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<th>Journal:</th>
<th>Organic &amp; Biomolecular Chemistry</th>
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<tr>
<td>Manuscript ID:</td>
<td>OB-ART-02-2015-000284.R1</td>
</tr>
<tr>
<td>Article Type:</td>
<td>Paper</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>17-Mar-2015</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
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<td>Schiesser, Carl; The University of Melbourne, School of Chemistry</td>
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Nitroxides as anti-biofilm compounds for the treatment of *Pseudomonas aeruginosa* and mixed-culture biofilms

Stefanie-Ann Alexander, a,b Caroline Kyi, a,b and Carl H. Schiesser* a,b

A series of 23 nitroxides (5-27) was tested for biofilm modulatory activity using a crystal violet staining technique. 3-(Dodecan-1-thyl)-4-(hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrroloxynyl (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. Moreover, the growth of biofilms and the production of harmful metabolites by microorganisms cause considerable aesthetic and structural damage to culturally significant materials. 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. Conventional methods for the control of biofilm formation and for the remedial treatment of biodeteriorated materials have largely focused on biocidal and antibacterial approaches, however these strategies have limited effectiveness due to the refractory nature of biofilms and the development of resistance.

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. 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. 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. 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. 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.

Anti-biofilm compounds, such as N-acyl homoserine lactone (AHL) analogues (1 – 3), 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
compounds have been found to increase the effectiveness of antimicrobial agents.\textsuperscript{12}

Another anti-biofilm compound that has been shown to modulate biofilm development is the signalling molecule, nitric oxide (NO).\textsuperscript{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 \textit{P. aeruginosa} biofilms while effecting their dispersal.\textsuperscript{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.\textsuperscript{15}

Nitroxides are related compound that are electronically similar to NO,\textsuperscript{16} and as such, it has been suggested that they may offer a more viable alternative to NO for the control of biofilms.\textsuperscript{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.\textsuperscript{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 \textit{P. aeruginosa}.\textsuperscript{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 \textit{P. aeruginosa}.\textsuperscript{17} Furthermore, they showed that these nitroxides were able to restore swarming motility in a mutant strain of \textit{P. aeruginosa} deficient in nitrite reductase (and therefore deficient in NO).\textsuperscript{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 \textit{P. aeruginosa} biofilms; Linington and co-workers identified that the antibiotic skylamycins are inhibitors of biofilm formation as well as inducers of biofilm detachment in \textit{P. aeruginosa}.\textsuperscript{19} Very recently, these same authors reported the development of benzox[1,4]oxazines (eg. 4) as biofilm inhibitor and dispersal agents against \textit{Vibrio cholerae}.\textsuperscript{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".\textsuperscript{20}

Herein we report our investigations into nitroxides as compounds that control the development of \textit{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.\textsuperscript{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.\textsuperscript{22}

Nitroxide design and synthesis

Nitroxides have been studied previously in biological systems as antioxidants\textsuperscript{23} or as spin labels.\textsuperscript{16a} Through the use of EPR experiments nitroxides such as 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolinoyl (6) were shown to pass through the membrane into the cytoplasmic space.\textsuperscript{21} With this in mind, intracellular-targeted nitroxides 5 – 18 were prepared in order to determine
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 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). 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 stearates28 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 1.

The synthesis of dodecanethiol-substituted pyrrolinoyl radicals 20 – 22 has been described in a previous communication.30 Installation of the dodecyl chain onto the pyrrolinoyl "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)-1-homoserine lactone (31).31

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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 assay22 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. 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 and 22) -mediated biofilm dispersal at 500 μM only - not shown. With the exception of the 1-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.

<table>
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<th>Nitroxides</th>
<th>% biofilm suppression</th>
<th>% enhancement in dispersal</th>
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<tr>
<td>5</td>
<td>15</td>
<td>not observed</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>not observed</td>
</tr>
<tr>
<td>9</td>
<td>13</td>
<td>not observed</td>
</tr>
<tr>
<td>14</td>
<td>11</td>
<td>not observed</td>
</tr>
<tr>
<td>17</td>
<td>14</td>
<td>not observed</td>
</tr>
<tr>
<td>18</td>
<td>12</td>
<td>not observed</td>
</tr>
<tr>
<td>20</td>
<td>not observed</td>
<td>21</td>
</tr>
<tr>
<td>22</td>
<td>70</td>
<td>57</td>
</tr>
<tr>
<td>26</td>
<td>28</td>
<td>not observed</td>
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*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 antibiofilm 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. Antibiofilm 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 measurements and biofilm biomass by crystal violet staining and subsequent OD measurements. * *, *** 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.
Biofilms that may be encountered outside the laboratory, in particular those found in association with biodeteriorated materials. To collect a sacrificial cultural objects and sonicated in saline solution were incubated in a series of media. The resulting cultures were then pooled to create a ‘Cultural Materials Organisms (CMO) broth. 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 anoxic 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-thyl)-4-(hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl (22) on cell motility

Cell motility - swimming, twitching, and swarming - 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. Contrastingly motility has also been suggested to be linked to the dispersal of single organisms from biofilms. 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, 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?

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<th>Swimming</th>
<th>Twitching</th>
<th>Swarming</th>
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<tr>
<td>P. aeruginosa</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>CMO</td>
<td>no</td>
<td>not observed</td>
<td>yes</td>
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* CMO did not exhibit twitching motility under these assay conditions.

Table 2

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. 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, CMO did not exhibit twitching motility under these assay conditions.

**In comparison to P. aeruginosa, CMO**

![Image](image_url)

**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).

**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-(dodecan-1-thyl)-4-(hydroxymethyl)-2,5,5-tetramethyl-1-pyrrolinoyl (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 antibiofilm 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$_2$Cl$_2$ (30 ml) and 4Å molecular sieves under argon was added 1-bromo-2,3,4,6-tetra-O-acetyl-α-D-glucopyranose (28) ($R_t$ 0.31 (EtOAc/Pet. spirits, 1:1); MS (ESI$^+$) $m/z$ 539 [M + Na$^+$]; IR (neat): $\nu_{max}$ 2977, 1745, 1366, 1210, 1031, 906 cm$^{-1}$; $\delta_{29}$ $^{13}$C$_{NMR}$ 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$_{36}$Na$_2$O$_{12}$ 568.23645 [M + Na$^+$], found 568.23625)

Compounds 29a was prepared by 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%). $R_t$ 0.79 (EtOAc/Pet. spirits, 1:1); MS (ESI$^+$) $m/z$ 560 [M + H$^+$]; IR (neat): $\nu_{max}$ 2971, 1755, 1742, 1376, 1229, 1209, 1172, 1116, 1080, 1034, 962 cm$^{-1}$; $\delta_{29}$ $^{13}$C$_{NMR}$ 171.37, 170.7, 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$_{36}$Na$_2$O$_{12}$ 568.23645 [M + Na$^+$], found 568.23625

3-Carboxy-β-(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$_3$OH (10 ml) was added sodium metal (2.44 mmol, 979 mg) and 3pcarboxyp(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl) (15) (3.75 (2H, s), 2.08 (3H, s), 2.05 (3H, s), 2.02 (3H, s), 2.01 (3H, s), 0.00 (3H, s), 1.31 (3H, s), 1.24 (3H, s), 1.20 (3H, s), 1.13 (6H, td, $J = 2.5, 7.1$ Hz); 1$^3$C NMR (CDCl$_3$, 125 MHz): $\delta_1$ 173.7, 170.7, 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$_{36}$Na$_2$O$_{12}$ 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)

To anhydrous CH$_2$Cl$_2$ (30 ml) and 4Å molecular sieves under argon was added 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) (2.86 mmol, 552 mg) and 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$_2$SO$_4$) 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%). $R_t$ 5.3 ($J = 15.73$ G; HRMS (ESI$^+$) $m/z$ 553.21283; HPLC purity analysis: 94% pure, retention time: 27.86 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 left 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%). $R_t$ 0.76 (EtOAc/Pet. spirits, 1:1); MS (ESI$^+$) $m/z$ 546 [M + H$^+$]; IR (neat): $\nu_{max}$ 2974, 1749, 1365, 1211, 1032, 905 cm$^{-1}$; $\delta_{29}$ $^{13}$C$_{NMR}$ 171.2, 170.8, 170.6, 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$_{36}$NO$_{12}$ 568.23645 [M + Na$^+$], found 568.23625
3-Carboxy-(β-L-glucopyranosyl)-2,2,5,5-tetramethyl-1-pyrrolidinyl (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. [α]<sub>23</sub><sup>sp3</sup> = −59.4 (c. 1.0, CH₃OH).

4-Carboxy-(β-D-glucopyranosyl)-2,2,6,6-tetramethyl-1-piperidinyl (16)

Compound 16 was prepared by using the procedure described for the preparation of 15. 4-Carboxy-(2,3,4,6-tetra-O-β-D-glucopyranosyl)-2,2,6,6-tetramethyl-1-piperidinyl (30) (0.39 mmol, 209 mg) and sodium metal (1.66 mmol, 38 mg) in CH₂OH (10 ml) followed by flash column chromatography (EtOAc/CH₂Cl₂; MS (ESI) <sup>+</sup> m/z 385 [M + Na]<sup>+</sup>; [α]<sub>R</sub> = 14.07 G; HPLC purity analysis: >99% pure, retention time: 19.64 min.

4-Carboxy-(β-D-glucopyranosyl)-2,2,6,6-tetramethyl-1-piperidinyl (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. [α]<sub>23</sub><sup>sp3</sup> = −40.9 (c. 1.0, CH₃OH).

4-O-Dodecyl-2,2,6,6-tetramethyl-1-piperidinyl (20)

To 4-hydroxy-2,2,6,6-tetramethylpiperidinyl (11)<sup>24h</sup> (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 CH₂Cl₂, washed with H₂O, dried (Na₂SO₄) and concentrated. Preparatory TLC (10% EtOAc in hexane) afforded 4-O-dodecyl-2,2,6,6-tetramethyl-1-piperidinyl (19) as a red oil (263.5 mg, 27%). R<sub>f</sub> 0.29 (hexane/EtOAc, 4:1); MS (ESI<sup>+</sup>) m/z 341 [M + H]<sup>+</sup>; IR (neat): ν<sub>max</sub> 2924, 2854, 1457, 1372, 1360, 1102, 1040, 722, 681 cm<sup>−1</sup>; EPR (CH₂Cl₂): triplet, g = 2.0663, a<sub>0</sub> = 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<sub>f</sub> 0.72 (EtOAc/hexane, 1:9); MS (ESI<sup>+</sup>) m/z 370 [M + H]<sup>+</sup>; IR (neat): ν<sub>max</sub> 2924, 2854, 1457, 1372, 1360, 1102, 1040, 961, 804, 726 cm<sup>−1</sup>; ¹H NMR (CDCl₃, 500 MHz): δ<sub>H</sub> 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); ¹³C NMR (CDCl₃, 125 MHz): δ<sub>C</sub> 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.

Acknowledgements

We thank the Australian Research Council through the Centre of Excellence Schemes and the Albert Shimmmins Memorial Fund for generous financial support.

Notes and references


