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ARTICLE TYPE

Controlling Biofilms on Cultural Materials: The Role of 3-(Dodecane-1-thiyl)-4-(hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl†

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3-(Dodecanethiyl)-4-(hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl (5) effectively disperses biofilms of relevance to cultural materials while preventing their formation.

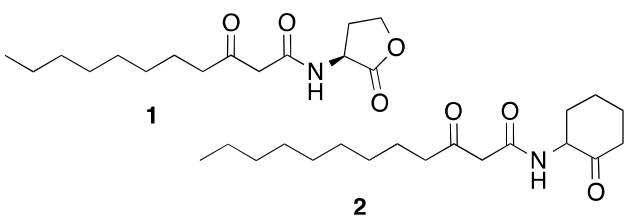
Bacterial biofilms present major challenges in areas as diverse as medicine, food, sewerage and cultural materials conservation and much research has been directed toward the control of these bacterial assemblagess.[1] In the context of cultural materials, these bacterial colonies can lead to biodeterioration of objects of significance and this can manifest itself in the form of undesirable changes to the material leading to disfigurement and degradation. Recent research into the mechanisms of biodeterioration of cultural materials suggest that bacterial microorganisms play a significant role as primary colonisers, modifying substrates and providing nutrients for successive colonisers that include mould.[2,3]

During the life-cycle of a biofilm, once a sufficient number of bacterial cells have attached themselves to a surface, the genes that trigger biofilm growth are activated, and the colony begins to produce a glycocalyx coating in order to protect itself from external threats and maintain the increasingly complex architecture of the biofilm.[4] The glycocalyx coating (exopolymeric matrix) consisting of polysaccharides, proteins and nucleic acids is the primary reason that harsh conditions (abrasion, biocides) are usually required to eradicate biofilms.[5–7] In dealing with cultural materials there is the added requirement that any remedial treatment not impact adversely on the object, and this necessitates an approach that minimises the use of physically and chemically harsh treatments.

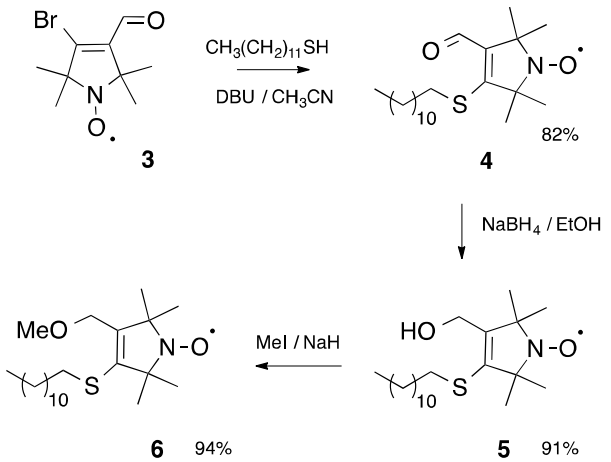
Bacteria are about 1000 times more vulnerable to biocides in their planktonic state,[8] and remediation techniques that induce biofilm dispersal prior to the application of a biocide are attractive because of the potential reduction in biocide load required to eradicate the colony. In addition, this approach offers the conservator a less-invasive and more sustainable protocol for dealing with biofilms on cultural heritage.

Nitric oxide (NO) is an important signalling molecule that is involved in the modulation of "quorum sensing" (QS), a method of cell-to-cell communication, that bacterial colonies employ in order to survive as communal organisms.[9,10] Controlled application of NO donors to biofilms has been shown to be an effective method for preventing biofilm formation and eliciting bacterial dispersal.[11]

Swarming motility, a form of surface translocation that depends on extensive flagellation, biosurfactants and cell-to-cell contact, is a distinct multicellular and cooperative growth state that has been associated with the propensity of microorganisms to form into, and disperse from biofilms.[12,13] There is also evidence to suggest that an increase in surface motility such as swarming, is associated with the prevention of biofilm development because hypermotile cells are unable to form complex biofilm architectures.[14] Furthermore, enhancement of surface-associated motilities in established biofilm communities may lead to dispersal.[15–17] Swarming motility and biofilm formation in *Pseudomonas aeruginosa* can be controlled by inhibiting the effect of QS autoinducers such as *N*-(3-oxododecanoyl)-L-homoserine lactone **1**.[5,18] High throughput screening identified a number of agonists and antagonists of the *P. aeruginosa* autoinducer **1**, with **2** being a particularly effective antagonist



1 **2**



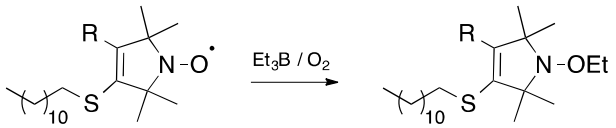
Scheme 1.

with anti-biofilm properties.[19]

Recently Hancock and co-workers showed that some nitroxides (aminoxyl radicals) are able to disperse biofilms and suggested that this was due to the structural similarity between these stable free radicals and NO itself.[20] Interestingly, despite observing both inhibition of biofilm formation and dispersal when treated with nitroxides, these authors reported no effect on the swarming motility of wild-type *P. aeruginosa*.[20]

Given the apparent anti-biofilm properties of nitroxides and the dispersal benefits that are derived from increases in bacterial swarming motility, we reasoned that a nitroxide that also inhibited the activity of *P. aeruginosa* autoinducer **1** would lead to a superior treatment option for biofilms associated with objects of cultural significance.

In previous work, we had prepared novel pyrrolinoxyl-substituted AT_1 receptor antagonists that proved to be effective antihypertensives.[21] With well-established chemistry available to us, we chose to prepare and examine the anti-biofilm properties of dodecanethiyl-substituted pyrrolinoxyl radicals **4** – **6**, compounds that combined some of the key structural features of known inhibitors with the nitroxide moiety; these compounds were prepared from the known aldehyde **3** as detailed in Scheme 1.



4 – 6 **7 – 9**

Scheme 2.

Treatment of **3** with 1-dodecanethiol in the presence of DBU afforded the sulfide **4** in good yield following chromatography. Subsequent reduction of the aldehyde moiety with sodium borohydride gave the alcohol **5** which was converted into the ether **6** by the action of iodomethane and base.

Because of their paramagnetic nature, all novel nitroxides were fully characterised by EPR spectroscopy, and by conversion into the corresponding ethoxylamine **7** – **9** by reaction with ethyl radicals generated from triethylborane and oxygen (Scheme 2);† the EPR spectrum of **5** is shown in Figure 1. The structure of

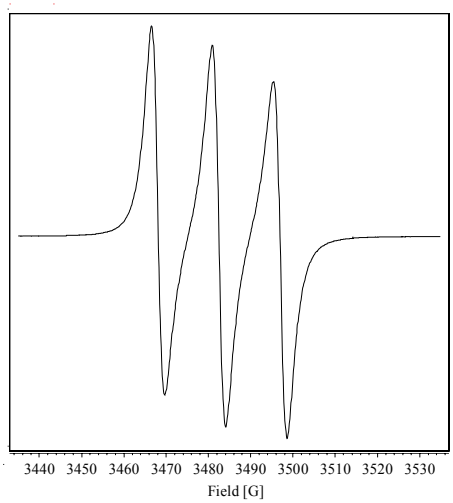


Figure 1. EPR spectrum of compound **5**.

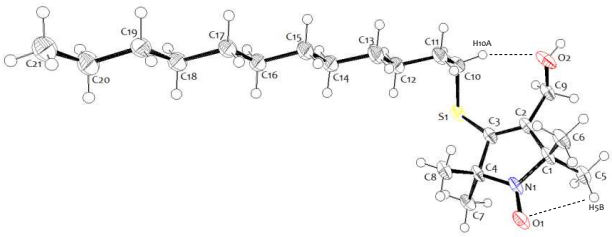
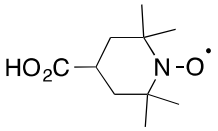


Figure 2. X-ray crystal structure of 3-(dodecane-1-thiyl)-4-(hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl (**5**) showing the atom numbering scheme and the intramolecular hydrogen bonds (dashed lines). Displacement ellipsoids are drawn in the 50% probability level and hydrogen atoms are shown as small spheres of arbitrary radii.

alcohol **5** was confirmed by X-ray crystallography and a perspective view of **5** showing the atom numbering scheme and the intramolecular bonds [C10-H10A...O2 = 2.52 Å and C5-H5B...O1 = 2.61 Å] is given in Figure 2. Nitroxide **5** was found to crystallize in the triclinic space group ($P\bar{1}$) at 130(1) K with bond distances and angles pertaining to the pyrroline ring similar to those found in literature molecules containing the same ring system.[22,23]

Batch-culture biofilm assays were performed to assess the efficacy of compounds **4** – **6** and **8** as anti-biofilm agents against the model organism *P. aeruginosa* PA01. To target the initial attachment phase of biofilm development, and following a method adapted from Barraud *et al*.,[24] **4** – **6**, **8** (500 nM – 5 mM) were added to the growth medium together with *P. aeruginosa* and incubated for 24h (preventative treatment model - full details in the ESI).† To assess the ability of these compounds to elicit dispersal of developed biofilms, a reactive treatment model was used in which biofilms were first grown over a 24h period prior to the addition of compound. Dispersal candidates were then added and the biofilm incubated for a further 30 minutes.†

In both treatment models, changes to the amounts of planktonic and biofilm mass were determined quantitatively by comparing the optical density of treated cells to DMSO treated control cells using a microplate fluorometer following well-establish protocols (see ESI).†[25–27] The results from these studies are summarised in Figure 3 together with that of "carboxy-TEMPO" **10** (control), a key compound used in the study of Hancock and coworkers.[20]



10

Inspection of Figure 1 reveals that while we do observe a decrease in biofilm mass in response to the control compound **10** (5 mM) in the preventive model, in our experiments this decrease is not accompanied by an increase in planktonic biomass suggesting that **10** is inhibiting planktonic cell growth, which is undesirable as this may, in the long term, lead to the development of tolerance towards **10**. Figure 3 also shows that **10** is not successful at inducing dispersal of a pre-formed *P. aeruginosa* biofilm. On the other hand, compounds **4** and **6** appear not to decrease biofilm biomass in either treatment model, but instead appear to stimulate planktonic bacterial growth. Further analysis

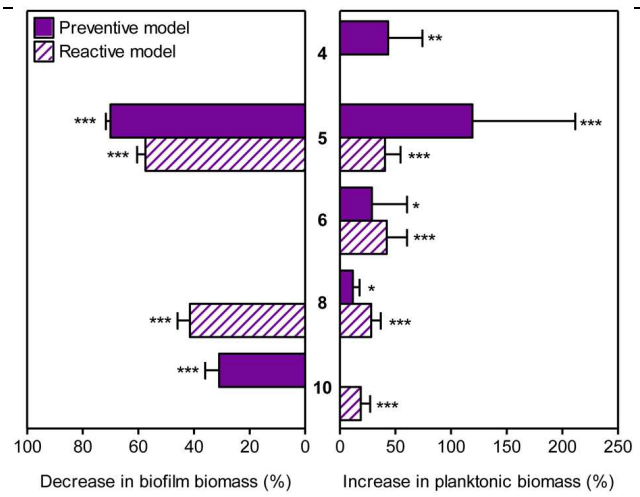


Figure 3. Performance of compounds **4** – **6**, **8**, **10** (5 mM) as anti-biofilm agents in both preventative and reactive treatment models.

(not shown - see ESI) reveals that these two compounds actually catalyse an increase in biofilm mass. Compounds **4**, **6** and **10** are essentially ineffective at eliciting any response at lower concentrations (data not shown - see ESI).†

To our surprise and delight, the alcohol **5** elicited a significant reduction in biofilm mass in both treatment models, while concomitantly stimulating an increase in planktonic biomass. Indeed, **5** began to supress biofilm development at 50 μM and exhibited dispersal at 500 μM (see ESI),† significantly lower concentrations than for which any effect was observable for the remaining compounds. This nitroxide, is therefore clearly able to inhibit formation and disperse *P. aeruginosa* biofilms in both models as it significantly reduces biofilm mass while increasing the number of planktonic cells. It is interesting to note that the ethoxylamine derivative of **5** (**8**) appears to induce biofilm dispersal (at 5 mM only) while not inhibiting biofilm formation, clearly highlighting the importance of the nitroxide moiety in the effectiveness of **5**.

To assess swarming motility, **5** was added to M9 minimum medium solidified with 0.5% agar. 2,3,5-Triphenyltetrazolium chloride (TTC, 1%) was added to the agar (as a vital dye) immediately before plate pouring to enhance visualization. *P. aeruginosa* were inoculated at the centre of the plate and the distances travelled by bacteria in response to **5** at 500 μM and 5 mM (5 days at ambient temperature) were compared to DMSO control samples.[29] Full details in ESI.† All experiments were performed in triplicate. As clearly evident in Figure 2, nitroxide **5** elicits a statistically significant increase in swarming motility in *P. aeruginosa*.

Since **5** proved effective against *P. aeruginosa*, we chose to examine its effectiveness against organisms associated with the biodeterioration of a cultural object. We identified the outdoor wooden sculpture *So Its Come To This* (1986) by Bruce Armstrong, currently located on the main campus of the University of Melbourne, as a suitable object for sampling and organisms were sampled (with permission) from a deteriorated area using a sterile cotton swab. These organisms were then incubated in separate wells together with nitroxide **5** (500 nM – 5 mM) (see ESI)† following the preventative treatment model protocol and the planktonic and biofilm masses quantified as

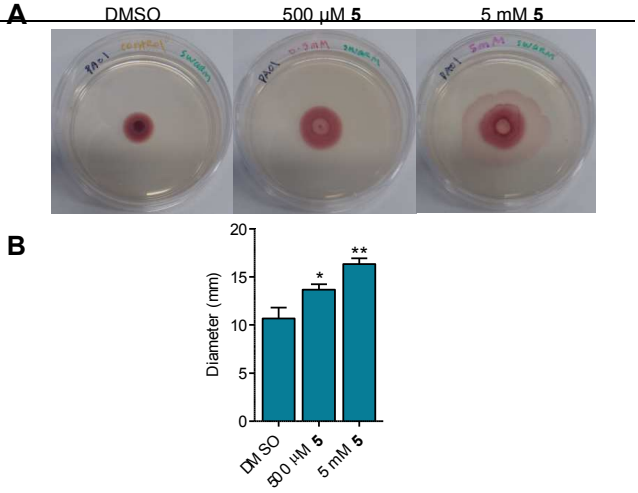


Figure 4. Images showing how nitroxide **5** enhances swarming behaviour in *P. aeruginosa*. **5** (500 μM and 5 mM) was added to swarming motility assay (0.5% agar) plates in triplicate (**A**); Migration pattern diameters were measured after 5 days of swarming under ambient conditions (**B**). For details, see text.

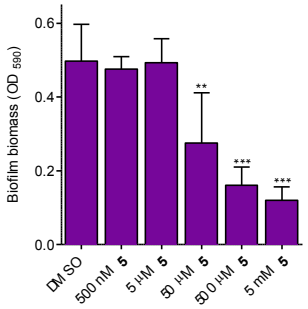


Figure 5. The performance of nitroxide **5** against biofilms grown from bacteria sampled from a deteriorated region of an outdoor wooden sculpture showing **5** reduces biofilm biomass.

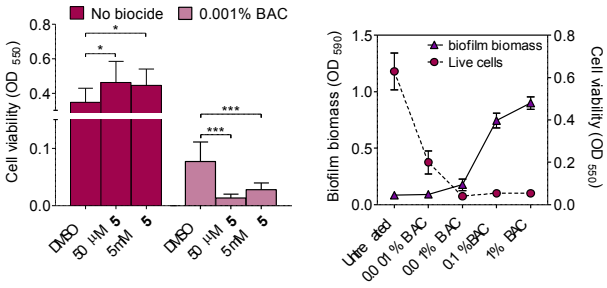


Figure 6. Summary of the performance of BAC and **5** against biofilms grown from bacteria sampled from a deteriorated region of an outdoor wooden sculpture. Nitroxide **5** increases planktonic biomass (no biocide) and effectively eradicates planktonic bacteria when used in combination with BAC (0.001% w/v) (left). BAC concentrations higher than 0.001% were identified to induce biofilm formation (right).

described above. The results of these assays (Figure 3) clearly show that **5** is able to disperse a biofilm of organisms found on cultural materials at concentrations of 50 μM and above. When combined with a commercially-available biocide commonly used in the treatment of cultural materials (benzalkonium chloride, BAC), biofilms were effectively eradicated after 24h treatment with **5** (50 μM) followed by 2h treatment with BAC (0.001 %

w/v) (Figure 4, left panel). It should be noted that this result was achieved using a concentration of BAC that is significantly lower than that (2 % w/v) recommended by other heritage professionals,[30] and that higher concentrations of BAC (>0.001% w/v) induced the formation of biofilms (Figure 4, right panel).

We speculate that the anti-biofilm activity of **5** and the enhancement of swarming motility in response to **5** are induced through similar mechanisms. For example, an enhancement in hypermotility will lead to cells that are less able to form complex biofilm architectures and are more likely to detach from pre-formed biofilms.[31] Alternatively, **5**–mediated stimulation of biosurfactant production and/or secretion through QS may decrease the surface tension between the migrating cells and the surface resulting in enhanced swarming motility and dispersal, leading to a decrease in biofilm formation.[32] Studies into the mode of action of **5** are currently underway and will be reported in due course.

In summary, 3-(dodecanethiyl)-4-(hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl **5** is an anti-biofilm compound that effectively disperses biofilms from *P. aeruginosa* while preventing biofilm formation. When applied to biofilms grown from bacteria associated with the biodeterioration of an outdoor wooden sculpture, **5** was also effective at inducing dispersal. In combination with low concentrations of biocide, these biofilms can be effectively eradicated.

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Notes and references

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†Electronic Supplementary Information (ESI): experimental details for the preparation of **4** – **9**; HPLC traces and EPR spectra for compounds 4 – **6**; ^{1}H and ^{13}C NMR spectra of compounds **7** – **9**; biological materials and methods for biofilm batch-culture assays and motility studies. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/b000000x/

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