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Synthesis and antibacterial evaluation of bis-thiazolium, bis-imidazolium and bis-triazolium derivatives

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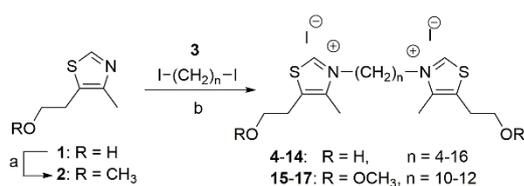
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Abstract: Because of the worldwide spreading of bacterial resistance, it is urgent to develop new compounds exhibiting potent antibacterial activity which would not be impaired by this phenomenon. Quaternary ammoniums compounds were used for many years as disinfectants, but recent advances showed that multicationic derivatives exhibit a much stronger activity and are less prone to induce bacterial resistance than commonly used monocations. In that sense, we prepared three series of new bis-cationic compounds: bis-thiazoliums, bis-imidazoliums, and bis-1,2,4-triazoliums. If some compounds of the first series showed fair antibacterial activity, most of those belonging to the two other series were highly potent with Minimum Inhibitory Concentrations close to 1 µg/mL. Some of them also exhibited low toxicity towards MRC-5 lung fibroblast eukaryotic model and we showed that this toxicity was clearly correlated with *clog P*. Finally, four selected compounds exhibited a clear bactericidal effect.

Very recently, the World Health Organization published^[1] a list of 12 bacteria whose resistance ability towards antibiotics is so high that they constitute a real threat towards human health, highlighting it is high time to react. A recent review chaired by Jim O'Neill^[2] also listed major recommendations in order to fight against antimicrobial resistance. Among them are exposed: improving hygiene, reduce unnecessary use of antibiotics, improve global surveillance of drug resistance, and better incentives to promote investment for new drugs. Developing new chemical entities is thus urgently required. Quaternary ammoniums compounds (QACs) were first discovered by Domagk in 1935^[3] and were used for many years as skin disinfectants. Mono-ammonium compounds such as benzalkonium chloride or cetylpyridinium chloride were followed by bis-ammoniums such as dequalinium chloride and chlorhexidine. Recent advances in this area indeed showed that bis- or multicationic compounds exhibit a much stronger activity than monocations. They also suggest that some derivatives of this class could be used for other purposes than simple topical administration.^[4-6] In particular, multicationic compounds are less prone to induce bacterial resistance than the classical monocationic compounds which are still commercially used.^[7,8] Furthermore, one general drawback of this class of compounds comes from the low selectivity towards eukaryotic cells. Thus, some efforts have been done in order to correlate the structural features of QACs and both their antibacterial activity and

eukaryotic toxicity.^[9,10] In addition, other recent efforts also led to the design of powerful molecules showing very low toxicity towards mammalian cells.^[11] Besides, QACs may exhibit anti-malarial activity and some bis-cationic derivatives proved to be very potent both in vitro and in vivo.^[12] Later, analogous compounds were prepared, where the cationic charges were harbored by thiazolium moieties.^[4,13] The latter compounds revealed as potent as the classical ammonium counterpart, but were much less toxic on a rodent model. We wondered whether these positively-charged heterocycles could also exhibit attractive antibacterial activity. Yet, some mono-thiazoliums showed low potency on reference bacterial strains,^[14] but we envisioned that dimeric analogues could be much more active. Thus, we prepared numerous derivatives and tested them against reference bacterial strains. The most active compound was selected for toxicity evaluation on eukaryotic cells (MRC-5). Moreover, we intended to extend this study to related 5-membered heterocycles such as imidazole derivatives. Some bis-imidazolium compounds were already prepared by others^[15,16] and showed interesting, yet moderate antibacterial activity. We envisioned replacing some alkyl moieties by aromatic groups in order to test their influence on both antibacterial activity and toxicity versus eukaryotic cells. Finally, 1,2,4-triazole analogs were also prepared, as in the frame of antifungal therapy, they led to more active compounds,^[17] and as interesting antibacterial activities have recently been reported.^[18] All these compounds were tested against reference bacterial strains but also against clinical isolates harboring various and different mechanisms of resistance to antibiotics. Furthermore, cytotoxicity on eukaryotic cells was determined for each compound.

Bis-thiazolium derivatives were easily prepared as previously described^[13] (Scheme 1) by *N*-alkylation of 4-methyl-5-(2-hydroxyethyl)thiazole with various α,ω -diiodoalkanes, the latter being prepared from commercial α,ω -dibromoalkanes.^[13] In contrast to this previous study, each even and odd linkers from $n = 4$ to $n = 12$ were prepared. As activity increased with n , 1,14-dibromotetradecane and 1,16-dibromohexadecane were prepared according to a reported procedure.^[19] Conversion to the diiodo counterparts allowed the preparation of longer analogs. Besides, *O*-methylation^[13] prior to dimerization afforded three more compounds.



Scheme 1. Synthesis of bis-thiazolium compounds. a) NaH/THF, CH₃I; b) CH₃CN, reflux, 72h.

The Minimum Inhibitory Concentrations (MICs) were evaluated against 4 reference strains: two Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) (Table 1).

Table 1. Minimum inhibitory concentration (MIC, µg/mL) values of prepared bis-thiazoliums

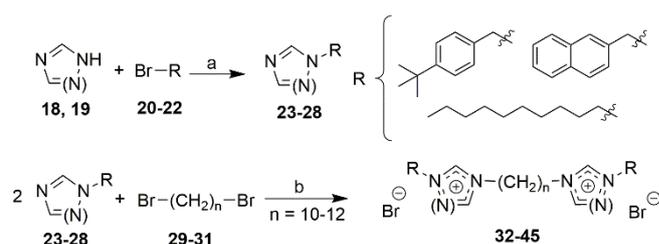
Entry ^[a]	n	<i>S.a.</i>	<i>E.f.</i>	<i>E.c.</i>	<i>P.a.</i>
Chx ^[b]	1		2	0.5	8
4	4	>256	>256	>256	>256
5	5	>256	>256	>256	>256
6	6	128	128	128	>256
7	7	>256	>256	>256	>256
8	8	>256	>256	>256	>256
9	9	>256	>256	>256	>256
10	10	64	128	64	128
11	11	256	>256	>256	>256
12	12	16	>256	16	>256
13	14	32	256	256	256
14	16	16	64	128	256
15	10	>256	>256	>256	>256
16	11	256	>256	256	>256
17	12	64	>256	128	>256

[a] All MIC data were acquired by compilation of the highest value of at least two independent trials. Twofold serial dilutions of the compounds were prepared from a stock solution at 1024 µg/mL. Each condition was repeated in 8 wells. For further details, see Supporting Information. Organisms used in this study: *S.a.* (*Staphylococcus aureus* ATCC 29213), *E.f.* (*Enterococcus faecalis* ATCC 29212), *E.c.* (*Escherichia coli* ATCC 25922), *P.a.* (*Pseudomonas aeruginosa* ATCC 27853). [b] Reference compound used: Chx (Chlorhexidine).

As a result, short-linker compounds exhibited no activity, which overall increased with the linker length. Surprisingly, compound **11** (n = 11) showed no potency while **10** (n = 10) and **12** (n = 12) were active against *E. coli* and *S. aureus* strains. Compound **12** was the most active compound of this series, with MICs reaching 16 µg/mL against these strains. No activity was

observed against *E. faecalis* and *P. aeruginosa*. Increasing the linker length did not bring significantly better activities. Compound **14** (n = 16) yet showed a better MIC than **12** against *E. faecalis* (64 µg/mL versus 256 µg/mL), but it was less potent against *E. coli* (128 µg/mL versus 16 µg/mL). Thus, we selected the lower molecular weight compound **12** for further consideration. In vitro cytotoxicity (IC₅₀) on eukaryotic lung fibroblasts (MRC-5 ATCC CCL-171) was determined for this molecule and was equal to 256 µg/mL, leading to a selectivity index (SI = IC₅₀/MIC) of 16 towards the sensitive strains. Surprisingly, compounds **15** to **17**, O-methylated analogues of compounds **10** to **12**, were much less active than their counterparts.

With these considerations in mind, we intended to prepare bis-imidazolium and bis-1,2,4-triazolium analogues. Prior to dimerization, we first alkylated the heterocycles with various agents. In addition to simple alkyl (i.e. decyl) chain, we also introduced aromatic substituents such as 4-*t*-butylbenzyl or 2-methylnaphthalenyl moieties. This was achieved using commercially available alkyl bromides in basic conditions (Scheme 2). In order to avoid any trace of over-alkylation, the heterocycles were introduced in excess compared to the alkylating agents (3:1 ratio). The desired compounds were obtained in 41-74% yield after column chromatography. In the conditions used (DMF, K₂CO₃, 80 °C), a highly regioselective 1-alkylation was observed in the case of the 1,2,4-triazole compounds **26-28**. This was easily determined by ¹H-NMR which showed unsymmetrical patterns. Only traces of the other regioisomer were observed in the case of compound **28** and they were easily removed by chromatography (see Supporting Information). Dimerization with α,ω-dibromoalkanes afforded the desired bis-imidazoliums or bis-1,2,4-triazoliums in excellent (75-98%) yields after crystallization (Scheme 2). Above-mentioned results lead us to choose n = 10 to 12 carbon units as linkers.



Scheme 2. Synthesis of bis-imidazolium and bis-1,2,4-triazolium compounds. a) K₂CO₃, DMF, Δ; b) CH₃CN, Δ.

MICs of the resulting 14 compounds were evaluated against the same 4 reference strains, but also against 4 clinical isolates harboring various resistance mechanisms (Table 2). Unfortunately, compound **41** was too highly lipophilic to be soluble in the conditions used, thus its antibacterial activity could not be evaluated. Globally, all the prepared bis-imidazolium and bis-1,2,4-triazolium compounds showed strong to very strong activities towards most of the tested strains with MICs around 1 µg/mL. Besides, both strains of *P. aeruginosa* appeared to be less sensitive but still, some compounds exhibited low to very low MICs. Such strong activity of our compounds against Gram-

negative bacteria is rare in this class of antibacterials and has to be highlighted. The major part of these compounds proved to be as active as the reference compound (chlorhexidine), and a few were more active. This is even more evident when taking into account the lower molecular weight of chlorhexidine (for MICs in $\mu\text{Mol/L}$, see Supporting Information). In addition, for most of the compounds and for each bacterial species, there is almost no difference in activity towards the reference strain compared to the resistant clinical isolate. This is of high importance considering the need for treatments devoted to resistant bacteria. There is no clear difference in antimicrobial activity between bis-imidazolium and bis-1,2,4-triazolium series. In either case, the positive charge is spread over the heterocyclic structure, as in QACs bearing a pyridinium structure.^[9] Considering compounds with the same linker length, derivatives bearing a 4-*tert*-butylbenzyl moiety are slightly more active than those with a 2-methylnaphthalenyl group (for example compare **33** to **36**, **34** to **37**, and **40** to **43**). Nevertheless, it is clear that independently of these two substituents, a longer linker is associated with a higher potency. Besides, for the compounds holding a decyl substituent (compounds **38** and **45**), only one linker ($n = 10$) was envisioned and remarkably, these compounds were the most active ones, especially against both strains of *Pseudomonas aeruginosa*. In order to evaluate the potential safety of our compounds, they were tested against MRC-5 eukaryotic lung fibroblast and the results are expressed as IC_{50} (Table 2). The obtained values are very different from each other. Clearly, compounds **38** and **45**, which showed the strongest antibacterial activity, also exhibited the highest toxicity towards these eukaryotic cells. Compounds with aromatic substituents are less toxic than those bearing an alkyl (decyl) group. A longer linker increases the toxicity and for a same linker length, 2-methylnaphthalenyl derivatives are less toxic than 4-*t*-butylbenzyl ones. No clear difference in toxicity is seen between imidazoliums and 1,2,4-triazoliums since 4-*t*-butylbenzyl-bis-imidazoliums are less toxic than their 1,2,4-bis-triazoliums counterparts (for example compare **32** to **39**), but 2-methylnaphthalenyl-bis-imidazoliums are more toxic than their 1,2,4-bis-triazoliums counterparts (compare **35** to **42**). The latter are the least toxic derivatives of the series, with compound **42** exhibiting an IC_{50} of 37.6 $\mu\text{g/mL}$. In order to confirm the low toxicity of this compound, it was further evaluated in a red blood cell lysis test. It showed that at roughly its IC_{50} (*i.e.* 40.0 $\mu\text{g/mL}$), no significant lysis was observed (see Supporting Information) confirming the high potential of this compound.

In order to rationalize the above results, we intended to search for a possible link between the antibacterial activities and the eukaryotic toxicities on the one hand, and the lipophilicities of the molecules on the other hand. For this purpose, we used calculated $\log P$ ($\text{clog } P$) as reported in Table 2. As above-mentioned, the more lipophilic 4-*tert*-butylbenzyl moiety is associated with a slightly higher activity than the less lipophilic 2-methylnaphthalenyl derivatives. In addition, the better activity seen with the compounds with longer linkers may be attributed to their higher lipophilicity. Finally, the two compounds exhibiting the highest activities, compounds **38** and **45** are highly lipophilic,

but not the highest ones. In addition, as reported above, no clear difference in activity was seen between bis-imidazolium and bis-1,2,4-triazolium series even though the former is less lipophilic than the latter. In order to clarify these facts and with the aim of linking them to the eukaryotic toxicities, we drew some graphics (Figure 1) showing the relationships between IC_{50} and $\text{clog } P$, and between SI ($\text{SI} = \text{IC}_{50}/\text{MIC}$) and $\text{clog } P$ for three bacterial strains. In the case of compounds bearing the less lipophilic 2-methylnaphthalenyl moiety, there is a clear linear correlation between IC_{50} and $\text{clog } P$ in the bis-imidazolium series (compounds **35**, **36**, and **37**) and also in the bis-1,2,4-triazolium series (compounds **42**, **43**, and **44**). In both cases, the slope is negative, as less lipophilic compounds (*i.e.* with shorter linker) show greater IC_{50} . In contrast, for these six compounds and for the same linker length, each bis-1,2,4-triazolium analogue is more lipophilic and shows a higher IC_{50} than its bis-imidazolium counterpart, with compound **42** being the least toxic one. Besides, considering compounds bearing the more lipophilic 4-*tert*-butylbenzyl moiety, all the compounds are more toxic than their 2-methylnaphthalenyl counterpart. Once again, a linear correlation is seen between IC_{50} and $\text{clog } P$ in the bis-imidazolium series, but with a lower (negative) slope than previously, and only two bis-1,2,4-triazoliums could be tested. In contrast with the previously evoked 2-methylnaphthalenyl series, 4-*tert*-butylbenzyl bis-imidazolium derivatives are less toxic than their bis-1,2,4-triazolium counterpart. Finally, both compounds harboring a linear alkyl chain as substituent (compounds **38** and **45**) are highly lipophilic and exhibit high toxicity. In order to study the potential therapeutic interest of our molecules, the selectivity indexes (SI) towards three bacterial strains were evaluated (Figure 1). Concerning *E. coli*, since most of the MIC values are equal to 1, SI values are the reflect of the IC_{50} s, except for compounds **34** ($\text{MIC} = 0.5$) and **43** ($\text{MIC} = 2$), with compound **42** showing the best SI of 37.6. Concerning *S. aureus*, it is interesting to have a look at bis-imidazolium compounds. The one bearing a 2-methylnaphthalenyl moiety shows the usual negative slope: a lower $\text{clog } P$ correlates with a higher SI. In contrast, the compounds bearing a 4-*tert*-butylbenzyl moiety show a positive slope: a higher $\text{clog } P$ correlates with a higher SI. As a result, compounds **34** (4-*tb*, $n = 12$) and **35** (2-*mn*, $n = 10$) exhibit almost the same SI (26.4 versus 25.2, respectively). This reflects the very low MIC of **34**. A similar tendency is observed for 1,2,4-bis-triazolium bearing a 4-*tert*-butylbenzyl moiety. For those bearing a 2-methylnaphthalenyl substituent, the MIC value (0.5) of **43** compensates its relatively low IC_{50} (20.7), yielding an interesting SI of 41.4, to be compared to the SI of the least toxic compound **42** (37.6). Concerning *E. faecalis*, most of the compounds show low SI, except compound **35**, which is a bis-imidazolium holding a 2-methylnaphthalenyl substituent. With a MIC of 0.5 and an IC_{50} of 25.2, it combines a high activity to a relatively low toxicity towards our eukaryotic model, thus its SI reaches 50.4, which is the best of the series.

Table 2. Minimum inhibitory concentration (MIC, $\mu\text{g/mL}$) and IC_{50} ($\mu\text{g/mL}$) values of prepared bis-imidazoliums and bis-triazoliums


Entry ^[a]	n	clog P	R ^[c]	S.a.	MRSA	E.f.	VRE	E.c.	ESBLE	P.a.	PAR	IC_{50} ^[e]
Chx ^[b]				1	1	2	2	0.5	1	8	16	22.3
32	10	2.68	4- <i>t</i> bb	1	1	1	2	1	2	16	64	15.2
33	11	3.08	4- <i>t</i> bb	0.5	0.5	1	1	1	1	16	16	9.6
34	12	3.48	4- <i>t</i> bb	0.25	0.5	0.5	0.5	0.5	1	4	8	6.6
35	10	1.43	2-mn	1	0.5	0.5	4	1	4	64	128	25.2
36	11	1.83	2-mn	1	1	1	1	1	1	32	64	13.3
37	12	2.23	2-mn	0.5	0.5	0.5	0.5	1	1	16	16	3.9
38	10	3.05	decyl	0.5	0.5	1	0.5	1	1	2	2	1.7
39	10	3.35	4- <i>t</i> bb	1	1	2	4	1	4	16	32	9.8
40	11	3.74	4- <i>t</i> bb	0.5	0.5	0.5	2	1	2	16	16	8.4
41	12	4.14	4- <i>t</i> bb	nd ^[d]	nd	nd	nd	nd	nd	nd	nd	nd
42	10	2.1	2-mn	1	1	2	2	1	2	64	64	37.6
43	11	2.49	2-mn	0.5	0.5	2	2	2	4	16	32	20.7
44	12	2.89	2-mn	1	1	1	1	1	1	8	16	8.5
45	10	3.71	decyl	0.5	0.5	0.5	0.5	0.5	1	2	2	1.8

[a] All MIC data were acquired by compilation of the highest value of at least two independent trials. Twofold serial dilutions of the compounds were prepared from a stock solution at 1024 $\mu\text{g/mL}$. Each condition was repeated in 8 wells. For further details, see Supporting Information. Organisms used in this study: S.a. (*Staphylococcus aureus* ATCC 29213), MRSA (Methicillin-Resistant *Staphylococcus aureus*, *mecA*), E.f. (*Enterococcus faecalis* ATCC 29212), VRE (Vancomycin-Resistant *Enterococcus faecalis*, *van A*), E.c. (*Escherichia coli* ATCC 25922), ESBLE (Extended-Spectrum β -Lactamase producing *Escherichia coli*), P.a. (*Pseudomonas aeruginosa* ATCC 27853), PAR (*Pseudomonas aeruginosa* Resistant, efflux pump). [b] Reference compound used: Chx (Chlorhexidine). [c] 4-*t*bb = 4-*tert*-Butylbenzyl; 2-mn = 2-Methylnaphthalenyl. [d] Not determined (insoluble compound). [e] IC_{50} ($\mu\text{g/mL}$) was determined on MRC-5 cells according to the literature.^[20,21]

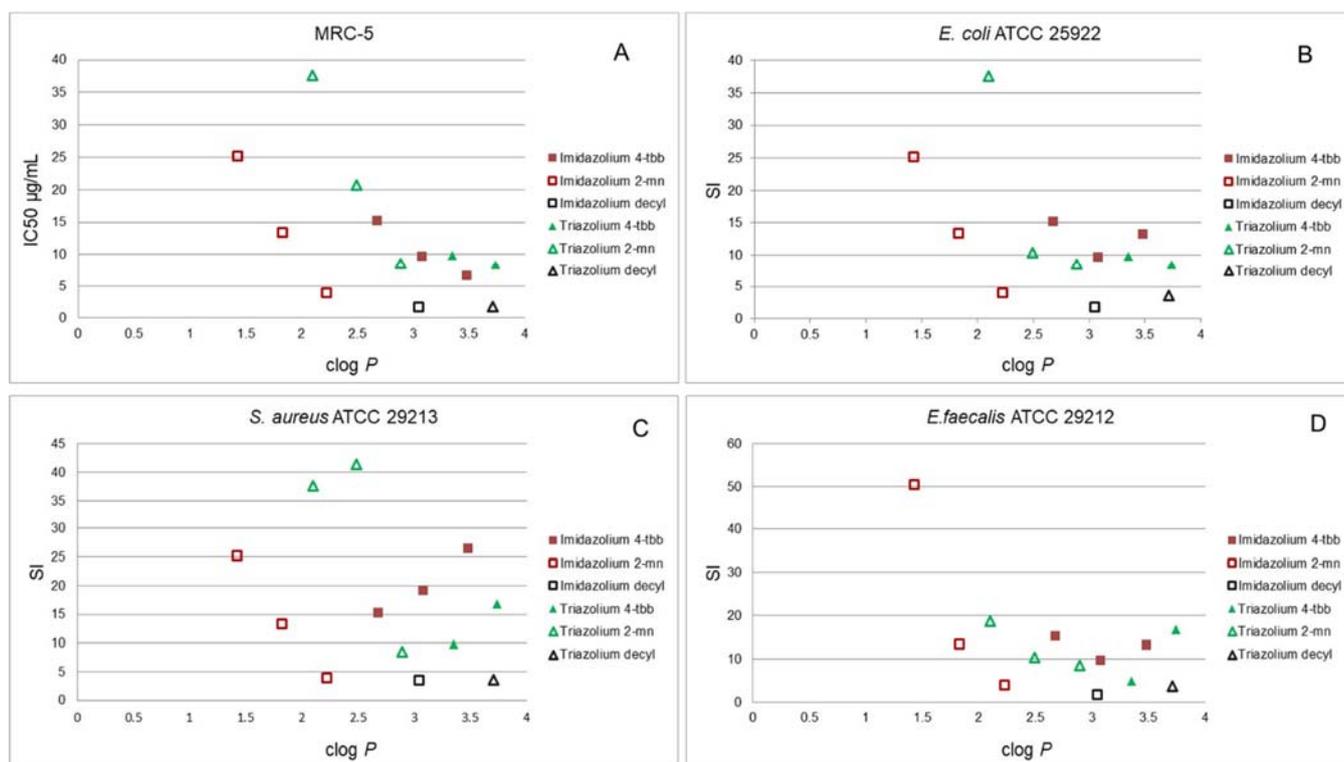


Figure 1. A) Toxicity (IC_{50} in $\mu\text{g/mL}$) of the various molecules on MRC-5 strains as a function of $\log P$. B) Selectivity index (SI) of the various molecules towards *E. coli* as a function of $\log P$. C) Selectivity index of the various molecules towards *S. aureus* as a function of $\log P$. D) Selectivity index of the various molecules towards *E. faecalis* as a function of $\log P$.

Based on these data, we decided to further characterize the antibacterial properties of some compounds. The bis-imidazolium and the bis-1,2,4-triazolium derivatives showing the best antimicrobial activities, lowest cytotoxicities or interesting SI were selected. Thus, we evaluated the bactericidal activity of compounds **35**, **42**, **43** and **45** on two reference bacterial strains, *E. coli* (Gram-negative) and *S. aureus* (Gram-positive). The bactericidal activity was defined as a ≥ 3 -log reduction of the initial inoculum with treatment compared with the untreated control, for a compound concentration not greater than $4 \times \text{MIC}$, after 24 h of incubation at 35°C .^[22] In the case of compounds **43** and **45**, the analysis of the Minimal Bactericidal Concentration (MBC) showed a clear bactericidal activity on both tested bacterial strains, with more than 3-log reduction of the initial inoculum, after 24 h of incubation (Table 3). In that instance, the MBC was attained independently of the compound concentration used in the assay, $1 \times \text{MIC}$ and $4 \times \text{MIC}$. Regarding compound **35** at $1 \times \text{MIC}$, we observed a killing effect for *E. coli* (starting inoculum of $4.2 \times 10^5 \pm 0.2 \times 10^5$ CFU/mL, breakpoint at 420 CFU/mL, mean of 770 CFU/mL obtained), although the bactericidal breakpoint is not reached. Nevertheless, the bactericidal breakpoint for *E. coli* is reached at $4 \times \text{MIC}$ (< 10 CFU/mL), while for *S. aureus*, both tested concentrations proved bactericidal. Similarly, for compound **42**, at $4 \times \text{MIC}$ the bactericidal breakpoint for *S. aureus* is not reached but a killing effect is also obtained (starting inoculum of $6.7 \times 10^5 \pm 0.5 \times 10^5$ CFU/mL, breakpoint at 670 CFU/mL, mean of 1600 CFU/mL obtained). On the opposite, the impact of the compound on *E. coli* led to more than $3 \log_{10}$ reduction of the initial inoculum. As

compound **42** was particularly interesting in terms of Selectivity Index, we went further and made a preliminary evaluation of the reduction of initial inoculum of *E. coli*. At the MIC concentration, the inoculum reduction was superior to $5 \log_{10}$, which emphasizes the high activity of this molecule.

Table 3. Reduction of initial bacterial inoculum after 24h of incubation with the considered compound at 35°C .

Compound	<i>S. aureus</i> ATCC 29213		<i>E. coli</i> ATCC 25922	
	1xMIC	4xMIC	1xMIC	4xMIC
35	$>3 \log_{10}$	$>3 \log_{10}$	$<3 \log_{10}$	$>3 \log_{10}$
42	$<3 \log_{10}$	$<3 \log_{10}$	$>3 \log_{10}$	$>3 \log_{10}$
43	$>3 \log_{10}$	$>3 \log_{10}$	$>3 \log_{10}$	$>3 \log_{10}$
45	$>3 \log_{10}$	$>3 \log_{10}$	$>3 \log_{10}$	$>3 \log_{10}$

In this study, we addressed the question of the rise in antibiotic-resistant bacteria by designing new antibacterial compounds intended to keep their activity towards clinically occurring resistant bacteria. With this aim, we prepared numerous bis-cationic compounds, among which bis-imidazolium and bis-1,2,4-triazolium derivatives appeared to show high antibacterial activity against two Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and two Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) reference bacterial strains. For most of these compounds, the MIC were around $1 \mu\text{g/mL}$, except against *Pseudomonas aeruginosa*. All these compounds were at least as potent as chlorhexidine, a molecule commonly

used as disinfectant. It is noteworthy that the antibacterial activity was maintained against resistant clinical isolates. Furthermore, four selected compounds exhibited low-dose bactericidal activity towards two representative bacterial strains. Interestingly, some of our compounds showed low toxicity towards eukaryotic MRC-5 lung fibroblast cells. Furthermore, we could demonstrate very interesting linear relationships between eukaryotic cytotoxicity and $\log P$ of our compounds, and between Selectivity Index (SI) and $\log P$. This study opens new perspectives in the design of new bis-cationic features with high antibacterial potency while keeping low eukaryotic toxicity.

Experimental Section

Full experimental details are provided in the Supporting Information

Acknowledgements

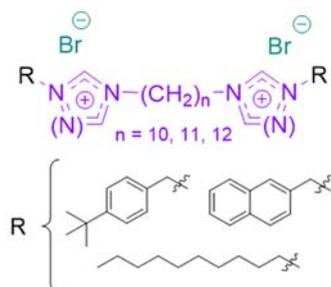
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Keywords: imidazoliums • 1,2,4-triazoliums • antibacterials • $\log P$ • cytotoxicity

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Entry for the Table of Contents



S. aureus MIC 0.25 → 1 µg/mL

E. coli MIC 0.5 → 2 µg/mL

Highly potent antibacterials. New dicationic compounds were prepared among which bis-imidazoliums and bis-1,2,4-triazoliums exhibited low to very low MICs towards a panel of bacterial strains. These activities were also observed towards resistant clinical isolates. Some derivatives displayed a low toxicity towards a model of eukaryotic cell. Very interesting correlations were found between these activities and the lipophilicity of the compounds.