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Article

A Study of Fluorinated β-Nitrostyrenes as Antimicrobial Agents

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Abstract: The effect of variously fluorine-substituted β-methyl-β-nitrostyrenes on their antimicrobial activity was investigated. Their efficacy was determined by minimum inhibition concentration (MIC) in cultures of Gram positive and Gram negative bacteria and a fungus. Highest activity against the Gram negative bacterium, *E.coli*, was achieved with 4-fluorine-aryl substituted β-methyl-β-nitrostyrenes, while most compounds gave excellent results against gram positive bacteria. Importantly, the addition of the β-methyl group profoundly enhanced the antibacterial activity of the compounds tested. The comparative K_D values for the most potent compounds against *E.coli* were much lower than those required for the gram positive and fungus counterparts. This investigation illustrated that fluorine substituted nitropropenylarenes have enhanced antimicrobial activity suitable for antibiotic applications.

Keywords: antimicrobial agents; Henry reaction; fluoro-β-methyl-β-nitrostyrenes; MIC; partition coefficients; log P values

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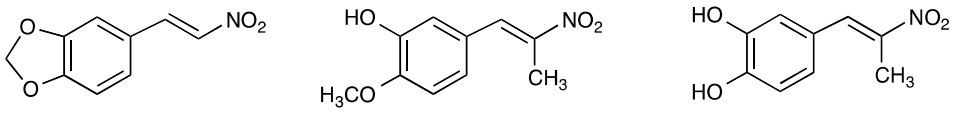
1. Introduction

Time is running out for antibiotics. The rising levels of antibacterial resistance, the decline in productive drug development and the reduction in the numbers of pharmaceutical companies engaging in research and development of anti-infective agents are huge public health concerns and pose problems for the effective management of microbial infections [1]. There is an urgent need to source

novel antimicrobial agents to combat human pathogens that are not susceptible to currently available antibiotics coupled with the strategic use of all available and emerging antimicrobial agents.

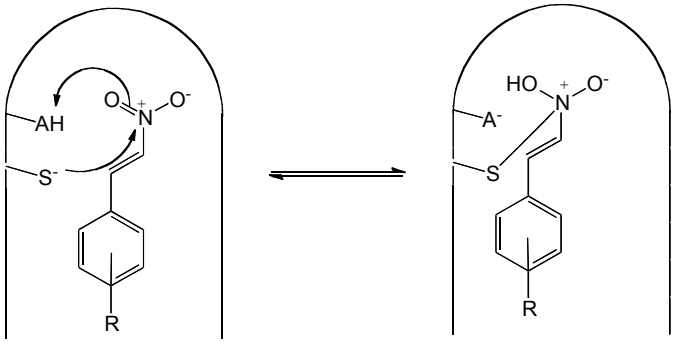
In 1952 Schales and Graefe [2] found that a range of substituted β-nitrostyrenes including nitropropenyl aromatic compounds were active against Gram positive and Gram negative bacteria and Denisenko *et al.* [3] who confirmed that **1** was as active as clinical antibiotics against bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *enterococci* (VRE). From the work of Milhazes *et al.* in 2006, the antibacterial activity against S *aureus* American Type Culture Collection (ATCC) 29213 of some aryl-hydroxy/methoxy substituted β-nitrostyrenes was found to be improved by the addition of a β-methyl group on the nitro alkene, with a 2 to 8 fold increase in the minimum inhibitory concentrations (MIC) relative to the β-nitrostyrene [4]. Interestingly, this MIC enhancement was most pronounced for Gram positive bacteria utilizing 3-hydroxy-4-methoxy-β-methyl-β-nitrostyrene **2** (MIC 32); whereas 3,4-dihydroxy-β-methyl-β-nitrostyrene **3** (MIC 64) showed the best result against all Gram negative bacteria except for *Pseudomonas aeruginosa.*



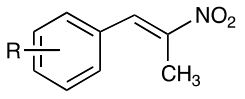
1 **2** **3**

Protein-tyrosine phosphatases (PTPases) are modulators of signal transduction pathways that regulate numerous cell functions. Malfunction of PTPases has been linked to a number of oncogenic and metabolic disease states. The PTPases are also utilized by microbes and viruses for pathogenic activity. Therefore, inhibitors of PTPases have potential as therapeutic antimicrobial agents [5–7]. Park and Pei in enzyme mechanistic studies found *trans*-β-nitrostyrene derivatives to be a new class of inhibitors of protein tyrosine phosphatase PTP1B [8]. They proposed that β-nitrostyrene is a reversible inhibitor of the tyrosine phosphatases PTP1B by their interaction and formation of a covalent complex with cysteine at the catalytic site.

Scheme 1. Park and Pei [8] mechanism of PTP1B inhibition by β-nitrostyrene. Reprinted with permission from Park, J.; Pei, D. Trans- β-nitrostyrene Derivatives as Slow-Binding Inhibitors of Protein Tyrosine Phosphatases. *Biochem.* **2004**, *43*, 15014–15021. Copyright ©2004, American Chemical Society.

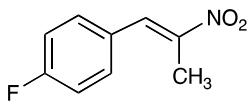


Their evidence suggested that in the absence of free thiols, nucleophilic attack by enzymic cysteine occurs on the side chain nitro group, as shown in Scheme 1. This rationale was based on the positive charge on the nitrogen atom being susceptible to nucleophilic attack, forming a reversible adduct that inhibited PTP1B. However, exactly the opposite is found in the literature. It has been reported [9] that β-nitrostyrenes reversibly add to the thiol group of cysteine and cysteine peptides under mild conditions. In contrast, sodium trimethylsilane thiolate at 185 °C for 24 h has been found to reduce nitro groups to amines [10]. The conjugate addition to β-nitroalkenes reflects the high reactivity of β-nitroalkenes as Michael acceptors [11], and it is widely recognized that nitro group olefins undergo rapid conjugate addition with thiol-type nucleophiles [12,13].



4

R = variable group



5

The antimicrobial activity of a range of β-methyl-β-nitrostyrene derivatives **4** against Gram positive, Gram negative bacteria and fungi were evaluated by Nicoletti *et al.* [14] who had previously found that:

(a) *E.coli* was suppressed effectively by chlorine or fluorine C_4-aryl-substituents relative to the unsubstituted or the 3-4-methylenedioxy ring compounds.

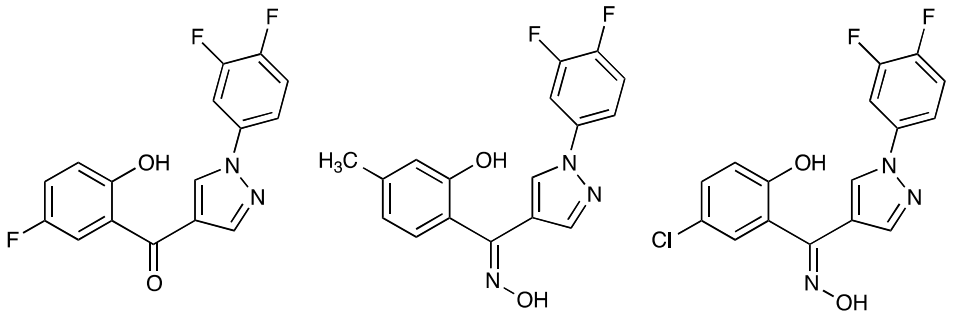
(b) *S.aureus* was suppressed effectively by a wide range of nitropropenyl arenes including β-methyl-β-nitrostyrene, the 4-fluorine and 4-chlorine substituted β-methyl-β-nitrostyrene, imidazolyl, benzothiazole and the 3,4-methylenedioxy derivatives. An important finding was that when two hydroxy groups were present on the ring in the 2- and 4- positions or the 2- or 5- positions, activity against this microorganism noticeably reduced. The fact that this also occurred with substitution by *N*,*N*-dimethyl and *N*,*N*-diethyl groups indicated that the more polar nature of these substituents was detrimental to activity. This was supported by the K_D values of the latter compounds being relatively low compared with the unsubstituted and halogenated-substituted compounds.

(c) *B.subtilis* was suppressed by a wide range of compounds in a similar fashion to *S.aureus*. The 2,4- and 2,5- dihydroxyaryl substituted compounds exhibited poor bacterial inhibition whereas the 3,4-dihydroxy derivative gave high activity.

(d) *C.albicans* was suppressed by the 3,4-dichloro, 4-chloro, 4-fluoro, derivatives. However 2,4-dihydroxy, 2,5-dihydroxy and the benzimidazole derivatives were very unreactive.

The introduction of 'fluorine functionality' into molecular architecture has proved to be invaluable for modulation of the molecular properties of medicinal compounds [15]. The substitution of hydrogen and other functional groups with fluorine can have multiple molecular impacts, including electronic, lipophilic, *H*-bonding, chemical, metabolic stability and steric factors, which can influence the pharmacodynamic and pharmacokinetic properties of pharmaceuticals [16]. When fluorine atoms or trifluoromethyl groups were introduced into either the peptidic chain or the C-terminal end of cationic pentapeptides, the antimicrobial activity always improved by diminishing the minimal inhibitory concentration (MIC) [17]. A recent report on the structure activity relationships of antitubercular

nitroimidazoles highlighted the high activity achieved by the inclusion of trifluoromethoxybenzyl substituents in the nitroimidazoles [18]. Carbon films with a high fluorine content exhibited excellent antibacterial properties against *E. coli* growth [19]. Furthermore, a simple super hydrophobic coating composed of nano-structured fluorinated silica colloids with fluoroalkoxysilane on a silane structure demonstrated that the adhesion of *Staphylococcus aureus* and *Pseudomonas aeruginosa* was reduced by 2.08 ± 0.25 and 1.76 ± 0.12 log over controls, respectively [20]. The fluorine containing 4-(substituted-2-hydroxybenzoyl) pyrazoles **6**, **7**, **8** showed promising antibacterial activity [21].



6 **7** **8**

MIC_{50} (microgram/mL)			
E. coli	62.5	25	12.5
S. aureus	100	250	100

Based on the discoveries that fluorine substituted β-nitrostyrene enhanced antimicrobial activity, this investigation was undertaken to study the effects on antimicrobial activity of difluoro-, trifluoromethyl- and trifluoromethoxy- substituted β-methyl-β-nitrostyrenes and related compounds that were prepared via the Henry reaction [22]. For an antimicrobial agent to be effective, it must penetrate the bacterial cell first to reach its target enzymes, therefore the lipophilicity of the prepared compounds was determined from the octanol-water partition coefficients. The antimicrobial activities of all products were measured by minimum inhibitory concentration (MIC). Three Gram positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and *Enterococcus faecalis*), one Gram negative bacterium (*Escherichia coli*) and a fungus (*Candida albicans*) were tested.

2. Experimental Section

2.1. Chemicals

Chemicals and solvents were typically sourced from Sigma-Aldrich Chemical Company, and were AR grade or better.

2.2. Instrumentation and Synthesis

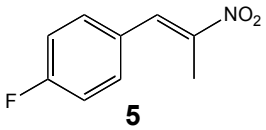
Gas chromatography and Mass Spectroscopy (GC/MS) spectra were obtained in either electron ionization (EI) or positive/negative electrospray (ESI) modes with the Varian Saturn 2200 GC/MS/MS (ion-trap) coupled to a Varian CP-3800 GC (FactorFour—Capillary Column; Stationary Phase: VF-5ms; L(m) ID(mm) × OD (mm): 30 × 0.25 × 0.39) or Micromass Platform II ESI/MS (240 V, 10 A). Resultant ions are expressed as m/z values.

NMR spectroscopy was performed on Bruker Advance 300 MHz or Bruker Avance 300 III MHz spectrometer. Some FIDs from Bruker Advance 300 MHz were processed using Mestrec23. ^{1}H chemical shifts were recorded as δ values in parts per million (ppm) downfield shifts, referenced internally to the residual $CHCl_3$ singlet at 7.26 ppm. Chemical shifts are presented in multiplicity, coupling constant(s) (*J* in Hz), integration and assignments. Abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, dd = doublet of doublet, m = multiplet. ^{13}C chemical shifts were ^{1}H decoupled and recorded as δ values in parts per million (ppm), referenced internally to the $CHCl_3$ triplet at 77.0 ppm. Assignments have typically been made using additional information from COSY and gCOSY spectra, DEPT, HMBC and HSQC experiments.

Flash Column Chromatography was performed using silica gel 60 Å, 0.04–0.06 mm (230–400 mesh ASTM).

Melting Points were determined on a Gallenkamp melting point apparatus, and were uncorrected.

2.2.1. Synthesis of 1-Fluoro-4-(Nitroprop-1-Enyl)Benzene **5**



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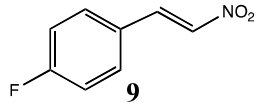
Compound ***5*** was prepared [23] in 30% yield, yellow crystals, mp. 65–66 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ_H (ppm): 8.06 (s, 1H, H-C=C), 7.46 (dd, *J* = 3.30, 5.37 Hz, 2H, Ar-H), 7.17 (t, *J* = 8.66 Hz, 2H, Ar-H), 2.46 (s, 3H, C-H, *E*), 1.59 (s, 3H, C-H, *Z*). ^{13}C NMR (300MHz, $CDCl_3$) δ_C (ppm): 165.2 (d, *J* = 252.21 Hz, 1C, C-F), 147.5 (C=C, β carbon), 132.5 (C=C, α carbon), 132.2 (Ar), 128.5 (Ar), 128.5 (Ar), 116.3 (Ar), 116.0 (Ar), 14 .0 (CH_3). GC/MS *m/z* (M^+) 181.

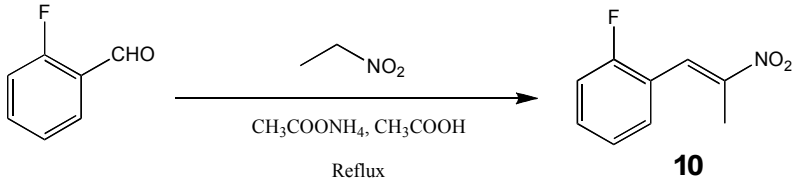
2.2.2. Synthesis of 4-fluoro-β-nitrostyrene **9** [4-fluoro-2-(nitroethen-1-enyl)benzene]



9

Following literature procedures [24], a stirred solution of acetic acid (33.5 mL) and ammonium acetate (4.4 g, 57.1 mmol) was added to nitromethane (10 g, 164.0 mmol) followed by 4-fluorobenzaldehyde (2.94g, 23.7mmol) and the solution was refluxed in an oil bath at 100 °C for 5.5 hr. The dark orange mixture was then cooled to room temperature and poured into water (100 mL). The pH of the solution was basified with sodium hydroxide solution (2 M), and the product was extracted with ethyl acetate (3 × 100 mL). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated under high vacuum. Recrystallization from 95% ethanol removed the brown oily impurities, giving pale yellow needles, 57% yield and mp. 99–100 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ_H (ppm) 7.94–7.89 (d, *J* = 13.81 Hz, 1H, αC-H), 7.52–7.44 (m 1H, βC-H), 7.52–7.44 (m, 2H, Ar-H), 7.11–7.05 (t, *J* = 8.40, 2H, Ar-H). ^{13}C NMR (300 MHz, $CDCl_3$): δ_H (ppm) 164.9 (d, *J* = 255.08 Hz, C-F), 137.8 (C=C, β carbon), 136.8 (C=C, α carbon), 131.2 (Ar), 126.3 (Ar), 116.7 (Ar). GC/MS *m/z* (M^+) 167.

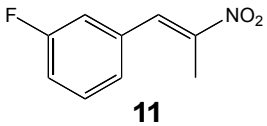
2.2.3. 1-Fluoro-2-(Nitroprop-1-Enyl)Benzene **10**



10

2-Fluoro-benzaldehyde (4.81 g, 38.8 mmol) was dissolved in nitroethane (4.01 g, 53.5 mmol, 20% excess), ammonium acetate (4.00 g, 52 mmol) and glacial acetic acid (5 mL) added and the mixture refluxed for 2 h in an oil bath at 100 °C. The orange mixture was then chilled and de-ionized water (6 mL) was then added. A small portion of the crude orange crystalline product obtained by filtration was taken for determination of melting point. The rest of the product was dissolved in hot ethanol (95%, 2 mL) and chilled for an hour to obtain the recrystallized product. The recrystallization procedure was repeated and light yellow crystals were obtained (2.38 g, 34% yield, mp 45–47 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ_H (ppm): 7.98 (s, 1H, H-C=C), 7.39–7.33 (t, *J* = 8.70 Hz, 1H, Ar-H), 7.19 (d, *J* = 8.67 Hz, 1H, Ar-H), 7.12 (d, *J* = 8.57 Hz, 1H, Ar-H), 7.04 (d, J = 8.76 Hz, 1H, Ar-H), 2.36 (s. 3H,C-H, *E*), 1.59 (s, 3H, C-H, *Z*). ^{13}C NMR (300MHz, $CDCl_3$) δ_C (ppm): 165.1 (d, *J* = 252.21 Hz, C-F), 147.5 (C=C, β carbon), 132.5 (C=C, α carbon), 132.2 (Ar), 132.0 (Ar), 128.7 (Ar), 116.4 (Ar), 116.2 (Ar), 14.0 (CH_3); GC/MS *m/z* (M^+) 181.

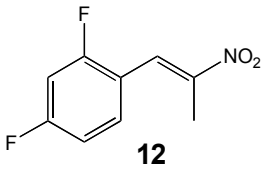
2.2.4. 1-Fluoro-3-(Nitroprop-1-Enyl)Benzene **11**



11

This compound was prepared [23] by reacting 3-fluorobenzaldehyde (1 g, 8.05 mmol) with ammonium acetate (0.19 g, 2.4 mmol) in nitroethane (4.98 g, 66.4 mmol), under reflux overnight (approximately 17 h) in an oil bath at 125 °C. The compound was identified by thin layer chromatography (TLC). The mixture was then concentrated under high vacuum to remove the excess nitroethane and then the yellow mixture was dissolved in chloroform (20 mL), washed with water (3 × 20 mL) and with sodium chloride solution (25%, 20mL). The mixture was dried over ($MgSO_4$) and concentrated under high vacuum. A yellow liquid was obtained (0.75 g) this being 52% of theoretical yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ_H (ppm): 8.04 (s, 1H, C=C-H), 7.46–7.39 (m, 1H, Ar-H), 7.46–7.39 (m, 1H, Ar-H), 7.22–7.19 (d, *J* = 7.80 Hz, 1H, Ar-H), 7.13–7.09 (d, *J* = 9.30 Hz, 1H, Ar-H coupled with F), 2.42 (s, 3H, CH_3). ^{13}C NMR (300MHz, $CDCl_3$) δ_C (ppm): 164.3 (d, *J* = 246.79 Hz, 1C, C-F), 148.7 (C=C, β carbon), 134.6 (Ar), 132.0 (Ar), 130.6 (C=C, α carbon), 130.5 (Ar), 125.9 (Ar), 125.7 (Ar), 13.9 (CH_3). GC/MS *m/z* (M^+) 181.

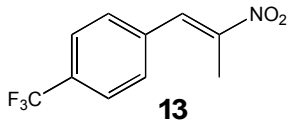
2.2.5. 1,3-Difluoro-4-(Nitroprop-1-Enyl)Benzene **12**



12

43% yield mp. 48–49 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ_H (ppm): 8.06 (s, 1H, C=C-H), 7.45–7.35 (dd, *J* = 8.30, 8.38Hz, coupling due to two fluorine atoms were coupling with this proton. 1H, Ar-H); 7.04–6.96 (dd, for the proton at the middle of two fluorine atoms it should split to a quartet as two fluorine atoms were coupling with it. *J* = 8.47, 8.50 Hz. 1H Ar-H. The other proton at position 6 should be a triplet as there is only fluorine atom coupling with it. t, *J* = 8.48 Hz, 1H, Ar-H), 2.38 (s 3H, CH_3, *E*), 1.57 (s 3H, CH_3, *Z*). ^{13}C NMR (300MHz, $CDCl_3$) δ_C (ppm): 163–162.3 (quartet due to two fluorine atoms coupling with the carbon. *J* = 222.02, 233.55Hz. 1C, F-C=C-C-F), 162.2–159 (q, *J* = 234,37 Hz. 1C, F-C=C-C-F), 149.5 (s, C=C α carbon), 134.1–131.1 (q, *J* = 234.64 Hz, 1C, F-C-C=C-C=C-F), 125.7–125.4 (s, C=C, β carbon), 117.0–116.5 (d, *J* = 9.88 Hz, 1C, F-C=C), 112.3–111.7 (q, *J* = 3.57, 21.68, 24.97Hz, 1C, F-C-=C-C-F), 104.8 – 104.4 (d, *J* = 25.80, 1C, F-C-C=C), 14.2–14.0 (CH_3). GC/MS *m/z* (M^+) 199.

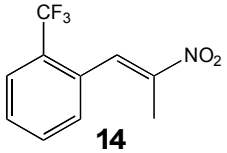
2.2.6. 1-Trifluoromethyl-4-(Nitroprop-1-Enyl)Benzene **13**



13

This compound was synthesized [25] using 4-trifluoromethylbenzaldehyde (1 g, 5.7 mmol) and ammonium acetate (0.38 g, 5.0 mmol) dissolved in nitroethane (20 mL, 280 mmol), heated to 100 °C and refluxed overnight. The excess nitroethane was removed under high vacuum and the yellow mixture was poured into water (20 mL) and extracted with ethyl acetate (3 × 20 mL). The combined organic extracts were washed with water (3 × 20 mL) and sodium chloride solution (25%, 20 mL) and then dried over anhydrous $MgSO_4$. The solvent was removed under high vacuum (enhanced with liquid nitrogen) to yield a yellow solid (1.16 g, 87% yield). The material was purified by washing with cold ethanol (95%) to yield **13** (0.748g, 42%), a yellow solid which had a melting point of 96–98 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ_H (ppm) 8.02 (s, 1H, C=C-H), 7.65 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.46 (d, *J* = 8.2 Hz, 2H, Ar-H), 2.37 (s, 3H, C-H, *E*), 1.59 (s, 3H, C-H, *Z*). ^{13}C NMR (300MHz, $CDCl_3$) δ_C (ppm): 148.9 (s, C=C, β carbon), 135.6 (Ar), 132.6 (s, C=C, α carbon), 131.4 (s, Ar-C-CF_3), 129.8 (Ar), 128.5 (Ar), 123.7 (q. *J* = 273.6 Hz, CF_3), 13.6 (CH_3). GC/MS *m/z* (M^+) 231.

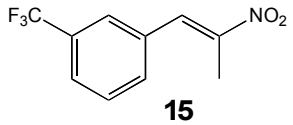
2.2.7. 1-Trifluoromethyl-2-(Nitroprop-1-Enyl)Benzene **14**



14

The product [25] was purified by flash column chromatography on silica gel with hexane/ethyl acetate (20/1) to obtain a yellow liquid 46% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ_H (ppm) 8.16 (s, 1H, C=C-H), 7.69–7.67 (d, J = 7.80 Hz, 1H, Ar-H, *trans*-compound), 7.61–7.53 (m, multiplets, overlapping of the *cis* and *trans*-compounds, 1H, Ar-H), 7.48–7.42 (m, 1H, Ar-H), 7.39–7.34 (t, J = 6.60 Hz, *Z*-conformer, 1H, Ar-H), 7.28–7.26 (d, J = 7.50 Hz, *Z*-conformer, 1H, Ar-H), 7.17–7.15 (d, J = 6.30 Hz, *E*-conformer, 1H, Ar-H), 2.31 (s, *E*-isomer, 3H, CH_3), 2.17 (s, *E*-conformer, 3H, CH_3). ^{13}C NMR 149.9 (s, 1C, C=C, β C), 148.9 (Ar), 132.0 (Ar), 130.2 (s, C=C, α carbon), 130.0 (*Z*-Ar), 129.3 (Ar), 129.0 (*E* Ar),128.4 (*Z*-Ar),127.2 (q, J = 264.5 Hz,1C, CF_3), 126.4 (*E*-Ar), 125.9 (Ar), 124.7 (Ar), 122.0 (Ar), 19.3 (s, 1C, *E*-CH_3), 13.7 (s, 1C, *Z* CH_3). GC/MS *m/z* (M^+) 231.

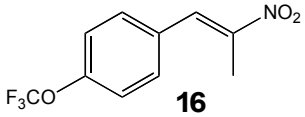
2.2.8. 1-Trifluoromethyl-3-(Nitroprop-1-Enyl)Benzene **15**



15

The product was purified by flash column chromatography on silica gel with hexane/ethyl acetate (20/1) to obtain a yellow liquid in yield of 46%. ^{1}H NMR (300 MHz, $CDCl_3$): δ_H (ppm) 8.07 (s, 1H, C=C-H), 7.44–4.38 (t, J = 8.66 Hz, 1H Ar-H), 7.27 (d, J = 7.91 Hz, 1H, Ar-H), 7.19 (d, 1H, Ar-H), 7.17 (s, 1H, r-H), 2.34 (s, 3H, CH_3). ^{13}C NMR 149.1 (s, C=C, β C), 133.3 (Ar), 132.8 (Ar), 131.6 (Ar), 131.2 (Ar), 130.3 (Ar), 129.5 (Ar), 126.3 (s, C=C, α carbon), 123.6 (q, J = A272.4 Hz, 1C, CF_3), 13.8 (s, CH_3). GC/MS *m/z* (M^+) 231.

2.2.9. 1-Trifluoromethoxy-4-(Nitroprop-1-Enyl)Benzene **16**



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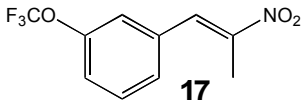
4-trifluoromethoxybenzaldehyde (1 g, 5.3 mmol), ammonium acetate (0.12 g, 1.6 mmol) and nitroethane (3.3 g, 43.3 mmol) were heated at 115 °C for 5 h. The yellow mixture was placed under high vacuum to remove excessive nitroethane and then dissolved in chloroform (10 mL), washed with water (3 × 20 mL) and washed again with saturated brine solution (25%, 2 × 20 mL). The washed mixture was then dried over $MgSO_4$, and then concentrated under high vacuum. 73% yield, yellow crystals mp 47–48 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ_H (ppm) 7.98 (s, 1H, C=C–H), 7.42 (d, J = 8.66 Hz, 2H, Ar-H), 7.24 (d, J = 8.29 Hz, 2H, Ar-H), 2.37 (s, 3H, CH_3). ^{13}C NMR 150.0 (s, O-C in Ar), 148.3 (s, C=C, β C), 131.9 (s, C=C, α C), 131.5 (Ar), 130.9 (Ar), 121.1 (Ar), 120.3 (q, J = 258.2 Hz, 1C, CF_3), 13.9 (s, CH_3).GC/MS *m/z* (M^+) 247.

2.2.10. 1-Trifluoromethoxy-3-(Nitroprop-1-Enyl)Benzene **17**



17

51% yield, yellow liquid. ^{1}H NMR (300 MHz, $CDCl_3$): δ_H (ppm) 8.05 (s, 1H, C=C–H), 7.45–7.49 (t, *J* = 8.70 Hz, 1H, Ar-H, *trans* compound), 7.40–7.36 (t, *J* = 6.60 Hz, 1H, Ar-H, *Z*- conformer), 7.31–7.28 (d, *J* = 7.20 Hz, 1H, Ar-H, *E*-conformer), 7.19–7.17 (d, *J* = 7.80 Hz, 1H, Ar-H, *Z*-conformer), 6.49 (s, 1H, Ar-H)2.45 (s, 1H, CH_3, *E*-conformer), 2.38 (s, 1H, CH_3, *Z*-isomer). ^{13}C NMR (300 MHz, $CDCl_3$): δ_H (ppm) 149.3 (s, C-OCF_3), 149.2 (s, C=C, β carbon), 134.4 (Ar), 131.7 (s, C=C, α carbon), 130.4 (*E*- Ar), 130.0 (Ar), 128.1 (*Z*-Ar), 126.2 (*E*-Ar), 124.1 (Ar), 122.6 (q, *J* = 258.0 Hz, 1C, OCF_3), 122.1 (Ar), 121.2 (*trans* Ar), 120.6 (*Z*-Ar), 19.9 (s, 1C, *Z*-CH_3), 13.9 (s, 1C, *E*-CH_3). **GC/MS** *m/z* (M^+) 247.

2.2.11. Minimum Inhibitory Concentrations

The strains used for biological tests were: *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231. Stocks of all microorganisms were kept in −80 °C in MHB (Oxoid, Cambridge, UK) with 20% v/v glycerol (BDH chemicals, Poole, UK). Strains were subcultured onto Nutrient agar (NA, Oxoid) and subcultures between three and nine used to prepare inocula. Inocula were prepared by adjusting a suspension to match McF 0.5 turbidity standard (equal to ~1.5×10^8 bacterial cells/mL or ~1.5×10^6 yeast cells/mL) and subsequent dilution to the required density. Broth microdilution testing was according to the National Committee for Clinical Laboratory Standards standard methods [26,27] in MHB (Oxoid) for bacteria or Sabouraud Liquid Medium (SLM, Oxoid) for *C. albicans*. Microplate assays were performed in clear, round-bottomed, 96-well plates (Sarstedt Australia, SA, AUS) with a total volume of 200 μL per well. Standard inoculum densities were approximately 1×10^5 Colony Forming Units per mL (CFU/mL) for bacteria and 1×10^4 CFU/mL for *C. albicans*. Inoculum densities were confirmed by serial dilution plating onto NA and incubation aerobically at 37 °C for 24 h.

The test β-nitrostyrene derivatives were added to plates at two times tested concentrations in 100 μL media. Ciprofloxacin was used as an internal positive control for bacteria and miconazole for *C.albicans*. Microplates were incubated 18–24 h at 37 °C aerobically before reading wells visually for turbidity. All assays included duplicated wells and were at least twice replicated. The geometric MIC (μg/mL) for each strain was adjusted to the nearest $\log_2$ dilution tested. MIC results were reported as MIC (μg/mL) for standards.

2.2.12. Octanol-Water Partition Coefficients

The lipophilicity level of each compound was determined by octanol-water partition coefficients. The buffer solution used in the determination of K_D was made by mixing sodium chloride (3.78 g, 65 mmol), disodium hydrogen orthophosphate (2.14 g, 18 mmol) and sodium dihydrogen orthophosphate (0.78 g, 5.5 mmol) in 500 mL water at room temperature (~22 °C) and had a pH of 7.5. Each compound (10 mg) was dissolved in octanol (2 mL) in a stoppered test tube, followed with the addition of the buffer solution (2 mL) and the tube was shaken over 48 hours. Finally the mixtures were allowed to separate into two layers. The top layers were removed and absorbance measured after dilution 1:20, 1:50 or 1:200 to 3 mL with octanol in a 1 cm cuvette path length at 370 nm for each diluted sample. The aqueous bottom layers were removed and the absorbance measured without dilution.

K_D measurements were according to the equation:

$$K_D = \frac{[\text{Octanol}]}{\text{Water}}$$

As the absorbance of the octanol and water layers is directly proportional to the concentration in each layer, the K_D value can be calculated from the relative absorbance of each layer.

3. Results and Discussion

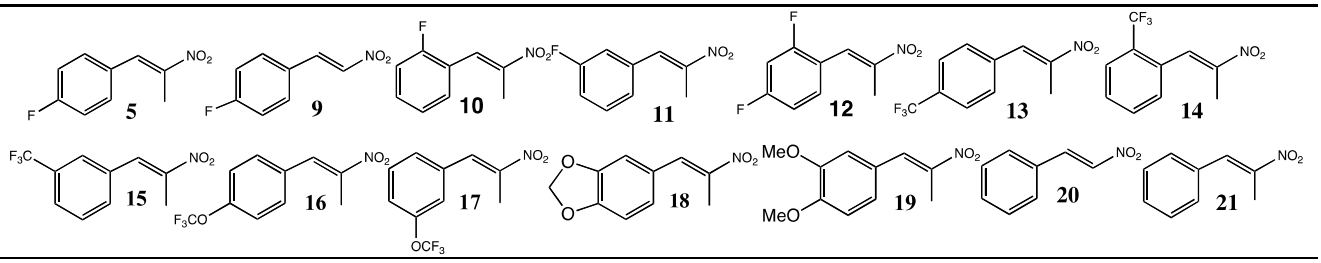
The preparation of the β-methyl-β-nitrostyrenes involved the Henry condensation reaction [22] of the aromatic aldehyde with nitroethane under basic conditions followed by dehydration of the resultant alcohol. The NMR analysis of the β-methyl-β-nitrostyrene fluorine derivatives indicated that the β-methyl-β-*E*-nitrostyrene conformation was predominant in most of the compounds prepared. For example, for 13 the *E*/*Z* ratio is 14/1.

The antibacterial activity of β-nitrostyrene derivatives and related compounds has been well known for a number of years. Their resurgence is attributed to the strong antibacterial/antifungal activity of some compounds with applications in veterinary and human medicine. It has been established from SAR studies that their lipohilicity plus the nucleophilic addition of thiol groups of some enzymes to the exocyclic double bond of these compounds are critical for the development of their antimicrobial activity. The antimicrobial efficacy of the β-nitrostyrenes was assessed by their activity against a panel of bacteria and a fungus (*Candida albicans*). The results appear as the minimum inhibitory concentration (MIC) for each compound. The partition coefficient (K_D) between octanol and water for each compound, representing its degree of lipophilicity, was also determined in order to access the extent of its interaction with the surface of the microorganism. The measured and calculated log P values are also included. Compounds with the incorporation of fluorine were of considerable interest as earlier studies had indicated an improvement of antibacterial activity [9].

The antibacterial assays shown in Table 1 indicated that the β-nitrostyrene compounds **20** and **9** displayed significantly lower activity towards all the tested bacteria relative to all the β-methyl-β-nitrostyrene analogues that were studied. This is exemplified by comparing the activity values of **20** and **21** as well as **9** and **5** that differ by at least an order of magnitude. The β-nitropropenyl side chain deviates 28° from coplanarity with the benzene ring [4] and the relative higher K_D values of the β-methyl-β-nitrostyrene compounds provides evidence that the steric effect and presence of the β-methyl group is substantial. However, the reasons for their enhanced antibacterial potency are unclear. It is also difficult to correlate the position of fluorine substitution on the benzene ring or the effect of multiple fluorine substituents with activities and K_D values of **5**, **10** **11** and **12**. The activity order of the four most potent antibacterial compounds **5** > **10** > **12** > **13** does not correlate with their K_D values. The data also provides evidence of the apparent tendency for fluorine functionalized β-methyl-β-nitrostyrenes to show considerable hydrophilic properties. The results also suggest that the more hydrophilic nature of the fluorine functionalized-β-methyl-β-nitrostyrenes with K_D values in the lowest range (23–132) is more effective against *E.coli.* It is interesting to note that substituent size appears to affect antibacterial activity against *E. coli.*, since for F-, the potency order is *p* > *o* > *m* and for CF_3-, its *p* > *m* > *o*. Of all the substances tested, compound **5** was most active against *E.coli* (27 μg/mL) and the MIC values against the other microorganisms were 6 μg/mL or less, making it also

the most effective against the three Gram positive bacteria and *C.albicans*. We deduce from this that for many Gram negative bacteria (such as *E. coli*) that are known to have polysaccharide structures, hydrophilic compounds more readily penetrate the bacterial cell wall to inhibit bacterial enzymes. From our results, the only Log *P* value for that can be cited for an effective fluorinated compound on *E.coli* (Gram negative) is 2.00 for compound **5**. For the Gram positive bacteria, a range of Log *P* values of 1.15–2.19 apparently relate to efficacy. For the non-fluorinated compounds, optimal Log *P* values cover a wider range 1.61–3.41, and becomes wider 1.15–3.41 with the inclusion of *C.albicans*.

Table 1. Antibacterial/fungus assays of various β-*E*-nitrostyrene compounds, their MIC [μg/mL] and K_D, logP values. MIC and K_D values are the means of two determinations.



Strain	5	9	10	11	12	13	14	15	16	17	18	19	20	21
S. aureus	2	128	3	8	2	2	16	16	2	16	3	2	256	8
B. subtilis	2	256	3	16	2	2	8	8	4	8	2	2	256	16
E. faecalis	5.5	64	5	16	6	4	32	16	8	16	4	5	128	16
E. coli	27	256	42	256	45	96	512	256	512	256	256	128	256	96
C. albicans	2	32	3	8	3	4	16	8	8	8	3	4	32	6
Meas. K_D	88	73	207	23	155	68	74	213	66	132	354	247	44	133
Meas. logP	1.94	1.86	2.32	1.36	2.19	1.83	1.87	2.33	1.82	2.12	2.55	2.39	1.64	2.12
Calc. logP	2.34	2.27	2.34	2.34	3.49	3.08	3.08	3.08	3.63	3.63	1.82	1.88	2.13	2.20

The plots of the MIC–K_D for the twelve β-methyl-β-nitrostyrenes investigated for their antibacterial activities against *E. coli* and *E. faecalis* presented in Figure 1 reflect the poor correlation between the effectiveness against *E.coli* and the partition coefficients of the compounds tested (r^2 = 0.0498 for *E.coli*, r^2 = 0.1416 for *E. faecalis*). There were no correlations between MIC value and K_D value for *S. aureus*, *B. subtilis*, and *C. albicans* (graphs not shown).

Figure 1. MIC–K_D plots for (**a**) *E. coli* and (**b**) *E. faecalis*.

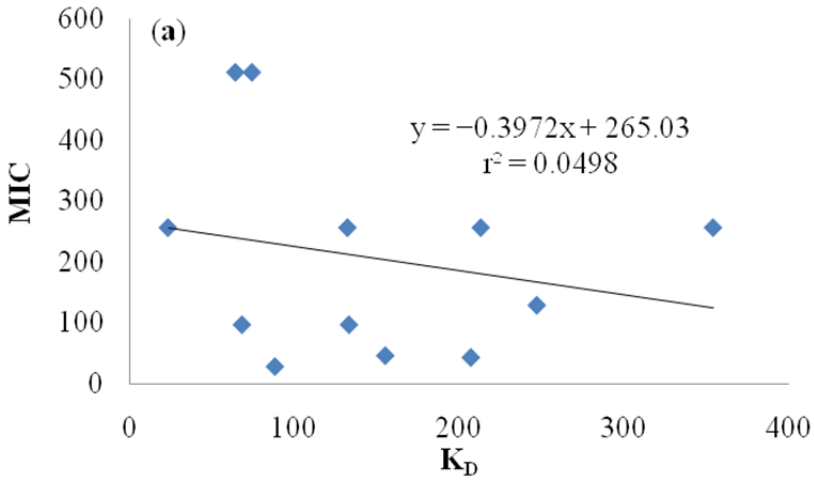
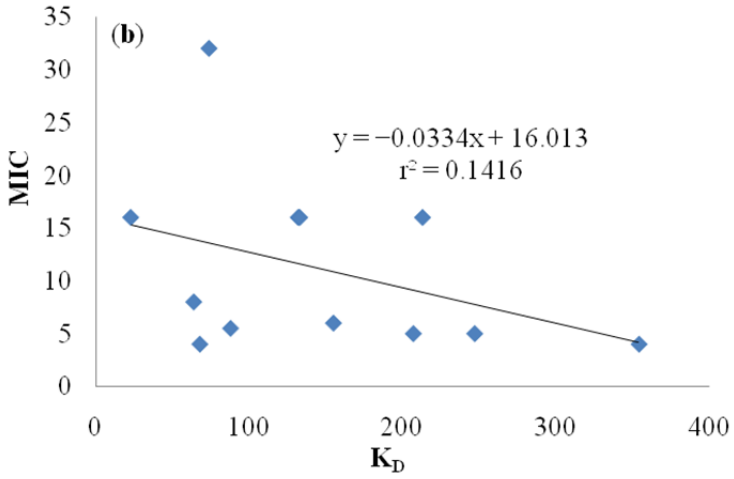
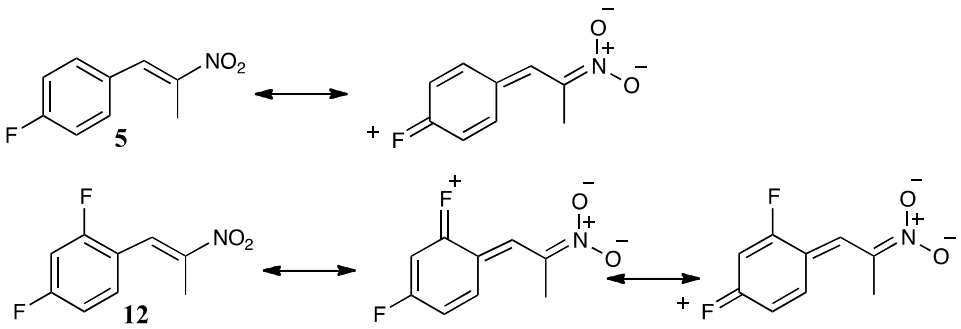


Figure 1. *Cont.*



The low K_D for fluorine containing compound **5** [K_D = 88] may be accounted by the presence of fluorine atom back bonding, leading to polar resonance structures and a gain in solvation energy in a hydrophilic compared to lipophilic medium, as shown in Scheme 2. Similarly, the 2,4-difluoro substituted compound **12** results in additional fluorine back bonding resonance structures with an overall increase in polarity and solvation in the aqueous solvent, significantly lowering its partition coefficient value.

Scheme 2. Fluorine back-bonding in fluorine substituted β-methyl-β-*E*-nitrostyrene compounds.



Can aromatic ring fluorine/hydrogen exchange influence membrane permeability? Generally, aromatic F/H substitution tends to increase compound lipophilicity, in direct contrast to what is observed. Alternatively, the reason for the effectiveness of fluorine substitution on the aromatic ring may be connected with the high electronegativity of fluorine, although size factors could also be important. Perhaps the electronegativity of fluorine could affect binding affinity to the binding site of the bacteria, thus causing inhibition of the enzyme. Further investigations with other fluorine-substituted compounds to determine the structural features required for the optimal anti-bacterial activity of β-methyl-β-nitrostyrene compounds are currently being carried out.

4. Conclusions

We have deployed the β-methyl-β-nitrostyrene scaffold for the development of fluorinated derivatives with enhanced antibacterial activity. While the screening of whole cells in MIC assays ensures cell

penetration and antimicrobial activity, it does not distinguish selective inhibitors from toxic compounds. In addition, the mechanism of antimicrobial action is unknown. The dependency between antimicrobial activity and lipophilicity K_D of fluorinated β-methyl-β-nitrostyrenes (FBNS) was examined for three Gram positive and one Gram negative bacteria and a fungus. The antimicrobial activity is rationalized with unspecific cytoplasmic membrane damaging effects that is near optimum at a lipophilicity (logP) of 2 (±0.5) for Gram-negative bacteria. For Gram-positive bacteria and fungus poor MIC *vs.* log K_D correlations were found. The disparity between measured and calculated log P values is also under investigation. There is, however, unequivocal literature evidence that both the β-nitrostyrenes and the β-methyl-β-nitrostyrenes are exceptional Michael acceptors. An expanded set of substituted β-methyl-β-nitrostyrene compounds will be investigated to determine the structural features required for the optimal anti-bacterial activity of β-methyl-β-nitrostyrene compounds.

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