[a] Isolated yield based on chloroquinoline derivative involved

As an extension of this reaction, various polyamines have been selected to perform the palladium catalyzed amination of 4,7-dichloroquinoline **8** under our best experimental conditions giving the monoarylated compound in yields ranging from 40 to 77% yield (Table 3).

Table 3. Palladium catalyzed amination of 4,7-dichloroquinoline **8** with various polyamines



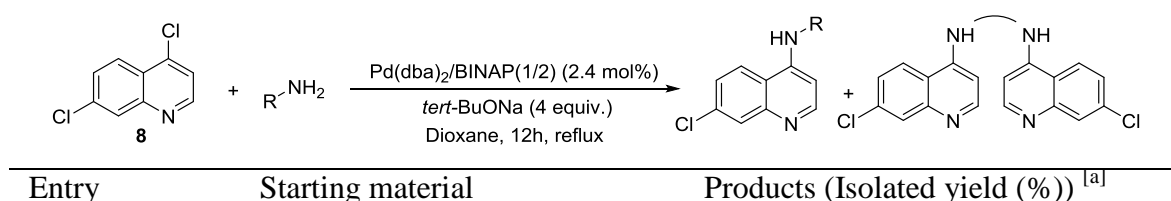
Entry	Polyamine	Products (Isolated yield (%)) ^[a]
1		
2		
3		
4		
5		
6		
7		
8		
9		

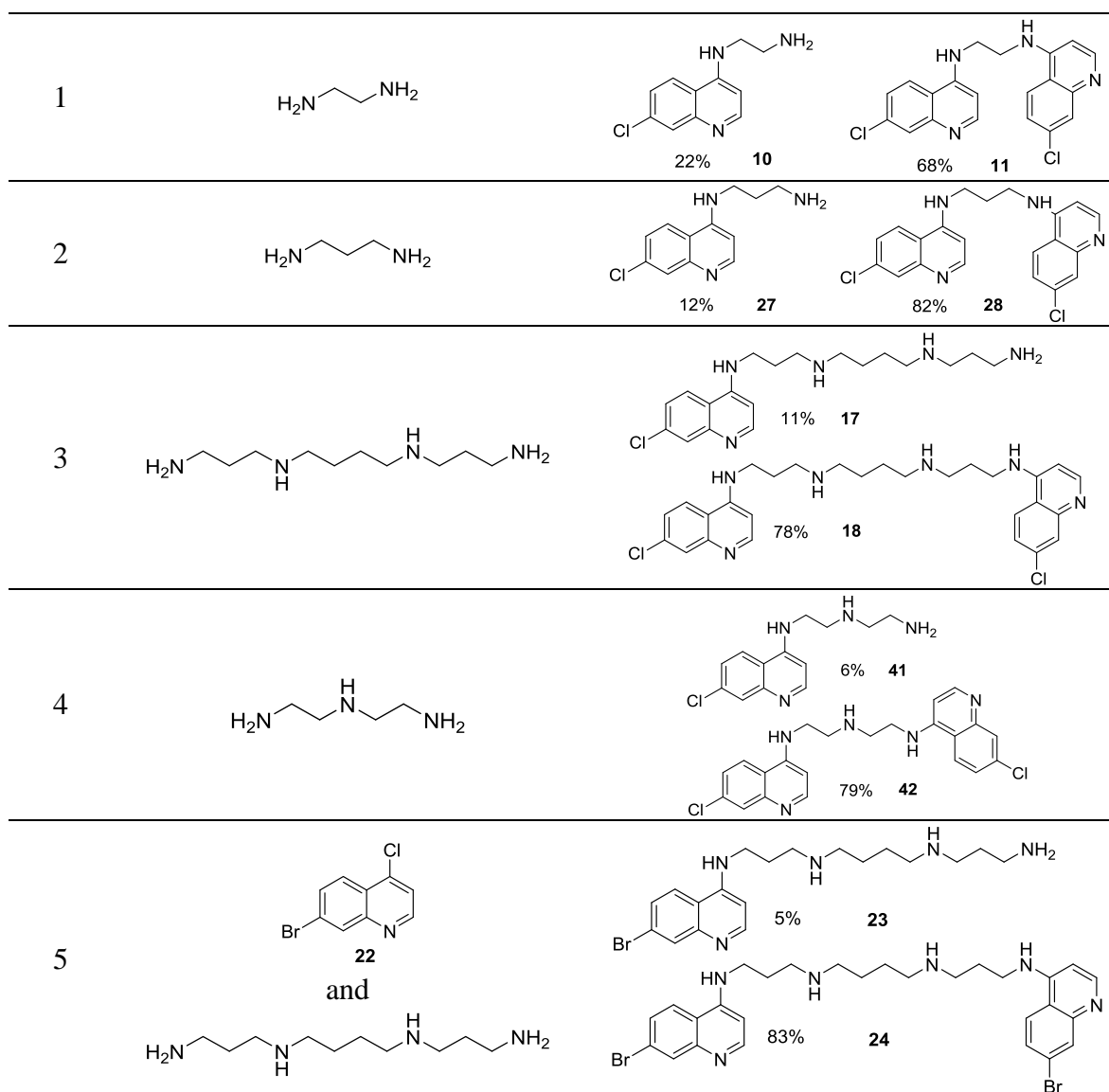


[a] Isolated yield based on 4,7-dichloroquinoline **8** involved.

In a complementary fashion, diarylated polyamine derivatives were prepared using 2 equivalents of 4,7 dichloroquinoline in the presence of 1 equivalent of the considered polyamine. Under these conditions, the di-adducts were obtained in isolated yields ranging from 68 to 83% depending on the nature of the polyamine (Table 4).

Table 4. Palladium catalyzed synthesis of various diarylated polyamine.





[a] Isolated yield based on 4,7-dichloroquinoline **8** involved.

Subsequent examination of the reaction between spermine (2 equiv.) and 4,7-dichloroquinoline **8** (1 equiv.) by HPLC analysis indicated the initial formation of the monoarylated derivative **17** reaching a plateau of 75% yield after 1 h of reaction time followed by an increase of the formation of the diarylated compound **18** (Figure 4A). When 2 equivalents of 4,7-dichloroquinoline are involved, the diarylated product **18** is predominantly formed in up to 80% yield after only 6 hours (Figure 4B)

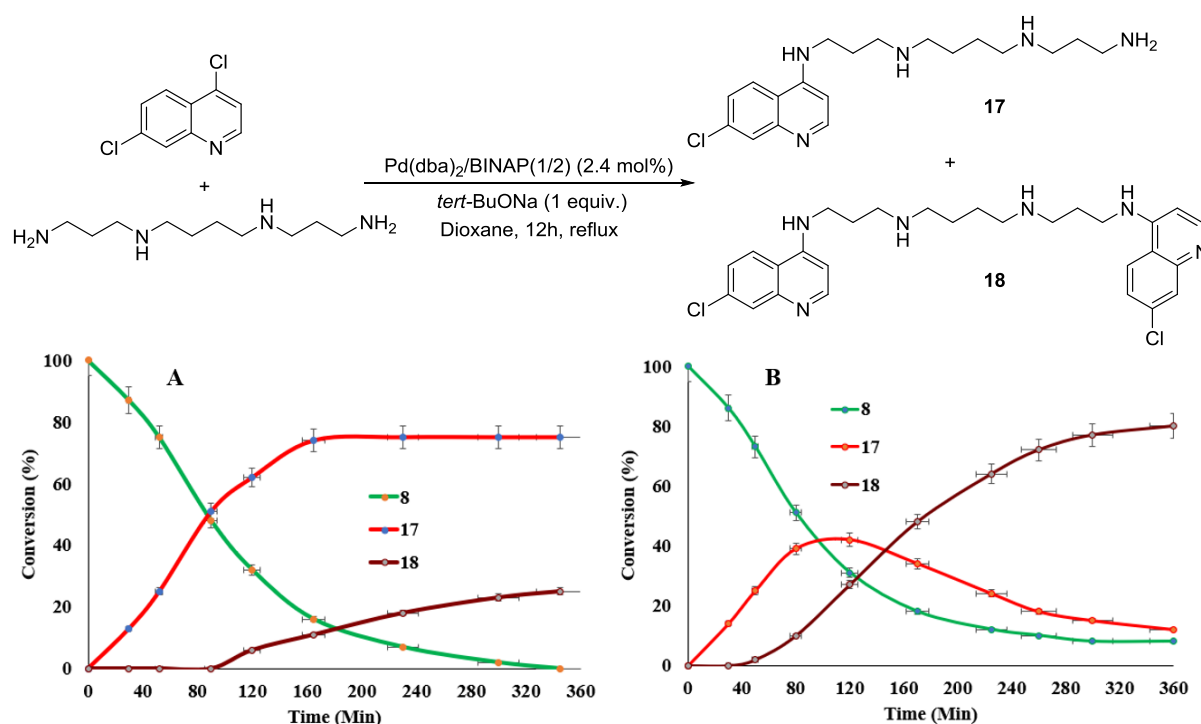


Figure 4. Conversion/time diagram for the palladium catalyzed reaction of 4,7 dichloroquinoline **8** in the presence of 1 (**A**) or 2 (**B**) equivalents of spermine.

The above-mentioned products constituted a library, which was evaluated for activity against both Gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus*, *Staphylococcus intermedius*) (Table 5). As a general trend, derivative **18** exhibited the most pronounced activity against these bacteria with MICs of 25 μM (15 $\mu\text{g/mL}$) but exhibited significant cytotoxicity towards CHO cells with an CC_{50} of around 2 μM . All the other derivatives did not present any activity ($\text{MIC} > 200 \mu\text{M}$) against the selected bacterial strains but CC_{50} ranging from 1 to 500 μM depending on the nature of the polyamine attached to the quinoline fragment as well as the type (Me, Cl, Br) and position (6-Br/7Br) of the quinoline substituent. Moreover, it clearly appeared that diarylated (bis quinoline) products **11**, **24**, **18**, **28** and **42** presented higher cytotoxicities (CC_{50} varying from 1 to 11 μM) compared to those of the monoarylated, thus limiting their potential use for a therapeutic approach.

Table 5. Antibacterial activities and cytotoxicity of polyamine quinoline hybrids

Cpd	MIC (μM)				$\text{CC}_{50} \pm \text{SD}$ (μM)	Cpd	MIC (μM)				$\text{CC}_{50} \pm \text{SD}$ (μM)
	P.a ^a	E.c ^b	S.a ^c	S.i ^d			P.a ^a	E.c ^b	S.a ^c	S.i ^d	
6	>200	>200	>200	>200	>500	29	>200	>200	>200	>200	241.25±15.17
7	>200	>200	>200	>200	73.25±3.46	30	>200	>200	>200	>200	143.71±12.63
10	>200	>200	>200	>200	113.26±9.47	31	>200	>200	>200	>200	29.81±3.37

11	>200	>200	>200	>200	4.04±1.34	32	>200	>200	>200	>200	>500
17	200	200	200	200	473.60±9.65	33	100	200	200	200	124.23±6.41
18	25	25	25	25	1.84±0.18	35	200	200	200	200	33.44±2.61
20	>200	200	>200	>200	76.03±4.56	37	200	200	200	200	83.22±4.89
21	200	200	100	100	129.19±8.94	39	200	200	200	200	>500
23	200	200	100	200	43.64±2.07	40	200	200	200	200	>500
24	100	200	100	100	120.19±8.36	41	>200	>200	>200	>200	>500
26	200	200	200	200	30.61±3.18	42	>200	>200	>200	>200	10.85±2.08
27	>200	>200	>200	>200	135.35±9.17	43	200	>200	>200	>200	>500
28	>200	>200	>200	>200	<1	45	>200	>200	>200	>200	242.92±8.12

^a *Pseudomonas aeruginosa* PA01 with streptomycin (MIC 21.5 µM) and colistine (MIC 1 µM) used as positive controls and values presented as the mean (n = 3). ^b *Escherichia coli* ATCC25922 with streptomycin (MIC 21.5 µM) and colistine (MIC 2 µM) used as positive controls and values presented as the mean (n = 3). ^c *Staphylococcus aureus* ATCC 25923 and *Staphylococcus intermedius sp* with streptomycin (MIC 21.5 µM) and chloramphenicol (MIC 1.5–3 µM) used as positive controls and values presented as the mean (n = 3).

In subsequent experiments, all derivatives were evaluated for their ability to enhance the antibiotic activity of doxycycline towards *P. aeruginosa* PA01. While doxycycline alone was totally ineffective against this bacterial strain (MIC 64 µg/mL), a combination of doxycycline at 2 µg/mL with several derivatives (**17**, **18**, **23**, **24**, **26**) was able to sensitize the action of the antibiotic (Table 6). The absence of such a phenomenon for numerous compounds implies a narrow structural specificity for the observed activity. Thus, we could mention that the presence of numerous nitrogen atoms (positive charges) lead to more efficient derivatives such as those presenting a spemine moiety.

Table 6. MIC of the compounds required to sensitize doxycycline activity at 2 µg/mL against *P. aeruginosa* PA01.

Cpd	Concentration (µM) (µg/mL)	Cpd	Concentration (µM) (µg/mL)
6	200 (64)	29	200 (50)
7	200 (67)	30	200 (53)
10	100 (22)	31	200 (72)
11	200 (76)	32	200 (73)

17	25 (9.07)	33	50 (17)
18	3,12 (1.63)	35	100 (31)
20	100 (34)	37	50 (15)
21	50 (24)	39	50 (15)
23	25 (10)	40	50 (17)
24	12,5 (7.6)	41	200 (53)
26	25 (10)	42	50 (21)
27	200 (47)	43	200 (61)
28	200 (79)	45	100 (33)

^a Concentration (μM) required to sensitize doxycycline activity at 2 $\mu\text{g}/\text{mL}$ (4.5 μM) against *P. aeruginosa* PA01.

As an extension of this study, we have investigated the use of various tetracycline antibiotics and determined the efficiency of two antibiotic-adjuvant (**17** and **24**) combinations against PA01 (Table 7). Among all the antibiotics tested, minocycline and tigecycline in combinations with derivative **24** at 3.125 and 6.25 μM respectively led to the best results enabling minocycline or tigecycline to become active at 2 $\mu\text{g}/\text{mL}$ against *P. aeruginosa* PA01. Altogether these results demonstrate that derivatives **24** represent a good candidate for circumventing *P. aeruginosa* tetracycline antibiotics resistance. A similar behavior was encountered with chloramphenicol, erythromycin and nalidixic acid used at 2 $\mu\text{g}/\text{mL}$ against *P. aeruginosa* PA01 but with higher concentrations of the adjuvant used. It is also worth noticing a successful attempt against *E. coli* by using a 12.5 μM concentration of **24**, to sensitize erythromycin activity at 2 $\mu\text{g}/\text{mL}$.

Table 7. MIC of **24** or **17** required to sensitize antibiotics activity at 2 $\mu\text{g}/\text{mL}$ against *P. aeruginosa* PA01.

Cpd	Concentration (μM)						
	Doxy	Mino	Tetra	Tige	Chlor	Ery	Nal
24	12.5	3.125	25	6.25	12.5	50	12.5
17	25	50	200	100	>200	>200	>200

^a Doxy: Doxycycline. ^b Mino: Minocycline. ^c Tetra: Tetracycline. ^d Tige: Tigecycline. ^e Chlor: Chloramphenicol. ^f Ery: Erythromycin. ^g Nal: Nalidixic acid.

Based on these results, the monoarylated compound **17** and the diarylated one **24** were selected for rationalization of their potent mechanism of action against bacteria and more precisely towards the membranes of bacteria (See also Figure S1 and S2).

Lipophilic polyamines such as naphthylacetylspermine,¹⁶ ianthelliformisamine¹⁰ or polyaminosterol derivatives^{17,18} are well-known for their capacities to increase the permeability of bacterial outer membranes enhancing subsequently the action of hydrophobic antibiotics. In this context, we have investigated the effect of **17** and **24** on bacterial membrane integrity of Gram-negative (*Pseudomonas aeruginosa* PA01) and Gram-positive (*Staphylococcus aureus* ATCC25923) bacteria by determining the permeabilization of the outer membrane and depolarization of the inner one. Briefly, Polymyxin B (PMB) and Polymyxin nonapeptide (NONA) were chosen for these studies as positive controls. In a first approach, a nitrocefin colorimetric method efficiently hydrolyzed by periplasmic β -lactamases was used to study the permeabilization of the outer membrane of PA01 and to determine if our compounds possess outer membrane-facilitating activity.^{19,20}

As shown in Figure 5, even when used at a low concentration polyamine quinoline **24** increases the rate of nitrocefin hydrolysis compared to that of the PMB treated control whereas a lower rate of hydrolysis is observed in the presence of derivative **17**. Thus, in this latter case, the rate of nitrocefin hydrolysis suggests a similar mode of action for **17** than NONA.

All these data suggest that compounds **24** and **17** possess a different behaviour towards the outer membrane of *P. aeruginosa* PA01. Polyamine quinoline **24** might disrupt the membrane integrity of the Gram-negative bacteria whereas compound **17** could interact with cell membranes leading to pore formation.

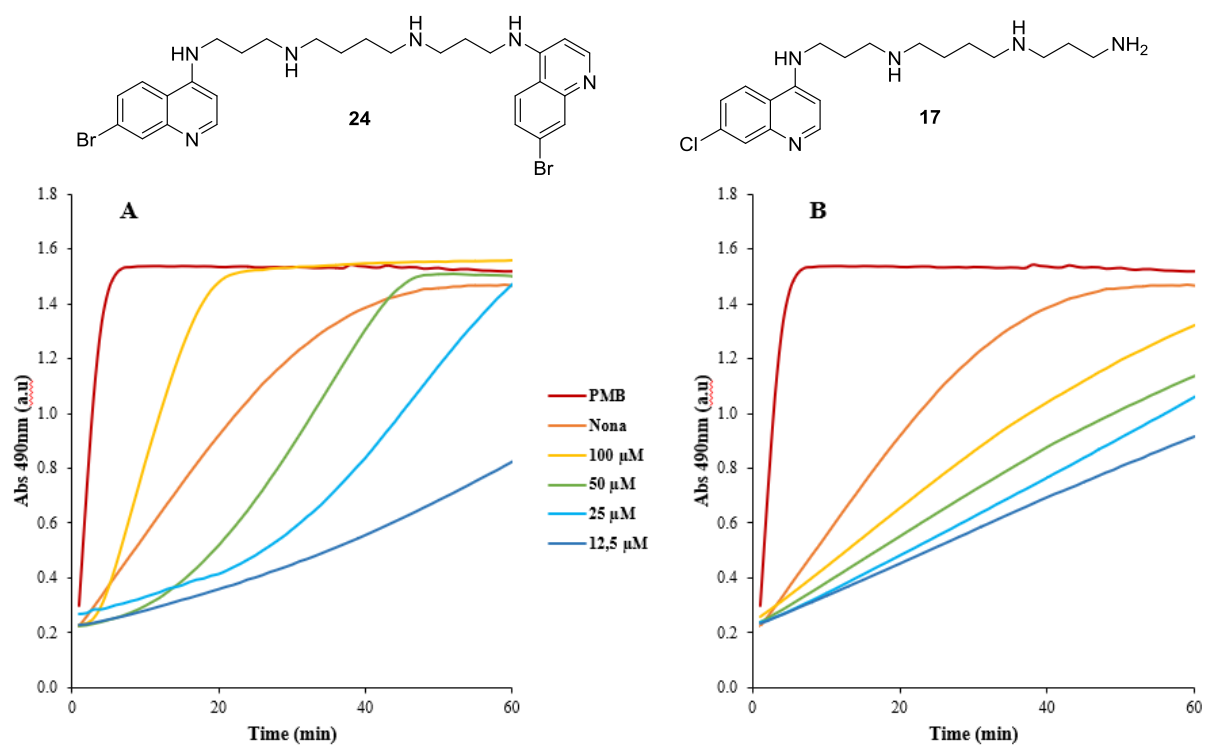


Figure 5. Dose dependent study of outer membrane permeabilization of *P. aeruginosa* (PA01) by evaluating the rate of nitrocefin hydrolysis in the presence of compound **24** (A) and **17** (B). Membrane permeation effect compared to PMB (200 μM) and PMBn (106.3 μM) To better understand whether **17** and **24** might act as disruptors of the transmembrane potential, a real-time efflux assay was used.²¹ This assay makes use of the membrane-potential-sensitive probe DiSC₃(5), which possesses a strong affinity for the bacterial inner membrane, where it concentrates by losing its fluorescence through self-quenching. If a strong depolarization of the membrane occurs, the dye is released, inducing a fluorescence rate which can be monitored. Closer examination of the membrane depolarization of *S. aureus* after only 10 min of incubation exhibited by polyamine quinoline derivatives identified a dose-dependent response, with a rapid and strong increase in fluorescence (RFU) after 10 minutes in the case of **24** whereas a lower depolarization effect was encountered with **17** in 36 and 10%, respectively (Figure 6). A strong depolarization effect of the inner membrane was also encountered in a dose dependent manner for **24** against Gram-negative *P. aeruginosa* PA01 bacterial strain whereas no effect was noticed even at high concentrations for **17**. Once again, a difference in terms of mechanism of action of both polyamine quinoline **17** and **24** can be underlined. Concerning derivative **24** and its ability to enhance doxycycline activity towards PA01 numerous questions remain suggesting that this restoration of

antibiotic susceptibility could occur via indirect inhibition of the efflux pump or by direct interaction of the molecule with the efflux pumps of the bacteria

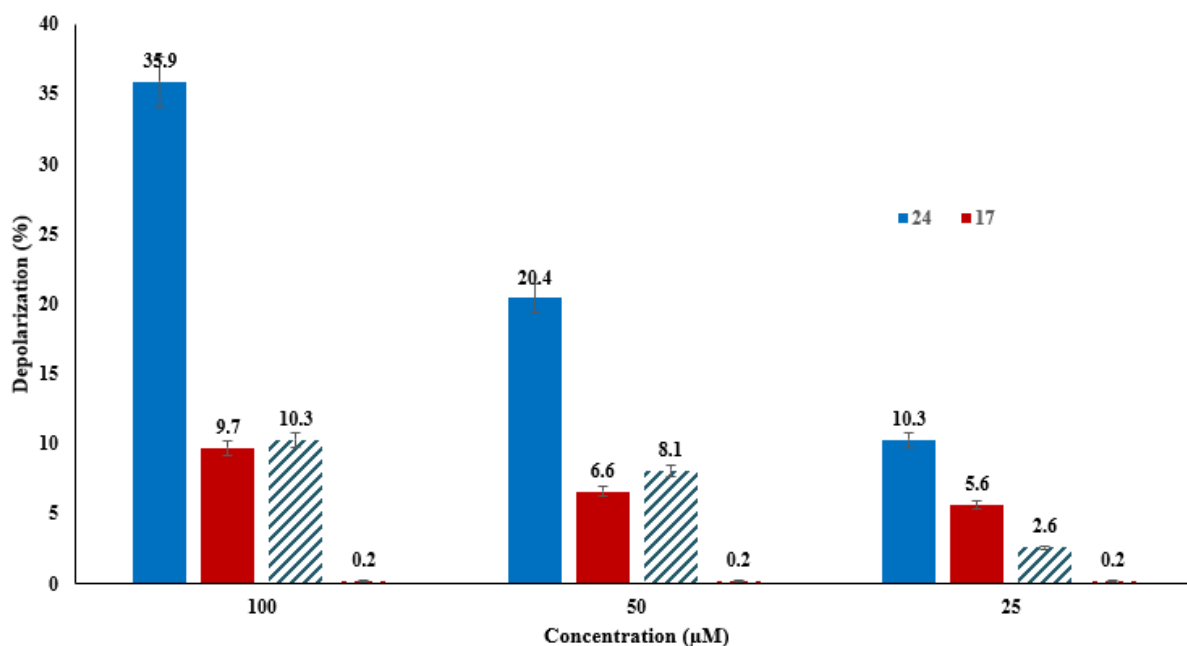


Figure 6. Dose dependent membrane depolarization of *P. aeruginosa* (PA01) and *S. aureus* by **24** and **17** as measured using DiSC₃(5) fluorescent dye release after 10 minutes.

3. Conclusion

Two derivatives **24** and **17** from our polyamine quinoline library were identified as moderate to excellent doxycycline enhancing agents towards *Pseudomonas aeruginosa* Gram-negative bacteria. Preliminary exploration of the findings identified that derivative **24** can disrupt the integrity of, and depolarize, bacterial membranes. Studies are under way to investigate more precisely the mechanism of action of monoarylated compound **17** against a larger panel of bacteria.

4. Experimental section

4.1. Materials

All solvents were purified according to previously reported procedures, and the reagents used are commercially available. Methanol, ethyl acetate, and dichloromethane were purchased from Fisher Scientific and used without further purification. Column chromatography was performed using Merck silica gel (70-230 mesh). ¹H NMR and ¹³C NMR spectra were recorded in MeOD using a Bruker AC 400 spectrometer (abbreviations: s: singlet, d: doublet, t: triplet, q: quadruplet, m: multiplet). All chemical shifts are presented in ppm. Mass spectroscopy analysis was performed at Spectropole (Analytical Laboratory, Aix-Marseille

university). The purity of the compounds was verified by analytical HPLC (C18 column, eluent MeOH-water-HCOOH, 2.3 mL/Min) with a PDA detector from 210 nm to 310 nm. All compounds showed purity greater than 95%, as determined by analytical HPLC-PDA at 254 nm.

4.2 General procedure for the synthesis of monoarylated compounds.

In a two-necked round flask, 4.8 mg Pd(dba)₂ ($8.2 \cdot 10^{-3}$ mmol) (2.4 mol%) was introduced in 8 mL of dioxane. BINAP (10.2 mg, $1.6 \cdot 10^{-2}$ mmol) was then added followed by 34 mg of *tert*-BuONa (0.17 mmol). The reaction was stirred for 10 minutes, then 4,7-dichloroquinoline (33 mg, 0.17 mmol) and spermine (34 mg, 0.17 mmol) were subsequently added. The mixture was stirred at reflux for 10 hours. After cooling, the solvent was removed *in vacuo* and the crude residue purified by column chromatography silica gel (eluent: ethylacetate, MeOH and CH₂Cl₂/MeOH/NH₄OH, 7:3:1) affording 39 mg of **17** (64% isolated yield). ¹H NMR (MeOD, 400 MHz) : δ = 8.34 (d, *J* = 8.0 Hz, 1H), 8.06 (d, *J* = 8.0 Hz, 1H), 7.76 (d, *J* = 4.0 Hz, 1H), 7.38 (dd, *J* = 12.0, 4.0 Hz, 1H), 6.51 (d, *J* = 4 Hz, 1H), 3.42 (t, *J* = 8.0 Hz, 2H), 2.56-2.78 (m, 10H), 1.91-1.98 (m; 2H), 1.63-1.70 (m, 2H), 1.52-1.56 (m, 2H). ¹³C NMR (MeOD, 100 MHz) : δ = 152.69, 152.48, 149.71, 136.30, 127.65, 125.97, 124.30, 118.80, 99.64, 50.58, 50.55, 48.45, 48.23, 42.30, 40.58, 33.05, 28.99, 28.31, 28.18. MS (ESI) C₁₉H₃₀ClN₅ *m/z* 364.4135 (100 %, (M + H⁺)).

A similar procedure was applied for the synthesis of the other monoarylated compounds

Compound 10: ¹H NMR (MeOD, 400 MHz) : δ = 8.27 (d, *J* = 8.0 Hz, 1H), 7.99 (d, *J* = 8.0 Hz, 1H), 7.69 (d, *J* = 4.0 Hz, 1H), 7.27 (m, 1H), 6.43 (d, *J* = 8 Hz, 1H), 3.33 (t, *J* = 8.0 Hz, 2H), 2.92 (t; *J* = 8 Hz, 2H). ¹³C NMR (MeOD, 100 MHz) : δ = 152.68, 152.32, 149.58, 136.21, 127.56, 125.91, 124.23, 118.71, 99.64, 46.40, 40.88. MS (ESI) C₁₁H₁₂ClN₃ *m/z* 222.0690 (100 %, (M + H⁺)).

Compound 15: ¹H NMR (CDCl₃, 400 MHz) : δ = 8.68 (d, *J* = 4.0 Hz, 1H), 8.12 (d, *J* = 8.0 Hz, 1H), 7.99 (d, *J* = 4.0 Hz, 1H), 7.40 (dd, *J* = 4.0, 12.0 Hz, 1H), 7.22 (d, *J* = 4.0 Hz, 1H), 4.21 (q, *J* = 8.0, 16.0 Hz, 2H), 1.54 (t, *J* = 8.0 Hz, 3H). ¹³C NMR (CDCl₃, 100 MHz) : δ = 161.68, 152.63, 149.81, 135.72, 127.91, 126.95, 123.64, 119.98, 100.97, 64.41, 14.52. MS (ESI) C₁₁H₁₀ClNO *m/z* 208.0451 (100 %, (M + H⁺)).

Compound 16: ¹H NMR (MeOD, 400 MHz) : δ = 8.20 (d, *J* = 8.0 Hz, 1H), 7.96 (d, *J* = 8.0 Hz, 1H), 7.60 (d, *J* = 4.0 Hz, 1H), 7.37 (dd, *J* = 8.0, 4.0 Hz, H), 6.31 (d, *J* = 8.0 Hz, 1H). ¹³C

NMR (MeOD, 100 MHz) : δ = 180.16, 142.18, 14.91, 139.54, 128.27, 125.94, 125.25, 118.76, 110.41. MS (ESI) C_9H_6ClNO m/z 180.0108 (100 %, (M + H⁺)).

Compound 20: ¹H NMR (MeOD, 400 MHz) : δ = 8.23 (d, J = 8 Hz, 1H), 7.90 (d, J = 8.0 Hz, 1H), 7.52 (s, 1H), 7.19 (dd, J = 8.0, 2.0 Hz, 1H), 6.35 (d, J = 4 Hz, 1H), 3.31 (t, J = 8.0 Hz, 3H), 2.43-2.70 (m, 10H), 2.40 (s, 3H), 1.78-1.90 (m, 2H), 1.41-1.65(m, 6H). ¹³C NMR (MeOD, 100 MHz) : δ = 152.51, 151.15, 149.05, 140.81, 127.88, 127.54, 122.07, 118.19, 98.73, 50.50, 50.41, 48.44, 48.16, 42.19, 40.55, 32.80, 29.01, 28.23, 28.10, 21.65. MS (ESI) $C_{20}H_{33}IN_5$ m/z 344.2770 (100 %, (M + H⁺)).

Compound 23: ¹H NMR (MeOD, 400 MHz) : δ = 8.43 (d, J = 8.0 Hz, 1H), 7.92 (d, J = 8.0 Hz, 1H), 7.19 (d, J = 8.0 Hz, 1H), 7.11 (dd, J = 8, 4.0 Hz, 1H), 6.86 (d, J = 4.0 Hz, 1H), 3.27 (t, J = 8.0 Hz, 2H), 2.49-2.78 (m, 10H), 1.83-1.93 (m, 2H), 1.44-1.71 (m, 6H). ¹³C NMR (MeOD, 100 MHz) : δ = 152.53, 152.36, 150.54, 143.86, 125.56, 121.30, 120.22, 117.87, 103.12, 50.52, 50.49, 48.43, 48.21, 42.26, 40.59, 33.17, 29.53, 28.24, 28.18. MS (ESI) $C_{19}H_{30}Br_2N_5$ m/z 408.1664 (100 %, (M + H⁺)).

Compound 26: ¹H NMR (MeOD, 400 MHz) : δ = 8.32 (d, J = 4 Hz, 1H), 7.74 (d, J = 8.0 Hz, 1H), 7.47 (d, J = 4.0 Hz, 1H), 7.27 (dd, J = 8.0, 2.0 Hz, 1H), 6.95 (d, J = 4 Hz, 1H), 3.29 (t, J = 8.0 Hz, 3H), 2.52-2.83 (m, 10H), 1.85-1.97 (m, 2H), 1.60-1.74 (m, 2H), 1.44-1.59 (m, 4H). ¹³C NMR (MeOD, 100 MHz) : δ = 149.79, 144.94, 143.97, 141.07, 130.26, 129.90, 124.09, 122.64, 93.38, 50.50, 50.43, 48.44, 48.18, 42.49, 40.55, 32.86, 29.47, 28.20, 28.13. MS (ESI) $C_{19}H_{30}BrN_5$ m/z 408.1664 (100 %, (M + H⁺)).

Compound 27: ¹H NMR (CDCl₃, 400 MHz) : δ = 8.35 (d, J = 4.0 Hz, 1H), 7.81 (d, J = 4.0 Hz, 1H), 7.60 (d, J = 8.0 Hz, 1H), 7.14 (dd, J = 12.0, 4.0 Hz, 1H), 6.16 (d, J = 4.0 Hz, 1H), 2.93-3.04 (m, 2H), 2.63 (t, J = 8.0 Hz, 2H), 1.49 (t, J = 4.0 Hz, 2H). ¹³C NMR (CDCl₃, 100 MHz) : δ = 151.86, 150.41, 148.96, 134.39, 128.01, 124.64, 122.25, 117.46, 98.15, 43.24, 41.18, 29.87. MS (ESI) $C_{12}H_{14}ClN_3$ m/z 236.0847 (100 %, (M + H⁺)).

Compound 29: ¹H NMR (MeOD, 400 MHz) : δ = 8.22 (d, J = 8.0 Hz, 1H), 7.96 (d, J = 8.0 Hz, 1H), 7.67 (d, J = 4.0 Hz, 1H), 7.25 (dd, J = 8.0, 4.0 Hz, 1H), 6.32 (d, J = 4.0 Hz, 1H), 3.21 (t, J = 8.0 Hz, 2H), 2.63 (t, J = 8.0 Hz, 2H), 1.63-1.67 (m, 4H). ¹³C NMR (MeOD, 100 MHz) : δ = 152.52, 152.31, 149.56, 136.12, 127.84, 125.78, 124.25, 118.66, 99.53, 43.83, 42.26, 31.29, 26.79. MS (ESI) $C_{13}H_{16}ClN_3$ m/z 250.1003 (100 %, (M + H⁺)).

Compound 30: ¹H NMR (MeOD, 400 MHz) : δ = 8.29 (d, J = 8.0 Hz, 1H), 8.04 (d, J = 8.0 Hz, 1H), 7.72 (d, J = 4.0 Hz, 1H), 7.31 (dd, J = 12.0, 4.0 Hz, 1H), 6.42 (d, J = 8.0 Hz, 1H), 3.29 (t, J = 8.0 Hz, 2H), 2.66 (t, J = 8.0 Hz, 2H), 1.40-1.75 (m, 6H). ¹³C NMR (MeOD, 100

MHz) : δ = 152.67, 152.36, 149.64, 136.21, 127.54, 125.86, 124.34, 118.73, 99.55, 43.89, 42.14, 32.74, 29.15, 25.46. MS (ESI) $C_{14}H_{18}ClN_3$ m/z 264.1160 (100 %, (M + H⁺)).

Compound 31: ¹H NMR (MeOD, 400 MHz) : δ = 8.26 (d, J = 8.0 Hz, 1H), 7.96 (d, J = 8.0 Hz, 1H), 7.68 (d, J = 4.0 Hz, 1H), 7.28 (dd, J = 12.0, 4.0 Hz, 1H), 6.40 (d, J = 8.0 Hz, 1H), 3.29 (t, J = 8.0 Hz, 2H), 2.76 (t, J = 8.0 Hz, 2H), 2.32-2.45 (m, 11H), 1.58-1.88 (m, 4H). ¹³C NMR (MeOD, 100 MHz) : δ = 152.61, 152.41, 149.56, 136.20, 127.61, 125.86, 124.37, 118.68, 99.59, 57.58, 57.15, 53.96, 53.91, 42.65, 40.61, 27.90, 25.93, 25.90. MS (ESI) $C_{19}H_{28}ClN_5$ m/z 362.2004 (100 %, (M + H⁺)).

Compound 32: ¹H NMR (MeOD, 400 MHz) : δ = 8.28 (d, J = 4.0 Hz, 1H), 8.06 (d, J = 8.0 Hz, 1H), 7.71 (d, J = 4.0 Hz, 1H), 7.33 (dd, J = 12.0, 4.0 Hz, 1H), 6.47 (d, J = 4.0 Hz, 1H), 3.42-3.54 (m, 10H), 2.97-2.90 (m, 3H), 1.79-1.97 (m, 5H), 1.52-1.63 (m, 5H). ¹³C NMR (MeOD, 100 MHz) : δ = 152.63, 151.99, 149.18, 136.14, 127.18, 125.78, 124.14, 118.46, 99.34, 71.74, 71.67, 69.50, 69.05, 62.47, 41.32, 39.16, 29.47, 29.01, 27.28. MS (ESI) $C_{19}H_{28}ClN_3O_2$ m/z 366.3101 (100 %, (M + H⁺)).

Compound 33: ¹H NMR (MeOD, 400 MHz) : δ = 8.36 (d, J = 4.0 Hz, 1H), 8.07 (d, J = 4.0 Hz, 1H), 7.77 (d, J = 4.0 Hz, 1H), 7.40 (dd, J = 12, 4.0 Hz, 1H), 6.54 (d, J = 8.0 Hz, 1H), 3.43 (t, J = 4.0 Hz, 2H), 2.75 (t, J = 8.0 Hz, 2H), 2.59-2.70 (m, 7H), 1.90-1.98 (m, 2H), 1.64-1.75 (m, 4H). ¹³C NMR (MeOD, 100 MHz) : δ = 152.72, 152.50, 149.72, 136.32, 127.65, 126.00, 124.30, 118.81, 99.64, 48.90, 48.84, 48.30, 42.35, 42.38, 40.59, 33.15, 30.21, 29.04. MS (ESI) $C_{18}H_{28}ClN_5$ m/z 350.3412 (100 %, (M + H⁺)).

Compound 35: ¹H NMR (MeOD, 400 MHz) : δ = 8.10 (d, J = 8.0 Hz, 1H), 8.01 (d, J = 12.0 Hz, 1H), 7.49 (d, J = 2.0 Hz, 1H), 7.18 (dd, J = 32.0, 4.0 Hz, 1H), 6.41 (d, J = 8.0 Hz, 1H), 3.27 (t, J = 4.0 Hz, 2H), 2.28 (m, 3H), 2.51 (m, 3H), 2.21 (s, 3H), 1.67-1.85 (m, 4H). ¹³C NMR (MeOD, 100 MHz) : δ = 154.27, 148.93, 145.53, 137.84, 126.74, 125.21, 124.41, 117.67, 99.69, 56.04, 55.91, 41.69, 39.65, 25.96, 24.83. MS (ESI) $C_{16}H_{23}ClN_4$ m/z 307.2334 (100 %, (M + H⁺)).

Compound 37: ¹H NMR (MeOD, 400 MHz) : δ = 8.34 (d, J = 4.0 Hz, 1H), 8.06 (d, J = 8.0 Hz, 1H), 7.77 (d, J = 2.0 Hz, 1H), 7.40 (dd, J = 8.0, 4.0 Hz, 1H), 6.53 (d, J = 4.0 Hz, 1H), 3.42 (t, J = 8.0 Hz, 2H), 2.60-2.80 (m, 6H), 1.88-1.98 (m, 2H), 1.65-1.72 (m, 2H). ¹³C NMR (MeOD, 100 MHz) : δ = 152.69, 152.48, 149.70, 136.31, 127.64, 125.98, 124.28, 118.79, 99.63, 48.42, 42.31, 40.61, 33.38, 29.05. MS (ESI) $C_{15}H_{21}ClN_4$ m/z 293.580 (100 %, (M + H⁺)).

Compound 39: ¹H NMR (MeOD, 400 MHz) : δ = 8.34 (d, J = 4.0 Hz, 1H), 8.06 (d, J = 8.0 Hz, 1H), 7.77 (d, J = 4.0 Hz, 1H), 7.40 (dd, J = 8.0, 4.0 Hz, 1H), 6.52 (d, J = 6.0 Hz, 1H),

3.41 (t, $J = 8.0$ Hz, 2H), 2.63-2.82 (m, 9H), 1.89-1.98 (m, 2H), 1.65-1.72 (m, 2H). ^{13}C NMR (MeOD, 100 MHz): $\delta = 152.69, 152.47, 149.69, 136.30, 127.63, 125.97, 124.27, 118.79, 99.62, 57.90, 54.01, 42.30, 40.39$. MS (ESI) $\text{C}_{15}\text{H}_{22}\text{ClN}_5$ m/z 308.2820 (100 %, (M + H⁺)).

Compound 40: ^1H NMR (MeOD, 400 MHz): $\delta = 8.35$ (d, $J = 8.0$ Hz, 1H), 8.05 (d, $J = 12.0$ Hz, 1H), 7.78 (d, $J = 2.0$ Hz, 1H), 7.42 (dd, $J = 12.0, 4.0$ Hz, 1H), 6.52 (d, $J = 8$ Hz, 1H), 3.41 (t, $J = 6$ Hz, 2H), 2.48-2.70 (m, 12H), 1.92 (m, 3H), 1.66 (m, 5H). ^{13}C NMR (MeOD, 100 MHz): $\delta = 152.72, 152.50, 149.71, 136.32, 127.67, 126.02, 124.30, 118.80, 99.64, 53.02, 52.94, 49.85, 42.69, 40.98, 30.46, 26.39$. MS (ESI) $\text{C}_{18}\text{H}_{28}\text{ClN}_5$ m/z 350.3314 (100 %, (M + H⁺)).

Compound 41: ^1H NMR (MeOD, 400 MHz): $\delta = 8.35$ (d, $J = 4.0$ Hz, 1H), 8.18 (d, $J = 8.0$ Hz, 1H), 7.76 (d, $J = 4.0$ Hz, 1H), 7.40 (dd, $J = 8.0, 4.0$ Hz, 1H), 6.56 (d, $J = 4.0$ Hz, 1H), 3.50 (t, $J = 4.0$ Hz, 2H), 2.98 (m, 4H), 2.90 (m, 2H). ^{13}C NMR (MeOD, 100 MHz): $\delta = 152.74, 152.27, 149.41, 136.35, 127.35, 126.02, 124.64, 118.72, 99.72, 52.25, 48.19, 43.51, 40.70$. MS (ESI) $\text{C}_{13}\text{H}_{17}\text{ClN}_4$ m/z 265.2665 (100 %, (M + H⁺)).

Compound 43: ^1H NMR (MeOD, 400 MHz): $\delta = 8.38$ (d, $J = 4.0$ Hz, 1H), 8.11 (d, $J = 8.0$ Hz, 1H), 7.80 (d, $J = 4.0$ Hz, 1H), 7.41 (dd, $J = 8.0, 4.0$ Hz, 1H), 6.57 (d, $J = 8.0$ Hz, 1H), 3.52 (t, $J = 6.0$ Hz, 2H), 2.98 (t, $J = 8.0$ Hz, 2H), 2.63-2.87 (m, 13H). ^{13}C NMR (MeOD, 100 MHz): $\delta = 152.72, 152.53, 149.67, 136.33, 127.67, 126.05, 124.27, 118.83, 99.74, 54.10, 52.64, 48.52, 41.89, 39.06$. MS (ESI) $\text{C}_{15}\text{H}_{22}\text{ClN}_5$ m/z 308.2739 (100 %, (M + H⁺)).

Compound 45: ^1H NMR (MeOD, 400 MHz): $\delta = 8.36$ (d, $J = 4.0$ Hz, 1H), 8.05 (d, $J = 8.0$ Hz, 1H), 7.74 (d, $J = 2.0$ Hz, 1H), 7.40 (dd, $J = 8.0, 2.0$ Hz, 1H), 6.50 (d, $J = 8.0$ Hz, 1H), 3.40 (m, 3H), 2.61-2.85 (m, 11H), 1.88-1.96 (m, 2H), 1.62-1.69 (m, 2H). ^{13}C NMR (MeOD, 100 MHz): $\delta = 152.68, 152.46, 149.68, 136.28, 127.63, 125.96, 124.30, 118.79, 99.67, 49.68, 48.44, 48.27, 42.21, 40.44, 32.60, 29.10$. MS (ESI) $\text{C}_{17}\text{H}_{26}\text{ClN}_5$ m/z 336.2957 (100 %, (M + H⁺)).

4.3 General procedure for the synthesis of diarylated compounds.

In a two-necked round flask, 4.8 mg Pd(dba)₂ ($8.2 \cdot 10^{-3}$ mmol) (2.4 mol%) was dissolved in 8 mL of dioxane. BINAP (10.2 mg, $1.6 \cdot 10^{-2}$ mmol) was then added as well as 34 mg of *t*-BuONa (0.35 mmol). The reaction was stirred for 10 minutes then 4,7-dichloroquinoline (66 mg, 0.34 mmol, 2 equiv.) and spermine (34 mg, 0.17 mmol, 1 equiv.) was subsequently added. The mixture was refluxed under stirring for 10 hours. After cooling, the solvent was removed *in vacuo* and the crude residue purified by column chromatography silica gel (eluent: ethylacetate, MeOH,) affording **18** in 78% isolated yield. ^1H NMR (MeOD, 400

MHz) : δ = 8.35 (d, J = 4.0 Hz, 2H), 8.02 (d, J = 8.0 Hz, 2H), 7.79 (d, J = 4.0 Hz, 2H), 7.36 (dd, J = 12.0, 4.0 Hz, 2H), 6.47 (d, J = 8.0 Hz, 2H), 3.41 (t, J = 8.0 Hz, 4H), 2.61-2.78 (m, 8H), 1.91-1.98 (m, 4H), 1.55-1.59 (m, 4H). ^{13}C NMR (MeOD, 100 MHz) : δ = 152.35, 152.13, 149.39, 136.10, 127.51, 125.80, 123.93, 118.53, 99.35, 50.37, 48.35, 42.20, 28.70, 28.14. MS (ESI) $\text{C}_{28}\text{H}_{34}\text{Cl}_2\text{N}_6$ m/z 525.4164 (100 %, (M + H⁺)).

A similar procedure was applied for the synthesis of the other diarylated compounds

Compound 11: ^1H NMR (MeOD, 400 MHz) : δ = 8.34 (d, J = 8.0 Hz, 2H), 8.27 (d, J = 8.0 Hz, 2H), 7.78 (d, J = 4.0 Hz, 2H), 7.53 (dd, J = 12.0, 4.0 Hz, 2H), 6.89 (d, J = 4.0 Hz, 2H), 3.89(s, 4H). ^{13}C NMR (MeOD, 100 MHz) : δ = 157.69, 146.03, 142.39, 139.28, 126.92, 123.77, 119.97, 114.00, 99.54, 41.51. MS (ESI) $\text{C}_{20}\text{H}_{16}\text{Cl}_2\text{N}_4$ m/z 383.0723 (100 %, (M + H⁺)).

Compound 21: ^1H NMR (MeOD, 400 MHz) : δ = 8.30 (m, 2H), 7.97 (m, 2H), 7.58 (m, 2H), 7.29 (m, 2H), 6.48 (m, 2H), 3.19-3.48 (m, 7H), 2.34-2.91 (m, 12H), 1.84-2.11 (m, 3H), 1.44-1.67 (m, 4H). ^{13}C NMR (MeOD, 100 MHz) : δ = 152.81, 151.09, 148.72, 142.90, 127.69, 127.54, 122.06, 118.12, 98.75, 50.30, 48.25, 42.07, 28.79, 28.05, 21.58. MS (ESI) $\text{C}_{30}\text{H}_{40}\text{N}_6$ m/z 484.3348 (100 %, (M + H⁺)).

Compound 24: ^1H NMR (MeOD, 400 MHz) : δ = 8.43 (d, J = 8.0 Hz, 2H), 7.94 (d, J = 8.0 Hz, 2H), 7.21 (d, J = 8.0 Hz, 2H), 7.12 (dd, J = 12.0, 4.0 Hz, 2H), 6.86 (d, J = 4.0 Hz, 2H), 3.27 (t, J = 8.0 Hz, 6H), 2.55-2.79 (m, 9H), 1.86-1.93 (m, 4H), 1.54-1.57 (m, 4H). ^{13}C NMR (MeOD, 100 MHz) : δ = 152.51, 152.35, 150.54, 143.90, 125.58, 121.29, 120.26, 117.89, 103.13, 50.40, 48.30, 42.20, 29.40, 28.14. MS (ESI) $\text{C}_{28}\text{H}_{34}\text{Br}_2\text{N}_6$ m/z 615.1212 (100 %, (M + H⁺)).

Compound 28: ^1H NMR (MeOD, 400 MHz) : δ = 8.28 (d, J = 8.0 Hz, 2H), 8.27 (s, 2H), 7.71 (d, J = 4.0 Hz, 2H), 7.44 (dd, J = 12.0, 4.0 Hz, 2H), 6.76 (d, J = 4.0 Hz, 2H), 3.69 (t, J = 8.0 Hz, 4H), 2.21-2.30 (m, 2H). ^{13}C NMR (MeOD, 100 MHz) : δ = 156.00, 146.03, 142.39, 139.63, 127.82, 125.78, 122.11, 117.32, 99.87, 42.15, 27.20. MS (ESI) $\text{C}_{21}\text{H}_{18}\text{Cl}_2\text{N}_4$ m/z 397.0879 (100 %, (M + H⁺)).

Compound 42: ^1H NMR (MeOD, 400 MHz) : δ = 8.21-8.23 (m, 2H), 7.83-7.87 (m, 2H), 7.64-7.67 (m, 2H), 7.15-7.19 (m, 2H), 6.39-6.42 (m, 2H), 3.36-3.47 (m, 4H), 3.32 (s, 2H), 2.92-2.97 (m, 5H). ^{13}C NMR (MeOD, 100 MHz) : δ = 152.55, 152.23, 149.41, 136.26, 127.53, 125.95, 124.02, 118.61, 99.64, 48.17, 43.25. MS (ESI) $\text{C}_{22}\text{H}_{21}\text{Cl}_2\text{N}_5$ m/z 426.1145 (100 %, (M + H⁺)).

5. Antimicrobial assays

5.1 Bacterial strains

Bacterial strains used in this study were: *S. aureus* (ATCC25923), *S. intermedius* (1051997), *E. coli* (ATCC25922) and *P. aeruginosa* (PA01). Strains were maintained at -80 °C in 15% (v/v) glycerol for cryoprotection. Bacteria were routinely grown in Mueller-Hinton (MH) broth at 37 °C.

5.2 Antibiotics

Doxycycline was purchased from TCI Europe and dissolved in water.

5.3 Antimicrobial evaluation

The susceptibility of bacterial strains to antibiotics and compounds was determined in microplates using the standard broth dilution method in accordance with the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM).²² Briefly, the Minimal Inhibitory Concentrations (MICs) were determined with an inoculum of 10^5 CFU in 200 μ L of MH broth containing two-fold serial dilutions of each drug. The MIC was defined as the lowest concentration of drug that completely inhibited visible growth after incubation for 18 h at 37 °C. To determine all MICs, the measurements were independently repeated in triplicate.

5.4 Determination of the MICs of antibiotics in the presence of synergizing compounds

Briefly, restoring enhancer concentrations were determined with an inoculum of 5×10^5 CFU in 200 μ L of MH broth containing two-fold serial dilutions of each derivative in the presence of doxycycline at 2 μ g/mL. The lowest concentration of the polyamine adjuvant that completely inhibited visible growth after incubation for 18 h at 37 °C was determined. These measurements were independently repeated in triplicate.

5.5 Outer membrane permeabilization assay

Nitrocefin was used as a chromogenic substrate of periplasmic β -lactamase to measure the outer membrane permeabilization. The nitrocefin hydrolysis assay is a colometric assay wherein a color change from yellow to red occurs when the chromogenic β -lactam is efficiently hydrolyzed by periplasmic β -lactamases. After an overnight culture of *Pseudomonas aeruginosa* PA01 at 37°C, 100 μ L of the suspension was added to 10 mL of MIII broth. Once the culture reached the mid-logarithmic phase ($OD_{600}=0.5$), cells were recovered by centrifugation ($3,600\times g$ for 20 min at 20°C) and washed twice with 20 mM

potassium phosphate buffer (pH 7.2) and 1 mM MgCl₂ (PPB). After the second centrifugation, the pellet was resuspended and adjusted at OD₆₀₀ 0.375. Then, 100 µL of the bacterial suspension was mixed with 50 µL of the compounds **17** or **24** at different concentrations ranging from 100 to 12.5 µM already set up in a 96-wells microplate. Polymyxin B (PMB) and polymyxin Nona (PMBn) were used as positive controls and PPB was used as a negative one. Finally, 50 µL of nitrocefin was added to obtain a final concentration of 50 µg/mL. Nitrocefin hydrolysis was monitored by measuring the increase in absorbance at 490 nm, using a M200 Pro Tecan spectrophotometer, for 1 hour with a 1-minute interval between each measurement. Experiments were performed in triplicate.

5.6 Membrane depolarization assays

Bacteria were grown in MH broth for 24 h at 37 °C and centrifuged at 10000 rpm at 20 °C. The supernatant was discarded, and the bacteria were washed twice with buffered sucrose solution (250 mM) and magnesium sulfate solution (5 mM). The fluorescent dye 3,3'-diethylthiacarbocyanine iodide was added to a final concentration of 3 µM and allowed to penetrate bacterial membranes during 1 h of incubation at 37 °C. Bacteria were then washed to remove the unbound dye before adding compounds **17** or **24** at different concentrations. Fluorescence measurements were performed using a M200 Pro Tecan spectrophotometer. The maximum RCF was that recorded with a pure solution of the fluorescent dye in buffer (3 µM).

5.7 Cytotoxicity toward Chinese Hamster Ovary Cells

CHO-K1 cells (ATCC-LGC Standards Sarl, Molsheim, France) were maintained in McCoy's 5A media supplemented with 10% bovine calf serum, 2 mM glutamine, and 100 (U/mL)/10 µg/mL penicillin/streptomycin. They were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. The cell lines were seeded in 96-well plates and incubated overnight. Various concentrations of compounds were incorporated in triplicate cultures, and the cells were incubated at 37 °C for 24 h. At the end of the incubation period, cells were submitted to three successive washes in phosphate buffer saline (PBS) and incubated in PBS containing 10% WST-1 for an additional 30 min. Cell viability was evaluated by measuring WST-1 absorbance at 450 nm in a microplate spectrophotometer. The results are expressed as the percentage of cell viability compared to that of the control (culture medium only), which corresponded to 100% cell viability. Dose–response curves were calculated by nonlinear

regression analysis using TableCurve V2 software. The inhibitory concentration 50% (CC₅₀) was defined as the concentration of saponin that induced a 50% decrease in cell viability.

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Supplementary information

Supplementary data related to this article can be found at

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