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(Article begins on next page)

# Anti-*Pseudomonas* activity of 3-nitro-4-phenylfuroxan

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17

## 18 **Abbreviations**

19 MGE (mobile genetic elements), GFP (green fluorescent protein), NO (nitric oxide), MIC (minimal  
20 inhibitory concentration), CLSI (Clinical and Laboratory Standard Institute), LB (Luria Bertani), CV  
21 (crystal violet), CLSM (Confocal Laser Scanning Microscopy), UV (Ultraviolet), HPLC (High  
22 pressure Liquid Chromatography), ANOVA (Analysis of Variance), DMSO (dimethyl sulfoxide).

23

## 24 **Abstract**

25 *Pseudomonas aeruginosa* is a microorganism that is well adapted to both clinical and industrial  
26 settings, where it can form adherent communities that are difficult to eradicate. New anti-  
27 *Pseudomonas* compounds and strategies are necessary, as the current antimicrobial approaches for  
28 the inhibition of biofilm formation and, above all, the eradication of formed biofilms are ineffective.

29 Compounds that belong to the furoxan-family, which are well-known NO donors, have recently  
30 been shown to display anti-*Pseudomonas* activity. The present study investigates three furoxan  
31 compounds that are substituted at the heteroring with electron-withdrawing groups (NO<sub>2</sub>, CN,  
32 CONH<sub>2</sub>) for their effects on *P. aeruginosa* PAO1 growth and biofilm formation/dispersal. Of the  
33 furoxans tested, only 3-nitro-4-phenylfuroxan (KN455) inhibited the growth of suspended *P.*  
34 *aeruginosa* PAO1 cultures. Furthermore, KN455 inhibited the formation of both younger and older  
35 biofilms with very high yields and thus proved itself to be toxic to planktonic subpopulations. It also  
36 displayed moderate eradicating power. The activity of KN455 does not appear to be related to its  
37 capacity to release small amounts of NO. Interestingly, the isomer 4-nitro-3-phenylfuroxan (KN454),  
38 included for comparison, displayed a comparable antibiofilm rate, but did not show the same  
39 antimicrobial activity against suspended cells and planktonic subpopulations. While hypotheses as to  
40 the mechanism of action have been formulated, further investigations are necessary to shed light onto  
41 the antimicrobial activity of this furoxan.

42

43

## 44 **Introduction**

45 *Pseudomonas aeruginosa* is a microorganism that is optimally adapted to a variety of  
46 environmental conditions and is ubiquitously present in soil, water and sewage as well as in human,  
47 animal and plant hosts [1]. *P. aeruginosa* is also known to be a human opportunistic pathogen as it  
48 plays an important role in infections such as pneumonia in cystic fibrosis patients, chronic-wound  
49 infections and medical-device-related infections [2, 3].

50 Treatment of *P. aeruginosa* infections is often a challenge due to its resistance to antibiotics,  
51 which is acquired via a number of mechanisms. *P. aeruginosa* secretes enzymes, including  $\beta$ -  
52 lactamases and aminoglycoside modifying enzymes, that inactivate antibiotic molecules. It can also  
53 reduce antimicrobial agent accumulation in the cell producing multidrug efflux pumps (i.e. MexAB-  
54 OprM, MexEF-OprN, MexCD-OprJ, MexXY-OprM, etc.) [4]. Chromosomal mutations in DNA  
55 gyrase and topoisomerase IV genes lead to resistance towards quinolones [5]. The isolation of  
56 multidrug-resistant strains is increasing in clinical settings, where antibiotic selection is routinely  
57 practiced. Furthermore, the transfer of mobile genetic elements (MGE) contributes to the spread of  
58 resistance determinants among strains that belong to the same species and those of different species  
59 [6]. *P. aeruginosa* is also able to contaminate disinfectants such as chlorhexidine, benzalkonium and  
60 triclosan [7].

61 The treatment of *P. aeruginosa* infections becomes more difficult when bacteria are encased in  
62 their self-made matrix. This tolerance is caused by restricted compound penetration through the  
63 exopolysaccharide matrix and decreased metabolism, which is due to low nutrient and oxygen levels  
64 and accumulated waste [8]. The inhibition of biofilm formation and the eradication of mature biofilms  
65 are goals that are important for a wide range of antimicrobial applications because microbial biofilms  
66 are not only a significant issue in clinical infections, but also in the biofouling of industry water  
67 systems. Anti-biofilm strategies either attempt to destroy the established community in its  
68 microenvironment or target a specific step within the biofilm cycle. Biofilm disruption and/or  
69 corrosion can be achieved by metal chelators, such as EGTA and EDTA [9], by surfactants, such as  
70 SDS [10], by D-amino acids [11], and, as found more recently, by photoantimicrobials [12,13].

71 Nitric oxide (NO) has recently emerged as an endogenous regulator of biofilms [14]. Low  
72 levels of NO induce a transition from the biofilm mode of growth to the planktonic form [15]. A  
73 number of NO donors, products that can release NO under physiological conditions, have been found  
74 to suppress biofilm formation and elicit dispersal events in *P. aeruginosa* [15, 16, 17]. Furoxans

75 (1,2,5-oxadiazole N-oxides) are an interesting class of NO donors. A recent study has described the  
76 effects of three furoxan derivatives that are able to spontaneously release nitrogen oxide species  
77 (NO<sub>x</sub>), namely NO and nitrite (NO<sub>2</sub><sup>-</sup>), on *P. aeruginosa* PAO1 biofilm formation and dispersion [18].  
78 The present work extends our research into the use of furoxan derivatives as potential PAO1  
79 antibiofilm tools, and focuses on the impairment of *P. aeruginosa* biofilms using furoxan derivatives,  
80 which release NO under the action of thiol cofactors. Phenylfuroxan derivatives with electron  
81 withdrawing substituents (NO<sub>2</sub>, CN, CONH<sub>2</sub>) at the 3 position of the heteroring (Figure 1) are  
82 investigated for their effect on *P. aeruginosa* biofilm formation in this work, as electron-withdrawing  
83 groups have been shown to facilitate the release of NO from furoxans [19, 20]. 3-Nitro-4-  
84 phenylfuroxan (KN455), 3-cyano-4-phenylfuroxan (CF357) and 4-phenyl-3-furoxancarboxamide  
85 (CF1656) have been selected for this study. The isomer 4-nitro-3-phenylfuroxan (KN454) is included  
86 for comparison, as is the related des-NO furazan, 3-nitro-4-phenylfurazan (CF389).

## 87 **Materials and methods**

### 88 *Synthesis of the tested compounds*

89 Compounds KN455 [21]), CF357 [22], CF1656 [23], CF389 and KN454 [24] were synthesized  
90 as previously described.

### 91 *Bacteria strains and growth conditions*

92 *Pseudomonas aeruginosa* PAO1 was chosen as the model biofilm microorganism [25]. PAO1  
93 was transformed using the pVO(GFP) expression vector; it is a pJB3 KmD derivative, in which the  
94 Green Fluorescent Protein (GFP) coding sequence was cloned under an arabinose inducible promoter  
95 (pAra). Both strains were grown overnight in LB medium at 37°C under 200 rpm shaking.

### 96 *Antimicrobial assays*

97 A modified disk diffusion assay (Kirby-Bauer test) was set up to test the sensitivity that *P.*  
98 *aeruginosa* PAO1 shows towards furoxans [26]. Microorganisms grown in LB overnight at 37°C  
99 were diluted 100-fold in phosphate buffer (KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> 10 mM, pH 7.4) and a 100 µl sample  
100 was plated onto LB Agar. Filter paper disks (bioMerieux) with 6 mm diameters were loaded with  
101 increasing volumes of furoxans/furazan (20, 15, 10 and 5 µl) at a concentration of 50 mM, and placed  
102 on LB Agar. A disk loaded with 20 µl DMSO was used as a control. The plates were incubated at  
103 37°C for 24 h and the antibacterial effect was evaluated by measuring the growth inhibition halo.

104 In order to evaluate the furoxans' Minimal Inhibitory Concentration (MIC) toward *P. aeruginosa*  
105 PAO1, according to guidelines from CLSI (Clinical and Laboratory Standard Institute) [27], bacteria  
106 were grown overnight in LB at 37°C and diluted 100-fold to give a cellular concentration of 10<sup>7</sup> CFU  
107 ml<sup>-1</sup>. Two-fold dilutions of KN455, CF357, CF1656, KN454 and CF389 (from 2 to 0.001 mM), were  
108 added to bacterial samples. The dilution series were observed for microbial growth after 24 hours of  
109 incubation at 37°C and MIC values were evaluated. MBC (Minimal Bactericidal Concentrations)  
110 values were determined by re-culturing dilutions that inhibited PAO1 growth (i.e. those at or above  
111 the MIC). Broth dilutions were streaked onto LB agar and incubated for 24 to 48 hours. The MBC  
112 was the lowest broth dilution of furoxan/furazan that prevented *P. aeruginosa* PAO1 growth on the  
113 agar plate. All experiments were performed in triplicate, at least.

#### 114 ***Biofilm assays***

115 Overnight-PAO1 cultures were diluted 1:200 times in M9 medium that was supplemented with  
116 glucose 10 mM and 0.2% w/v casamino acid, used as carbon and nitrogen sources respectively, to  
117 obtain homogenous and repeatable biofilms [28, 29, 30]. One ml of the diluted culture was added to  
118 each well of a 24-well polystyrene plate. The furoxans were administered at three different  
119 concentrations (5, 50 and 500 µM) and dimethylsulfoxide (DMSO) 0.5 % was used as a solvent  
120 control. L-cysteine 5 mM, NO scavenger 2-phenyl-4,4,5,5,-tetrahydroimidazole-3-oxide-1-oxyl  
121 (PTIO) 1 mM and sodium nitrite, at 0.01, 0.1 and 0.5 mM, were added when indicated. The cells were  
122 incubated at 37°C under 200 rpm shaking for 6 h to evaluate the inhibitory effect of furoxans. The  
123 biofilm dispersal effect of the furoxans was observed upon administering compounds for 1 h, after 5  
124 h of untreated biofilm growth [18].

125 Planktonic biomass was spectrophotometrically quantified for each well via a measurement of  
126 absorbance at 600 nm, while adherent biomass was quantified via crystal violet (CV) staining. Briefly,  
127 planktonic biomass was removed and collected, and wells were washed once with one ml phosphate  
128 buffer. One ml of 0.1% CV was added to each well for approximately 20 min to stain the biofilm,  
129 after which the CV was removed and each well washed twice with 1 ml phosphate buffer. The  
130 remaining CV, which indicated the amount of biofilm present, was dissolved in acetic acid 30 % for  
131 10 min. The amount of solubilized dye was spectroscopically measured at 595 nm.

132 In order to evaluate the effect of the different treatments on the cellular viability of suspended  
133 and adherent populations, the planktonic phase was axenically collected and adherent cells were  
134 recovered by scraping and suspended in 1 ml of phosphate buffer. Viable counts - expressed as colony  
135 forming units per mL, CFU ml<sup>-1</sup>, in cell suspensions and as CFU per well in adherent biomass - were

136 estimated using a plate count technique; a volume (0.01 ml) of undiluted or serially diluted samples  
137 was plated onto LB Agar plates and incubated for 24 h at 37°C. All experiments were independently  
138 repeated at least three times.

### 139 *Confocal analyses*

140 Overnight PAO1- GFP tagged cultures were diluted 1:200 times in M9 medium that was  
141 supplemented with glucose 10 mM and 0.2% w/v casamino acid. Five ml of the diluted culture was  
142 added to a glass coverslip and placed into a well of a 6-well polystyrene plate. Volumes of the  
143 furoxans, 500 µM, and DMSO 0.5 % were added to the bacteria and subsequently incubated at 37°C.  
144 After either 24 h or 48 h of incubation, cells were induced with arabinose 1 mM to express green  
145 fluorescent protein. The coverslip was placed on a microscope glass slide for the acquisition of the  
146 adherent biofilm image. In order to observe the suspended bacteria, cells were placed between the  
147 glass and coverslip after 24 hours of treatment with furoxan/furazan 125 µM and DMSO 0.5 % as  
148 control. All microscopy observation and image acquisition was performed on a Leica TCS SP5  
149 confocal laser scanning microscope (CLSM) (Leica Microsystems, Wetzlar, Germany) equipped with  
150 a detector and filter set for GFP monitoring. Images were obtained using a 63/1.3 objective lens.  
151 Simulated 3D images were generated using the free, open-source software ImageJ (National Institute  
152 of Health, USA).

### 153 *NO and NO<sub>2</sub><sup>-</sup> detection*

#### 154 *Nitrite measurements by Griess reaction*

155 The total release of NO<sub>x</sub> was evaluated as nitrite (NO<sub>2</sub><sup>-</sup>) using the Griess reaction. Compounds were  
156 incubated at 37°C in 50 mM phosphate buffer, pH 7.4 at 0.1 mM concentration (thus final 1% DMSO)  
157 either in the absence or in the presence of L-cys at 0.1, 0.5 or 1 mM (1, 5 and 10 times mol/mol  
158 excess). The presence of nitrite in the reaction mixture was determined using the Griess assay at  
159 regular time intervals; 1 ml of the reaction mixture was treated with 250 µl of the Griess reagent (4%  
160 w/v sulphanilamide, 0.2 % w/v N-naphthylethylenediamine dihydrochloride, 1.47 M phosphoric  
161 acid). Absorbance was measured at 540 nm on a UV spectrophotometer after 10 min at room  
162 temperature (UV-2501PC, Shimadzu). A calibration curve was obtained using standard solutions of  
163 sodium nitrite in a concentration range of 1-80 µM ( $r^2 > 0.99$ ). The yield in nitrite was expressed as  
164 NO<sub>2</sub><sup>-</sup> percentage (mol/mol, relative to the initial compound concentration) ± SEM.

#### 165 *NO measurements using the DAN (2,3-diaminonaphthalene) method*

166 NO release from furoxan compounds was quantified using a 2,3-diaminonaphthalene (DAN)-based  
167 chemical assay. This assay is based on the immediate reaction of NO with oxygen (O<sub>2</sub>) to form  
168 dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>), which then reacts with non-fluorescent DAN to form the highly  
169 fluorescent 2,3-naphthotriazole (NAT), which can be quantified by RP-HPLC. Compounds were  
170 incubated at 37°C in 50 mM phosphate buffer, pH 7.4 at 0.1 mM concentration (then final 1% DMSO)  
171 with 0.2 mM (2 times mol/mol excess) DAN, either in the absence or in the presence of L-cys at 0.5  
172 mM. The presence of NAT in the reaction mixture was evaluated using HPLC analysis at fixed time  
173 points, according to a previously published protocol [31]. HPLC analyses were performed using a HP  
174 1200 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) that was equipped with a  
175 quaternary pump (model G1311A), a membrane degasser (G1322A), a UV detector, MWD (model  
176 G1365D), and a fluorescence detector (model G1321A), all integrated into the system. Data analyses  
177 were performed on a HP ChemStation system (Agilent Technologies). The sample was eluted on a  
178 Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 μm; Agilent); the injection volume was 20 μl.  
179 The mobile phase consisted of 65% 15 mM potassium phosphate buffer (pH 8.0) and 35% acetonitrile  
180 at a flow rate of 1.00 ml min<sup>-1</sup>. The fluorescence signals were obtained at excitation and emission  
181 wavelengths of 355 and 460 nm, respectively (gain factor = 10). Data manipulation was performed  
182 using an Agilent ChemStation. The values obtained from integrating the NAT peak were interpolated  
183 into a calibration line that was prepared using NaNO<sub>2</sub> (in acidic conditions) as a standard. Briefly,  
184 sodium nitrite standard solutions were acidified with HCl (pH 2) in the presence of an excess of DAN  
185 (0.2 mM). The reaction mixture was diluted in a phosphate buffer after 10 min (at pH = 7.4 and NO  
186 final concentration 1 to 80 μM), and analysed by HPLC.

### 187 *Statistical analyses*

188 Experiments were repeated at least three times on separate dates. Mean and SD calculations  
189 were performed using Microsoft Excel 2010. Data were analysed by means of one-way ANOVA  
190 (Origin\_ 7.0 SR0; Origin lab) and the statistical significance of all treatments was estimated (P < 0.05  
191 and P < 0.01).

## 192 **Results**

### 193 *Effect of furoxans on P. aeruginosa PAO1 growth*

194 The furoxans KN455, CF357 and CF1656, which bear different substituents on the 3 position  
195 of the furoxan heteroring (Figure 1), were investigated for their effect on *P. aeruginosa* PAO1 growth,  
196 both in solid and liquid media. As a first step, a Kirby-Bauer test was performed to assay the activity

197 of the three compounds. The DMSO used to dissolve the furoxans did not influence bacterial growth.  
198 Of the three compounds, only KN455 provided a reproducible inhibition growth halo ( $12.3 \pm 0.57$   
199 mm). In order to investigate the possible involvement of nitric oxide (NO) in the observed inhibitory  
200 activity, the corresponding furazan CF389, which is known not to release NO, was included in the  
201 analysis and no inhibition halo was observed (Figure 2). Furthermore, the corresponding 4-nitro  
202 isomer (KN454) was assayed to evaluate whether the nitro position on the heteroring influenced  
203 antimicrobial activity. In fact, KN454 failed to show any antimicrobial activity (Figure 2) and 3-nitro-  
204 4-phenylfuroxan was the only active compound. Furthermore, its activity was dose-dependent (data  
205 not shown).

206 The MIC value of KN455 was  $125 \mu\text{M}$ , and the MBC value  $250 \mu\text{M}$ , meaning that its Killing  
207 Quotient, calculated as MBC/MIC ratio, was lower than 4. This would indicate that the effect of  
208 KN455 is bactericidal. The MIC value of its isomer KN454 was  $1 \text{ mM}$ , whereas the MIC values of  
209 furoxans CF357 and CF1656, and furazan CF389 were all higher than  $2 \text{ mM}$ .

210 Confocal analyses were performed to evaluate the effect of KN455 on suspended PAO1 cells.  
211 The PAO1 strain was transformed with the Green Fluorescent Protein-gene-carrying plasmid, pVO,  
212 under the control of the *araBAD* promoter. Upon induction with arabinose, the cultures produced  
213 enough GFP to give a fluorescent signal, while cells that were not induced with arabinose were not  
214 fluorescent (data not shown). Upon 24 hours of treatment with KN455  $125 \mu\text{M}$  (MIC), few survivors  
215 were detected and most showed morphological changes compared to untreated (not shown) and  
216 DMSO 0.5 % treated cells (3A). As seen in Figure 3B, the surviving bacteria are longer than the  
217 untreated cells. In fact, the binary division process seems to be impaired, septation appears to be  
218 incomplete and giant cells are present in the treated group. Moreover, some of the filamentous rods  
219 show bright fluorescence while others show less pronounced GFP fluorescence. CF389 (Figure 3C)  
220 and KN454  $125 \mu\text{M}$  (data not shown) did not impaired the cellular viability as KN455, however few  
221 long cells were detectable. The administration of CF1556 (Figure 3D) and CF357 (data not shown)  
222 did not cause any change in cellular density, GFP expression and bacterial morphology.

### 223 ***Effect of furoxans on the inhibition of P. aeruginosa biofilm formation***

224 As it is known that furoxans release NO, the effect of increasing furoxan concentration (5, 50  
225 and  $500 \mu\text{M}$ ) on PAO1 biofilm formation was investigated. Compounds were added to minimal  
226 medium together with PAO1 and the adherent biomass was examined using crystal violet staining  
227 after 6 hours of incubation. Compounds were dissolved in DMSO 0.5 %, without significantly  
228 changing the amount of adherent biomass ( $p = 0.61$ ) compared to untreated controls (data not shown).

229 The nitro substituted furoxan, KN455, provided the most significant and dose-dependent biofilm  
230 inhibition of the three different furoxans tested, giving significant inhibition rates at the highest  
231 concentrations of 50 and 500  $\mu\text{M}$ , respectively (Figure 4A). The CN substituted furoxan, CF357,  
232 inhibited biofilm formation in a significant manner ( $p = 0.014$ ) at 500  $\mu\text{M}$  (Figure 4B), while the  
233  $\text{CONH}_2$  substituted analogue, CF1656, did not show any anti-biofilm effect at any tested  
234 concentration (Figure 4C). As the nitro derivative showed the highest inhibitory activity, its 4-nitro  
235 isomer KN454 and the corresponding des-NO furazan analogue, CF389, were also tested for their  
236 ability to inhibit biofilm formation. The rates of inhibition caused by KN454 and CF389 at the highest  
237 tested concentrations were statistically significant as shown in figures 4D and 4E, respectively (data  
238 not shown).

239 The viability of sessile biofilm cells was checked and KN455, alone among the tested furoxans,  
240 displayed a toxic effect against this sub-population (Figure 5A). A  $\sim 3$ -log unit decrease, compared to  
241 untreated cells, was observed. The corresponding 4-nitro isomer, KN454, and furazan, CF389, caused  
242 2-log unit decreases when administered at 500  $\mu\text{M}$ .

243 The planktonic counterparts of each biofilm were also evaluated as they play an important role  
244 in bacterial dissemination and equilibrium maintenance in the biofilm community. DMSO 0.5 % did  
245 not influence the cellular viability of planktonic biomass, compared to untreated cells (data not  
246 shown), while KN455 provided a much more significant decrease in viable cells ( $p = 3.2 \times 10^{-7}$ ) than  
247 the untreated samples (Figure 5B). Furoxans, CF357 and CF1656, did not alter the viability rate of  
248 cells (Figure 5B). Furazan CF389 and furoxan KN454 only caused a 1-log unit decrease, compared  
249 to untreated control biofilms.

250 Confocal analyses of 24- and 48-hour-old biofilms showed that the inhibitory effect of KN455  
251 500  $\mu\text{M}$  was also reliable when used in prolonged treatment (Figure 6). The GFP signal of a furoxan-  
252 incubated biofilm was lower than those of untreated and DMSO 0.5 % v/v treated biofilms. KN455's  
253 inhibitory effect was also evident against a 48-hour-old biofilm, which was noticeably thicker than  
254 the 24-hour-old biofilm (Figure 6). The corresponding furazan CF389 was not as effective as KN455,  
255 especially against 48 hour old biofilm (figure 6).

### 256 *Effect of furoxans on biofilm eradication*

257 While the inhibition of biofilm formation is an important goal, biofilm eradication is much more  
258 difficult to achieve and antimicrobials usually fail. The compounds were administered at 500  $\mu\text{M}$  for  
259 1h to cells grown for 5h to assay biofilm eradication power. The solvent was not toxic to cells (data  
260 not shown). KN455 showed the most statistically significant ( $p = 9.65 \times 10^{-4}$ ) eradication power, of  
261 the furoxans tested (Figure 7). The decreases in adherent biomass caused by isomer KN454 and des-

262 NO furazan CF389 were not significant. The solvent was not toxic to cells in tests on planktonic  
263 counterparts and the biomass changes observed upon furoxan treatment were not significant (data not  
264 shown), compared to control samples DMSO treated biofilms.

265

### 266 *Insight into anti-Pseudomonas activity of KN455*

267 KN455 was the most effective anti-*Pseudomonas* agent of the furoxans tested as it inhibited  
268 both cellular growth and biofilm formation, and even showed moderate biofilm eradicating power.  
269 The kinetics of NO<sub>x</sub> release from KN455 were compared to those of the other furoxans, CF357 and  
270 CF1656, because it this thought that NO may play the role of anti-biofilm messenger. Furthermore,  
271 the isomer KN454 and corresponding furazan CF389, which were included in the antimicrobial and  
272 antibiofilm assays, are also included in Figure 8, as % mol/mol. The amounts of NO<sub>2</sub><sup>-</sup> detected for 3-  
273 nitro-4-phenylfuroxan (KN455) were about 130 mol/mol after 1 hour. The amount of NO<sub>2</sub><sup>-</sup> produced  
274 by KN455 is the sum of the NO<sub>2</sub><sup>-</sup> formed by the nucleophilic substitution of the nitro group (see  
275 supporting information, S1, pathway a), and of the NO<sub>2</sub><sup>-</sup> produced by the aerobic oxidation of the NO  
276 released following the ring opening of the tetrahedral intermediate precursors (see supporting  
277 information, S1, pathways b, R=NO<sub>2</sub>, R = CyS). DAN (2,3-diaminonaphtalene) assays (Fang *et al.*  
278 2009), which allow NO centre dots to be specifically detected, were carried out to shed further light  
279 onto this process. NO<sub>2</sub><sup>-</sup> was the dominant species produced by KN455 after 1 h, whereas NO only  
280 corresponded to 5% of total NO<sub>x</sub>. The amount of NO<sub>2</sub><sup>-</sup> detected for the 4-nitro isomer KN454 was  
281 about 80 % mol/mol after 1 hour (Figure 8). For the *des*NO-nitrofurazan CF389, which cannot release  
282 NO from the heterocycle core, the reaction rate was slower and the amount of NO<sub>2</sub><sup>-</sup> detected was 25  
283 % mol/mol after 1 hour (Figure 8). 3-cyanofuroxan, CF357, and 3-carbamoylfuroxan, CF1656,  
284 produced nitrite in good and modest amounts, respectively. However, it is worth noting that these  
285 yields derived exclusively from the oxidation of NO (DAN test).

286 The highest rate of NO release was displayed by CF357, which, however, showed low anti-  
287 biofilm activity. Furoxans KN455 and CF1656, which release the same very low NO amount, showed  
288 different effects on microbial growth and biofilm formation. These data seem to rule out the possible  
289 involvement of NO in KN455 toxicity.

290 In order to investigate this hypothesis, biofilm formation experiments were repeated in the  
291 presence of PTIO, a known NO radical scavenger, and no changes were observed (data not shown).  
292 The possible involvement of nitrite, the other species released in large amounts by KN455, was also  
293 evaluated. The biofilm inhibition effect induced by acidified sodium nitrite was checked and  
294 compared to that of equimolar increasing concentrations of KN455. Sodium nitrite was less active  
295 than KN455 at the tested concentrations (Figure 9).

296 Since previous studies have shown that thiol groups, such as those present in L-cysteine (L-  
297 cys), can promote the liberation of NO<sub>x</sub> (NO and NO<sub>2</sub><sup>-</sup>) from furoxan compounds (Feelisch M. *et al.*  
298 1992), PAO1 was treated with increasing concentrations of KN455 in the presence of an excess of  
299 cysteine 5mM. L-Cysteine decreased the anti-biofilm activity of 50 μM and 500 μM KN455 (Figure  
300 10). Furthermore, L-cysteine abolished the anti-biofilm activity of KN455-related compounds,  
301 KN454 and CF389 (data not shown).

302 The reaction between L-cysteine and KN455 was monitored by HPLC in order to more deeply  
303 probe the role played by thiol groups. HPLC/mass data (Figure S2) indicate that, after 20 min, the  
304 compound was almost quantitatively transformed into 3-cysteinyl-4-phenylfuroxan (282 M+1, 120  
305 M-cysteinyl), which is the product of the nucleophilic substitution of the 3-nitro group with cysteine  
306 (see supporting information S1, pathway a). Over time, this latter compound was largely transformed  
307 into unidentified products and NO<sub>2</sub><sup>-</sup> (see supporting information S1, pathway b). The 4-nitro isomer,  
308 KN454, also reacted with L-cysteine giving rise to the corresponding cysteinyl derivatives (see  
309 supporting information S3). Des-NO furazan CF389, which cannot release NO from its heterocycle  
310 core, behaved similarly and afforded the cysteinyl substituted product. 3-Cyanofuroxan, CF357, and  
311 3-carbamoylfuroxan, CF1656, produced nitrite and unidentified products (see supporting information  
312 S4).

## 313 Discussion

314 Previous studies have shown that thiol groups, such as those present in L-cysteine (L-cys), can  
315 promote the liberation of NO<sub>x</sub> from furoxan compounds and increase their activity [18]. In this study,  
316 three phenylfuroxan derivatives that can release varying amounts of NO<sub>x</sub> were assayed for their  
317 antimicrobial activity against *P. aeruginosa* PAO1, and it would appear that the substituent influences  
318 furoxan antimicrobial activity; the NO<sub>2</sub>-substituted analogue, KN455, inhibited the growth of PAO1,  
319 whereas CN- and CONH<sub>2</sub>-substituted furoxans did not show any antimicrobial activity in the solid  
320 media. Furthermore, KN455 activity was bactericidal. Interestingly, the position of the nitro group  
321 on the furoxan skeleton significantly influences antimicrobial activity against *P. aeruginosa* PAO1;  
322 KN454 did not show any inhibition halo in the solid media and its MIC value was four-times higher  
323 than that of KN455.

324 Furthermore, the chosen furoxans showed different NO<sub>x</sub> release rates. As NO is emerging as  
325 an anti-biofilm messenger [32], the biofilm formation inhibition activity of the furoxans was  
326 investigated and correlated with NO<sub>x</sub> release. KN455, which is characterized by very low NO release,  
327 proved itself to be the most active compound in inhibiting biofilm formation, whereas CF357 and

328 CF1656, which can release good and very low amounts of NO, respectively, showed weak or null  
329 activity even at the highest concentrations tested. CF389, which is known not to release NO, and  
330 KN454, which releases nitrite, showed inhibitory activity. It would thus appear that nitric oxide does  
331 not play a role in furoxan biofilm inhibition. Indeed, KN455 was very toxic and no viable cells were  
332 recovered from the planktonic subpopulation of the six-hour-old biofilms. By contrast, the 4-nitro  
333 isomer KN454 was not toxic to the planktonic subpopulation, and only caused a slight decrease in  
334 cellular viability. The two furoxans showed different selectivities against biofilm cellular  
335 subpopulations; KN455 inhibited the growth of both planktonic and adherent cells, while KN454 was  
336 selectively active against adherent bacteria.

337 Furthermore, the furoxans release different amounts of nitrite (KN455 > CF357 > CF1656),  
338 which has been reported to inhibit staphylococcal biofilm formation [33, 34]. Under the tested  
339 conditions, sodium nitrite alone did inhibit *P. aeruginosa* PAO1 biofilm formation, but showed lower  
340 activity than equimolar concentrations of KN455. Although the nitrite-effect cannot be ruled out,  
341 other mechanisms should be considered.

342 L-cysteine prevented KN455 from inhibiting biofilm formation. It is reasonable to connect  
343 KN455 activity with its capacity to react with the thiol groups (or other nucleophilic centres) present  
344 on functional targets that are involved in cellular division and/or biofilm formation. This may also  
345 explain the activity of the 4-nitro isomer, KN454, and of the related des-NO furazan, CF389, which  
346 behave similarly in their reactions with cysteine.

347 Cysteine has also been demonstrated to act as an antioxidant, supporting the hypothesis that  
348 furoxan and furazan can elicit oxidative stress [35]. This is in agreement with the morphological  
349 changes observed upon furoxan treatment. Indeed, filamentation is a typical stress response that  
350 occurs when cell growth continues in the absence of cell division [36]. Both highly fluorescent giant  
351 cells and other cells that showed less pronounced GFP fluorescence were observed in  
352 furoxan/furazan-treated samples. GFP expression in the first group of cells was compatible with  
353 functional transcriptomic and expression machinery. The longest bacteria showed fuzzy fluorescence,  
354 which is compatible with GFP that has been broken down following decreased protein synthesis  
355 and/or permeability stress. However, the impairment of divisome machinery and cell wall septation  
356 seems to precede a pre-death phase. The low fluorescence of filamentous cells can probably be related  
357 to decreased metabolic activity before cellular death, as the formation of multinucleate giant cells  
358 may be a pre-death phase. Both nitro-furoxans, KN455 and KN454, and furazan, CF389, seem to  
359 elicit cellular stress, which induces cellular elongation.

360 In conclusion, nitrofuraxan KN455 quite potently inhibits *P. aeruginosa* PAO1 growth and  
361 prevents PAO1 biofilm formation. This activity does not appear to be related to its capacity to release  
362 small amounts of nitric oxide, rather than nitrite. Oxidative stress and/or the impairment of diverse  
363 machinery, which is ascribable to its strong electrophilic properties, may contribute to this anti-  
364 *Pseudomonas* activity. Additional work is necessary to investigate the intriguing differences found  
365 between furaxan KN455 and the isomer KN454, as well as to identify their target/s.

366

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371 The authors report no potential conflict of interest.

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470

## 471 CAPTIONS

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473

474 **Figure 1.** Schematic representation of the furoxans and furazan investigated.

475 **Figure 2.** Kirby-Bauer disk diffusion tests. Effect of 20 µl of DMSO 0.5 %, KN455, KN454 and  
476 CF389 at a concentration of 50 mM on the growth of *P. aeruginosa* PAO1.

477 **Figure 3.** Confocal images of *P. aeruginosa* PAO1 that was GFP-tagged after 24 hours of treatment  
478 with DMSO 0.5 % (A) and KN455 (B), CF389 (C) and CF1656 (D) 125 µM, respectively (bar 10  
479 µm). GFP expression was induced by arabinose 1 mM, one hour before confocal analyses.

480 **Figure 4.** Effect of phenyl-R-substituted furoxans/furazan on the inhibition of *P. aeruginosa* PAO1  
481 biofilm formation. The effect was evaluated by CV staining OD595 values upon treatment for 6 hours  
482 with KN455 (A), CF357 (B), CF1656 (C), KN454 (D) and CF389 (E) at increasing concentrations,  
483 with bars representing data from at least three biological replicates and error bars representing  
484 standard deviation from the mean \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.001$ .

485 **Figure 5.** Viable counts of the adherent (A) and planktonic (B) phases upon the inhibition of *P.*  
486 *aeruginosa* PAO1 biofilm formation exerted by furoxans/furazan 500 µM. The data expressed as  
487 CFU well<sup>-1</sup> and CFU ml<sup>-1</sup> of survivors, respectively, are the means of at least three independent  
488 experiments ± SD \*  $p < 0.05$ , \*\*  $p < 0.001$  and \*\*\*  $p < 10^{-6}$ .

489 **Figure 6.** Projections and sections of CLSM images of *P. aeruginosa* PAO1 GFP-tagged biofilms  
490 that were subjected to furoxan/furazan and solvent treatments. Bacteria were treated for 24 hours  
491 (left) or 48 hours (right) with KN455 and CF389 500 µM or DMSO 0.5 %. The live cells that  
492 express GFP are green.

493 **Figure 7.** *P. aeruginosa* biofilm dispersal upon treatment with furoxans at 500 µM for 1h. The bars  
494 represent data from three biological replicates and error bars represent the standard deviation from  
495 the mean \*,  $p \leq 0.05$ .

496

497 **Figure 8.** NO and total NO<sub>2</sub><sup>-</sup> released by furoxans (KN455, KN454, CF357,CF1656) and furazan  
498 CF389 in the presence of L-cysteine (5:1 molar excess) at a selected time point (60 min). The results  
499 are expressed as percentage (% mol/mol) of NO or total NO<sub>2</sub><sup>-</sup> released with respect to the quantity of  
500 parent compound. Bars represent data from three or more replicates and error bars represent standard  
501 deviations from the mean.

502 **Figure 9.** *P. aeruginosa* biofilm inhibition upon treatment with KN455 and Sodium Nitrite for 6  
503 hours at increasing concentrations. The error bars represent the standard deviation from the mean of  
504 three biological replicates.

505 **Figure 10.** *P. aeruginosa* biofilm inhibition upon treatment with KN455 500 μM for 6 hours in the  
506 presence and absence of L-cysteine 5 mM. The bars represent OD595 values upon Crystal Violet  
507 staining of three biological replicates, whereas error bars represent the standard deviation from the  
508 mean.

509