β-Nitrostyrene derivatives as potential antibacterial agents: A structure–property–activity relationship study

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Abstract—A multidisciplinary project was developed, combining the synthesis of a series of β-nitrostyrene derivatives and the determination of their physicochemical parameters (redox potentials, partition coefficients), to the evaluation of the corresponding antibacterial activity. A complete conformational analysis was also performed, in order to get relevant structural information. Subsequently, a structure–property–activity (SPAR) approach was applied, through linear regression analysis, aiming at obtaining a putative correlation between the physicochemical parameters of the compounds investigated and their antibacterial activity (both against standard strains and clinical isolates). The β-nitrostyrene compounds displayed a lower activity towards all the tested bacteria relative to the β-methyl-β-nitrostyrene analogues. This was observed particularly for the 3-hydroxy-4-methoxy-β-methyl-β-nitrostyrene (**IVb**) against the Gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium*). The SPAR results revealed the existence of a clear correlation between the redox potentials and the antibacterial activity of the series of β-nitrostyrene derivatives under study.
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1. Introduction

Over the last few years, compounds comprising a β-nitrostyrene moiety have been recognized to have a series of relevant biological activities. In fact, some β-nitrostyrene derivatives have recently been tested as pro-apoptotic anticancer agents and the β-nitrostyrene moiety was identified as the pharmacophore for this activity.[1] These compounds have also been described as highly potent and selective human telomerase inhibitors[2] as well as cytotoxic for human cancer cell lines.[3] Moreover, it was shown that they are able to downregulate the production of some interleukins, thus affecting the immune response in humans.[3] The nitrovinyl side chain attached to the aromatic ring was recognized to be an essential chemical feature in this type of compounds and a critical conformational pattern for their biological activity.[1,4]

Although the antifungic and antibacterial properties of this type of compounds have been studied since the 1940s, the work in this area during the last decade is rather scarce. In general, the nitrostyrene derivatives were found to be more effective against Gram-positive than Gram-negative bacteria.[5–7]

Drug discovery studies have been significantly intensified in recent years, aiming at the search for more effective antibacterial agents displaying activity against Gram-positive and Gram-negative bacteria resistant to the antimicrobials currently used.[8,9] The search for novel chemotherapeutic agents, as well as the attempt to modify and improve the currently available ones, has been a priority mainly as a consequence of the rapid spread of multidrug-resistant bacterial pathogens. The development of new antibacterial drugs is presently based on structure–activity relationship (SAR), structure–property–activity relationship (SPAR) and quantitative structure–activity relationship (QSAR) studies.[9,10]

The aim of the present work is to be aware of the significance of β-nitrostyrene as a putative pharmacophore for antibacterial activity. The antibacterial activity of a series of compounds was evaluated against several type strains and clinical isolates of different bacterial species pathogenic to humans. Simultaneously, relevant physicochemical parameters, such as redox potentials (E_p) and partition coefficients ($\log P$), were determined and/or calculated. A complete conformational analysis of the β-nitrostyrene derivatives under study was also performed through theoretical methods—ab initio molecular orbital calculations—gathering information of the utmost relevance for correlating structural features with the physicochemical properties and antimicrobial activity of this class of compounds.

The compounds studied are chemically related to the parent compound with slight modifications in their aromatic substitution pattern: β-nitrostyrene (**Ia**), 3,4-dihydroxy-β-nitrostyrene (**IIa**), 3,4-dimethoxy-β-nitrostyrene (**IIIa**), 3-hydroxy-4-methoxy-β-nitrostyrene (**IVa**), 4-hydroxy-3-methoxy-β-nitrostyrene (**Va**) and 3,4-methylenedioxy-β-nitrostyrene (**VIa**) (Table 1). In addition, another series of compounds was synthesized, displaying a methyl group in the β-carbon of the vinyl side chain, the aromatic part being kept unchanged. The following compounds were thus obtained: β-methyl-β-nitrostyrene (**Ib**), 3,4-dihydroxy-β-methyl-β-nitrostyrene (**IIb**), 3,4-dimethoxy-β-methyl-β-nitrostyrene (**IIIb**), 3-hydroxy-4-methoxy-β-methyl-β-nitrostyrene (**IVb**), 4-hydroxy-3-methoxy-β-methyl-β-nitrostyrene (**Vb**) and 3,4-methylenedioxy-β-methyl-β-nitrostyrene (**VIb**) (Table 1).

2. Results and discussion

2.1. Chemistry

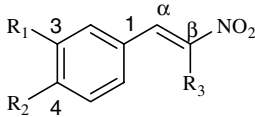β-Nitrostyrene and β-methyl-β-nitrostyrene derivatives were prepared by a nitroaldol condensation reaction (Henry reaction).[11] The compounds were synthesized by the reaction of the corresponding benzaldehydes—with the same aromatic substitution pattern of the desired product—and nitromethane or nitroethane, respectively, for β-nitrostyrenes and β-methyl-β-nitrostyrenes, using ammonium acetate as the catalyst (Scheme 1). These experimental conditions led to moderate/ high yields. The final products were identified by NMR (^{1}H NMR and ^{13}C NMR), FT-IR and MS-EI spectroscopic techniques. The purity of the target compounds was determined by two different HPLC systems (see Section 4). The structures of the synthesized compounds are depicted in Table 1.

Several β-nitrostyrene derivatives were synthesized in order to study the influence of different aromatic substitution patterns, which confer diverse electronic environments, in the antibacterial activity. In this kind of systems, the nitro group is relatively distant from the aromatic ring and conjugated with an ethylenic double bond, thus allowing long-distance transmission of electronic effects.[12] One methyl group was also added to the β-carbon of the double bond, to verify the influence of conformational and steric parameters on the redox and antibacterial profiles of this chemical family.

2.2. Conformational analysis

In order to establish the effect of the structural preferences of β-nitrostyrene derivatives on their biological activity, a complete conformational analysis was carried out, through theoretical (ab initio) methods. The effects of three structural parameters on the overall stability of these molecules were investigated: (i) relative orientation of the hydroxy and/or methoxy group(s) in the aromatic ring; (ii) orientation of the benzene and the nitro group relative to the double bond, $C_\alpha = C_\beta$, defining either a *Z* or an *E* configuration; (iii) position of the NO_2 and

Table 1. β-Nitrostyrene and β-methyl-β-nitrostyrene derivatives synthesized in the present work

Compound	R_1	R_2	R_3
Ia	H	H	H
Ib	H	H	CH_3
IIa	OH	OH	H
IIb	OH	OH	CH_3
IIIa	OCH_3	OCH_3	H
IIIb	OCH_3	OCH_3	CH_3
IVa	OH	OCH_3	H
IVb	OH	OCH_3	CH_3
Va	OCH_3	OH	H
Vb	OCH_3	OH	CH_3
VIa	–OCH_2O–		H
VIb	–OCH_2O–		CH_3
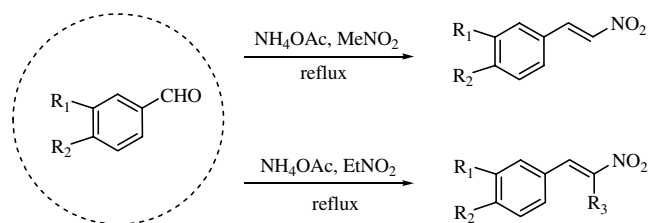
Scheme 1.

methyl group (in β-methyl-β-nitrostyrenes) relative to the ring. For all the geometries obtained calculation of the harmonic vibrational frequencies was also performed, allowing to determine which ones were real energy minima in the potential energy surface.

Since the more substituted β-nitrostyrene derivatives display a higher degree of freedom, several low energy conformers were calculated for them. Two stable geometries were obtained for compounds **Ia** and **Ib** (only one of them being populated at room temperature), 4 conformers were found for **VIa** and **VIb**, 8 were determined for **Va**, 10 for **IIb**, 11 for **IVb** and 12 for **IIa**, **IIIa**, **IIIb**, **IVa** and **Vb** (two of these having significant populations at room temperature). Figure 1 comprises the most stable structures calculated for each of the compounds presently studied.

The conformational behaviour of these phenolic compounds is mainly determined by electronic effects, as well as by the possibility of occurrence of (C)H···O intramolecular hydrogen bonds, which are medium strength, directional interactions (with a preference for linearity) known to be relevant for the overall stability of this kind of systems.[13] The most stable calculated structures were found to display a planar or quasi-planar geometry and an *E* conformation, which corresponds to a more effective π-electron delocalization, as well as to a minimization of repulsive effects, relative to the *Z* conformers. In fact, even for those compounds where two conformers exist at room temperature, they differ only in the value of the C_1–C_α torsion angle: either 0° or 180°. By comparing the β-nitrostyrenes with the β-methyl-β-nitrostyrenes, it was verified that the almost perfect planarity detected for the former is lost upon methyl substitution in the double bond C_β, the aromatic ring being tilted relative to the carbon side chain, the

angle around C_1–C_α changing from 0° to 28°. Concerning the relative orientation of the ring substituent groups, it was found that it is identical whenever two OHs or one OH and one OMe are present, in order to allow the formation of intramolecular hydrogen bonds. When there are two OMe substituents, an opposite orientation is favoured (Fig. 1), in view of the minimization of OCH_3···H_3CO steric repulsions.

2.3. Antibacterial activity

The antibacterial properties of the β-nitrostyrene derivatives investigated were measured against selected type strains of Gram-positive (*Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212) and Gram-negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) bacteria. *Salmonella typhimurium* FFUP1 and *Acinetobacter baumannii* FFUP10 are clinical isolates susceptible to all antibiotics, according to CLSI (formerly NCCLS) recommendations.[14]

Table 2 comprises the in vitro results of antibacterial activity for the β-nitrostyrenes under study, expressed as minimum inhibitory concentrations (MICs). The lowest antibacterial activity was observed for the β-nitrostyrene series, with MICs ranging from 128 to ⩾512 mg/L. In general, from the activity measured for the β-methyl-β-nitrostyrenes it is possible to conclude that the inclusion of a methyl group in the molecule's side chain leads to an increase of the inhibitory effect—MICs are between 16 and 256 mg/L. This activity enhancement due to the methyl substitution was found to be more pronounced for Gram-positive bacteria (*S. aureus* and *Enterococcus* spp.)—MICs ranging from 16 to 32 mg/L. Among the β-methyl-β-nitrostyrenes, compound **IVb** displayed the highest activity against Gram-positive
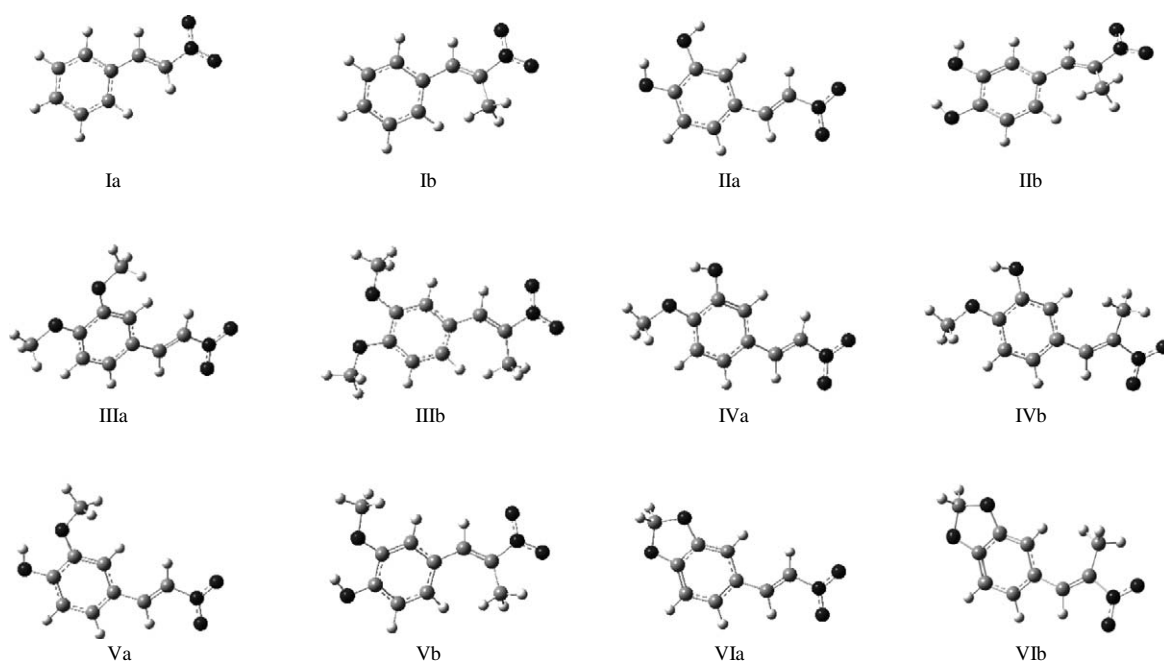

Figure 1. Schematic representation of the most stable geometries calculated (B3LYP/6-31G∗∗) for the β-nitrostyrene derivatives under study.

Table 2. In vitro antibacterial activity of the β-nitrostyrene derivatives against several bacterial strains

Bacterial strain	Minimum inhibitory concentration, MIC (mg/L)											
	Ia	Ib	IIa	IIb	IIIa	IIIb	IVa	IVb	Va	Vb	VIa	VIb
Escherichia coli ATCC 25922	256	128	256	64	⩾512	256	512	128	512	256	128	256
Salmonella typhimurium FFUP1	256	128	256	64	⩾512	256	512	128	512	256	128	256
Pseudomonas aeruginosa ATCC 27853	256	256	256	256	512	256	256	256	512	256	128	256
Acinetobacter baumannii FFUP10	256	128	256	64	512	256	256	128	256	256	128	256
Enterococcus faecalis ATCC 29212	256	64	256	64	512	32	256	16	256	32	128	32
Staphylococcus aureus ATCC 29213	128	64	256	64	512	32	256	16	256	32	128	32

bacteria. Interestingly, compound **IIb** showed the highest activity against all the tested Gram-negative bacteria (MIC of 64 mg/L), except for *P. aeruginosa*.

The antibacterial activity of the compounds under study was also evaluated against clinical isolates with different resistance profiles to several antimicrobial agents, including sulfonamide-resistant *Salmonella* and ampicillin, aminoglycoside, tetracycline, macrolide and glycopeptide-resistant *Enterococcus* (Table 3). The results obtained corroborate previously reported patterns observed for these type strains. It may then be concluded that the activity of β-nitrostyrene derivatives against antibiotic-resistant *Salmonella* and *Enterococcus* is not affected by their resistance mechanisms, which are capable of inactivating several antimicrobial agents.

Based on the presently obtained results, a series of other compounds based on the same structural templates were designed, aiming at the screening of their antibacterial activity. These derivatives display different aromatic ring substituents (changing their number and polarity degree), thus promoting changes in their electronic characteristics and lipophilicity. Neither of these chemical modifications led to an increase of the activity (data not shown). In order to check the relevance of the nitro group in the antimicrobial activity of this kind of systems, the amine analogues of some of the β-nitrostyrene and β-methyl-β-nitrostyrenes presently studied were synthesized and tested. In addition, various saturated analogues were also obtained, in order to check the relevance of the ethylenic double bond on the antibacterial properties. In both cases, no significant activity was observed (data not shown). These results reinforce the mechanistic hypothesis proposed in the present work.

2.4. Electrochemical and partition coefficient measurements

As the antibacterial activity and the mechanism of action of the nitro compounds, commonly used in therapy, are largely a function of their redox potential, it was found important to study the physicochemical properties of the synthesized β-nitrostyrene derivatives (Table 1).[15,16]

Thus, the standard redox potentials were determined at physiological pH 7.3, using differential pulse polarography, as well as cyclic and square wave voltammetry, with a glassy carbon working electrode.

A well-defined cathodic peak can be observed at physiological pH for all the compounds studied using differential pulse polarography (Fig. 2). This wave is due to a four-electron, four-proton reduction of the nitro group yielding the corresponding hydroxylamine derivative, according to the following equation:[12,17]

$$R_1R_2\text{–Ph–CH}\text{=}CR_3\text{–NO}_2 + 4e^- + 4H^+$$
$$\rightarrow R_1R_2\text{–Ph–CH}\text{=}CR_3\text{–NHOH} + H_2O$$
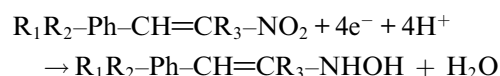
Cyclic voltammograms were recorded at different sweep rates. All compounds were found to produce a well-defined and irreversible peak at physiological pH (Fig. 3). This peak corresponds to the above-described wave observed by differential pulse polarography. From the dependence of the peak current on the sweep rate, it was possible to conclude that the process follows a mixed diffusion–adsorption control, since the experimentally obtained slopes ($d \log I_p / d \log v$) display values between 0.5 and 1.[18]

Table 3. In vitro antibacterial activity of the β-nitrostyrene derivatives against several clinical isolates

| Clinical isolates	Minimum inhibitory concentration, MIC (mg/L)											
	Ia	Ib	IIa	IIb	IIIa	IIIb	IVa	IVb	Va	Vb	VIa	VIb
Salmonella typhimurium FFUP40	256	128	256	64	⩾512	256	512	128	512	256	128	256
Salmonella enteritidis 09 FF-D	256	128	256	64	⩾512	256	512	128	512	256	128	256
Enterococcus faecalis H 318	256	64	256	64	512	32	256	16	256	32	128	32
Enterococcus faecalis H 318	256	64	256	64	512	32	256	16	256	32	128	32
Enterococcus faecalis H 181A	256	64	256	64	512	32	256	16	256	32	128	32
Enterococcus faecalis 536540	256	64	256	64	512	32	256	16	256	32	128	32
Enterococcus faecium Med150	256	64	256	64	512	32	256	16	256	32	128	32
Enterococcus faecium Med100 F3	128	64	256	64	512	32	256	16	256	32	128	32
Enterococcus faecium KE77F2	128	64	256	64	512	32	256	32	256	32	128	32
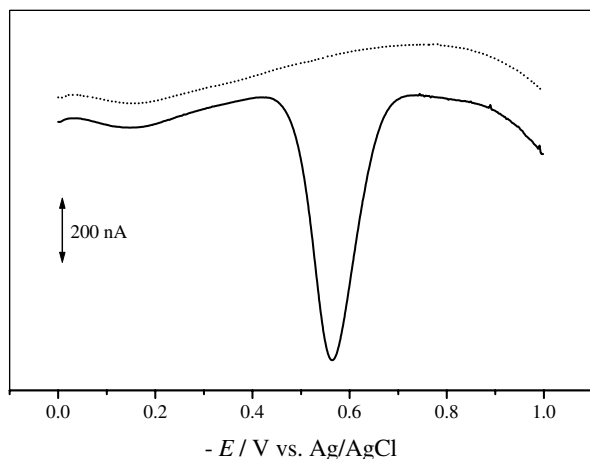
Figure 2. Differential pulse voltammogram for reduction of **VIb**, in pH 7.3 supporting electrolyte, in the absence (······) and presence (—) of 100 μM **VIb**. Scan rate 5 mV s^{-1}.

The results obtained are in agreement with the data reported for other β-nitrostyrene derivatives.[12,17] The shifts detected in the standard redox potential values (Table 4) for the different β-nitrostyrene derivatives are probably due to two distinct substituent effects: (i) substitution of H$_\beta$ by a methyl group; (ii) different *meta* and *para* substitutions on the aromatic ring.

The redox potential values presented in Table 4 evidence that in all cases the β-methyl-β-nitrostyrene derivatives are reduced at more negative potentials than their corresponding β-nitrostyrenes. Hence, the reduction potential seems to be highly sensitive to a methyl substitution in the β-position. A shift of several tens of millivolts towards more negative potentials is observed when going from β-nitrostyrene to β-methyl-β-nitrostyrene derivatives, which can be attributed to a lack of planarity due to β-methyl substitution.[12] In fact, this distortion of the coplanar arrangement in β-nitrostyrenes leads to a decrease of the resonance interaction between the electroactive nitro group and the aromatic ring, thus causing the observed shift towards negative potentials.[12] This conformational change upon β-methyl substitution is corroborated, in the present study, by the theoretical results that yielded a deviation of about 28° between the planar β-nitrostyrenes and the corresponding β-methyl-β-nitrostyrenes. Also, when such kind of distortion takes place an accumulation of charge on the C$_\alpha$ atom was verified—for example, from +0.050 to −0.008 for compounds **Ia** and **Ib**, respectively—which is probably due to the less effective electronic delocalization to the nitro group in the latter.

The substitution on the aromatic ring in *meta* and especially in *para* position also has a marked effect on the redox potentials. In fact, an increase in the electron-donor properties of the *para* substituent significantly increases the magnitude of the reduction potential, that is, reduction is unlikely to occur (Table 4). As expected, this effect is more pronounced in the β-nitrostyrenes than in the β-methyl-β-nitrostyrenes, due to the decrease in the conjugation coupled with the increase in the C$_1$–C$_\alpha$ tor-

sion angle,[12] also predicted by the ab initio calculations. Although the increase in the electron-donor properties of the *meta* substituent influences the reduction potential of the compounds, this effect is less pronounced than the one resulting from a *para*-substitution. Moreover, it is more significant for β-nitrostyrenes than for the β-methyl-β-nitrostyrenes, as expected.

In view of better understanding of the overall properties of the compounds, the lipophilicity, expressed as the octanol–water partition coefficient and herein called log P, was calculated according to Parham et al.[19] Log P between 1.5 and 2.5 were obtained (Table 4).

2.5. Structure–property–activity relationship (SPAR)

In this work, two types of bioisosteric replacements (modifications in the substitution pattern of both the unsaturated side chain and the aromatic ring) were performed in the parent compound—β-nitrostyrene—and the physicochemical properties, as well as antibacterial activity of the series, were examined in order to perform structure–property–activity relationship studies. The chemical structure of each compound was analyzed through three types of parameters: steric interference, electronic distribution and hydrophobicity, since these appear to significantly influence other biological activities.[20,21]

In these SPAR studies, the antimicrobial activities obtained for the nitrostyrene compounds against the *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 strains were represented as log 1/MIC. These values were then plotted against E_p and log P (Figs. 4 and 5, respectively). These results strongly suggest a correlation between antibacterial activity and redox potential. In turn, no correlation was found between antibacterial activity and lipophilicity. A statistical treatment of the redox potential and partition coefficient results was performed, in view of achieving a better understanding of these findings. An analysis of variance test was carried out, in order to compare the different E_p and log P values for the β-nitrostyrenes and the β-methyl-β-nitrostyrenes. Since the experimental values of F (between 1.1 and 2.6) were always lower than the theoretical value ($F_{5,5} = 7.1$; $p = 0.05$), it was concluded that there were no significant differences between the variances obtained for those two series of compounds, for neither of the two parameters studied. A comparison of the experimental mean values for the two series of nitrostyrene derivatives was also performed, using the *t* test. Comparison of the redox potentials of β-nitrostyrenes and β-methyl-β-nitrostyrenes yielded *t* test values ($t = 4.2$) higher than the theoretical one ($t = 2.6$; $p = 0.05$). There was no statistical evidence linking the presence of a β-methyl group to a significant effect on the partition coefficient value. Therefore, it may be concluded that the redox potential might be an important parameter ruling the antibacterial activity of β-nitrostyrene derivatives. In fact, it was verified that the more negative the E_p the stronger the antibacterial promoting effect.

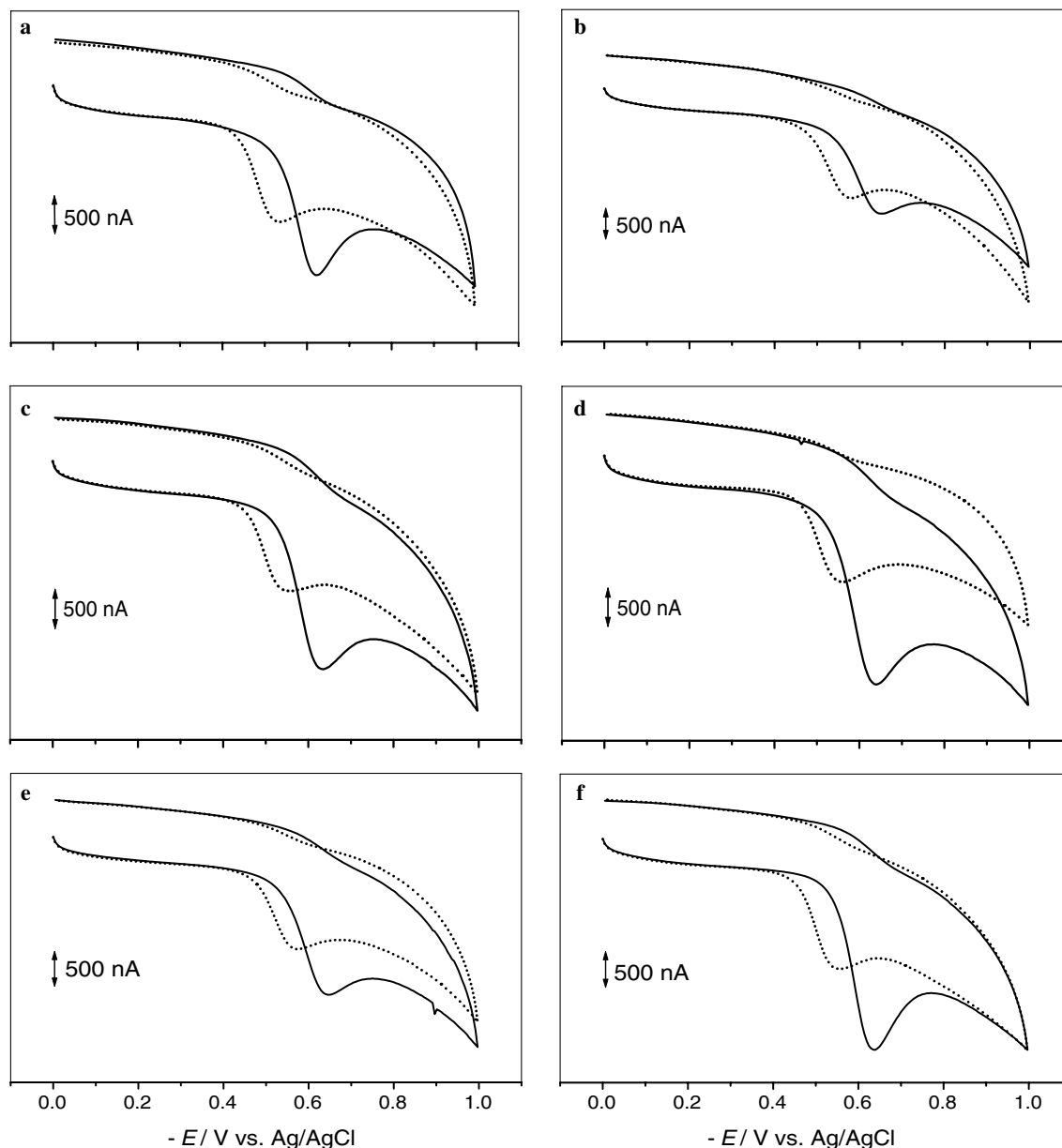In the light of the results presently obtained, it may be suggested that the mode of action of this kind of β-nitro-

Figure 3. Cyclic voltammograms for several β-nitrostyrene ($\cdots\cdots$) and β-methyl-β-nitrostyrene derivatives (—), in 100 μM solution, at physiological pH. (a) **Ia**, **Ib**; (b) **IIa**, **IIb**; (c) **IIIa**, **IIIb**; (d) **IVa**, **IVb**; (e) **Va**, **Vb**; (f) **VIa**, **VIb**. Scan rate 75 mV s^{-1}.

styrene derivatives is closely related to their electrophilicity, thus being a consequence of their ability to accept electrons, which enables the reduction of the nitro group. The antimicrobial action of nitro compounds is generally believed to result from a 4-electron reduction of the nitro group, carried on by bacterial nitroreductases, to short-lived redox active intermediates, like hydroxylamine adducts, known to be biologically active.[15,16,22,23]

A possible explanation for the higher antimicrobial activity verified for compound **IIb** (one of the β-methyl-β-nitrostyrene derivatives) against some of the Gram-negative bacteria, including members of the *Enterobacteriaceae* family, may be its rather high hydrophilicity (calculated log P value of 1.51). In fact, these bacterial strains are known to uptake hydrophilic drugs

(<650 Da) through porine channels located on their outer membranes.[24] As shown in Figure 1 and Table 4, the conformational and chemical characteristics of compound **IIb** are in complete accordance with these requisites. Nevertheless, further work is needed in order to prove this hypothesis.

3. Conclusions

The increasing resistance to available antibiotics against clinically important bacteria represents a serious problem in public health which requires the development of new therapeutic alternatives.

The results on the antibacterial activity of β-nitrostyrene derivatives, namely β-methyl-β-nitrostyrenes, towards

Table 4. Redox potential and partition coefficient values for the β-nitrostyrene and β-methyl-β-nitrostyrene derivatives studied in this work

Compound	E_p (V)	$\Delta E_p{}^a$ (mV)	Log P
Ia	−0.455	92	2.28
Ib	−0.547		2.40
IIa	−0.497	77	1.46
IIb	−0.574		1.51
IIIa	−0.471	87	2.34
IIIb	−0.558		2.50
IVa	−0.477	87	1.78
IVb	−0.564		2.11
Va	−0.492	77	1.79
Vb	−0.569		2.15
VIa	−0.473	92	1.85
VIb	−0.565		2.07

^{a} E_p difference between the β-nitrostyrenes and the corresponding β-methyl-β-nitrostyrenes.

standard strains suggest that these could constitute a group of promising agents for the most clinically relevant Gram-positive bacteria, *S. aureus* and *Enterococcus* spp. This propensity is in accordance with previously obtained results with similar compounds.[5–7] The high selectivity exhibited by these systems is unequivocally related to their structural features.

Even though further research must be conducted in order to study other physicochemical parameters, the redox potential value seems to be a crucial parameter for the displayed antimicrobial activity. It was verified that the conformational behaviour of these molecules is mainly determined by the stabilizing effect of π-electron delocalization, which is reflected in the most stable geometries calculated for the β-nitrostyrene derivatives, and is in accordance with the electrochemical data.

The results gathered along this work provide a rational approach that will hopefully allow the design of selective and effective antibacterial agents. The β-nitrostyrene family seems to represent a novel class of antibacterial agents possessing a surprisingly small pharmacophore. Changes in the ethylenic side chain of the β-nitrostyrenes must be performed, namely in the length and volume of its substituents, in order to check its influence in the activity versus electrochemical properties.

4. Experimental

4.1. Chemicals

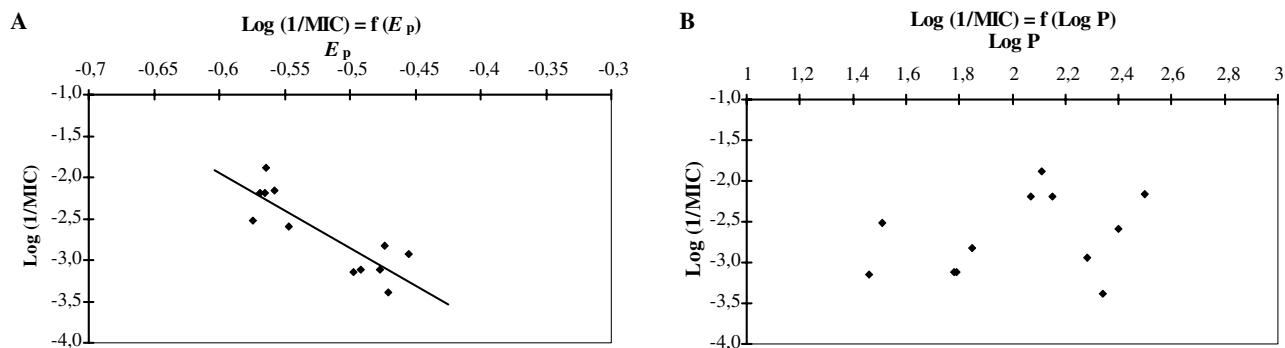β-Nitrostyrene (**Ia**), ammonium acetate, nitroethane and nitromethane were purchased from Sigma–Aldrich

Figure 4. Regression plots of log(1/MIC) towards *Staphylococcus aureus* ATCC 29213, for the β-nitrostyrene derivatives tested: (A) standard redox potential values at physiological pH; (B) log P values.
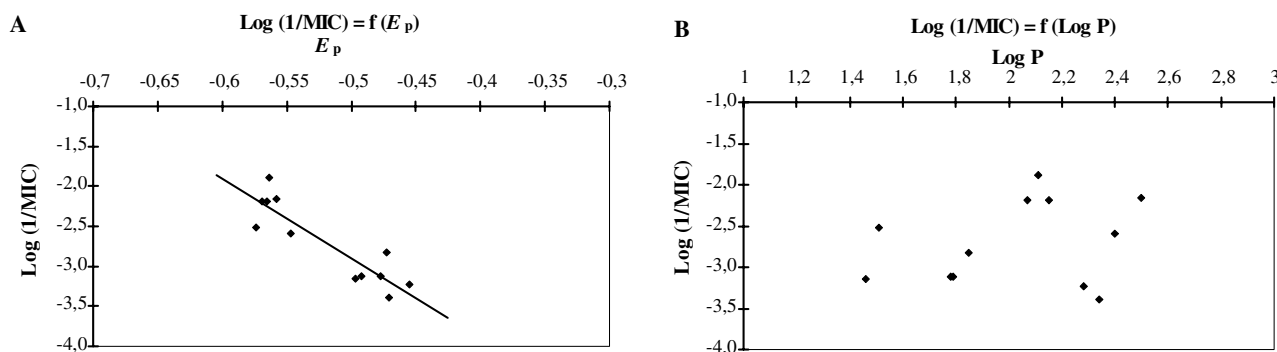
Figure 5. Regression plots of log(1/MIC) towards *Enterococcus faecalis* ATCC 29212, for the β-nitrostyrene derivatives tested: (A) standard redox potential values at physiological pH; (B) log P values.

Química SA. (Sintra, Portugal). All other reagents and solvents (pro analysis grade) were acquired from Merck (Lisbon, Portugal). Deionized water (conductivity <0.1 μS cm^{-1}) was used in all experiments. Trypticase soy broth was obtained from Oxoid (Basingstone, UK) and Mueller–Hinton II from Biomérieux (Marcy L'Etoile, France). All reagents were used without further purification.

4.2. Analysis

^{1}H and ^{13}C NMR data were acquired, at room temperature, on a Brüker AMX 300 spectrometer operating at 300.13 and 75.47 MHz, respectively. Dimethylsulfoxide-d_6 was used as a solvent; chemical shifts are expressed in δ (ppm) values, relative to tetramethylsilane (TMS) (as internal reference); coupling constants (J) are given in Hz. Assignments were also made from Distortionless Enhancement by Polarization Transfer (DEPT) experiments (underlined values). Electron impact mass spectra (EI-MS) were obtained on a VG AutoSpec instrument; data are reported as m/z (% of relative intensity for the most important fragments). Melting points were measured on a Köfler microscope (Reichert Thermovar) and are uncorrected.

The purity of the final products (higher than 98%) was verified using two different high-performance liquid chromatography (HPLC) systems, one equipped with UV and the other with an electrochemical detector. HPLC-UV chromatograms were obtained in a Jasco instrument (pumps model 880-PU and solvent mixing model 880-30, Tokyo, Japan), equipped with a Nucleosil RP-18 column (250 mm × 4.6 mm, 5 μm, Macherey-Nagel, Düren, Germany) and UV detection at 214 nm (Jasco model 875-UV). The mobile phase was acetonitrile/water, at a flow rate of 1 mL/min. A linear gradient of acetonitrile from 10% to 100% during a period of 35 min was used. The chromatographic data were processed in a Compaq computer, fitted with CSW 1.7 software (DataApex, Czech Republic).

A Waters 2690 Alliance system equipped with a CONCORDE Electrochemical Detector (Waters Corporation, Milford, USA). The electrochemical cell was a VT-03 flow cell (Antec Leyden, Zoeterwoude, Netherlands) with a confined wall-jet design, in a three-electrode configuration: a 2 mm diameter glassy carbon working electrode, an in situ Ag/AgCl reference electrode and a stainless steel auxiliary electrode. The electrochemical detector was operated in reductive amperometric mode with the working potentials between −450 and −600 mV. The HPLC separation was carried out in a reverse-phase LC-18-S Supelcosil analytical column (150 mm × 4.6 mm, 5 μm, Supelco, Bellefonte, USA). A 50 mM phosphate buffer of pH 6.8 with 6% MeOH was used as mobile phase, in isocratic mode at 1 mL/min flow rate. Chromatograms were acquired in a Millenium 32 Chromatography Manager (Waters Corporation, Milford, USA).

4.3. General synthetic procedure and structural elucidation

The method of Parker et al.,[25] slightly modified, was used for the synthesis of the compounds. The benzaldehyde with the same aromatic substitution pattern of the desired final product (25.0 mmol) and ammonium acetate (6.5 mmol) were dissolved in nitromethane (50 mL)—for the β-nitrostyrene derivatives (**IIIa**, **IVa**, **Va** and **VIa**)—or in nitroethane (50 mL)—for the β-methyl-β-nitrostyrene derivatives (compounds **IIIb**, **IVb** and **Vb**), and refluxed for 6 h. The reactions were followed by thin-layer chromatography (TLC). After cooling the reaction mixtures to room temperature, the solvents were partially evaporated, diluted with diethyl ether and washed twice with water. The organic layer was dried over anhydrous magnesium sulfate, filtered and concentrated. The remaining residues were recrystallized from diethyl ether/petroleum ether (40–60 °C) or from methanol/water.

The synthesized compounds were identified by both NMR and EI-MS. In the present study, the nitro compounds were systematically characterized, although some of them had been previously reported.[26–30] The syntheses of the remaining compounds used in this study (**Ib**, **IIa**, **IIb** and **VIb**) are described elsewhere.[31,32]

4.3.1. 3,4-Dimethoxy-β-nitrostyrene (IIIa). Yield 77%; ^{1}H NMR δ: 3.82 (3H, s, 3-OCH_3), 3.83 (3H, s, 4-OCH_3), 7.06 (1H, d, $J = 8.4$, H(5)), 7.43 (1H, d, $J = 8.4$, H(6)), 7.49 (1H, s, H(2)), 8.07 (1H, d, $J = 13.4$, H(α)), 8.22 (1H, d, $J = 13.5$, H(β)); ^{13}C NMR δ: 55.8 (4-OCH_3), 55.9 (3-OCH_3), 111.2, 111.8 C(2) and C(5), 123.0 C(1), 125.9 C(6), 136.0 C(α), 140.1 C(β), 149.2 C(4), 152.7 C(3); EI-MS m/z (%): 209 (M$^{+\cdot}$, 100), 162 (53), 147 (19), 119 (15), 91 (11), 77 (11), 63 (7); mp 142–144 °C.

4.3.2. 3,4-Dimethoxy-β-methyl-β-nitrostyrene (IIIb). Yield 68%; ^{1}H NMR δ: 2.44 (3H, s, CH_3), 3.80 (3H, s, 3-OCH_3), 3.82 (3H, s, 4-OCH_3), 7.08 (1H, d, $J = 8.5$, H(5)), 7.22 (1H, s, H(2)), 7.23 (1H, d, $J = 8.5$, H(6)), 8.08 (1H, s, H(α)); ^{13}C NMR δ: 14.0 CH$_3$, 55.6 (4-OCH_3), 55.7 (3-OCH_3), 111.8, 113.9 C(2) and C(5), 124.3 C(6), 124.4 C(1), 133.8 C(α), 145.6 C(β), 148.7 C(4), 150.7 C(3); EI-MS m/z (%): 223 (M$^{+\cdot}$, 100), 176 (78), 165 (22), 161 (37), 146 (47), 131 (46), 119 (36), 103 (37), 91 (47), 77 (38), 65 (34); mp 71–72 °C.

4.3.3. 3-Hydroxy-4-methoxy-β-nitrostyrene (IVa). Yield 92%; ^{1}H NMR δ: 3.84 (3H, s, OCH_3), 7.02 (1H, d, $J = 8.4$, H(5)), 7.24 (1H, d, $J = 2.0$, H(2)), 7.32 (1H, dd, $J = 8.4$; 2.1, H(6)), 8.01 (2H, s, H(α), H(β)), 9.35 (1H, br s, OH); ^{13}C NMR δ: 55.8 (OCH_3), 112.0, 115.6 C(2) and C(5), 122.9 C(1), 123.6 C(6), 135.6 C(α), 139.9 C(β), 146.8 C(3), 151.6 C(4); EI-MS m/z (%): 195 (M$^{+\cdot}$, 100), 148 (53), 133 (44), 105 (25), 89 (31), 77 (25), 63 (14); mp 158–161 °C.

4.3.4. 3-Hydroxy-4-methoxy-β-methyl-β-nitrostyrene (IVb). Yield 97%; ^{1}H NMR δ: 2.40 (3H, s, CH_3), 3.83 (3H, s, OCH_3), 7.04 (1H, d, $J = 8.2$, H(5)), 7.06–7.10

(2H, m, H(2), H(6)), 7.98 (1H, s, H(α)), 9.39 (1H, br s, OH); ^{13}C NMR δ: 14.0 CH$_3$, 55.6 (OCH$_3$), 112.1, 116.9 C(2) and C(5), 123.5 C(6), 124.5 C(1), 133.7 C(α), 145.2 C(β), 146.6 C(3), 149.8 C (4); EI-MS *m/z* (%): 209 (M$^{+\cdot}$, 100), 162 (56), 147 (53), 131 (23), 119 (29), 103 (75), 91 (50), 83 (62), 77 (31), 65 (29); mp 79–81 °C.

4.3.5. 4-Hydroxy-3-methoxy-β-nitrostyrene (Va).
Yield 91%; ^{1}H NMR δ: 3.82 (3H, s, OCH_3), 6.84 (1H, d, $J = 8.2$, H(5)), 7.30 (1H, dd, $J = 8.2$; 1.9, H(6)), 7.47 (1H, d, $J = 1.8$, H(2)), 8.02 (1H, d, $J = 13.4$, H(α)), 8.16 (1H, d, $J = 13.4$, H(β)), 10.15 (1H, br s, OH); ^{13}C NMR δ: 55.8 (OCH$_3$), 112.2, 115.8 C(2) and C(5), 121.5 C(1), 126.0 C(6), 134.9 C(α), 140.3 C(β), 148.2 C(4), 151.5 C(3); EI-MS *m/z* (%): 195 (M$^{+\cdot}$, 100), 148 (77), 133 (46), 105 (28), 89 (38), 78 (28), 63 (19); mp 168–171 °C.

4.3.6. 4-Hydroxy-3-methoxy-β-methyl-β-nitrostyrene (Vb).
Yield 95%; ^{1}H NMR δ: 2.44 (3H, s, CH_3), 3.81 (3H, s, OCH_3), 6.89 (1H, d, $J = 8.2$, H(5)), 7.12 (1H, dd, $J = 8.2$; 1.9, H(6)), 7.20 (1H, d, $J = 1.9$, H(2)), 8.05 (1H, s, H(α)), 9.85 (1H, br s, OH); ^{13}C NMR δ: 14.0 CH$_3$, 55.7 (OCH$_3$), 114.8, 115.9 C(2) and C(5), 123.0 C(1), 124.8 C(6), 134.2 C(α), 144.7 C(β), 147.7 C(4), 149.2 C(3); EI-MS *m/z* (%): 209 (M$^{+\cdot}$, 100), 162 (81), 147 (53), 131 (33), 119 (30), 103 (84), 91 (56), 77 (36), 65 (32); mp 97–99 °C.

4.3.7. 3,4-Methylenedioxy-β-nitrostyrene (VIa).
Yield 91%; ^{1}H NMR δ: 6.09 (2H, s, CH_2), 7.00 (1H, d, $J = 8.0$, H(5)), 7.34 (1H, dd, $J = 8.0$; 1.7, H(6)), 7.46 (1H, d, $J = 1.6$, H(2)), 8.01 (1H, d, $J = 13.5$, H(α)), 8.07 (1H, d, $J = 13.5$, H(β)); ^{13}C NMR δ: 102.3 CH$_2$, 107.8, 109.2 C(2) and C(5), 124.6 C(1), 128.0 C(6), 136.4 C(α), 139.9 C(β), 148.5 C(4), 151.2 C(3); EI-MS *m/z* (%): 193 (M$^{+\cdot}$, 100), 146 (92), 117 (17), 89 (70), 71 (24), 63 (46); mp 164–165 °C.

4.4. Ab initio MO calculations

The ab initio calculations—full geometry optimization and calculation of the harmonic vibrational frequencies—were performed using the GAUSSIAN 98W program,[33] within the Density Functional Theory (DFT) approach, in order to properly account for the electron correlation effects. The widely employed hybrid method denoted by B3LYP,[34–39] which includes a mixture of HF and DFT exchange terms and the gradient-corrected correlation functional of Lee, Yang and Parr,[40,41] as proposed and parameterized by Becke,[42,43] was used, along with the double-zeta split valence basis set 6-31G**.[44,45]

Molecular geometries were fully optimized by the Berny algorithm, using redundant internal coordinates:[46] The bond lengths to within ca. 0.1 pm and the bond angles to within ca. 0.1°. The final root-mean-square (rms) gradients were always less than 3×10^{-4} hartree bohr^{-1} or hartree radian^{-1}. No geometrical constraints were imposed on the molecules under study. The ZPE scaling was employed for all minima in the potential energy surface.

4.5. Antibacterial activity

4.5.1. Test strains and medium.
The antibacterial activity, expressed as MIC values (minimum inhibitory concentration), was determined on both clinical isolates and bacterial strains obtained from the American Type Culture Collection (ATCC) and from the Microbiology Laboratory of Faculty of Pharmacy of University of Porto (FFUP). All bacterial strains were maintained as frozen stocks at −80 °C in protected vials. The stock cultures were propagated overnight in trypticase soy broth at 35 °C, before experimental use.

The Mueller–Hinton II culture medium, containing beef infusion 300 g/L, bio-Case 17.5 g/L, starch 1.5 g/L and agar 17 g/L, was used in the antibacterial assays.

4.5.2. Antibacterial assays.
The antibacterial activity of the compounds was evaluated through the agar dilution procedure outlined by the National Committee for Clinical Laboratory Standards (NCCLS).[14] Stock solutions of the β-nitrostyrene derivatives were prepared either in ethanol or water depending on the compounds solubility, and dilutions were carried out with sterilized distilled water. Different concentrations were obtained by 2-fold plate-dilution of a stock containing 1280 mg/L of test substance, ranging from 16 to 512 mg/L, prepared immediately before use and sterilized by filtration through a 0.2 μm membrane filter.

The inoculated plates were incubated overnight at 35 °C. All the experiments were conducted in triplicate. The lowest concentration of the compounds that prevented visible growth was considered to be the MIC value. The solvent was found to have no effect on the microorganisms, for all the concentrations tested (data not shown).

4.6. Electrochemical determinations

4.6.1. Preparation of solutions.
Ten millimole stock solutions of the β-nitrostyrene derivatives were prepared by dissolving an appropriate amount in ethanol. The polarographic working solutions were prepared, in the electrochemical cell, by diluting 100 μL of the stock solution in 10 mL of supporting electrolyte, in order to get a final concentration of 0.1 mM.

The pH 7.3 supporting electrolyte was prepared by diluting 6.2 mL of 0.2 M dipotassium hydrogen phosphate and 43.8 mL of 0.2 M potassium dihydrogen phosphate to 100 mL.

4.6.2. Voltammetric measurements.
Differential pulse polarograms, cyclic and square wave voltammograms were obtained in a PalmSens potentiostat/galvanostat (Palm Instruments, The Netherlands) and a one-compartment glass electrochemical cell. A 2 mm diameter glassy carbon working electrode (GCE), a platinum wire counterelectrode and an Ag/AgCl saturated KCl reference electrode were used. pH measurements were carried out in a Metrohm E520 pH-meter with a glass electrode (Metrohm, Switzerland).

All measurements were performed at room temperature and purified nitrogen was used for oxygen displacement.

4.7. Log *P* calculations

Estimated partition coefficients (log *P*) were calculated according to the procedure of Parham et al.[19] This method uses an artificial neural network (ANN) analysis based on several property descriptors, which was trained with a large data set including more than 2000 compounds. Property descriptors encompass *Electrotopological state* (E-state) structure descriptors that encode the electron accessibility for each atom (i.e., the potential for non-covalent intermolecular interaction), *Molecular connectivity χ* indices which encode skeletal variation information and K shape indices describing the overall shape of the molecule.

4.8. Statistical analysis

The reported results are means of at least three independent determinations, with a divergence of not more than one MIC value.

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