New polyaminoisoprenyl antibiotics enhancers against two multidrug resistant Gram-negative bacteria *from Enterobacter and Salmonella species*

Aurélie Lieutaud[1], Cyril Pieri[1], Jean Michel Bolla[1]*and Jean Michel Brunel[1]*

[1] Aix Marseille Univ, INSERM, SSA, MCT, 13385 Marseille, France.

Keywords : Gram-negative bacteria, Multi-drug resistance, Efflux pump, Antibiotic enhancers, Polyaminoisoprenyl derivatives.

Abstract

A series consisting of new polyaminoisoprenyl derivatives were prepared in moderate to good chemical yields varying from 32 to 64% according two synthetic pathways: 1) using a titanium reductive amination reaction affording a 50/50 mixture of *cis* and *trans* isomers 2) a direct nucleophilic substitution leading to a stereoselective synthesis of the compounds of interest. These compounds were then successfully evaluated for their *in vitro* antibiotic enhancer properties against resistant Gram-negative bacteria of four antibiotics belonging to four different families. The mechanism of action against *Enterobacter aerogenes* of one of the most efficient of these chemosensitizing agents was precisely evaluated by using fluorescent dyes to measure outer-membrane permeability and to determine membrane depolarization. The weak cytotoxicity encountered led us to perform an *in vivo* experiment dealing with the treatment of mice infected with *Salmonella* Typhimurium and affording preliminary promising results in terms of tolerance and efficiency of the polyaminoisoprenyl derivative **5r** / doxycycline combination.

Introduction

Over the last decades, antibiotic resistance has become one of the predominant general health concerns principally due to the overuse of antibiotics, the ageing of the population mostly subjected to infections, the increase of efficiency of intensive care units taking in charge patients in decreased physiological state.[1-3] In this context and closely associated to the failure in the discovery of new antibiotics, there is a need to improve all the stages of infectious diseases treatments including the management of different steps of antibiotic usage and the improvement of the efficiency of well-known antibiotics.[4-5] Amongst the human pathogenic bacteria, the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp*) first described by Rice *et al.*, is the most problematic for the clinicians because of their ability to evade and escape common therapies.[6] This ESKAPE group was further considered as of major importance by the WHO.[7] On the other hand, bacteria may be intrinsically resistant or may acquire resistance by *de novo* mutation or *via* the acquisition of resistance genes from other organisms. Furthermore, acquired resistance mechanisms permit to a bacterium to produce enzymes able to destroy the drug, to produce target protection proteins that prevent the drug binding, the expression of efflux systems that avoid the drug to reach its intracellular target, the target site modification, the production of an alternative metabolic pathway to circumvent the action of the drug. Thus, efflux mechanisms constitute important determinants of resistance to antibiotics in human pathogens.[8-11] Additionally, susceptible Gram-negative bacteria can become resistant particularly by changing the permeability of their membranes or by reducing the number of channels available for drugs to diffuse through.[12-13] Given the clinical significance of antimicrobial exporters[9], it is clear that efflux must be considered in formulating strategies for treatment of drug-resistant infections, by the development of new agents less impacted by efflux or by targeting efflux directly with efflux inhibitors restoring the activity of

several drugs on acquired MultiDrugResistant (MDR) bugs and also allowing the susceptibility of drugs on naturally resistant bacteria.[14]

In this context, we have previously reported the ability of a natural compound, namely geraniol, to decrease the antibiotic resistance of a Gram-negative MDR Enterobacteria strain.[15] This compound has been determined to be a substrate of the AcrAB-TolC pump and conjointly inhibiting the transport of other molecules including antibiotics by this pump.[16] Furthermore, we recently reported the synthesis of polyaminogeranic acid derivatives and their efficient use as new chemosensitizers inducing a significant decrease of antibiotic resistance in Gram-negative bacterial MDR strains.[17] On the other hand, we were also able to demonstrate that water-soluble geraniol parent amino derivative, geranylamine as well as NV716 was effective as an efflux pump inhibitor (Figure 1).[18-19]

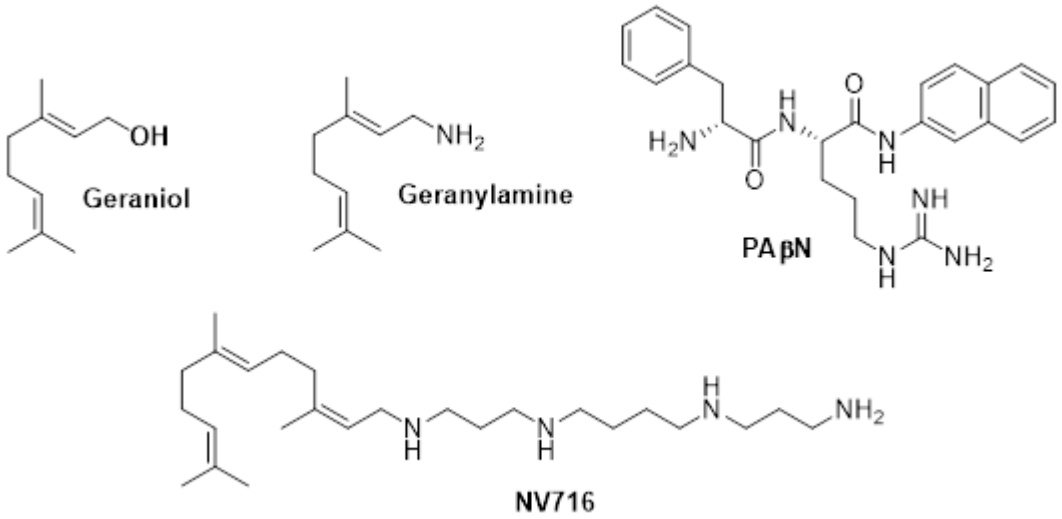


Figure 1. Structure of Geraniol, Geranylamine, PAβN and NV716

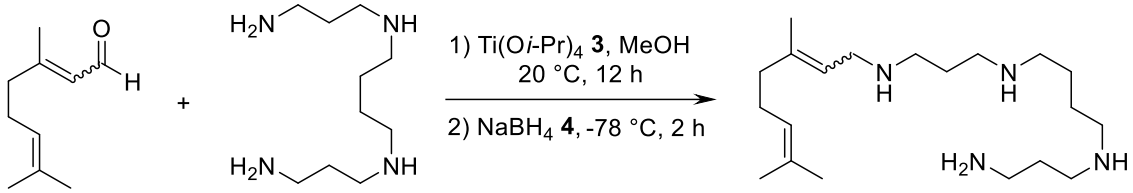
In this context, we will report herein the synthesis, biological evaluation, and structure activity relationships analysis of a series of new polyaminoisoprenyl antibiotic enhancers against multidrug resistant Gram-negative bacteria *Enterobacter aerogenes* and *Salmonella* enterica serovar Typhimurium.

Results and discussion

Chemistry

Using an efficient titanium reductive amination developed in our laboratory[20-21], we have envisioned a one-step synthesis procedure for the preparation of new polyaminoisoprenyl derivatives in a Z/E mixture from both citral and farnesal according to the following synthetic pathway (Table 1).

Table 1. Titanium (IV) reductive amination reaction of citral with spermine under various experimental conditions.



1 2 5p

Entry	Titanium source	Solvent	Yield (%)[c]
1[a]	Ti(O*i*-Pr)$_4$	MeOH	64
2[b]	Ti(O*i*-Pr)$_4$	MeOH	51
3[a]	Ti(O*i*-Pr)$_4$	CH_2Cl_2	30
4[a]	Ti(O*i*-Pr)$_4$	Toluene	19
5[a]	Ti(O*i*-Pr)$_4$	THF	33
6[a]	Ti(OEt)$_4$	MeOH	39
7[a]	Ti(OBu)$_4$	MeOH	30
8[a]	Ti(O*t*-Bu)$_4$	MeOH	21

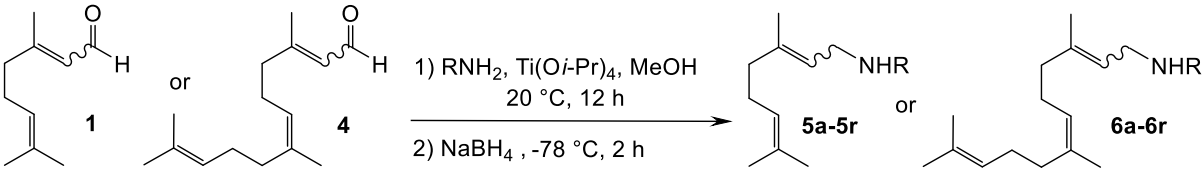
[a] Reaction performed at -78ºC for 12 h in MeOH on a 0.39 mmol scale of citral, Ti(O*i*-Pr)$_4$ (2.02 mmol) and the amine (6 mmol). [b] Reaction performed at 0 °C. [c] Isolated overall yield.

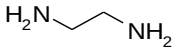
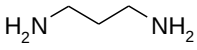
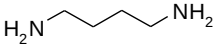
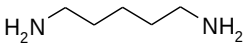
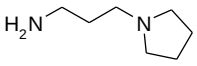
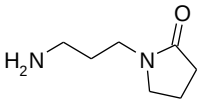
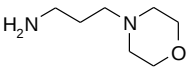
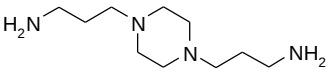
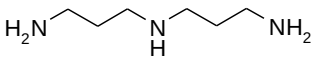
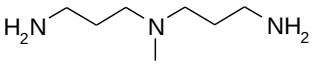
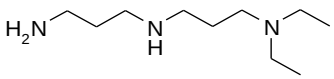
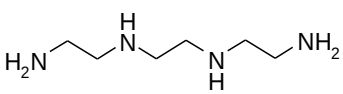
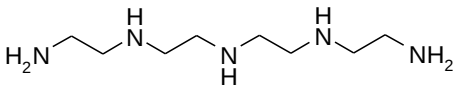
First, it clearly appears that isolated yields of compound **5p** are highly solvent-dependent. Thus, the expected amino derivative **5p** was obtained in 64% yield performing the reaction in MeOH (Table 1, entry 1), whereas only moderate yields varying from 19-33% were encountered performing the reaction in CH_2Cl_2, toluene and THF, respectively (Table 1, entries 3-5). Influence of the nature of the titanium source involved was also investigated and chemical yield variations from 21 to 64% were obtained (Table 1, entries 1, 6-8), best result being observed using Ti(O*i*-Pr)$_4$ as titanium source. Furthermore, under these best experimental conditions,

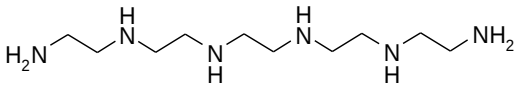
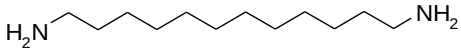
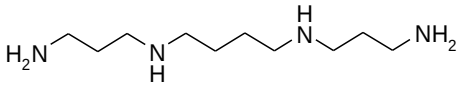
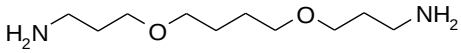
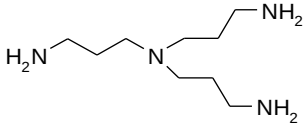
increasing of the reaction temperature from -78 to 0°C led to a slight decrease of the chemical yield encountered from 64 to 51%, respectively (Table 1, entries 1 and 2).

Under the best experimental conditions (Table 1, entry 1), we have envisioned the use of numerous different natural (such as cadaverine, putrescine, spermine...) or synthetic polyamines in the titanium reductive amination of citral and farnesal and the results are summarized in Table 2.

Table 2. Polyaminoisoprenyl derivatives obtained from citral (**5a-5r**) or farnesal (**6a-6r**).



RNH_2	Isolated Yield (%)		IC_{50} (µM) CHO	
			5a-5r	**6a-6r**
	**5a** 49	**6a** 63	36.24	32.30
	**5b** 33	**6b** 49	>150	32.06
	**5c** (NP)	**6c** 58	-	18.06
	**5d** (NP)	**6d** 38	-	38.38
	**5e** 61	**6e**71	>150	85.33
	**5f** 51	**6f** (NP)	>150	-
	**5g** 52	**6g** 56	>150	57.64
	**5h** (NP)	**6h** 62	-	33.75
	**5i** (NP)	**6i** 52	-	30.19
	**5j** 42	**6j** 58	>150	10.47
	**5k** 49	**6k** (NP)	30.92	-
	**5l** 64	**6l** (NP)	>150	-
	**5m** (NP)	**6m** 32	-	43.50

	**5n** 58	**6n** 47	>150	41.71
	**5o** (NP**)**	**6o** 48	-	25.32
	**5p** 64	**6p** 72	>150	142.79
	**5q** (NP**)**	**6q** 43	-	>150
	**5r** 49	**6r** 63	126.82	>150

NP : not prepared

Whatever the nature of the amines, the expected polyaminoisoprenyl products were obtained mixture of the *Z/E* isomers in a 50/50 ratio in moderate to good chemical yields varying from 32 to 72%. It is noteworthy that these reactions have not been yet optimized, the moderate results being explained by the difficulty to purify the expected polar products.

Antimicrobial activities

In the context of our studies, all the synthesized compounds were firstly screened for their antimicrobial activity against Gram-negative bacterial strains (Table S-1). Two pairs of enterobacterial strains were challenged, one *E. aerogenes* MDR strain Ea289 over-expressing the efflux pump AcrAB-TolC and its Δ*acrB* mutant, and one *S.* Typhimurium MDR strain BN10055 over-expressing the same efflux pump and its Δ*acrB* mutant. The data presented in Table S-1 demonstrated that the MICs of the polyaminoisoprenyl derivatives vary from a maximum of over 500 µM for **5l** and **5n** against the four strains to a minimum of 7.8 µM for compounds **6n** and **6o** against the Enterobacter strain Ea289Δ*acrB*, and 3.9 µM for **6o** against Salmonella strain BN10055Δ*acrB.* Additionally, except for **6o** no significant differences are noticed against both Salmonella strains indicating that none of the compounds is a substrate of the efflux pump AcrAB-TolC.

In this study, our goal was to identify molecules capable of decreasing antibiotic resistance of Gram-negative MDR strains. Thus, we chose drugs for which these strains had high MIC values and our compounds were tested for their ability to decrease resistance of the MDR strains to erythromycin, doxycycline, nalidixic acid and chloramphenicol in a synergistic assay. The compounds were used at a sub-inhibitory concentration corresponding to a quarter of their respective MICs to avoid an intrinsic action and to ascertain that the effect observed resulted from the combination of the molecules used. For the two MDR strains EA289 and BN10055 we determined the ratio between the MIC of each antibiotic when applied alone and the MIC of the antibiotic in the presence of a desired compound. The cumulative MIC ratios for each compound are presented in Figure 2A and 2B, respectively.

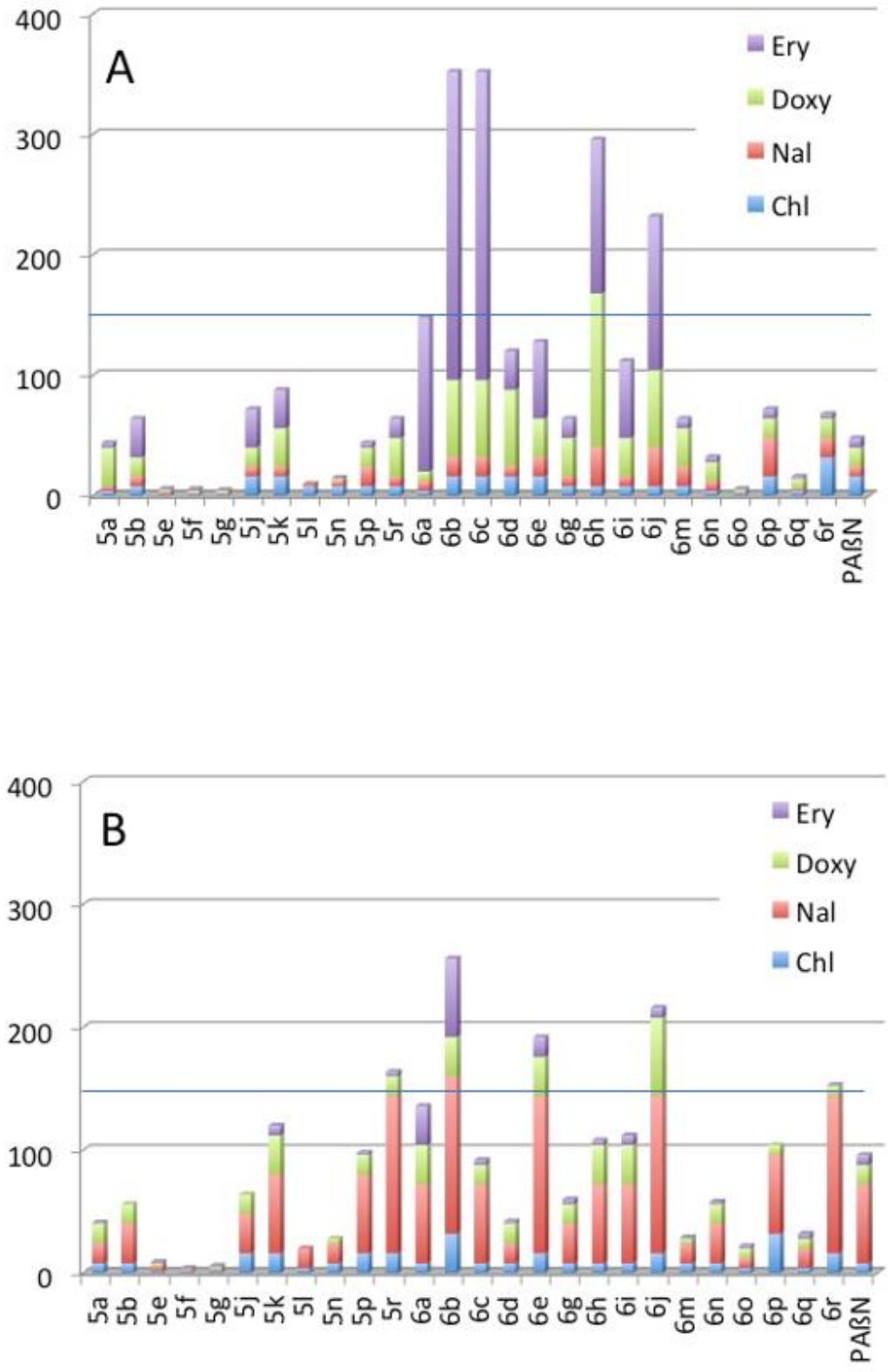


Figure 2. Cumulative MIC ratios for each compound for their ability to decrease resistance of MDR strains EA289 (Figure 2A) and BN10055 (Figure 2B) to erythromycin, doxycycline, nalidixic acid and chloramphenicol in a synergistic assay.

Although three compounds (**5e**, **5f**, and **5g**) are ineffective, the twenty-three other ones exhibited an efficiency at least in one of the combinations with the antibiotics. In the case of Enterobacter, 4 compounds (**6b**, **6c**, **6h**, **6j**) showed a cumulative score over 150 (Figure 2A) while 5 others (**6b**, **6j**, **6e**, **5r**, **6r**) had the same behavior in the case of Salmonella (Figure 2B). In this context, the data of Figure 2A and 2B highlighted that **6b** and **6j** are the most efficient and promising compounds.

We also observed, that several compounds decreased the MIC of the antibiotic under the threshold of susceptibility according to the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM)[22], (≤ 2 mg/L for the four antibiotics tested) (Table 3).

Table 3: Decrease of chloramphenicol, nalidixic acid, doxycycline and erythromycin resistance in the presence of the polyaminoisoprenyl enhancers **5a-6r**.

	Gain factor[a]							
Adjuvant	Ea289				BN10055			
	Chl	Nal	Doxy	Ery	Chl	Nal	Doxy	Ery
No[b]	1024	4096	32	256	1024	4096	64	256
5a	4	4	32	4	8	16	16	1
5b	8	8	16	32	8	32	16	
5e	1	2	2	1	2	4	2	1
5f	1	1	2	2	1	1	1	1
5g	1	1	2	1	2	1	2	1
5j	16	8	16	32	16	32	16	Nd
5k	16	8	32	32	16	64	32	8
5l	8	2	Nd[c]	Nd[c]	4	16	Nd[c]	Nd[c]
5n	8	4	2	1	8	16	4	Nd[c]
5p	8	16	16	4	16	64	16	2
5r	8	8	32	16	16	128	16	4
6a	4	8	8	128	8	64	32	32
6b	16	16	64	256	32	128	32	64
6c	16	16	64	256	8	64	16	4
6d	16	8	64	32	8	16	16	2
6e	16	16	32	64	16	128	32	16
6g	8	8	32	16	8	32	16	4
6h	8	32	128	128	8	64	32	4
6i	8	8	32	64	8	64	32	8

6j	8	32	64	128	16	128	64	8
6m	8	16	32	8	8	16	4	1
6n	4	8	16	4	8	32	16	2
6o	2	1	2	1	4	8	8	2
6p	16	32	16	8	32	64	8	Nd[c]
6q	4	2	8	2	4	16	8	4
6r	32	16	16	4	16	128	8	1
PAßN	16	8	16	8	8	64	16	8

[a] Gain factor determined as the ratio of the MIC of the antibiotic alone for each strain to the MIC of the same antibiotic in the presence of the considered compound at a concentration corresponding to its MIC/4. A gain factor greater than 1 indicates an improvement of activity. [b] MIC determined for the antibiotics alone. [c] Nd, Not determined.

This is particularly the case against Ea289 for most compounds in combination with doxycycline. Concerning erythromycin five compounds were observed to decrease the MIC of Ea289 under the threshold. To a lesser extent, the same observations can be drawn for the combination of seven compounds and doxycycline against Salmonella. Noticeably, derivatives **5k**, **6b**, **6e**, **6h**, **6i** and **6j** decrease the MIC of doxycycline towards these two strains. Surprisingly, whereas these compounds in the presence of erythromycin led to an efficient combination against Enterobacter, none of these significantly improve the MIC of erythromycin against Salmonella.

Taken together these results suggested that the two compounds **6b** and **6j** might be the most active. However, this analysis did not consider the concentration used to get the synergy with the different antibiotics. Consequently, in order to generate comparable data we decided to correlate the efficiency according to the concentration used by introducing an “Efficiency Parameter” (EP) that corresponds to the sum of gain for each compound obtained for the four antibiotics tested and divided by the concentration of the molecule used in the assay (Table S-2). It is noteworthy that the gain factor was defined as corresponding to the ratio of the MIC of the antibiotic tested alone to the MIC of the antibiotic obtained in the presence of the considered adjuvant.

This EP varies from 0.01 to 7.17 in arbitrary units allowing us to classify the compounds into three groups: one group corresponding to non-efficient molecules, a second group with molecules with an average score of EP varying from 0.01 to 0.57 and a third one with an EP higher than 1 and reaching 7.17 in the best case (Table S-2).

It is noticeable that PAßN, the reference inhibitor of Gram-negative efflux has through this classification an efficiency of 0.47 making it part of the second group. The third group comprised 13 compounds including the three compounds previously identified. This third group appeared as the most efficient. To better characterize our compounds and considering this kind of molecules as putative drug adjuvants, the cytotoxicity of each molecule has been investigated.

Cytotoxicity was assayed with Chinese hamster ovary (CHO) cells for all the compounds which present IC_{50} ranging from 1 to 150 µM. A major part of our compounds exhibited an IC_{50} comprised between 18.06 and 142.79 µM while 10 compounds had IC_{50} over 150 µM suggesting that they were minimally toxic. A toxicity index varying from 0.06 to 19.2 was subsequently defined as the ratio of IC_{50} to the concentration used leading to a decrease of MIC of at least 8 times. Thus, we were able to directly compare the EP of each compound versus its toxicity index as plotted in Figure 3.

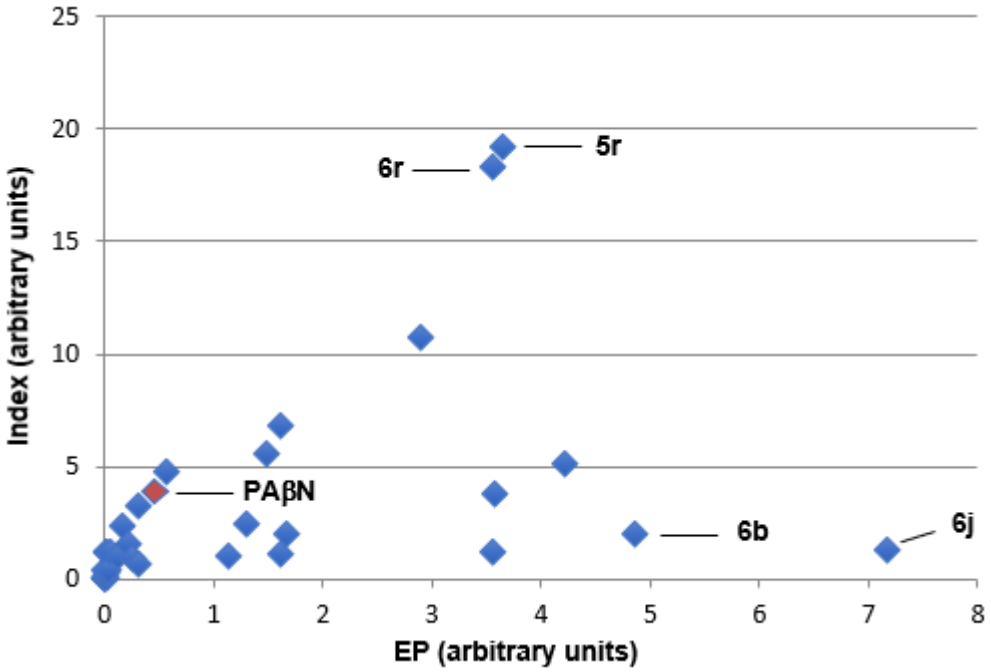
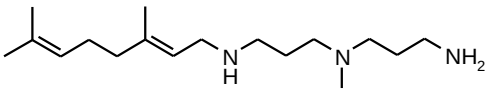
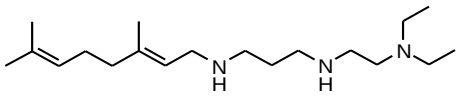
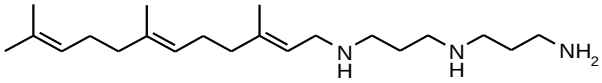
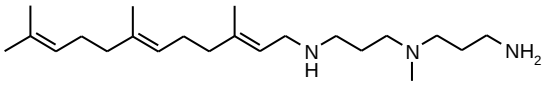
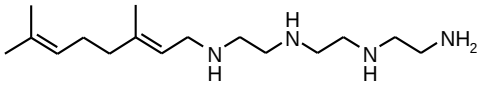
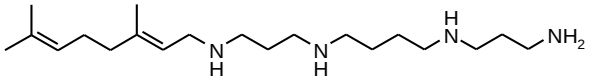
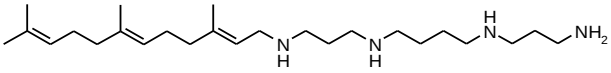
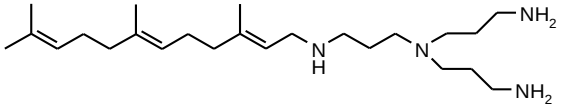
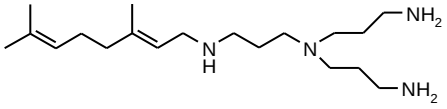
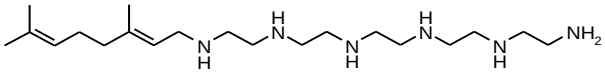
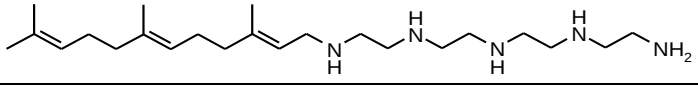
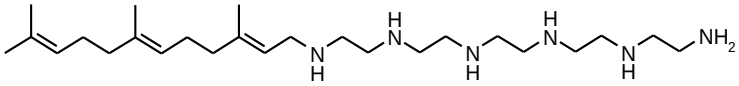


Figure 3. Efficiency/toxicity index correlation for all the polyaminoisoprenyl derivatives

It is noteworthy that two compounds **5r** and **6r** exhibited the best cytotoxicity index and **6j** and **6b** the best efficiency, respectively, and that they were used at similar concentration against the considered bacteria in order to compare their efficiency in the same range of concentration (see Table S-2). In this context, **5r** and **6r** showed efficiency index about 2 times lower than **6j** but presented a better toxicity index. By comparison, PAßN (red dot on Figure 3) presented a moderate to weak efficiency index of 0.47 and a toxicity index of 3.95. Thus, six compounds namely **6p**, **6r**, **5p**, **5r**, **6n**, and **6m** were identified to possess better parameters than PAßN. Additionally, **5r** was identified as the most potent compound by considering all these criteria.

We suppose, as already postulated by others[23] that the death process could involve electrostatic interactions and is related to the high density of charges exposed at the surface of bacterial membranes. Thus, the electrostatic compensation of the negative charges of the bacterial envelope could be provided by the cationic charges of the substrate leading for the bacteria to a loss of their natural counterions. Based on the number of positive charges involved (number of nitrogen atoms present in the structure) the different compounds were classified and three groups of molecules emerged (Table 4).

Table 4. Stucture-activity relationships analysis

Name	Structure	Efficiency[a]	Index[b]
5j		0.01	0.06
5k		1.66	1.98
6i		3.58	3.86
6j		7.17	1.34
5l		0.00	0.06
5p		0.57	4.80
6p		1.61	9.15
6r		3.54	19.20
5r		3.65	16.23
5n		0.00	0.06
6m		1.49	5.37
6n		2.88	10.69

[a] Efficiency parameter that corresponds to the sum of gain factor for each compound reported for the four antibiotics tested and divided by the concentration of the molecule used in the assay.[b] Index parameter is determined by the ratio of the IC_{50} to the concentration giving a decrease of MIC of at least 8 times for almost 3 antibiotics on both species.

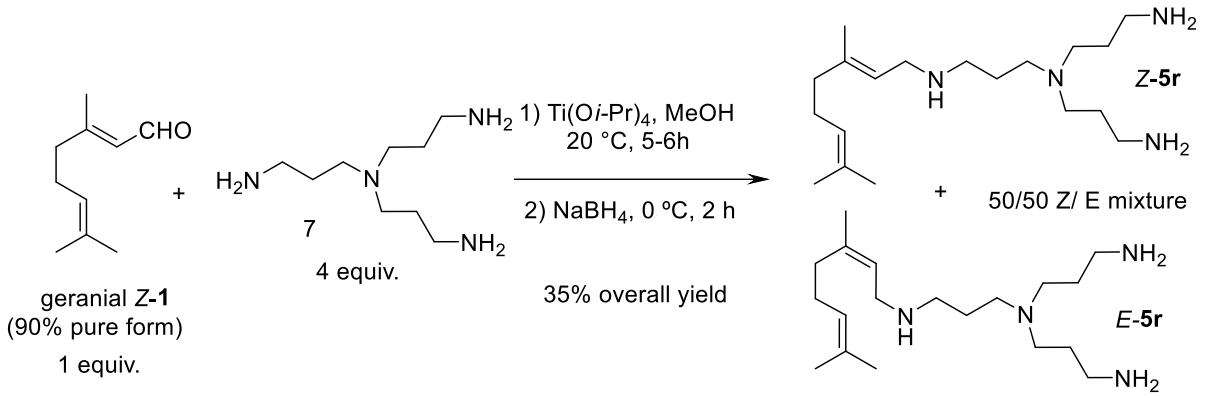
The most efficient compound **6j** was encountered in the first group with an efficiency score of 7.17. This compound is a farnesyl derivative, with a triterpene structure linked to a linear triamine (three positive charges). Otherwise, the parent geranyl compound **5j**, was neither efficient nor showed a good index of toxicity indicating that the length of the terpene moiety is

of major importance. While the activity of **6i** is near half of the activity of **6j**, its index of toxicity is about 3 times higher suggesting that the nature of the amino-methyl group plays an important role for toxicity.

On the other hand, some compounds including four amino groups such as **6r** and **5r** in their close present interesting results in terms of activity and toxicity. By comparing **5l** and **5p** it clearly appears that the number of carbon atoms between the amino groups remains crucial on the outcome of the observed antimicrobial activities whereas the number of positive charges is conserved. Furthermore, comparison of the structure for derivatives **6p** and **5p** lead to the conclusion that the length of the terpene chain involved is of major importance for the activity encountered whereas the polyamine chain is conserved. Moreover, the presence of a ramified amine in compounds **6r** and **5r** improve their efficiency and toxicity index with respect to their parent derivatives **5p** and **6p** involving a linear spermine group. In the third group, the importance of the length of the terpene moiety with respect to the encountered efficiency was confirmed by studying typically derivatives **5n** and **6m.** It is noteworthy that an attempt to correlate the efficiency or cytotoxicity of the different compounds with their intrinsic lipophilicity by considering the LogP parameter, which reflects the true behavior and bioavailability of an ionizable compound in a solution at a given pH, failed (Table S-2).

All these derivatives have been prepared as a 50/50 mixture of *cis* and *trans* isomers. At this stage, it appears of interest to determine the impact of each isomer on the observed biological activities. In a first approach and due to its interesting biological data, the synthesis of pure *Z*-**5r** and *E*-**5r** derivatives has been envisioned with the use of a titanium amination reaction involving pure isomers of neral or geranial as starting materials.

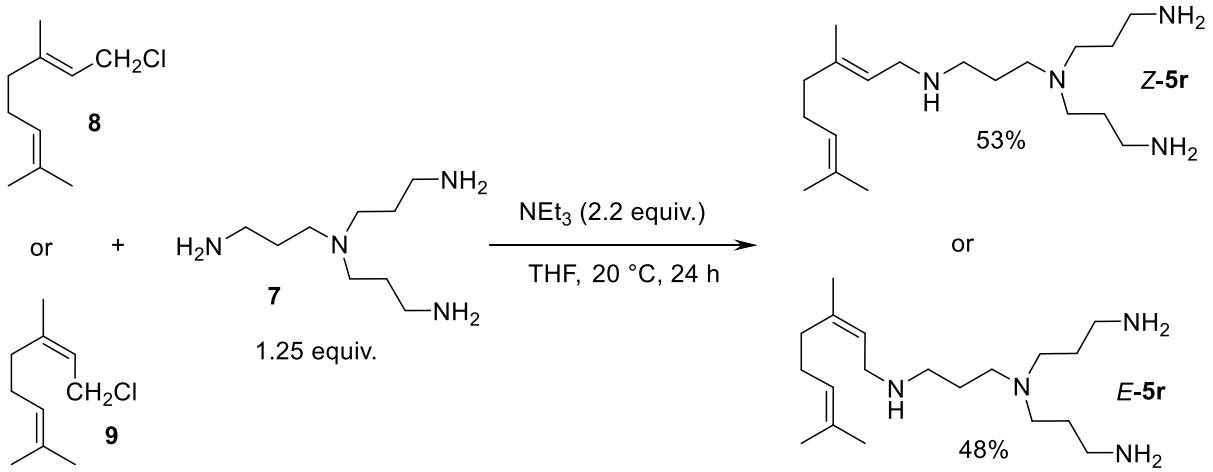
Surprisingly, a subsequent titanium amination reaction in methanol in the presence of tris(3-aminopropyl)amine **7** led to a mixture of the *Z/E* isomers of **5r** in a 50/50 ratio in a non-optimized 35% isolated yield (Scheme 1).



Scheme 1. Titanium reductive amination of geranial *Z*-**1** with tris(3-aminopropyl) amine **7**.

Thus, whatever the experimental conditions applied (data not shown) it was impossible to obtain the expected polyamino derivatives in a pure stereoselective form. We have been able to demonstrate that this racemization occurs by mixing the pure aldehyde and the amine in the absence of titanium isopropoxide. Indeed, after two days the corresponding imines were obtained as a mixture of the two isomers suggesting an isomerization process during the imine formation.[24-30]

These disappointing results led us to envision another strategy involving a direct nucleophilic substitution of the considered polyamine on geranyl chloride **8** and neryl chloride **9** derivatives (Scheme 2).



Scheme 2. Synthesis of pure *Z*-**5r** and *E*-**5r** by nucleophilic substitution of tris(3-aminopropyl)amine **7** on geranyl chloride **8** and neryl chloride **9** derivatives.

Under these experimental conditions, the expected products were obtained as pure isomers *Z*-**5r** and *E*-**5r** in 53 and 48% isolated yield, respectively. Their biological efficiency and cytotoxicity have been evaluated involving different salt formulations as summarized in Table S-3).

It clearly appears that no significant differences were obtained in terms of biological activities and cytotoxicity for the different isomers and formulations used.

Animal study

Because of these outcomes, we have used a mixture of *Z*-**5r**/*E*-**5r** to investigate the potent mechanism of action of these derivatives against Gram-negative bacteria and evaluate their efficiency in a very preliminary *in vivo* animal model. Thus, to ascertain the *in vivo* activity of compound **5r**, mice were used as animal model to determine their tolerance to this compound as well as the *in vivo* activity of **5r**/doxycycline combination against *S.* Typhimurium infection.[31] In a first approach, the results indicate a good tolerance of the mice towards **5r** to a concentration of 50 µM and they did not show signs of acute toxicity (Figure 4A). On the other, the treatment by a doxycycline/**5r** combination (64 mg/Kg for doxycycline and 0.5 mg/Kg for **5r**, respectively) of *S.* Typhimurium infected mice lead to a 43% survival rate whereas all the mice died by using a treatment involving saline solution or doxycycline alone as illustrated in Figure 4B.

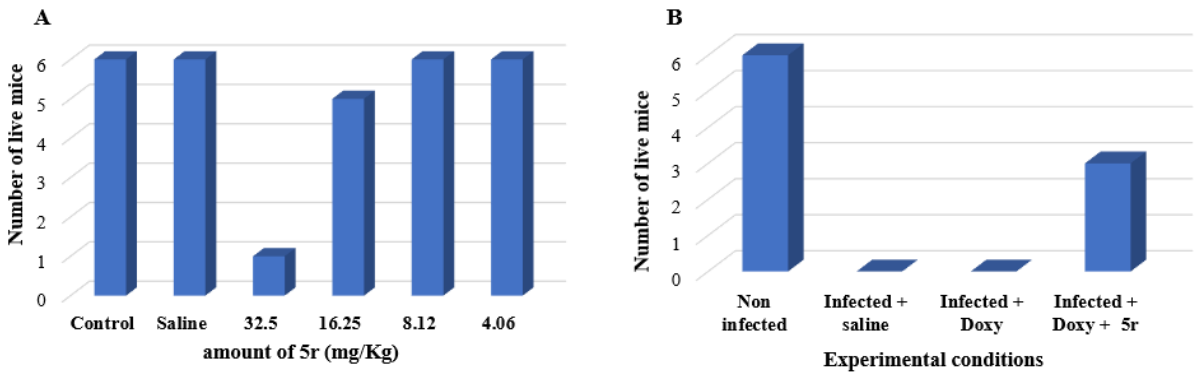


Figure 4. A) Mice survival 24h after administration with saline or **5r** (4.06, 8.12, 16.25, 32.5 mg/Kg). B) Survival of mice infected with *S.* Typhimurium following administration with

saline, doxycycline alone or a doxycycline/**5r** combination solution (64 mg/Kg for doxycycline and 0.5 mg/Kg for **5r**, respectively). Survival of mice number corresponds to the number of mice euthanized.

These results, even if there are preliminary, tend to demonstrate the potent *in vivo* enhancing activity of polyaminoisoprenyl compounds when associated with doxycycline against *S.* Typhimurium. Nevertheless, since the mechanism of action of these compounds remained unclear, we undertook to perform four different experiments to determine how derivative **5r** (as a Z/E mixture) targeted our Gram-negative *E. aerogenes* EA289 bacteria model, a kanamycine-susceptible derivative of the MDR clinical isolate Ea27.[32]

Mechanism of action

We have previously determined that compound **5r** strongly decrease the MIC of four antibiotics against MDR Enterobacter and Salmonella strains, and that this activity was not or weakly observed against their isogenic Δ*AcrAB* derivatives. This observation suggests that the efflux pump AcrAB-TolC could be the target of the involved compound. Nevertheless, such an inhibition of the efflux pump may result from various actions including either direct interaction with the pump by blocking the antibiotic transfer or competing with the antibiotic during its transfer, by energy disruption or by inhibition of the pump assembly. MIC determination involves the incubation of bacteria in the presence of the selected compound and of the antibiotic for about 18 hours. Thus, the observed synergy may result from the combination of numerous other effects. To eliminate such unwanted observations, we set up a series of real time assays allowing us to follow the interaction of the compound more accurately with the bacteria (Figure 5)

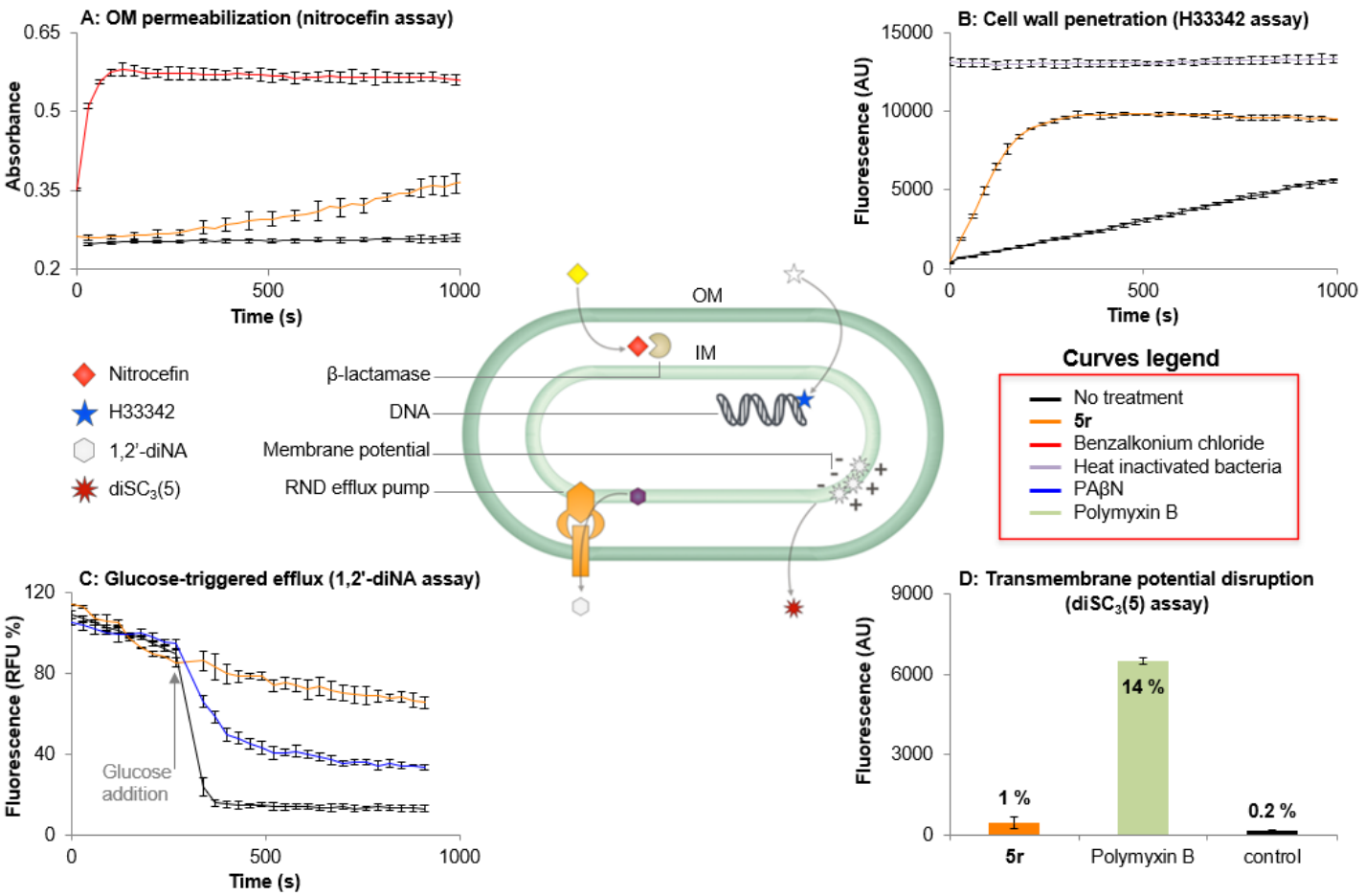


Figure 5. Evaluation of the possible modes of action of **5r** against EA289: A) Outer membrane permeabilization assay by evaluating the rate of nitrocefin hydrolysis. B) H33342 accumulation assay (cell wall penetration) C) Efflux performance of the bacteria by evaluating glucose-triggered 1,2'-diNA efflux. D) Transmembrane potential disruption assay ($diSC_3(5)$ assay).

Each experiment was performed using standard molecules as positive control, at a concentration of 100 µM.[33] In a first attempt we have envisioned to determine if a permeabilization and/or disruption of the outer membrane could occur. Thus, outer-membrane permeabilization was followed by monitoring the nitrocefin hydrolysis in the periplasm by the constitutively expressed ß-lactamase since an important color change from yellow to red would be observed in the positive.[34-35] It clearly appears that during the first 200 seconds of incubation, no outer membrane permeabilization was observed by using **5r** whereas in the same time benzalkonium chloride induced a complete outer membrane disruption (Figure 5A). Thus, even after 15 minutes, a slight effect was observed for **5r** without reaching the maximum observed for benzalkonium chloride.

Furthermore, membrane perturbation can also be observed by monitoring H33342 dye entrance into the bacteria (Figure 5B). Fluorescence of this compound correlates with its binding to DNA in the cytoplasm of the bacteria. Thus, even if the H33342 dye entrance occurred in non-treated bacteria, this kinetic is widely increased by adding compound **5r**. Taken together, these data suggested that derivative **5r** was unable to strongly disrupt outer membrane integrity but allowed the diffusion of molecules such as H33342 into the bacteria. Furthermore, these observations for nitrocefin and H33342 are in full agreement with the fact that their respective LogD are -0.95 and 3.76 suggesting a facilitated diffusion of H33342 relative to nitrocefin through the membranes of the bacteria. Moreover, since the bacterial strain considered is devoid of porins[36] which are used by ß-lactam compounds such as nitrocefin to cross the outer membrane of Gram-negative bacteria, our results confirm the property of **5r** to disrupt the integrity of the outer membrane and improve its permeability.

On the other hand, we investigated the ability of derivative **5r** to act as an inhibitor of the AcrAB-TolC efflux pump. In this context, the bacteria were loaded with 1,2'dNA dye, a

substrate of the AcrAB-TolC efflux pump.[37] Bacteria were then incubated with the compound (100 µM) before addition of glucose as energy source. The active transport of 87% of the dye is observed for non-treated bacteria (Figure 5C, black curve). By using PAßN, a well-known reference for efflux inhibition, a 25% retention of the dye within the bacterial membranes was observed (Figure 5C, blue curve), whereas addition of derivative **5r** led to a stronger inhibition, resulting in 68% retention of the dye (Figure 5C, orange curve).

RND efflux pumps, such as the AcrAB-TolC considered here, use the proton gradient across the inner membrane as energy source. Thus, we asked if the efflux inhibition observed in Figure 5C by **5r**, may result in the disruption of the transmembrane potential. For that, we used the membrane potential sensitive probe 3,3′-Dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) which concentrated across the inner membrane and self-quenched its fluorescence. If a compound impairs the membrane potential, DiSC3(5) is released into the medium leading to a fluorescence increase. Figure 5D summarizes the results obtained by using compound **5r** and polymyxin B, known to strongly disrupt the membrane. A decrease of 14% of the membrane potential was observed with polymyxin B, while no change was encountered by using compound **5r** suggesting that this later did not inhibit the efflux transport observed in Figure 5C by disrupting the transmembrane potential.

All these data suggest that the observed synergy, between compound **5r** and the tested intra-cytoplasmic targeted antibiotics, results from a dual effect *i.e.* a specific OM permeabilization combined with an AcrAB-TolC pump impairment. Thus, although we cannot exclude the impairment of the pump, this latter could be the result of a side effect of the outer membrane permeabilization. Nevertheless, a specific inhibition of AcrAB-TolC remains possible and researches in this regard are under current investigation.

3. Conclusion

An original chemical strategy has been developed affording new polyaminoisoprenyl compounds in moderate to good yields. Amongst all the synthetized derivatives, some of them exhibited a strong effect against resistant Gram-negative bacteria of four antibiotics belonging to four different families. Thus, this activity was correlated to the ability of the polyaminoisoprenyl derivatives to alter bacterial outer membrane integrity. Studies are now underway to determine if this restoration of antibiotic susceptibility occurs also by a direct interaction of the molecule with the efflux pump or by another mechanism.

4. Experimental section

4.1 Materials

All the solvents were purified according to reported procedures, and the reagents used were commercially available. Methanol, ethyl acetate and dichloromethane were purchased from Sigma-Aldrich and used without further purification. Column chromatography was performed on Merck silica gel (70-230 mesh). ^{1}H NMR and ^{13}C NMR spectra were recorded in MeOD on a Bruker AC 300 spectrometer working at 300 MHz and 75 MHz, respectively (the usual abbreviations are used: s: singlet, d: doublet, t: triplet, q: quadruplet, m: multiplet). All chemical shifts are given in ppm. Mass spectroscopy analysis has been performed by the Spectropole (Analytical Laboratory) of Aix-Marseille University (Marseille). The purity of the compounds was checked by analytical HPLC (C18 column, eluent CH_3CN-water-TFA (90:10:0.025,v/v/v), 0.5-1 mL/Min) with PDA detector spanning from 210 nm to 310 nm. All compounds possessed purity above 95%, as determined by analytical HPLC-PDA at 210 nm.

All bacterial experiments were performed on the BAC-Screen platform (UMR-MD1), with a Freedom EVO 150 liquid handling system (Tecan Lyon-France) and were independently repeated at least three times. Antibiotics (chloramphenicol, doxycycline and nalidixic acid)

were purchased from Sigma (St Quentin Fallavier-France); erythromycin-lactobionate was purchased from Amdipharm ltd (Dublin-Ireland). They were dissolved in ethanol, water or dimethyl sulfoxide (DMSO), as further precised. The chemicals benzalkonium chloride, phenylalanine-arginine beta-naphthylamide (PAßN), polymyxin-B and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Nitrocefin was purchased from Oxoid (Basingstoke, England), 1,2'-Dinaphthylamine (1,2'-diNA) was purchased from T.C.I (Zwijndrecht, Belgium), 3,3' -Dipropylthiadicarbocyanine iodide (diSC_3(5)) was purchased from Anaspec (Freemont, USA), Hoechst 33342 (H33342) was purchased from Molecular probes (Eugene, USA). Benzalkonium chloride, PAßN and polymyxin-B were dissolved in double distilled water and stored at -20°C until use. Nitrocefin was dissolved in DMSO 5%. 1,2'-diNA, diSC_3(5), H33342 were dissolved in DMSO 100%.

4.2 General procedure for the synthesis of compounds 5a-6r

The general synthetic pathway is illustrated for the preparation of compound **5p**.

A mixture of citral (345 mg, 2.27 mmol), titanium(IV)isopropoxide (645 mg, 2.27 mmol) and spermine (2.27 mmol) in absolute methanol (5 mL) was stirred at room temperature for 12 hours. Sodium borohydride (172 mg, 4.5 mmol) was then added at 0ºC and the resulting mixture was stirred for an additional 2 hours. The reaction was then quenched by adding water (1 mL). Stirring was maintained at room temperature for 20 minutes. After filtration over a pad of Celite washing with methanol and ethylacetate, the solvents were removed under vaccuum and the crude amine was purified by flash chromatography on silicagel. using CH_2Cl_2/MeOH/ NH_4OH (7/3/1) as eluent affording the expected coupling product **5p** in 64% yield.

Compound 5a, 49% yield; Yellow solid; ^{1}H NMR (MeOD): δ = 5.08-4.90 (m, 2H), 3.01-2.95 (m, 2H), 2.68-2.64 (m, 5H), 2.09-2.03 (m, 3H), 1.62-1.40 (m, 12H). ^{13}C (MeOD): δ = 142.24,

142.16, 132.80, 132.78, 123.05, 116.84, 116.78, 51.76, 51.72, 44.19, 41.20, 31.80, 26.20, 26.14, 25.70, 17.80, 17.14. $C_{12}H_{24}N_2$ m/z 197.1945 (100%, ($M+H^+$)).

Compound 5b, 33% yield; Yellow solid; ^{1}H NMR (MeOD): δ = 5.05-4.93 (m, 2H), 2.93-2.78 (m, 4H), 2.18-1.92 (m, 5H), 1.63-1.18 (m, 15H). ^{13}C (MeOD): δ = 136.20, 133.14, 124.83, 120.12, 47.18, 44.12, 40.60, 39.17, 33.14, 26.82, 25.38, 18.01, 17.84. $C_{13}H_{26}N_2$ m/z 211.2075 (100%, ($M+H^+$)).

Compound 5e, 61% yield; Yellow solid; Mixture of Z/E isomers; ^{1}H NMR (MeOD): δ = 5.08-4.82 (m, 2H), 2.95-2.70 (m, 4H), 2.59-2.40 (m, 6H), 2.02-2.00 (m, 4H), 1.83-1.66 (m, 4H), 1.61-1.51 (m, 12H). ^{13}C (MeOD): δ = 142.21, 131.83, 131.81, 122.95, 117.35, 53.70, 51.29, 48.22, 44.01, 43.67, 31.78, 27.61, 26.15, 25.82, 23.47, 17.51, 15.92. $C_{17}H_{32}N_2$ m/z 265.2578 (100%, ($M+H^+$)).

Compound 5f, 51% yield; Yellow solid; Mixture of Z/E isomers; ^{1}H NMR (MeOD): δ = 5.08-4.96 (m, 2H), 3.33-2.95 (m, 8H), 2.76-2.71 (m, 2H), 2.23-2.03 (m, 6H), 1.82-1.59 (m, 12H). ^{13}C (MeOD): δ = 177.21, 177.15, 143.24, 141.24, 141,21, 132.80, 123.05, 117.32, 115.34, 48.12, 48.09, 46.94, 46.89, 44.82, 44.01, 31.80, 31.78, 31.09, 29.78, 28.07, 27.52, 26.82, 19.43, 18.03, 17.95, 16.45. $C_{17}H_{30}N_2O$ m/z 279.2364 (100%, ($M+H^+$)).

Compound 5g, 52% yield; Yellow solid; ^{1}H NMR (MeOD): δ = 5.05-4.81 (m, 2H), 3.51-3.33 (m, 4H), 2.93-2.71 (m, 6H), 2.27-2.01 (m, 6H), 1.62-1.53 (m, 14H). ^{13}C (MeOD): δ = 141.84, 131.75, 131,21, 123.05, 114.32, 114.12, 66.85, 66.79, 52.81, 51.82, 48.19, 48.09, 43.13, 31.79, 31.09, 27.49, 26.18, 25,23, 17.46, 16.83. $C_{17}H_{32}NO$ m/z 281.2516 (100%, ($M+H^+$)).

Compound 5j, 42% yield; Yellow solid; Mixture of Z/E isomers; ^{1}H NMR (MeOD): δ = 5.04-4.97 (m, 2H), 2.91-2.75 (m, 5H), 2.18-1.97 (m, 7H), 1.67-1.08 (m, 21H). ^{13}C (MeOD): δ = 136.77, 132.43, 131.45, 125.41, 118.12, 55.84, 55.54, 44.12, 39.68, 39.45, 39.37, 38.12, 30.60, 27.07, 26.62, 25.70, 17.18, 16.95, 14.15. $C_{17}H_{35}N_3$ m/z 282.2846 (100%, ($M+H^+$)).

Compound 5k, 49% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.15-4.95 (m, 2H), 2.97-2.68 (m, 6H), 2.50-2.48 (m, 4H), 2.09-1.98 (m, 4H), 1.68-1.48 (m, 19H), 0.95 (t, *J* = 6Hz, 6H). ^{13}C (MeOD): δ = 136.10, 136.04, 132.80, 124.05, 123.92, 117.32, 49.51, 47.93, 47.67, 47.30, 44.00, 40.78, 39.68, 26.62, 25.92, 25.70, 24.63, 17.80, 17.03, 16.90, 16.13. $C_{20}H_{41}N_3$ m/z 324.3334 (100%, ($M+H^+$)).

Compound 5l, 64% yield; Yellow solid; Mixture of Z/E isomers; ^{1}H NMR (MeOD): δ = 5.06-4.94 (m, 2H), 3.04-2.56 (m, 16H), 2.03-1.99 (m, 2H), 1.64-1.58 (m, 14H). ^{13}C (MeOD): δ = 142.32, 142.29, 131.95, 123.05, 116.82, 51.32, 51.24, 49.32, 48.19, 48.03, 48.01, 44.12, 41.12, 31.79, 31.67, 26.15, 26.10, , 17.75, 17.69, 16.83. $C_{16}H_{34}N_4$ m/z 283.2817 (100%, ($M+H^+$)).

Compound 5n, 58% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.07-4.92 (m, 2H), 3.02-2.52 (m, 27H), 2.09-1.58 (m, 15H). ^{13}C (MeOD): δ = 141.34, 141.29, 131.77, 131.54, 124.56, 122.95, 118.89, 117.02, 117.01, 52.46, 51.30, 51.14, 49.64, 48.19, 48.12, 44.15, 42.45, 41.03, 41.01, 31.77, 26.18, 17.96,17.79, 16.92. $C_{20}H_{44}N_6$ m/z 369.3661 (100%, ($M+H^+$)).

Compound 5p, 64% yield; Mixture of Z/E isomers, white solid; ^{1}H NMR (MeOD): δ = 5.27-5.10 (m, 3H), 3.36-3.19 (m, 4H), 2.74-2.63 (m, 10H), 2.12-2.08 (m, 6H), 1.76-1.31 (m, 22H). ^{13}C (MeOD): δ = 140.12, 133.18, 132.82, 125.60, 124.18, 123.30, 50.83, 48.38, 47.98, 47.94, 41.39, 41.22, 33.49, 30.40, 30.30, 28.56, 27.98, 27.95, 26.42, 26.38, 24.14, 18.24, 16.83. $C_{20}H_{42}N_4$ m/z 339.3482 (100%, ($M+H^+$)).

Compound 5r, 49% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.27-5.10 (m, 3H), 3.34-3.19 (m, 4H), 2.76-2.53 (m, 9H), 2.12-2.09 (m, 6H), 1.77-1.63 (m, 20H). ^{13}C (MeOD): δ = 141.10, 140.53, 133.66, 133.29, 125.99, 125.90, 124.60, 123.71, 54.95, 53.97, 53.50, 49.15, 48.42, 48.37, 41.80, 41.60, 33.88, 30.97, 28.37, 28.34, 26.87-26.48, 19.33, 18.72, 17.32. $C_{19}H_{40}N_4$ m/z 325.3326 (100%, ($M+H^+$)).

Compound 6a, 63% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.30-4.99 (m, 3H), 3.01-2.92 (m, 2H), 2.70-2.67 (m, 5H), 2.09-1.93 (m, 7H), 1.62-1.49 (m, 15H). ^{13}C (MeOD): δ = 140.02, 139.84, 137.15, 137.09, 136.12, 125.17, 124.23, 116.80, 116.78, 51.78, 51.54, 44.19, 41.20, 39.90, 35.48, 26.82, 26.76, 26.32, 25.66, 23.30, 17.62, 16.82. $C_{17}H_{32}N_2$ m/z 265.2578 (100%, ($M+H^+$)).

Compound 6b, 49% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.18-5.01 (m, 4H), 3.22-3.08 (m, 3H), 2.84-2.64 (m, 4H), 2.09-1.90 (m, 8H), 1.66-1.02 (m, 18H). ^{13}C (MeOD): δ = 141.15, 141.09, 136.77, 136.64, 136.44, 136.33, 132.43, 132.17, 125.41, 125.37, 54.34, 47.25, 47.17, 41.33, 40.90, 40.80, 40.14, 32.95, 30.53, 28.86, 27.73, 27.77, 27.65, 27.42, 26.02, 25.97, 23.73, 17.82, 16.52, 16.25. $C_{18}H_{34}N_2$ m/z 279.2745 (100%, ($M+H^+$)).

Compound 6c, 58% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.19-4.99 (m, 3H), 2.73-2.56 (m, 4H), 3.32-3.06 (m, 3H), 2.73-2.56 (m, 4H), 2.03-1.80 (m, 9H), 1.67-1.09 (m, 20H). ^{13}C (MeOD): δ = 141.52, 141.41, 136.54, 136.42, 132.45, 132.24, 132.18, 125.73, 125.41, 125.37, 125.05, 121.15, 120.11, 120.02, 54.446, 46.88, 41.15, 40.90, 40.80, 33.38, 33.07, 32.96, 28.79, 28.73, 27.83, 27.41, 27.34, 26.05, 26.00, 24.39, 23.82, 23.76, 22.17, 17.86, 16.60, 16.19. $C_{19}H_{36}N_2$ m/z 293.2884 (100%, ($M+H^+$)).

Compound 6d, 38% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.28-5.11 (m, 4H), 3.43-3.19 (m, 3H), 2.75-2.61 (m, 4H), 2.14-2.06 (m, 9H), 1.77-1.40 (m, 20H). ^{13}C (MeOD): δ = 140.67, 140.58, 136.61, 136.28, 132.21, 132.15, 125.83, 125.40, 125.37, 125.13, 125.00, 121.92, 121.28, 47.39, 41.87, 40.89, 40.17, 33.03, 27.83, 27.76, 27.38, 25.94, 25.55, 23.73, 17.79, 16.45, 16.12. $C_{20}H_{38}N_2$ m/z 307.3052 (100%, ($M+H^+$)).

Compound 6e, 71% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.14-4.92 (m, 3H), 2.93-2.68 (m, 6H), 2.56-2.48 (m, 4H), 2.09-1.98 (m, 6H), 1.68-1.48 (m, 21H). ^{13}C (MeOD): δ = 140.12, 140.00, 136.10, 136.01, , 124.03, 123.92, 117.42, 54.70, 51.38, 48.19,

43.27, 39.72, 31.40, 27.50, 26.34, 25.42, 25.34, 25.20, 23.56, 17.62, 16.83, 15.18. $C_{22}H_{40}N_2$ m/z 333.3194 (100%, ($M+H^+$)).

Compound 6g, 56% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.09-4.92 (m, 3H), 2.91-2.61 (m, 6H), 2.56-2.48 (m, 4H), 2.08-1.98 (m, 6H), 1.67-1.46 (m, 21H). ^{13}C (MeOD): δ = 140.32, 140.14, 139.86, 137.61, 136.68, 132.48, 132.25, 125.83, 125.43, 125.32, 66.70, 66.32, 53.73, 51.92, 48.19, 44.00, 39.37, 31.20, 27.49, 26.82, 26.30, 25.66, 17.62, 16.90, 15.84. $C_{22}H_{40}N_2O$ m/z 349.3120 (100%, ($M+H^+$)).

Compound 6h, 62% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.09-4.92 (m, 3H), 2.91-2.61 (m, 6H), 2.56-2.48 (m, 4H), 2.08-1.98 (m, 6H), 1.67-1.46 (m, 21H). ^{13}C (MeOD): δ = 140.11, 139.95, 137.89, 136.58, 132.58, 132.15, 125.99, 125.43, 125.17, 52.36, 52.03, 51.68, 51.56, 48.19, 43.82, 40.29, 39.77, 31.02, 27.34, 26.82, 26.78, 26.28, 25.89, 25.66, 24.32, 17.54, 16.90, 15.84. $C_{25}H_{48}N_4$ m/z 405.3875 (100%, ($M+H^+$)).

Compound 6i, 52% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.17-4.97 (m, 3H), 3.20-3.18 (m, 4H), 2.66-2.54 (m, 7H), 2.02-1.90 (m, 10H), 1.63-1.24 (m, 24H). ^{13}C (MeOD): δ = 140.12, 140.00, 137.62, 136.68, 132.58, 132.26, 125.83, 125.43, 125.37, 125.07, 47.43, 47.28, 41.14, 40.90, 40.78, 31.96, 28.84, 27.78, 27.44, 26.03, 25.99, 22.74, 17.84, 16.57, 16.21. $C_{21}H_{41}N_3$ m/z 335.3342 (100%, ($M+H^+$)).

Compound 6j, 58% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.29-5.14 (m, 4H), 3.34-3.31 (m, 3H), 2.79-2.63 (m, 4H), 2.48-2.45 (m, 4H), 2.26 (s, 3H), 2.14-1.93 (m, 8H), 1.77-1.63 (m, 18H). ^{13}C (MeOD): δ = 140.97, 137.51, 137.3, 133.23, 132.96, 126.74, 126.29, 126.24, 126.02, 125.82, 57.79, 57.49, 50.90, 49.16, 48.35, 43.18, 41.75, 41.64, 29.53, 28.70, 28.28, 26.88, 26.83, 24.61, 18.70, 17.04. $C_{22}H_{43}N_3$ m/z 349.3463 (100%, ($M+H^+$)).

Compound 6m, 32% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.12-4.89 (m, 3H), 3.05-2.48 (m, 21H), 2.10-1.61 (m, 23H). ^{13}C (MeOD): δ = 136.22, 136.11, 135.60, 135.45, 131.09, 125.07, 124.66, 124.18, 116.78, 116.67, 51.32, 49.69, 49.62, 48.22,

48.06, 44.17, 39.53, 39.03, 26.85, 26.54, 25.89, 23.42, 17.61, 16.82. $C_{23}H_{47}N_5$ m/z 394.3786 (100%, ($M+H^+$)).

Compound 6n, 47% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.12-4.89 (m, 3H), 3.07-2.53 (m, 25H), 2.11-1.58 (m, 24H). ^{13}C (MeOD): δ = 136.57, 136.10, 135.32, 135.15, 131.09, 125.17, 124.86, 124.78, 116.74, 116.69, 51.39, 49.75, 49.67, 48.19, 48.16, 44.20, 41.09, 39.78, 39.57, 26.95, 26.32, 25.47, 23.25, 17.52, 16.92. $C_{25}H_{52}N_6$ m/z 437.4235 (100%, ($M+H^+$)).

Compound 6o, 48% yield; Mixture of Z/E isomers, Yellow solid; ^{1}H NMR (MeOD): δ = 5.03-4.94 (m, 3H), 2.97-2.95 (m, 2H), 2.67-2.2.61 (m, 4H), 2.01-1.90 (m, 6H), 1.61-1.32 (m, 37H). ^{13}C (MeOD): δ = 136.10, 135.00, 134.89, 131.09, 125.17, 125.09, 124.36, 117.24, 117.12, 48.36, 44.27, 42.60, 41.12, 34.55, 29.67, 29.37, 28.87, 28.45, 28.02, 27.52, 27.49, 27.42, 23.12, 17.60, 16.43. $C_{27}H_{52}N_2$ m/z 405.4110 (100%, ($M+H^+$)).

Compound 6p, 72% yield; Yellow solid; ^{1}H NMR (MeOD): δ = 5.05-4.93 (m, 3H), 2.93-2.57 (m, 14H), 2.19-1.92 (m, 10H), 1.63-0.97 (m, 23H). ^{13}C (MeOD): δ = 142.24, 134.83, 131.09, 124.86, 124.17, 117.32, 47.90, 47.69, 47.64, 46.61, 44.01, 40.60, 39.83, 33.97, 31.20, 26.97, 26.30, 25.66, 25.11, 24.90, 17.62, 16.90, 15.93. $C_{25}H_{50}N_4$ m/z 407.4033 (100%, ($M+H^+$)).

Compound 6q, 43% yield; Yellow solid; Mixture of Z/E isomers, ^{1}H NMR (MeOD): δ = 5.03-4.95 (m, 3H), 3.40-2.88 (m, 14H), 2.17-1.45 (m, 31H). ^{13}C (MeOD): δ = 136.20, 136.14, 134.98, 134.83, 131.02, 125.18, 125.07, 124.84, 117.12, 70.64, 69.07, 69.00, 68.87, 46.73,44.25, 39.72, 39.57, 38.86, 33.61, 27.14, 27.09, 26.32, 25.62, 23.33, 17.62, 16.27. $C_{25}H_{48}N_2O_2$ m/z 409.3715 (100%, ($M+H^+$)).

Compound 6r, 63% yield; Yellow solid; ^{1}H NMR (MeOD): δ = 5.03-4.95 (m, 3H), 2.93-2.64 (m, 8H), 2.36-1.92 (m, 14H), 1.65-1.39 (m, 23H). ^{13}C (MeOD): δ = 142.31, 134.93, 131.09, 124.76, 124.17, 117.39, 52.88, 51.68, 48.19, 44.00, 40.30, 39.33, 31.20, 30.98, 27.67, 26.93, 26.30, 25.66, 17.62, 16.90, 15.32. $C_{24}H_{48}N_4$ m/z 393.3869 (100%, ($M+H^+$)).

Preparation of geranyl chloride (*trans* (1-Chloro-3,7-dimethyl-octa-2,6-diene)) 7

To a solution of geraniol (40.1 g, 0.26 mol) and anhydrous LiCl (23 g, 0.54 mol) in CH_2Cl_2 (250 mL) are added dropwise triethylamine (50 mL, 0.37 mol) and mesyl chloride (30 mL, 0.39 mol). The reaction is stirred at room temperature for 16 h. and then washed with 10 % HCl (2 x 100 mL), sat. Na_2CO_3 (2 x 100 mL) and brine (2 x 100 mL). The organic layer is dried (Na_2SO_4) and the solvents removed *in vacuo*. The crude dark oil is distillated under reduced pressure to afford the pure expected *trans* 1-Chloro-3,7-dimethyl-octa-2,6-diene as a colorless oil. 27.5 g (61 %). ^{1}H NMR ($CDCl_3$, 400 MHz) : δ = 5.48-5.43 (m, 1H), 5.10-5.07 (m, 1H), 4.10-4.12 (d, J = 6.0 Hz, 2H), 2.14-2.04 (m, 4H), 1.74 (s, 3H), 1.1.69 (s, 3H), 1.61 (s, 3H).

Preparation of neryl chloride (*cis* (1-Chloro-3,7-dimethyl-octa-2,6-diene)) 8

To a solution of nerol (40.1 g, 0.26 mol) and anhydrous LiCl (23 g, 0.54 mol) in CH_2Cl_2 (250 mL) are added dropwise triethylamine (50 mL, 0.37 mol) and mesyl chloride (30 mL, 0.39 mol). The reaction is stirred at room temperature for 16 h. and then washed with 10 % HCl (2 x 100 mL), sat. Na_2CO_3 (2 x 100 mL) and brine (2 x 100 mL). The organic layer is dried (Na_2SO_4) and the solvents removed *in vacuo*. The crude dark oil is distillated under reduced pressure to afford the pure expected *cis* 1-Chloro-3,7-dimethyl-octa-2,6-diene **8** as a colorless oil. 26 g (58%). ^{1}H NMR ($CDCl_3$, 400 MHz) : δ = 5.40-5.35 (m, 1H), 5.01-4.99 (m, 1H), 4.04-4.02 (d, J = 8.0 Hz, 2H), 2.04-1.97 (m, 4H), 1.66 (s, 3H), 1.62 (s, 3H), 1.53 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) : δ = 142.69, 131.90, 123.55, 120.27, 41.08, 39.41, 26.19, 26.52, 17.64, 16.04.

Scale-up synthesis of *cis*-5r tartrate salt

To a solution of tris-(3-aminopropyl)amine (50 mL, 0.25 mol) and triethylamine (60 mL, 0.44 mol) in distillated THF (100 mL) is added dropwise neryl chloride **8** (35 g, 0.20 mol) in distillated THF (200 mL). The reaction mixture is stirred at room temperature for 24 h. and evaporated to dryness. The crude residue is purified by column chromatography (eluant CH_2Cl_2/MeOH/conc.NH_4OH, 7:3:1) to afford the pure desired *cis* {3-[Bis-(3-amino-propyl)-amino]-propyl}-(3,7-dimethyl-octa-2,6-dienyl)-amine **5r** as a pale yellow oil. 31.1 g (48%). Then, a solution of *cis* {3-[Bis-(3-amino-propyl)-amino]-propyl}-(3,7-dimethyl-octa-2,6-dienyl)-amine **5r** (23.1 g, 0.07 mol) and tartaric acid (42.8 g, 0.28 mol) in anhydrous methanol (200 ml) is stirred at room temperature for 16 h. Methanol is evaporated until residual volume of about 50 mL is reached. Diethyl ether is added (about 500 mL) and the resulting precipitate is filtered off to give the pure desired salt as a white solid. 48.4 g (74%). ^{1}H NMR (D_2O, 400 MHz) : δ = 5.24-5.20 (m, 1H), 5.10 (m, 1H), 4.50 (s, 6H), 3.66-3.61 (m, 2H), 3.30-3.24 (m, 6H), 3.06-3.02 (m, 6H), 2.10 (m, 9H), 1.75 (s, 3H), 1.63 (s, 3H), 1.56 (s, 3H). ^{13}C NMR (D_2O, 75 MHz) : δ = 146.96, 134.12, 123.34, 113.42, 72.75, 49.86, 45.00, 43.04, 36.37, 31.31, 25.58, 24.82, 22.62, 21.49, 16.69. MS (ESI) $C_{19}H_{40}N_4$ m/z 325.4 (100 %, ($M+H^+$)).

Scale-up synthesis of *trans*-5r

To a solution of tris-(3-aminopropyl)amine (50 ml, 0.25 mol) and triethylamine (60 mL, 0.44 mol) in distillated THF (100 mL) is added dropwise geranyl chloride **7** (35 g, 0.20 mol) in distillated THF (200 mL). The reaction mixture is stirred at room temperature for 24 h. and evaporated to dryness. The crude residue is purified by column chromatography (eluant CH_2Cl_2/MeOH/conc.NH_4OH, 7:3:1) to afford the pure desired *trans* {3-[Bis-(3-amino-propyl)-amino]-propyl}-(3,7-dimethyl-octa-2,6-dienyl)-amine **5r** as a pale yellow oil. 35.2 g (54%). ^{1}H NMR (MeOD, 400 MHz) : δ = 5.30 (t, J = 7.0 Hz, 1H), 5.14 (t, *J* = 6.9 Hz, 1H), 3.39 (d, *J* = 7.0 Hz, 2H), 2.85 (t, *J* = 7.2 Hz, 4H), 2.75 (t, *J* = 7.4 Hz, 2H), 2.58 (t, *J* = 6.9 Hz, 4H), 2.56 (t,

J = 7.0 Hz, 2H), 2.08-2.18 (m, 4H), 1.93 (s, 3H), 1.73-1.80 (m, 6H), 1.73 (s, 3H), 1.71 (s, 3H), 1.64 (s, 3H). ^{13}C NMR (MeOD, 75 MHz) : δ = 177.01, 143.60, 133.51, 125.79, 120.49, 72.75, 53.46, 53.25, 48.28, 47.78, 41.59, 40.88, 28.32, 28.25, 26.89, 26.78, 25.25, 18.66, 17.40. MS (ESI) $C_{19}H_{40}N_4$ m/z 325.4 (100 %, ($M+H^+$)).

Scale-up synthesis of *trans*-5r tartrate salt

A solution of *trans* {3-[Bis-(3-amino-propyl)-amino]-propyl}-(3,7-dimethyl-octa-2,6-dienyl)-amine **5r** (23.1 g, 0.07 mol) and tartaric acid (42.8 g, 0.28 mol) in anhydrous methanol (200 mL) is stirred at room temperature for 16 h. Methanol is evaporated until residual volume of about 50 mL is reached. Diethyl ether is added (about 500 mL) and the resulting precipitate is filtered off to give the pure desired salt as a white solid. 50.1 g (76%). ^{1}H NMR (D_2O, 250 MHz) : δ = 5.17 (m, 1H), 5.06 (m, 1H), 4.48 (s, 5H), 3.61-3.58 (d, *J*=7.3 Hz, 2H), 3.24-3.20 (m, 6H), 3.04-2.97 (m, 6H), 2.03 (m, 10H), 1.62 (s, 3H), 1.58 (s, 3H), 1.51 (s, 3H). ^{13}C NMR (D_2O, 62.5 MHz) : δ = 177.05, 147.84, 134.29, 124.39, 113.47, 73.48, 67.19 (reference: 1,4-dioxane), 50.49, 45.72, 43.68, 39.43, 37.09, 26.11, 25.51, 22.14, 21.24, 17.61, 16.36. MS (ESI) $C_{19}H_{40}N_4$ m/z 325.4 (100 %, ($M+H^+$)).

4.3 Bacterial strains and growth conditions

Four bacterial strains were used in this study. The *Enterobacter aerogenes* Ea289 strain is a Kan^s derivative of the MDR clinical isolate Ea27 isolated from a patient and multiresistant. This parent strain was determined to be common to several site of nosocomial infections amongst different Intensive Care units in France.[38] To allow genetic modification (mutagenesis of AcrAB and of TolC coding genes) the kanamycin resistance from EA27 was removed by plasmid elimination giving EA289. The *acrB* mutant Ea289Δ*acrB* strain was then constructed from the Ea289 strain.[32]

The *Salmonella enterica* serovar Typhimurium strain BN10055 and its Δ*acrB* derivative were previously described.[31] The strains were maintained at -80°C in 15% (v/v) glycerol for cryo-protection. Bacteria were routinely grown in Mueller-Hinton (MHII) broth at 37°C.

4.4 Antibiotic susceptibility testing

Susceptibilities to antibiotics and compounds were determined in microplates by 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 µL of MH broth containing two-fold serial dilutions of each molecule. The MIC was defined as the lowest concentration of a molecule that completely inhibited visible growth after incubation for 18 hours at 37°C. Compounds were all solubilized in DMSO at high concentration, in order to apply up to 5% DMSO on bacteria. This concentration did not detrimentally affect bacterial growth of the strains used in this study. All MIC determinations were repeated at least three times in independent experiments.

4.5 Effect of compounds on antibiotic resistance

Each compound was tested for its ability to reduce antibiotic resistance. It was assayed at MIC/4 in order to avoid a direct killing effect on bacteria. The MIC of the antibiotics supplemented with the compound was compared to the MIC of the antibiotic alone. For each strain, the ratio of the MIC of an antibiotic tested alone, to its MIC obtained in the presence of the compound determined the MIC ratio. Compounds allowing a MIC ratio ≥ 4 were considered as efficient synergistic agents with the antibiotic considered on the strain tested.

4.6. Membrane depolarization assays

Bacteria were re-suspended at $OD_{600\ nm}$= 0.25 in Hepes 5 mM, EDTA 10 mM pH 7.0 and then washed in Hepes 5 mM pH 7.0 with 3-3'-Dipropylthiadicarbocyanin iodide ($diSC_3(5)$) 8 µM. The membrane potential-sensitive cyanine dye $diSC_3(5)$ distributes between cells and the medium depending on the cytoplasmic membrane potential gradient. Released diSC3(5) was quantified by measuring the fluorescence (λ_{ex}= 622 nm; λ_{em}= 690 nm) 300 s after the addition of compound **5r** (100 µM). A control experiment was performed for every tested condition where the cells were treated with SDS 0.5 % bacteria to normalize the results. Cell suspension was added at 100 µl/well and the fluorescence read every 30 s at 37°C. An Infinite M200Pro reader (Tecan) was used. Assays were performed in Greiner Bio-One 96 well plates, ref 675076 (half area, black with solid bottom).

4.7. Nitrocefin hydrolysis assay

Bacteria were re-suspended at $OD_{600\ nm}$= 0.25 in Potassium Phosphate buffer K_2HPO_4 20 mM $MgCl_2$ 1 mM pH 7.0 (PPB), supplemented with CCCP 5 µM. Bacteria were mixed with nitrocefin 50 µg/mL before addition of compound **5r** (100 µM). Nitrocefin hydrolysis was followed by monitoring the absorbance (λ_{abs}= 490 nm). Cell suspension was added at 100 µL per well and the absorbance read every 30 s at 37°C. An Infinite M200Pro reader (Tecan) was used. Assays were performed in Greiner Bio-One 96 well plates, ref 675101 (half area, clear with flat bottom).

4.8. Cell wall penetration

Bacteria were re-suspended at $OD_{600\ nm}$= 0.25 in PPB supplemented with with CCCP 5 µM. Bacteria were mixed with H33432 2.5 µM before addition of compound **5r** (100 µM). H33342 binding to double stranded DNA was followed by monitoring the fluorescence (λ_{ex}= 350 nm; λ_{em}= 450 nm). Cell suspension was added at 100 µL per well and the fluorescence read every

30 s at 37°C. An Infinite M200Pro reader (Tecan) was used. Assays were performed in Greiner Bio-One 96 well plates, ref 675076 (half area, black with solid bottom).

4.9. Glucose-triggered 1,2'-diNA efflux assays

Bacteria were grown until the stationary phase was reached, collected by centrifugation, and re-suspended at $OD_{600\ nm}$= 0.25 in PPB supplemented with CCCP 5 µM, and incubated overnight with 1,2'-Dinaphthylamine (1,2'-diNA) 32 µM at 37°C. Before addition of compound **5r** (100 µM), the cells were washed in PPB. Glucose 50 mM was added at 300 s to initiate bacterial energization. Membrane incorporated 1,2'-diNA was followed by monitoring the fluorescence (λ_{ex}= 370 nm; λ_{em}= 420 nm). Cell suspension was added at 100 µL/well and the fluorescence read every 30 s at 37°C. An Infinite M200Pro reader (Tecan) was used. Assays were performed in Greiner Bio-One 96 well plates, ref 675076 (half area, black with solid bottom).

4.10 Cytotoxicity assays

Cytotoxicity assessment was performed on the referenced Chinese Hamster Ovary cell line (CHO-K1, ATCC-LGC Promochem, Molsheim France). Cells were maintained in McCoy's 5A medium (Sigma) supplemented with 10% foetal calf serum, 1 mM glutamine and penicillin-streptomycin (100 $U.mL^{-1}$ and 10 $\mu g.mL^{-1}$ respectively) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The cytotoxic effects of compounds were assessed by the colorimetric WST-1 cell proliferation assay. Briefly, a range of compounds concentrations from 30 µM to 1200 µM was incorporated in triplicate cultures, and cells were incubated at 37 °C for 24 h. At the end of the incubation period, cultures were submitted to three successive washes in phosphate buffer saline (PBS) and incubated in fresh culture medium containing 10% WST-1 for an additional 30 min. Cell viability was evaluated by the assessment of WST-1 absorbance

at 450 nm in a microplate spectrophotometer MRX1 II (Dynex technologies, Chantilly, VA, USA). The Inhibitory Concentration 50% (IC_{50}) was chosen to evaluate the cytotoxicity of compounds. IC_{50} was defined as the concentration of compounds that induced a 50% decrease of viable cells.

4.11 Animals

We used 5-6 weeks old female BALB/cByJ mice (SAS Janvier, Le-Genest-St-Isle, France). During all experiments, the mice were housed in a ventilated pressurized cabinet (A-BOX 160, Noroit, Rezé, France) with food and water available ad libitum. All experiments were performed according to the guidelines of the Ethics Committee for animal treatment at the Centre de Recherche de Tours (INRA, UE1277, 37380 Nouzilly). Ethical dossier number N°6646.

4.12 Model of *S*. Typhimurium mice infection

To assess the severity of infection with or without treatment 12 mice per groups were infected, followed and sacrificed 1 day (n = 3), 3 days (n = 3) or 7 days (n = 6) after inoculation. Six mice were used as controls and six were inoculated with saline. For infection, the animals were anaesthetized using Sevofluranew (Abbott, Rungis, France). Inoculation was performed intraperitoneally by injected 200 µL of a bacterial solution (10^2 CFU/mL) of *S.* Typhimurium then followed, each day, by administration of a saline, doxycycline alone or a doxycycline/**5r** combination solution (64 mg/Kg for doxycycline and 0.5 mg/Kg for **5r**, respectively). The animals were weighed every day until sacrifice or death. Euthanasia was performed using an overdose of thiopental,

4.13 *In vivo* toxicity assay

6 x 6 Mice weighing around 20 g were intraperitoneally injected with 200 µL of **5r** aqueous solution (12.5, 25, 50, 100 µM) or saline. The animals were weighed every day, checked 3 times per day and sacrificed after 48 hours. Euthanasia was performed using an overdose of thiopental.

Authors information

Corresponding author

Dr Jean Michel Bolla and Dr Jean Michel Brunel share senior co-authorship.
E-mails: jean-michel.bolla@univ-amu.fr and bruneljm@yahoo.fr

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Supporting Information

Table S-1: MIC of the different compounds against enterobacteria
Table S-2: Efficiency Parameter (EP) of the different molecules used in the assay
Table S-3. MIC of doxycycline in a synergistic assay involving *Z/E*-**5r** mixture, *Z*-**5r**, *E*-**5r**, *Z*-**5r** (Hydrochloride salt), *Z*-**5r** (tartrate salt) against MDR strain EA289 (Average of 3 experiments)
Table S-4. Molecular formula strings of **8**, *Z*-**5r**, *E*-**5r** and **6j**
Fig. S-1 ^{1}H and ^{13}C NMR spectra of nerylchloride **8**
Fig. S-2 ^{1}H and ^{13}C of Z/E mixture of **5r**
Fig. S-3 ^{1}H and ^{13}C of Z/E mixture of **6j**
Fig. S-4 ^{1}H and ^{13}C of *cis*-**5r**
Fig. S-5 ^{1}H and ^{13}C of *cis*-**5r** tatrate salt
Fig. S-6 ^{1}H and ^{13}C of *trans*-**5r**
Fig. S-7 ^{1}H and ^{13}C of *trans*-**5r** tartrate salt

Abbreviations

WHO, World Health Organization; EP, Efficiency Parameter; CHO, Chinese Hamster Ovary; PAβN, Phenyl-Arginine-Beta-Naphthylamide

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