Nitroxides as anti-biofilm compounds for the treatment of Pseudomonas aeruginosa and mixed-culture biofilms

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ARTICLE

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A series of 23 nitroxides (**5-27**) was tested for biofilm modulatory activity using a crystal violet staining technique. 3-(Dodecane-1-thiyl)-4-(hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl (**22**) was found to significantly suppress biofilm formation and elicit dispersal events in both *Pseudomonas aeruginosa* and mixed-culture biofilms. Twitching and swarming motilities were enhanced by nitroxide **22**, leaving the planktonic-specific swimming motility unaffected and suggesting that the mechanism of **22**-mediated biofilm modulation is linked to the hyperactivation of surface-associated cell motilities. Preliminary structure-activity relationship studies identify the dodecanethiyl chain, hydroxymethyl substituent and the free radical moiety to be structural features pertinent to the anti-biofilm activity of **22**.

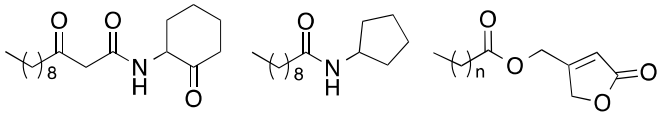
Introduction

Bacterial biofilms are notoriously problematic in many diverse areas such as medicine, food and water treatment.[1] Moreover, the growth of biofilms and the production of harmful metabolites by microorganisms cause considerable aesthetic and structural damage to culturally significant materials.[2] In addition, the gram negative bacterium *Pseudomonas aeruginosa*, one of the most extensively studied model organisms for bacterial biofilm formation, has been identified to be associated with biodeterioration of cultural materials.[3] Conventional methods for the control of biofilm formation and for the remedial treatment of biodeteriorated materials have largely focused on biocidal and antibacterial approaches,[4] however these strategies have limited effectiveness due to the refractory nature of biofilms and the development of resistance.[5]

The growth of biofilms and their tolerance toward exogenous physiochemical pressures represent remarkable adaptations by microorganisms to managing changing and often hostile environmental parameters. As a consequence, the propensity of microorganisms to exist as sessile cells within the physically-protective environment of a biofilm is more probable than the free swimming planktonic phenotype.[6] This is most likely a result of the 1000-fold increased sensitivity of planktonic bacteria, in comparison to their sessile counterparts, to conventional biocidal and antibiotic treatments.[7] This increased sensitivity can be attributed to both the increased physical protection afforded by the glycocalyx (a matrix of biomolecules secreted by colonizing bacteria) in addition to the higher metabolic activity of planktonic cells.[8] Significant differences in structure, function and gene expression between planktonic and biofilm cells, therefore translate into differences in biocidal and antibacterial sensitivity.

Pivotal to the continual maintenance of a community of microorganisms is the microbial cell-to-cell communication system, quorum sensing (QS). Utilizing biochemical messenger molecules (autoinducers), QS allows the population to function in unison by initiating concentration-dependent signal transduction cascades that culminate in population wide changes in gene expression.[9] Dispersal events, which are suspected to be QS controlled and synchronized, are characterized by the transformation of sessile cells into planktonic bacteria. In recent years there has been an abundance of research focusing on the manipulation of QS, and the use of anti-biofilm compounds, as strategies for controlling biofilm formation with enhanced effectiveness, less selective pressure and decreased resistance.[10]

Anti-biofilm compounds, such as N-acyl homoserine lactone (AHL) analogues (**1** – **3**),[11] have been found to prevent biofilm formation and/or induce dispersal events through the manipulation of intrinsic bacterial processes. By initiating cellular change and movement of microorganisms away from the protective barrier produced by the glycocalyx, anti-biofilm



1 **2** **3**

compounds have been found to increase the effectiveness of antimicrobial agents.[12]

Another anti-biofilm compound that has been shown to modulate biofilm development is the signalling molecule, nitric oxide (NO).[13] The use of NO donors, such as diazeniumdiolates, provide useful methods for introducing NO into biological systems. These donors have been employed successfully to prevent the formation of *P. aeruginosa* biofilms while effecting their dispersal.[14] Although NO-donor compounds offer a controlled method of NO application, their half-lives (which range from seconds to hours), in addition to their cost, render them impractical for many applications.[15]

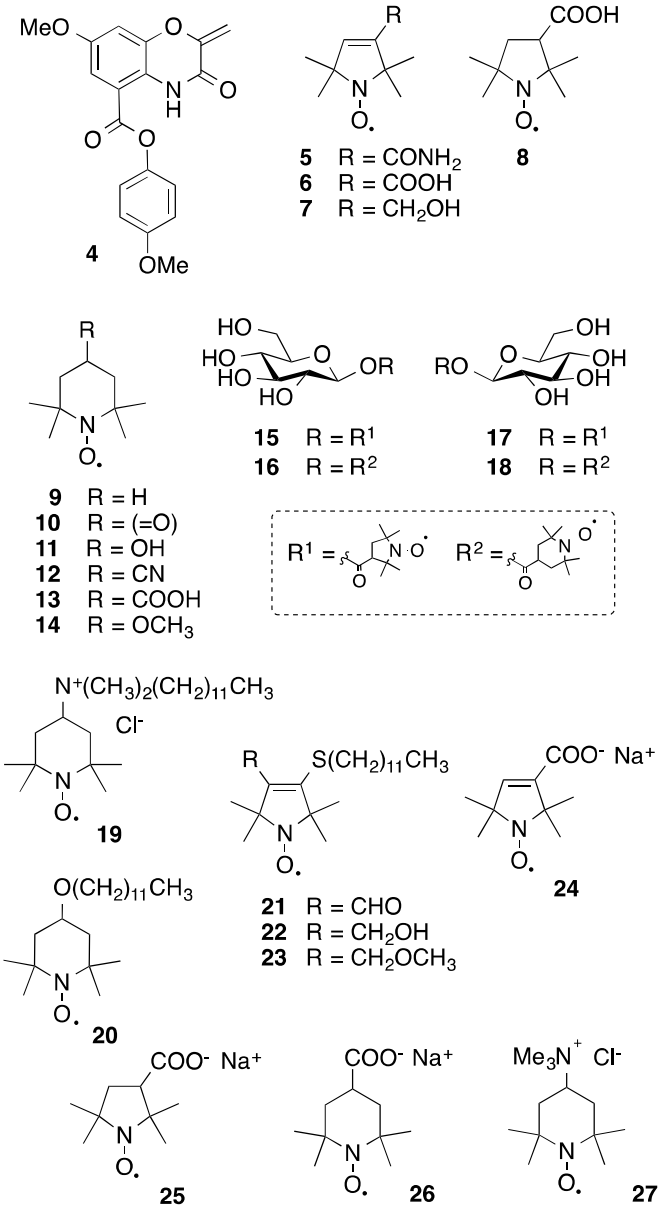
Nitroxides are related compound that are electronically similar to NO,[16] and as such, it has been suggested that they may offer a more viable alternative to NO for the control of biofilms.[17] Nitroxides are also readily prepared and handled, and, as a consequence, the chemical and biological reactivity, cell permeability and solubility of these compounds can be tailored for the desired application.

The biological potential of nitroxides was first recognized in 1964 by Emmerson and Howard-Flanders when they reported that nitroxides sensitized bacteria to subsequent treatment with radiation.[18] They speculated that given other free radical species such as oxygen and NO behaved as radiation sensitizers, so too should nitroxides.

During the course of our work, a report appeared describing the use of nitroxides as anti-biofilm agents against *P. aeruginosa*.[17] The authors described the use of three nitroxides to inhibit the initial stages of biofilm formation and initiate dispersal events in pre-established wild-type *P. aeruginosa*.[17] Furthermore, they showed that these nitroxides were able to restore swarming motility in a mutant strain of *P. aeruginosa* deficient in nitrite reductase (and therefore deficient in NO).[17] The authors went on to suggest that nitroxides are able to mimic the swarming activity response elicited by NO and as a consequence mimic other biological effects of NO, including biofilm dispersal.

We are aware of only one other report describing molecules capable of dispersing preformed *P. aeruginosa* biofilms; Linington and co-workers identified that the antibiotic skyllamycins are inhibitors of biofilm formation as well as inducers of biofilm detachment in *P. aeruginosa*.[19] Very recently, these same authors reported the development of benzo[1,4]oxazines (eg. **4**) as biofilm inhibitor and dispersal agents against *Vibrio cholerae*.[20] In this communication **4** is described as being among "just a handful of compounds capable of inducing the dispersal of mature surface-associated biofilms".[20]

Herein we report our investigations into nitroxides as compounds that control the development of *P. aeruginosa* and mixed-culture biofilms.

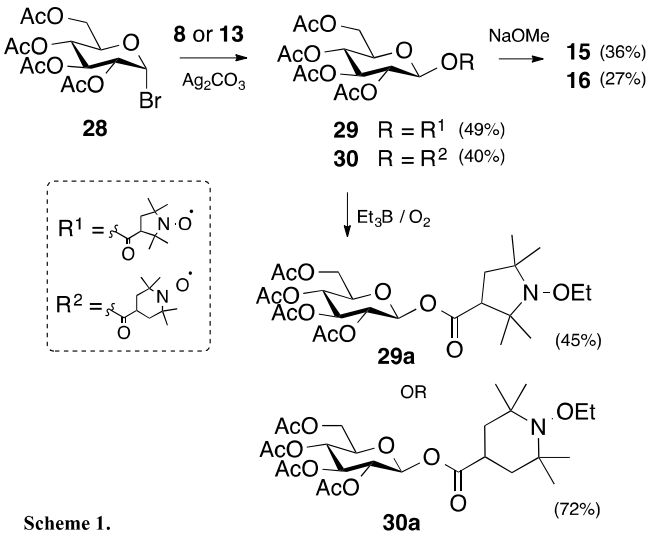


Results and Discussion

To assess nitroxides as a class of anti-biofilm agent, a library of nitroxides was constructed comprising molecules with varying charges and hydrophilicities in order to target extracellular, intracellular and intermembrane cellular localizations.[21] The approach adopted involved the design and synthesis of 23[§] nitroxides (**5** – **27**) which were then screened for biofilm modulatory activity using a crystal violet staining technique.[22]

Nitroxide design and synthesis

Nitroxides have been studied previously in biological systems as antioxidants[23] or as spin labels.[16a] Through the use of EPR experiments nitroxides such as 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolinoxyl (**6**) were shown to pass through the membrane into the cytoplasmic space.[21] With this in mind, intracellular-targeted nitroxides **5** – **18** were prepared in order to determine



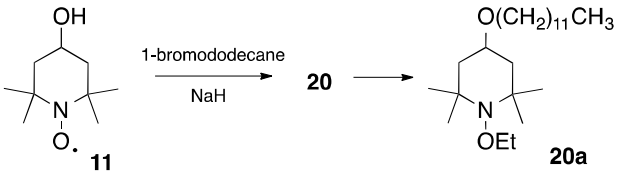
Scheme 1.

if nitroxides in this cellular environment confer anti-biofilm activity. In addition to the preparation of nitroxides **5** – **14** (prepared by well-established procedures),[24] we sought to take advantage of the improved solubility and cellular targeting of carbohydrate-based molecules, and explore the impact of monosaccharide-linked nitroxides on biofilm modulation. Carboxylic acids **8** and **13** were tethered to the glucosyl donor **28**[25] through an anomeric 1-*O*-acyl linkage furnishing the protected glucosyl nitroxides **29** and **30** in moderate yields (49% and 40% respectively). Deacetylation of **29** and **30** with sodium methoxide gave the corresponding free glycosides **15** and **16** (Scheme 1). To investigate the impact of replacing natural D-glucose with unnatural L-glucose on biological activity, the L-glucosyl nitroxides **17** and **18** were also prepared.†

Due to the paramagnetic nature of nitroxides, NMR analysis of this class of compound typically results in peak broadening which limits the elucidation of detailed structural information. Nitroxides **29** and **30** were therefore characterized as their ethoxylamine derivatives (**29a** and **30a**), prepared by the action of triethyl borane in the presence of oxygen (Scheme 1).

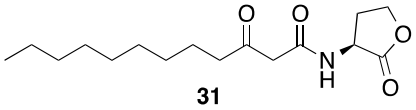
Nitroxides that carry a charge are unable to diffuse freely through the phospholipid bi-layer and so are expected to reside in the extracellular space.[21] Four charged nitroxides were prepared – sodium carboxylates **24** – **26** and the quaternary ammonium chloride **27** – in order to target the extracellular space. Carboxylic acids **6**, **8** and **13** were converted to their sodium carboxylates using one equivalent of sodium hydride in anhydrous THF. Nitroxide **27** was prepared following a literature procedure.[26]

Nitroxide probes with long hydrocarbon "tails" are able to localize within, and interact with, the cellular membrane.[27] For example, nitroxides based on fatty acids, such as doxyl stearates[28] are found to localize within the membrane bi-layer with their polar carboxyl group at the surface and their long hydrocarbon chains inserted into the hydrophobic phospholipid bilayer.[21,28] On the other hand, nitroxides with a charged head group such as quaternary ammonium salt **19**, should localize at the surface of the membrane with only its hydrocarbon tail penetrating into the lipophilic portion of the membrane. As such, we also endeavoured to synthesize a range of nitroxides, (**19** – **23**) with dodecyl substituents, both charged and uncharged in order to target the intermembrane space. 4-(*N*-dodecyl-*N,N*-dimethyl)ammonio-2,2,6,6-tetramethyl-1-piperidinoxyl chloride (**19**) was prepared using procedures described elsewhere.[27,29]



Scheme 2.

Treatment of 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinoxyl (**11**) with sodium hydride in THF followed by 1-bromododecane afforded 4-dodecyloxy-2,2,6,6-tetramethyl-1-piperidinoxyl (**20**) in 27% yield after heating at 70˚C for 48 hours (Scheme 2). The ethoxylamine adduct **20a** was obtained in modest yield (39%) from **20** by radical ethylation, as described for the preparation of **29a** and **30a**.



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The synthesis of dodecanethiyl-substituted pyrrolinoxyl radicals **20** – **22** has been described in a previous communication.[30] Installation of the dodecyl chain onto the pyrrolinoxyl "core" not only drives the nitroxide to localize within and interact with the hydrophobic phospholipid bilayer, but also creates compounds that bear structural similarity to the native *las*-dependent AHL autoinducer of *P. aeruginosa*, *N*-(3-oxododecanoyl)-l-homoserine lactone (**31**).[31]

In addition to its implication in the activation of phenotypes such as glycocalyx production, virulence factor production and motility, AHL **31** is also thought to be involved in the differentiation stage of biofilm development.[32] A number of studies have demonstrated that exogenously applied AHL **31** increases biofilm formation in a range of bacterial species.[33] As such, antagonists of the **31**-mediated QS system, for example *N*-(2-oxocyclohexyl)-3-oxododecamide (**1**), have been shown to inhibit the development of biofilms.[11a]

Effect of nitroxides 5 – 27 on biofilm formation and dispersal in *P. aeruginosa*

Nitroxides **5** – **27** were initially screened for biofilm inhibiting and dispersing activity against *P. aeruginosa* using an adaptation of the traditional crystal violet biofilm assay[22] the details of which are described in the ESI.† The anti-biofilm activity of the nitroxides were assessed at concentrations in the range 500 nM to 5 mM and changes in the amount of planktonic and biofilm masses in response to added compound were determined quantitatively by comparing the optical

density (OD) of treated cells to DMSO-treated controls using a microplate fluorimeter.[22] This approach enabled us to identify eight nitroxides (**5**, **6**, **9**, **14**, **17**, **18**, **22** and **26**) that suppressed biofilm formation in *P. aeruginosa* at 5 mM as determined by both a decrease in biofilm biomass and a concomitant increase in planktonic biomass. Two nitroxides (**20** and **22**) elicited biofilm dispersal events (Fig. 1 and Table 1) (**20** -mediated biofilm dispersal at 500 μM only - not shown). With the exception of the L-glucosylnitroxides **17** and **18**, that interestingly performed better as anti-biofilm agents than their D-isomers (**15** and **16**) there seemed to be no structural correlation between the remaining biologically active nitroxides.

Table 1 Nitroxides with anti-biofilm activity at 5 mM.

Nitroxides	% biofilm suppression	% enhancement in dispersal
5	15	not observed
6	14	not observed
9	13	not observed
14	11	not observed
17	14	not observed
18	12	not observed
20 [a]	not observed	21
22	70	57
26	28	not observed

[a] **20**-mediated dispersal was observed at 500 μM only.

To our delight, nitroxide **22** displayed superior biological activity towards *P. aeruginosa* biofilms effectively, repeatedly, and across a wide concentration range suppressing biofilm formation and eliciting dispersal (Fig. 2). The greatest effect was observed at 5 mM in which biofilm biomass was reduced by 60-70% and planktonic biomass increased by 50-120%. This concomitant increase in planktonic biomass is consistent with a transition of sessile biofilm cells to the planktonic phase and furthermore suggests that **22** is acting through a non-biocidal mechanism.

During the course of our experiments we noted that the anti-biofilm activity of **22** was dependent on a number of structural features. Nitroxide **7** which lacked a dodecanethiyl group at position 3 on the pyrroline ring was found to neither inhibit biofilm formation nor disperse a pre-established biofilm. Anti-biofilm activity is also lost by exchanging the hydroxymethyl substituent at position 4 of the pyrroline ring with an aldehyde (**21**) or a methoxymethyl substituent (**23**) suggesting that the biofilm modulatory capacity of **22** is not due to a surfactant effect. To determine the importance of the free radical moiety for biological activity of **22**, *P. aeruginosa* was treated with the ethoxylamine derivative of **22**. Ethoxylamine **22a** was unable to suppress biofilm formation but did induce dispersal at 5 mM as evidenced by the 41% decrease in biofilm biomass and a concomitant 28% increase in planktonic biomass compared to the DMSO control (data not shown). As such, the free radical moiety in **22** is likely to be important to the ability of **22** to inhibit biofilm formation and in the efficacy of **22**-induced dispersal of *P. aeruginosa* biofilms.

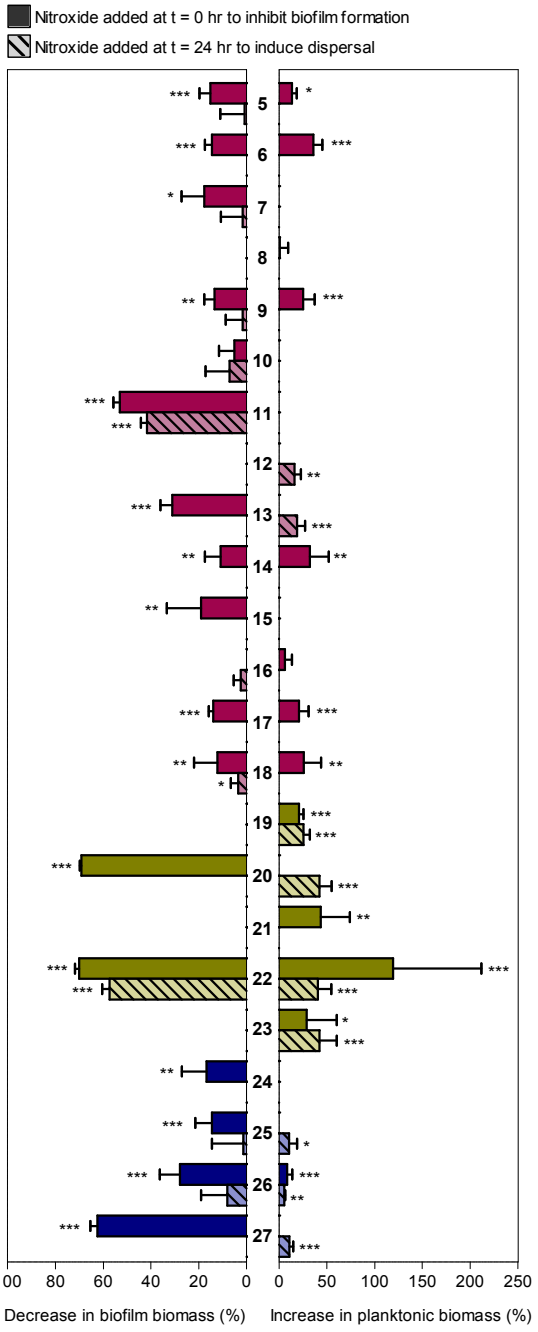

Fig. 1 Collective results of nitroxide candidates **5-27** which showed decreases in biofilm biomass and increases in planktonic biomass at 5 mM in the crystal violet biofilm assay. *P. aeruginosa* was grown in 24 well plates in the presence of nitroxide (5 mM) for 24 hr to examine nitroxide-mediated biofilm inhibition. Alternatively, the nitroxide candidate was added as a 30 min treatment to 24 hr old biofilms to examine nitroxide-induced dispersal. Planktonic biomass was quantified by OD_{600} measurements and biofilm biomass by crystal violet staining and subsequent OD_{590}. *, **, *** denote statistically significant differences compared to DMSO control wells where $p<0.05$, 0.01 and 0.001, respectively (Mann-Whitney t-test: two-tailed, unpaired, equal variance, n = 8); the results of one representative experiment are presented, each was repeated twice.

Effect of 3-(dodecane-1-thiyl)-4-(hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl (22) on biofilm formation and dispersal of mixed-culture biofilms

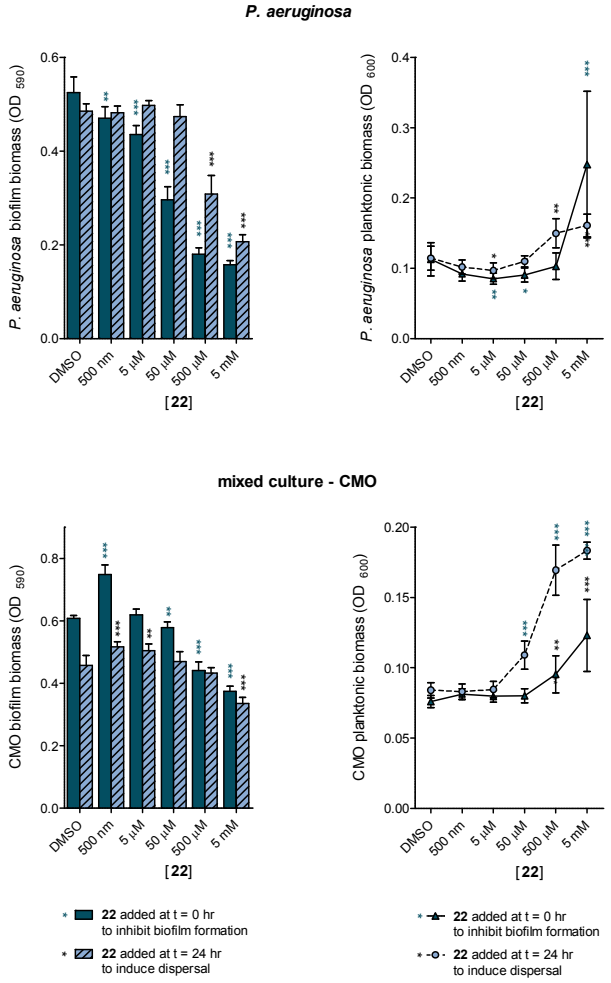

Fig. 2 Nitroxide **22** has anti-biofilm activity, inhibiting biofilm formation and inducing dispersal of *P. aeruginosa* and mixed-culture CMO biofilms. *P. aeruginosa* or CMO was grown in 24 well plates in the presence of **22** for 24 hr to examine **22**-mediated biofilm inhibition. Alternatively, **22** was added as a 30 min treatment to 24 hr old biofilms to examine **22**-induced dispersal. Planktonic biomass was quantified by OD_{600} measurements and biofilm biomass by crystal violet staining and subsequent OD_{590}. *, **, *** denote statistically significant differences compared to DMSO control wells where $p<0.05$, 0.01 and 0.001, respectively (Mann-Whitney t-test: two-tailed, unpaired, equal variance, n = 8); the results of one representative experiment are presented, each was repeated twice.

To extend our investigation into the biofilm modulatory effects of **22** and to confirm their relevance to the treatment of mixed biofilms that may be encountered outside the laboratory, in particular those found in association with biodeteriorated cultural heritage, we sought to determine whether or not nitroxide **22** also exhibited anti-biofilm activity towards organisms derived from cultural materials. Standardized samples of stone, paper and canvas were removed from a collection of sacrificial cultural objects and sonicated in saline solution to retrieve surface-associated microorganisms. To increase cell numbers and cell viability, aliquots of the saline solution were incubated in a series of media. The resulting cultures were then pooled to create a 'Cultural Materials Organisms (CMO) broth.[34] † This mixed-culture of CMO was used in the crystal violet biofilm assay in place of *P. aeruginosa*. A decrease in CMO biofilm biomass of 30-40% was observed in response to 5 mM **22** indicating that nitroxide **22** has greater anti-biofilm activity towards *P. aeruginosa* biofilms (which decreased biofilm biomass by 60-70% under the same experimental conditions) than the more complex mixed-culture CMO biofilms (Fig. 2). This may be attributed to the more complex composition of organisms present in the CMO, compared with the anexic model biofilms. These organisms likely have varied growth rates, metabolic processes, nutrient requirements and glycocalyx composition that alter their sensitivity to **22**.

Effect of 3-(dodecane-1-thiyl)-4-(hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl (22) on cell motility

Cell motility - swimming,[35] twitching,[36] and swarming[37] - are integral cellular functions during biofilm formation in many bacterial species such as *P. aeruginosa*. Loss of motility appendages, such as flagella and type IV pili, in mutant *P. aeruginosa* confers significant reductions in surface attachment rates.[38] Contrastingly motility has also been suggested to be linked to the dispersal of single organisms from biofilms.[39] We therefore sought to investigate if **22** elicits its anti-biofilm activity by interfering with cellular motility by determining the impact of **22** on normal swimming, twitching and swarming motility in *P. aeruginosa* and in CMO.

Utilizing modified motility procedures,[40] M9 minimal media doped with **22** (500 µM and 5 mM) were solidified with agar (0.2% for swimming, 0.5% for swarming and 2% for twitching assays).† *P. aeruginosa* or CMO were inoculated at the centre of the plate and the distance travelled by the microorganisms in response to **22** was compared to DMSO controls.

Table 2 Does **22** interfere with normal swimming, twitching and swarming motility in *P. aeruginosa* and CMO?

	Swimming	Twitching	Swarming
P. aeruginosa	no	yes	yes
CMO	no	not observed[a]	yes

[a] CMO did not exhibit twitching motility under these assay conditions.

Swimming motility, dominant in the planktonic mode of growth was unaffected by nitroxide **22** in either microorganism community (Table 2). Alternatively surface-associated twitching motility in *P. aeruginosa* was enhanced by 18% and 22% in response to 500 µM and 5 mM **22** respectively (Fig. 3A). Twitching plates (not shown) also showed characteristic concentric rings corresponding to periodic rounds of colony expansion and consolidation.[36] In plates doped with **22**, a longer period of colony expansion was observed as indicated by light growth radiating from the centre inoculation point, with a single area of consolidation at the twitch-advancing edge. For

reasons that may be related to nutritional status, cell density or specific intracellular signals,[41] CMO did not exhibit twitching motility under these assay conditions.

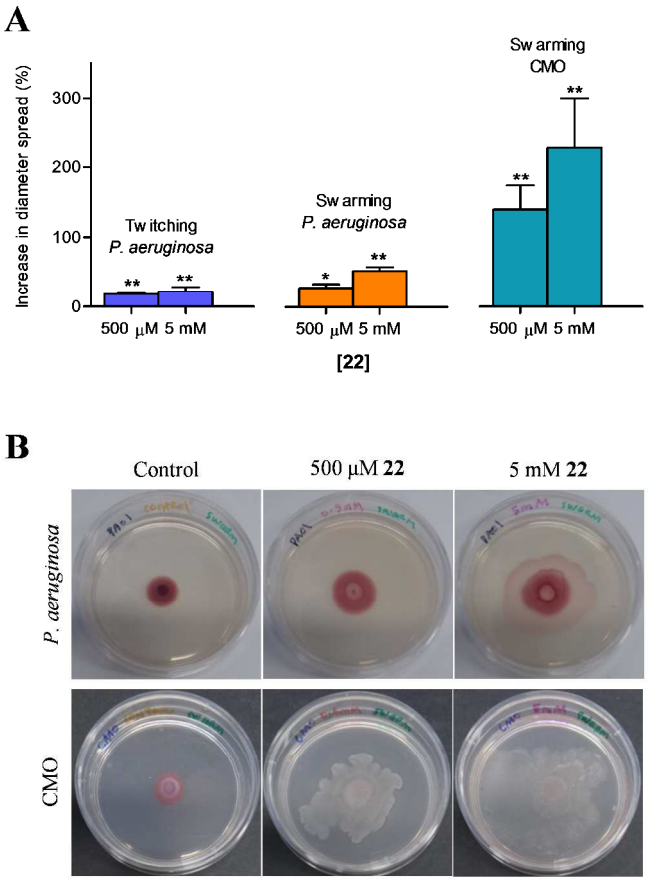

Fig. 3 (**A**) Nitroxide **22** more strongly enhances swarming than twitching in *P. aeruginosa* and **22**-induced CMO swarming is up to 4-fold more enhanced compared to the DMSO control than **22**-induced *P. aeruginosa* swarming. (**B**) Nitroxide **22** enhances swarming motility behaviour in both *P. aeruginosa* and CMO. **22** (500 μM and 5 mM) was added to swarming motility assay agar (0.5%) plates in triplicate. Migration pattern diameters were measured after 5 days of swarming under ambient conditions. *, ** denote statistically significant differences compared to DMSO control plates where $p<0.05$ and 0.01 respectively (Mann-Whitney t-test: two-tailed, unpaired, equal variance). Average +/- standard deviation. Representative images shown are of 2 independent swarming experiments performed per condition.

The diameter of swarm-mediated motility was significantly lengthened by **22** in a concentration-dependent manner in both organism types during a 5 day incubation period under ambient conditions (Fig. 3). In comparison to untreated controls, *P. aeruginosa* displayed a 28% and 53% increase in swarm spread in response to 500 μM and 5 mM **22** respectively (Fig. 3A). Furthermore, the same concentrations of **22** induced a spread-zone increase of 139% and 227% in CMO (Fig. 3A). Under these experimental conditions, the *P. aeruginosa* swarming colonies display a concentric pattern instead of the familiar dendritic pattern often displayed by wild-type *P. aeruginosa*, with regions of dispersal and biofilm consolidation corresponding to the attached bacteria differentiating into motile and non-motile subpopulations respectively (Fig. 3B).[42]

In comparison to *P. aeruginosa*, CMO swarm plates formed branched fractal patterns radiating out from the initial inoculation point in response to **22** (Fig. 3B). This difference in CMO swarming pattern, and the more pronounced swarming by CMO exhibited in response to **22** compared to anexic model organism, P. aeruginosa, is speculated to be attributed to an increased sensitivity of the organisms present in CMO to the biological action of **22**.

The action of **22** on surface-associated motilities, twitching and swarming, offers insight into the anti-biofilm mechanism of **22** observed using the crystal violet biofilm assay. Surface-associated motility has been implicated in biofilm architecture, with limited surface motility resulting in increases in biofilm biomass and more extensive three dimensional architectures.[43] The ability of **22** to suppress biofilm formation may be explained by an overstimulation of twitching and swarming motilities leading to hypermotile cells that are unable to form aggregates or other complex biofilm architectures such as mushroom caps or pillars. Furthermore, **22**-induced dispersal can also be explained by the overstimulation of twitching and swarming motilities leading to detachment of sessile cells from the established biofilm community.[44] The presence of a dodecyl substituent on nitroxide **22**, a structural characteristic shared with the native *las* autoinducer **31**, and antagonists of LasR such as **1**, suggests that **22** may elicit its biological effects through the modulation of *las*-specific QS systems.[45] The importance of the free radical moiety in the biological activity of **22** however also suggests a NO-mimetic mechanism may be at play.

Conclusions

A chemically diverse library of 23 nitroxides was prepared and screened utilizing a crystal violet biofilm assay, in an attempt to discover novel anti-biofilm agents capable of suppressing biofilm formation and inducing dispersal of pre-established biofilms. One nitroxide, 3-(dodecane-1-thiyl)-4-(hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl (**22**) was shown to possess the desired anti-biofilm activity - biofilm inhibition and dispersal – in both *P. aeruginosa* and mixed-culture biofilms derived from cultural material (CMO). Cell motility assays revealed that surface-associated cell motilities – twitching and swarming – closely linked to the biofilm phenotype, were exclusively enhanced by lead nitroxide **22**, leaving the planktonic-specific swimming motility, unaffected. The capability of **22** to selectively enhance twitching and swarming motilities over swimming motility, a vital process for cell survival, may result in less selective pressure and therefore decreased resistance in response to **22**. We speculate that the anti-biofilm activity of **22** is a consequence of motility enhancement, creating hypermotile cells that i) are unable to form surface-associated microcolonies, and ii) are able to more easily detach from pre-formed biofilms. The results presented here have revealed new biological features of nitroxides and highlighted their potential as anti-biofilm agents that can inhibit

biofilm formation and trigger dispersal. While the exact anti-biofilm mechanism of lead nitroxide **22** remains unclear, our findings provide us with insight that will guide further chemical modifications of, and microbiological studies with, **22** and analogues of **22**.

Experimental

(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl)-3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinoxyl (29)

To anhydrous CH_2Cl_2 (30 ml) and 4Å molecular sieves under argon was added 1-bromo-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (**28**)[46] (2.38 mmol, 979 mg) and 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinoxyl (**8**) (2.86 mmol, 532 mg). After stirring at room temperature for 30 minutes, silver carbonate (2.62 mmol, 722 mg) was added and the reaction mixture was stirred in the dark at room temperature for a further 24 hours. The reaction mixture was then filtered through a wet celite pad and the filtrate was washed with sat. aq. $NaHCO_3$ (1 x 15 ml) and sat. aq. NaCl (1 x 15 ml). The organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. The crude material was purified by flash column chromatography (EtOAc/Pet. spirits, 1:1) to give the title compound **29** as a sticky yellow syrup (602 mg, 49%). R_f 0.31 (EtOAc/Pet. spirits, 1:1); MS (ESI^+) *m/z* 539 $[M + Na]^+$; IR (neat): ν_{max} 2977, 1745, 1366, 1210, 1031, 906 cm^{-1}; $[\alpha]^{23}_{589}$ -5.3 (*c.* 1.0, $CHCl_3$); EPR (CH_2Cl_2): triplet, g = 2.0061, a_N = 14.56 G; HRMS (ESI^+) calc. for $C_{23}H_{36}NO_{12}$ 518.22320 $[MH + H]^+$, found 518.22320; HPLC purity analysis: >99% pure, retention time: 27.86 min.

(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-4-Carboxy-2,2,6,6-tetramethyl-1-piperidinoxyl (30)

Compound **30** was prepared by using the procedure described for **29** above. 1-Bromo-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (**28**) (2.39 mmol, 983 mg), 4-carboxy-2,2,6,6-tetramethyl-1-piperidinoxyl (**13**) (2.87 mmol, 574 mg) and silver carbonate (2.63 mmol, 725 mg) in CH_2Cl_2 (30 ml) followed by flash column chromatography (EtOAc/Pet. spirits, 1:1) furnished the title compound **30** as a sticky orange syrup (514 mg, 40%). R_f 0.46 (EtOAc/Pet. spirits, 1:1); MS (ESI^+) *m/z* 553 $[M + Na]^+$; IR (neat): ν_{max} 2974, 2937, 1747, 1442, 1371, 1215, 1164, 1060, 1033, 915 cm^{-1}; $[\alpha]^{24}_{589}$ -1.6 (*c.* 1.0, $CHCl_3$); EPR (CH_2Cl_2): triplet, g = 2.0063, coupling constant a_N = 15.73 G; HRMS (ESI^+) calc. for $C_{24}H_{36}NO_{12}Na$ 553.21297 $[M + Na]^+$, found 553.21283; HPLC purity analysis: 94% pure, retention time: 23.19 min.

(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-3-carboxy-1-ethoxy-2,2,5,5-tetramethylpyrrolidine (29a)

To a solution of nitroxide **29** (0.58 mmol, 300 mg) in anhydrous THF (3 ml) was added triethylborane (1.0 M in THF, 1.7 ml). The solution was allowed to stir under a saturated oxygen atmosphere for 15 minutes. The reaction mixture was concentrated *in vacuo*, and purified by flash column chromatography (EtOAc/Pet. spirits, 1:1) to give the title compound **29a** as a colourless syrup (143 mg, 45%). R_f 0.76 (EtOAc/Pet. spirits, 1:1); MS (ESI^+) *m/z* 546 $[M + H]^+$; IR (neat): ν_{max} 2974, 1749, 1365, 1211, 1032, 905 cm^{-1}; $[\alpha]^{23}_{589}$ -4.1 (*c.* 1.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz): δ_H 5.72 (1H, d, *J* = 8.2 Hz), 5.27-5.08 (3H, m), 4.27 (1H, ddd, *J* = 4.7, 12.5, 17.5 Hz), 4.10 (1H, ddd, *J* = 2.3, 4.4, 12.7 Hz), 3.86-3.80 (1H, m), 3.75 (2H, tt, *J* = 5.4, 10.6 Hz), 2.08 (3H, s), 2.05 (3H, s), 2.02 (3H, s), 2.01 (3H, s), 2.00 (3H, s), 1.31 (3H, s), 1.24 (3H, s), 1.20 (3H, s), 1.13 (6H, td, *J* = 2.7, 7.1 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_c 171.3, 170.73, 170.67, 170.3, 170.2, 169.53, 169.47, 169.3, 169.2, 91.7, 91.6, 73.2, 73.0, 72.8, 72.7, 72.3, 70.3, 70.2, 68.2, 67.8, 61.7, 61.5, 20.84, 20.79, 20.73, 20.71, 14.4; HRMS (ESI^+) calc. for $C_{25}H_{39}NO_{12}Na$ 568.23645 $[M + Na]^+$, found 568.23625

(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-4-Carboxy-1-ethoxy-2,2,6,6-tetramethylpiperidine (30a)

Compound **30a** was prepared by using the procedure described for **29a** above. Nitroxide **30** (0.22 mmol, 114 mg) and triethylborane (1.0 M in THF, 0.67 ml) in THF (3 ml) followed by flash column chromatography (EtOAc/Pet. spirits, 1:1) afforded the title compound **30a** as a colourless syrup (87 mg, 72%). R_f 0.79 (EtOAc/Pet. spirits, 1:1); MS (ESI^+) *m/z* 560 $[M + H]^+$; IR (neat): ν_{max} 2971, 1755, 1742, 1376, 1229, 1209, 1172, 1116, 1080, 1034, 962 cm^{-1}; $[\alpha]^{24}_{589}$ -2.0 (*c.* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz): δ_H 5.72 (1H, d, *J* = 8.3 Hz), 5.42 (1H, t, *J* = 9.4 Hz), 5.16-5.10 (2H, m), 4.29 (1H, dd, *J* = 4.5, 12.5 Hz), 4.10 (1H, dd, *J* = 2.2, 12.5 Hz), 3.84 (1H, ddd, *J* = 2.3, 4.5, 10.1 Hz), 3.75 (2H, q, *J* = 7.1 Hz), 2.66 (1H, tt, *J* = 3.5, 12.7 Hz), 2.08 (3H, s), 2.02 (3H, s), 2.01 (3H, s), 2.00 (3H, s), 1.73-1.49 (4H, m), 1.18 (3H, s), 1.16 (3H, s), 1.11-1.08 (9H, m); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_c 173.7, 170.7, 170.2, 169.5, 169.2, 91.8, 72.9, 72.8, 72.5, 70.3, 68.0, 61.6, 59.2, 59.1, 41.7, 41.0, 34.9, 32.90, 32.86, 20.9, 20.72, 20.70, 20.65, 20.4, 20.3, 13.7; HRMS (ESI^+) calc. for $C_{26}H_{41}NO_{12}Na$ 582.25210 $[M + Na]^+$, found 582.25202.

3-Carboxy-(β-D-glucopyranosyl)-2,2,5,5-tetramethyl-1-pyrrolidinoxyl (15)

To a cooled (0°C) solution of 3-carboxy-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-2,2,5,5-tetramethyl-1-pyrrolidinoxyl (**29**) (0.58 mmol, 300 mg) in anhydrous CH_3OH (10 ml) was added sodium metal (2.44 mmol, 56 mg) portion-wise. The solution was then stirred at room temperature for 15 minutes and quenched with Amberlite resin (H^+ form) until the solution was acidic (pH 1). The reaction mixture was filtered through celite, evaporated *in vacuo* and purified by flash column chromatography ($EtOAc/CH_3OH/H_2O$, 7:2:1) to give the title compound **15** as an orange syrup (73 mg, 36%). R_f 0.26 ($EtOAc/CH_3OH/H_2O$, 7:2:1); MS (ESI^+) *m/z* 350 $[MH + H]^+$; IR (neat): ν_{max} 3298, 1661, 1399, 1030 cm^{-1}; $[\alpha]^{23}_{589}$ +65.2 (*c.* 0.7, CH_3OH); EPR (CH_2Cl_2): triplet, g = 2.0061, a_N = 14.84 G; HRMS (ESI^+) calc. for $C_{15}H_{28}NO_8$ 350.18094 $[MH + H]^+$, found 350.18086; HPLC purity analysis: 97% pure, retention time: 9.95 min. Data are in agreement with the literature.[47]

3-Carboxy-(β-L-glucopyranosyl)-2,2,5,5-tetramethyl-1-pyrrolidinoxyl (17) was prepared from 1-bromo-2,3,4,6-tetra-*O*-acetyl-α-L-glucopyranose in identical fashion to that for **15**. All characterization data for **17** - including IR spectra, ESI-MS, high resolution ESI-MS and EPR spectra - are identical to **15**. $[\alpha]^{23}_{589}$ –59.4 (*c.* 1.0, CH_3OH)

4-Carboxy-(β-D-glucopyranosyl)-2,2,6,6-tetramethyl-1-piperidinoxyl (16)
Compound **16** was prepared by using the procedure described for the preparation of **15**. 4-Carboxy-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-2,2,6,6-tetramethyl-1-piperidinoxyl (**30**) (0.39 mmol, 209 mg) and sodium metal (1.66 mmol, 38 mg) in CH_3OH (10 ml) followed by flash column chromatography ($EtOAc/CH_3OH/H_2O$, 7:2:1) furnished the title compound **16** as an orange syrup (39 mg, 27%). R_f 0.27 ($EtOAc/CH_3OH/H_2O$, 7:2:1); MS (ESI^+) *m/z* 385 $[M + Na]^+$; IR (neat): ν_{max} 3299, 2927, 1360, 1007 cm^{-1}; $[\alpha]^{23}_{589}$ +37.2 (*c.* 1.0, CH_3OH); EPR (CH_2Cl_2): triplet, g = 2.0060, a_N = 14.07 G; HPLC purity analysis: 97% pure, retention time: 11.15 min.

4-Carboxy-(β-D-glucopyranosyl)-2,2,6,6-tetramethyl-1-piperidinoxyl (18) was prepared from 1-bromo-2,3,4,6-tetra-*O*-acetyl-α-L-glucopyranose in identical fashion to that for **16**. All characterization data for **18** - including IR spectra, ESI-MS, high resolution ESI-MS and EPR spectra - are identical to **16**. $[\alpha]^{23}_{589}$ –40.9 (*c.* 1.0, CH_3OH)

4-*O*-Dodecyl-2,2,6,6-tetramethyl-1-piperidinoxyl (20)
To 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinoxyl (**11**)[24g,24h] (2.9 mmol, 500 mg) in anhydrous THF (15 ml) was added NaH (60 % mineral oil, 5.8 mmol, 140 mg). The reaction mixture was refluxed for 30 minutes, at which time 1-bromododecane (4.4 mmol, 1.0 ml) was added. After refluxing for 48 hours, the solvent was removed *in vacuo* and the resultant residue was dissolved in CH_2Cl_2, washed with H_2O, dried (Na_2SO_4) and concentrated. Preparatory TLC (10% EtOAc in hexane) afforded 4-*O*-dodecyl-2,2,6,6-tetramethyl-1-piperidinoxyl (**19**) as a red oil (263.5 mg, 27%). R_f 0.29 (hexane/EtOAc, 4:1); MS (ESI^+) *m/z* 341 $[M + H]^+$; IR (neat): ν_{max} 29245, 2854, 1739, 1461, 1375, 1363, 1218, 1178, 1102, 722, 681 cm^{-1}; EPR (CH_2Cl_2): triplet, g = 2.0063, a_N = 15.62 G; HPLC purity analysis: >99% pure, retention time: 19.64 min.

4-*O*-Dodecyl-1-ethoxy-2,2,6,6-tetramethylpiperidine (20a)
Compound **20a** was prepared by using the procedure described for **29a** above. Nitroxide **20** (0.28 mmol, 95 mg) and triethylborane (1.0 M in THF, 0.84 mmol, 0.8 ml) in THF (10 ml) and purification by preparatory TLC (EtOAc/hexane, 1:9) afforded the title compound **20a** as a colourless oil (40 mg, 39%). R_f 0.72 (EtOAc/hexane, 1:9); MS ($ESI)^+$ *m/z* 370 $[M + H]^+$; IR (neat): ν_{max} 2924, 2854, 1457, 1372, 1360, 1102, 1040, 961, 804, 726 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ_H 3.76 (2H, q, *J* = 7.1 Hz), 3.50 (1H, tt, *J* = 4.1, 11.3 Hz), 3.39 (2H, t, *J* = 6.8 Hz), 1.84-1.79 (2H, m), 1.56-1.50 (2H, m), 1.39 (2H, t, *J* = 11.9 Hz), 1.32-1.25 (18H, m), 1.17 (6H, s), 1.13 (6H, s), 1.10 (3H, t, *J* = 7.1 Hz), 0.88 (3H, t, *J* = 7.0 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 72.4, 70.5, 68.4, 59.9, 45.2, 33.4, 32.1, 30.4, 29.82, 29.80, 29.76 (2C), 29.6, 29.5, 26.4, 22.8, 21.2, 14.3, 13.8.

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Notes and references

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† Electronic Supplementary Information (ESI) available: General synthetic information; HPLC traces and EPR spectra for compounds **29**, **30**, **15**, **16** and **20**; ^1H and ^{13}C NMR spectra of compounds **29a**, **30a** and **20a**; biological materials and methods. For ESI in electronic format see DOI: 10.1039/b000000x/

§ 2,2,6,6-Tetramethyl-1-piperidinoxyl (TEMPO) **9** was obtained from commercial sources (Sigma).

‡ Motility media vary in agar concentration depending on the motility of interest: 0.2% (w/v) for swimming, 0.5% (w/v) for swarming and 1% (w/v) for twitching motilities.

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