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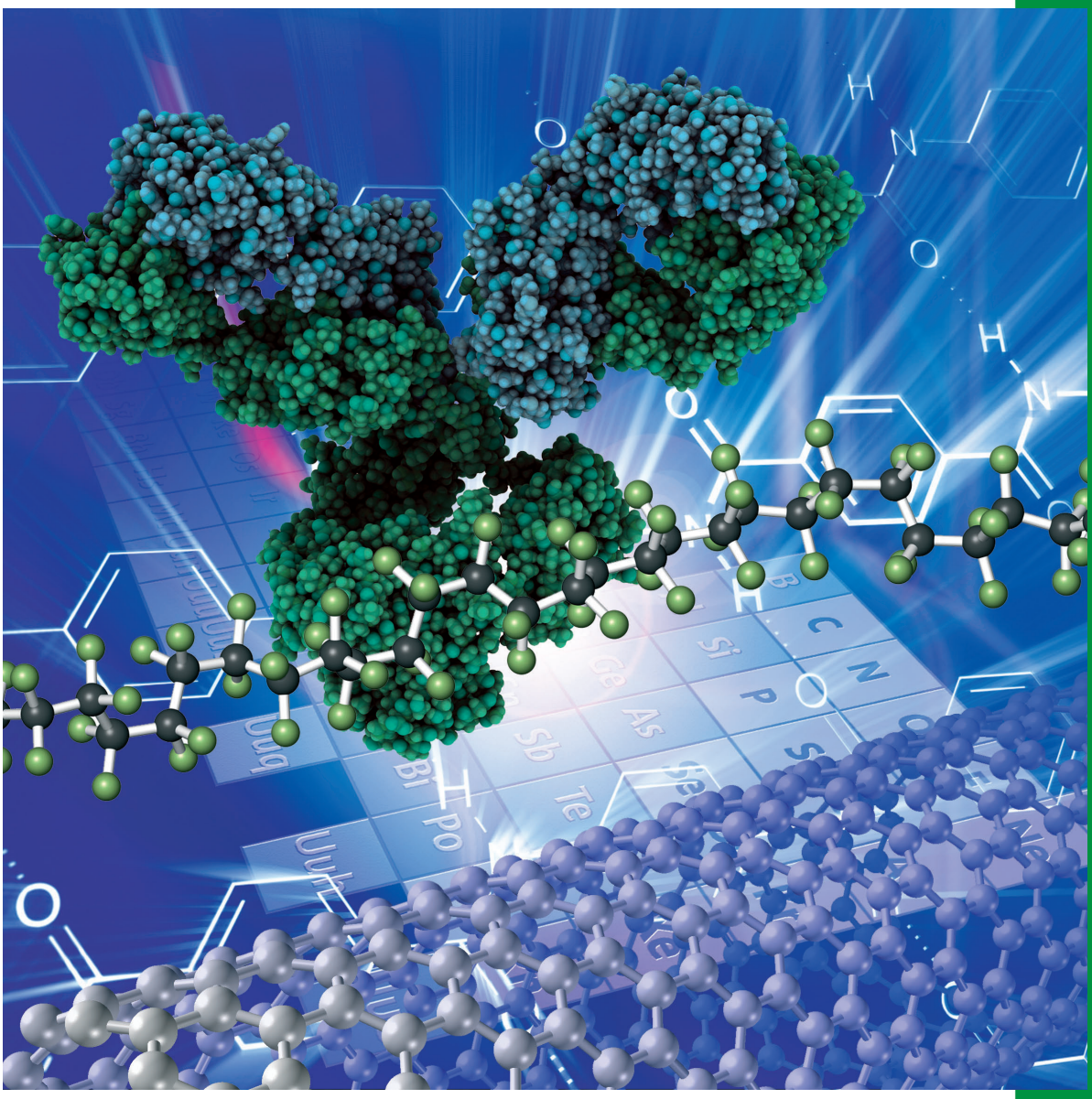

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Structure-Activity Relationships for Poly(phenylene) vinylene Derivatives as Antibacterial Agents

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A series of poly(phenylene)vinylene (PPV) derivatives with different geometries and peripheral functional groups have been synthesized by Horner-Wadsworth-Emmons reaction. Compounds bearing carboxylic acid or quaternary ammonium functional groups prepared by this metal-free protocol were weakly active against both Gram-negative and Gram-positive bacteria, which is in contrast to previously reported results. The activity of polyanionic derivatives was higher for Gram-negative bacteria and showed bacteriostatic behavior, whereas polycationic derivatives were clearly more active against Gram-positive bacteria and behaved as bactericidal agents. The spatial disposition of the active groups seems to play a key role, with the highest activity observed for a C_{3v} geometry. The presence of quaternary ammonium functional groups also facilitated the internalization of the molecules into the bacteria. Levels of cytotoxicity similar to that of the solvent were obtained for most compounds against COS-1 and VERO cells.

Introduction

Antibiotic resistance is an increasingly serious global concern.[1] Bacteria acquire resistance naturally over time, whether by spontaneous genetic changes or by genetic exchange with other bacteria, which leads to a low-level of natural selection.[2] Predictably, the misuse and rampant overuse of antibiotics in both humans and animals has clearly contributed to accelerate this process. Since the 1980's there has been a decline in the development of new antibiotics.[3] Concomitantly, there has also been an increasing number of infections that do not respond to any available treatments. Hence, there is an urgent need to rekindle the discovery of new, effective, broad-spectrum antibacterial drugs by the scientific community.[4] Nevertheless, the development of new antibiotic pipelines against highly resistant bacteria (superbugs) is particularly challenging.[5]

Dendrimers that contain covalently bonded antibacterial moieties provide an alternative to conventional, low molecular weight antimicrobial agents.[6] Dendrimers can interact in a multivalent fashion with several bacterial targets, thus forcing the bacteria to make several changes to reduce their susceptibility and, consequently, diminishing the likehood of resistance. Dendrimers that are highly functionalized with either cationic or anionic surface groups are among the most widely used as it has been well established that they show antibacterial activity.[7]

Antibacterial dendrimers generally contain positively charged groups that are shielded to some extent by membrane-disrupting alkyl chains. The principal mode of action is comparable to that of more conventional bacteriostatic quaternary ammonium compounds, i.e., the electrostatic interaction with the negatively charged bacteria disrupts and eventually disintegrates the prokaryotic bacterial membrane.[8] Different dendrimer scaffolds containing quaternary ammonium groups at their periphery have been shown to possess activity against both Gram-positive and Gram-negative strains of pathogenic bacteria.[9] Nevertheless, cationic dendrimers have repeatedly shown cytotoxicity against a variety of eukaryotic cell lines.[10]

In contrast to the above, dendrimers with terminal anionic charges are generally noncytotoxic[11] and they also exhibit membrane disruptive features. Thus, carboxylic acid-terminated poly(amidoamine) (PAMAM) dendrimers have been used against Gram-negative *Escherichia coli*,[12] while amphiphilic dendrimers containing multiple carboxylate groups have shown activity against Gram-positive bacteria.[13]

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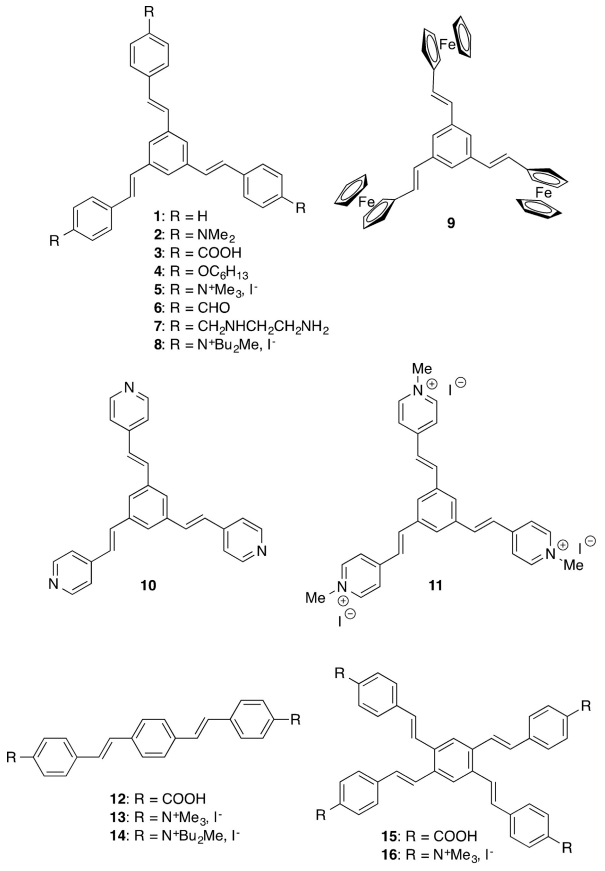
Although the multivalency of dendrimers seems to play an important role in the antibactericidal activity, it is also known that biopermeability is a key factor. Structures with hydrophobic units permeate through the cell wall more easily.[7c] Nevertheless, structure-property relationships are difficult to establish and predict in materials with flexible branches. Dendrimers with a rigid and well-defined framework have the advantage of enabling structure-property relationships to be more easily understood.

Tris(stilbene) compounds are simple aromatic systems that can be envisaged as first generation poly(phenylene)vinylene (PPV) dendrimers with a rigid conjugated scaffold. These compounds can be easily synthesized through a variety of strategies,[14] and recently they have been reported as a new class of potent Gram-positive antibacterial agents.[15] Specifically, some derivatives bearing carboxylic acid groups at the periphery exhibited good activity against several bacterial strains, including multidrug-resistant *Staphylococcus aureus* (MRSA), at similar concentrations to those required for frontline antibiotics. In contrast, with some exceptions, these compounds cannot penetrate the Gram-negative outer membrane and they show little activity against this type of bacterium. Antibacterial properties have also been demonstrated in even simpler stilbene derivatives and related phenols.[16] Nevertheless, little is known about the structure-activity relationships of PPV derivatives as antibacterial agents.

Thus, we decided to prepare a larger family of PPV compounds with two, three or four branches emanating from the benzene central core and bearing different peripheral moieties (Scheme 1) and explore the structure-activity relationships of this set of compounds. Our studies demonstrated that the geometry of the molecule has a marked influence on the antibacterial activity.



Scheme 1. Structures of compounds **1–16**.

Results and Discussion

Synthesis and Characterization of PPV Derivatives

The synthesis of the target compounds was based on the HWE reaction for the formation of double bonds (Scheme S1 in the Supporting Information). This methodology, which has been extensively developed by some of us since 1999,[14] has several advantages compared to other methods to generate double bonds, such as the Heck–Mizoroki reaction. Firstly, the *trans* stereochemistry of the double bonds located at the core was preserved throughout the synthetic methodology. This stereochemistry was unequivocally established on the basis of the coupling constant for the vinylic protons in the ^{1}H NMR spectra ($J \approx 16$ Hz). This is an important issue because when establishing a structure-activity relationship it is important to compare molecules with defined structures, including stereochemistry. Secondly, the HWE reaction usually requires an easy workup in a catalyst-free approach, thus avoiding an increase in production costs and possible interferences in the biological properties. In the pharmaceutical industry, synthetic processes must also provide drug ingredients with very high purity. Therefore, procedures that do not need the use of chromatographic techniques or the use of transition metals are preferable to meet the stringent specifications for materials subjected to clinical testing.

Additional chemical modification was performed to obtain the polycationic compounds by quaternization of either the tertiary amines or pyridine nitrogen atoms with methyl iodide (Scheme S2 in the Supporting Information). Derivatives **8**, **13**, and **16** are described for the first time, whereas **14** has been previously reported as triflate salt.[17] All new compounds were characterized by a variety of analytical techniques.

Antibacterial Activity

The potential antibacterial activity of all the prepared compounds was evaluated by screening against *E. coli* and *E. faecalis*, Gram-negative and Gram-positive bacteria, respectively. So far, only compounds **3**, **12**, and **15** had been studied.[15] The antibacterial activity was determined by the reduction of the counted colony forming units (CFU/mL). In order to establish a correlation between the structure and the antibacterial activity, three sets of stilbene derivatives were considered according to the hypothetical charge of the peripheral groups in the *in vitro* media.

The results for compounds with neutral groups (**1**, **2**, **4**, **9**, and **10**) are shown in Figures 1a (*E. coli*) and 1d (*E. faecalis*). In

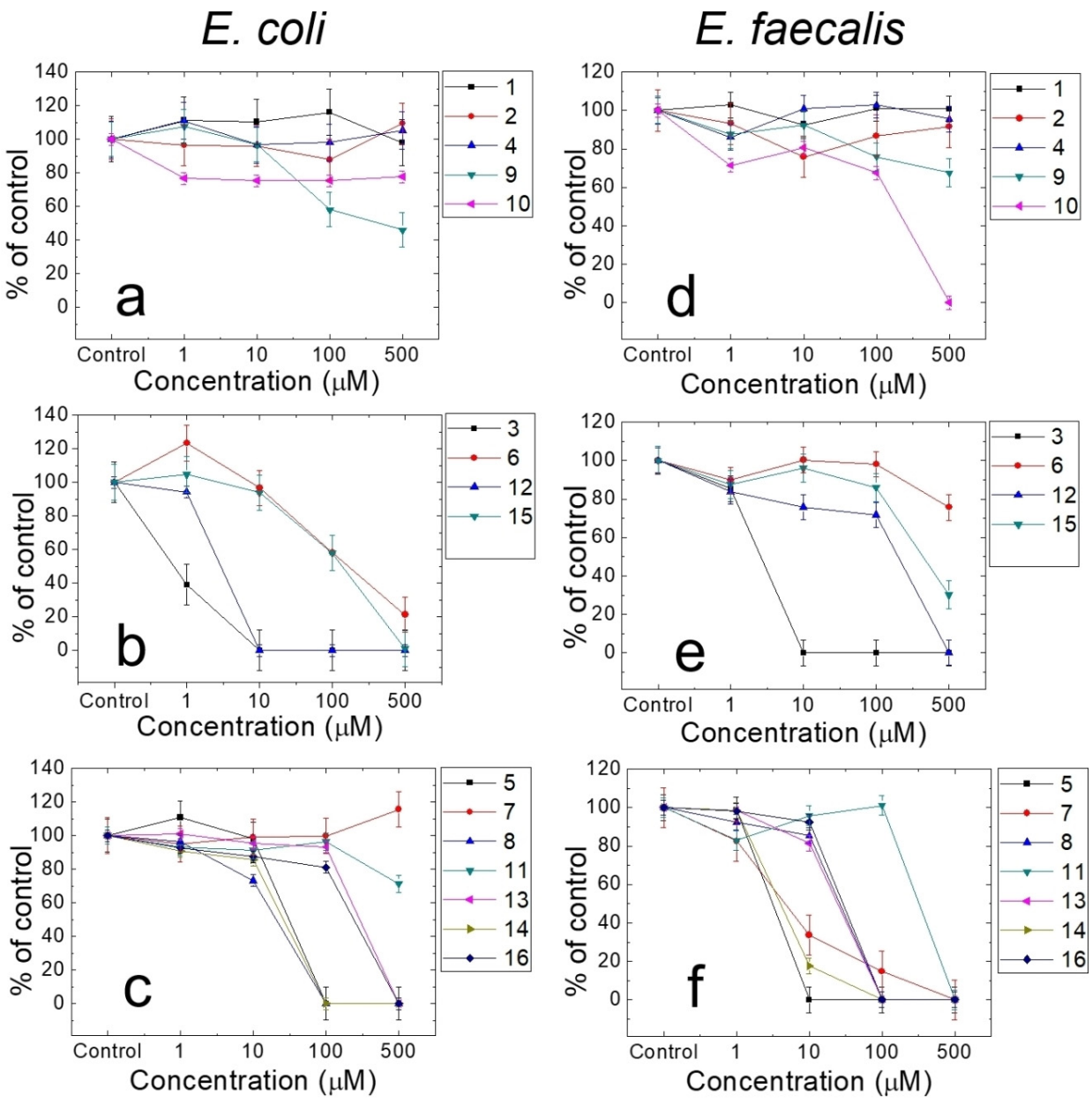


Figure 1. Antibacterial activity against *E. coli* (a, b and c) and *E. faecalis* (d, e and f) determined by the relative reduction of counted colony forming units (CFU) with respect to the control experiment.

both strains, compounds **1**, **2**, and **4** did not exhibit an inhibitory effect. The reduction of counted CFU was similar to that obtained in the dimethyl sulfoxide (DMSO) control experiment. These experiments rule out the possibility that the styrylbenzene core alone was responsible for the antibacterial activity. Thus, the functional groups present on the periphery and their relative positions generated by the rigid conjugated core should play an important role. On the other hand, ferrocene derivatives have previously been described as possible antibacterial agents.[18] Nevertheless, despite bearing three ferrocene units, compound **9** showed only slight antibacterial activity for *E. coli*, with 50% of the CFU reached at 100 mM, and a value very close to the standard deviation of the DMSO control experiments for *E. faecalis*. Thus, this molecule cannot be considered as a good candidate for active antibacterial drugs. Pyridine derivative **10** also displayed low antibacterial activity and it only became active against *E. faecalis* at high concentrations.

Antibacterial activities for compounds containing carboxylic acid functional groups at the periphery, i.e., **3**, **12**, and **15** as well as compound **6** bearing formyl groups, are shown in Figures 1b and 1e. In a moderate way, all of these molecules were active against both bacteria, although the activity was clearly higher for *E. coli*. These results are in marked contrast to those in a previous report, in which it was found that compounds **3**, **12**, and **15** did not inhibit the growth of *E. coli*

bacteria.[15] The number and nature of the peripheral groups are important for antibacterial activity, but the geometry, spatial disposition, and polarity of the molecule also seem to be significant factors. In fact, the highest activity was observed for compound **3**, which contains three carboxylic acid groups in a rigid C_{3v} geometry, followed by compound **12** with only two groups in a linear distribution, and finally **15** with four groups in a cross shape. Despite the fact that compound **6** does not have a carboxylic acid functional group, its behavior is similar to that of the polyacid compounds. A possible explanation for this is that *in situ* oxidation of the formyl groups to carboxylic groups occurs due to the bacterial benzaldehyde dehydrogenases, thus generating an active species. The lower activity of the prodrug **6** in comparison to **3** might be due either to a partial oxidation or a lower kinetic rate, which would lead to a less active compound within the bacteria.

Finally, the activities of polycationic compounds are depicted in Figures 1c and 1f. In contrast to the polyanionic derivatives, polycationic derivatives were more active against *E. faecalis* (Gram-positive) than *E. coli* (Gram-negative), probably due to a stronger interaction with the negatively charged peptidoglycan layer in the bacterial cell wall. As expected, the presence of cationic groups at the periphery is required for antibacterial activity. Thus, while compound **2** exhibited negligible activity against *E. faecalis* (Figure 1d), compound **7** was active as it corresponds to aliphatic amines that can be partially protonated at physiological pH. Once again, the molecule bearing three quaternary ammonium groups in a C_{3v} geometry, compound **5**, was the most active. Meanwhile, compounds **13** and **16** displayed a similar activity despite the different numbers of trimethylammonium groups, i.e., two and four, respectively. Once more, the geometry and spatial disposition of the charged groups seem to play an important role. Many of the properties and applications of polystilbenes depend on self-assembly processes in which they form complex supramolecular structures. The geometry of the molecule strongly influences the nature of the aggregates, a factor that might be behind the higher antibacterial activity for the C_{3v} symmetry.[19]

As mentioned in the Introduction, the hydrophobicity of the structure is also a factor to be considered. In this context, the linear molecule **13** exhibited a lower activity than **14**, where two methyl groups have been substituted by two butyl chains to give a more hydrophobic compound. Nevertheless, this effect was not as noticeable between the molecules with a C_{3v} geometry (**5** and **8**). Some improvement was observed for *E. coli* on increasing the hydrophobicity in **8** but this was not the case for *E. faecalis*, where the less hydrophobic compound **5** was more active.

Since pyridines are among the most common aromatic rings present in bioactive molecules, in addition to the aminostyryl derivatives, pyridine (**10**) and pyridinium (**11**) derivatives were also evaluated. The pyridin-4-ylvinyl compound **10** displayed antibacterial activity against *E. faecalis* but not against *E. coli*, probably due to their basic nature (Figures 1a and 1d). This issue was confirmed because the fully positively charged structure **11** followed a similar trend (Figures 1c and 1f).

Susceptibility test

In an effort to gain a better understanding of the different behavior between polyanionic and polycationic compounds, we determined the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of acids **3** and **15** as well as trimethylammonium derivatives **5** and **16**. These compounds were tested against *E. coli* and *K. pneumoniae*, as Gram-negatives, and *E. faecalis* and *S. epidermidis*, as Gram-positives (Table 1). The antiseptic Cetrimide, which is a mixture of different quaternary ammonium salts including cetrimonium bromide, was also used for comparison purposes.

Table 1. MICs and MBCs (μg/mL) of acids **3** and **15** and trimethylammonium derivatives **5** and **16**.

Compd	Gram-negative				Gram-positive			
	Escherichia coli		*Klebsiella pneumoniae*		*Enterococcus faecalis*		*Staphylococcus epidermidis*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
3	512	> 2048	512	> 2048	256	> 2048	256	> 2048
15	128	1024	512	> 2048	512	> 2048	512	> 2048
5	128	128	256	256	128	128	128	128
16	512	512	1024	1024	512	1024	256	256
Cetrimide	29,7	116	29,3	29	12,1	29	1,8	3,6

These results confirmed those previously obtained in the CFU experiments. Polyanionic stilbenes are better antibacterial agents for *E. coli* and *K. pneumoniae* (Gram-negative), while polycationic derivatives show higher activities for *E. faecalis* and *S. epidermidis* (Gram-positive). The spatial disposition of the active groups plays a key role and a C_{3v} symmetry seems to be advantageous. Additionally, compounds bearing carboxylic acid groups behave as bacteriostatic agents whereas compounds bearing quaternary ammonium groups behave as bactericidal agents. The molecules tested are less potent than cetrimide, showing weak antibacterial activity, with MIC no less than 128 μg/mL. Only compound **5** is comparable in bactericidal activity against *E. coli* (MBC of 128 μg/mL vs 116 μg/mL).[20] As mentioned above, it has been reported that acid derivatives **3** and **15** have bacteriostatic activity for Gram-positive bacteria (MIC value of **3** against *S. epidermidis*: 4 μg/mL),[15] but negligible activity for Gram-negative bacteria. Beyond a different protocol for the preparation of these compounds that, in our case, avoids the use of metal catalysts, we currently do not have a satisfactory explanation for these distinct results.

Internalization experiments

PPV derivatives emit blue fluorescence. Taking advantage of this property, the internalization of the molecules was quantified by fluorometry. The fluorescence intensity ratio

before sonication between the cells and the supernatant is represented in Figure 2a. The relative intensity of the bars can

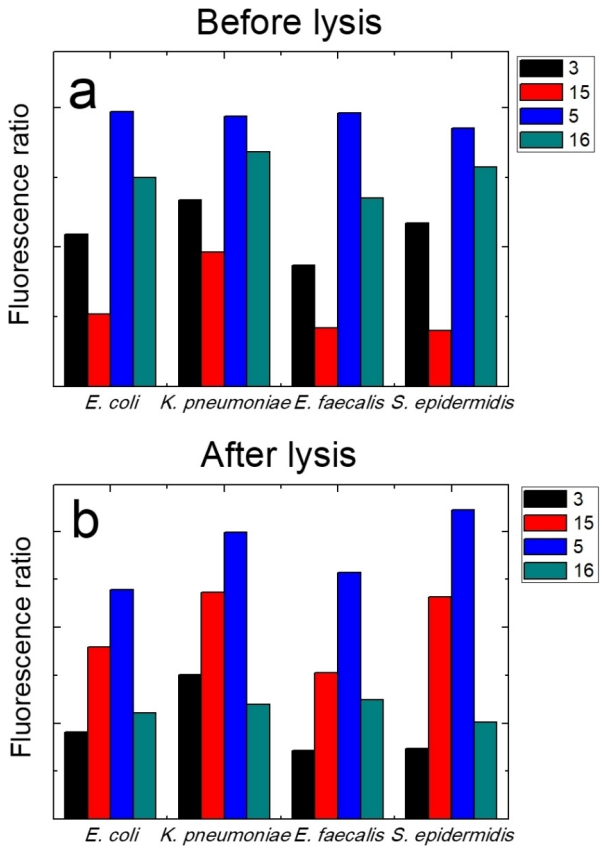


Figure 2. Internalization experiments with four different bacterial strains. Fluorescence intensity ratio between cell and supernatant, before (a) and after (b) sonication is represented for acids 3 and 15, and trimethylammonium derivatives 5 and 16.

be correlated with the amount of compound that interacts with the bacteria. Although changes in the fluorescence properties of the molecules due to aggregation with biological molecules cannot be ruled out, comparison between them rules out this issue and higher bars signify greater interaction with the bacteria. In all cases, the greater interaction is obtained for compounds **5** and **16**, which contain quaternary ammonium functional groups. Furthermore, and as discussed previously, molecules with a C_{3v} symmetry (**3** and **5**) lead to better results than the corresponding counterparts **15** and **16**.

The release of the compounds after inducing lysis of the bacterial walls by sonication is represented in Figure 2b. Compound **15**, which showed the lowest internalization, retains the interaction with the biomolecules and the cell remains. In contrast, the concentrations of compounds **3** and **16**, with higher internalization, are higher in the supernatant, which indicates a release of the molecules from the cell remains. The behavior of compound **5** is particularly interesting as it shows the best internalization but remains strongly attached to the biomolecules of the cell. The electrostatic interactions between the positive charges of compound **5** and the negatively charged bacterial cellular membrane, which results in the permeation and ultimately the death of the cells, seems to be very strong.

The internalization of the compounds was confirmed by fluorescence microscopy after cellular uptake (Figure 3). An *E.*

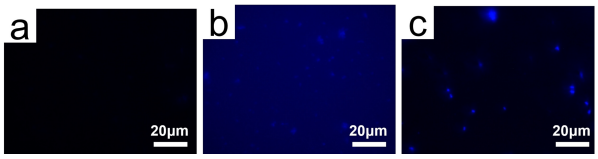


Figure 3. Fluorescence microscopy images for *E. faecalis* after 1 h of incubation with (a) phosphate-buffered saline control, (b) compound **3**, and (c) compound **5**. Scales correspond to a magnification of 40×.

faecalis cell culture did not show fluorescence after incubation only with phosphate-buffered saline (PBS) as control. Nevertheless, after incubation with compound **5**, a strong blue fluorescence was observed from inside the bacteria cells (Figure 3c). The lower internalization of compound **3** resulted in a distribution through the PBS media that consequently led to a blue background (Figure 3b).

We also investigated the cytotoxicity of selected compounds **3**–**7** and **15** against COS-1 and VERO cells, at similar concentrations to those used in the antibacterial activity experiments (Figure S1 in the Supporting Information). Most of the compounds showed cytotoxicity comparable to that of DMSO, the solvent used to dissolve the samples. Only compound **15** clearly affected the growth of eukaryotic cells. More specifically, COS-1 cells were affected by a concentration of 100 mM and VERO cells by a concentration of 10 mM. It is worth highlighting that the cytotoxicity of compound **7** was lower than that of DMSO. Although an appropriate explanation would require further experiments, we recently demonstrated that this compound shows a high tendency to aggregate and form micelles in water. It is possible in this case that an encapsulation effect over the DMSO occurs and this would explain the protective behavior.[19]

Conclusions

A large family of PPV derivatives with different geometries and peripheral functional groups was synthesized following a HWE protocol that avoided the use of metal catalysts. In contrast to previously reported results, our investigation revealed that compounds with carboxylic acid or quaternary ammonium functional groups had weak antibacterial activity against *E. coli* and *K. pneumoniae* (Gram-negative) and *E. faecalis* and *S. epidermidis* (Gram-positive) with MIC no less than 128 μg/mL. Polyanionic compounds behaved as bacteriostatic agents and were more active against *E. coli* and *K. pneumoniae* (Gram-negative), whereas polycationic compounds behaved as bactericidal agents and were more active against *E. faecalis* and *S. epidermidis* (Gram-positive). Not only the number and nature of

the peripheral groups, but also the geometry, spatial disposition, and polarity of the molecules were significant factors for the antibacterial activity. The spatial disposition of the active groups plays a key role and the C_{3v} geometry seems to be advantageous. Additionally, compounds that contain quaternary ammonium functional groups are internalized better into the bacteria. On the other hand, most of the compounds showed cytotoxicity comparable to that observed for the solvent used in this study (DMSO).

Although the compounds studied in this work were weakly active, stilbenes have recently emerged as a new class of poorly understood antibacterial compounds. The development of this type of structures as antibacterial agents might provide considerable benefits due to a novel mode of action, thus reducing the likelihood of cross-resistance.[21]

Supporting Information Summary

Experimental section, general procedures and description of the techniques used. Synthetic procedures and full characterization for compounds **5**, **8**, **11**, **13**, **14**, **16**, and **20**. Cytotoxicity activity against VERO and COS-1 cells using the MTT cell survival assay. ^{1}H NMR and ^{13}C NMR spectra.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: antibacterial · biologically active compounds · internalization · poly(phenylene)vinylenes · structure-activity relationships

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