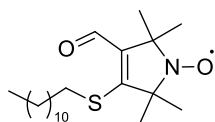


**Controlling Biofilms on Cultural Materials:
The Role of 3-(Dodecane-1-thiyl)-4-(hydroxymethyl)-2,2,5,5-
tetramethyl-1-Pyrrolinoxyl†**

Stefanie-Ann Alexander,^{a,b} Emma M. Rouse,^c Nicole Tse^c and Carl H. Schiesser^{*ab}

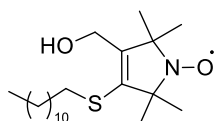
Electronic Supplementary Information

Experimental procedures for compounds **4** – **9**; HPLC traces and EPR spectra for compounds **4** – **6**; ¹H and ¹³C NMR spectra of compounds **7** - **9**; biological materials and methods and biofilm batch-culture assay results for compounds **4** - **6**, **8** and **10** (18 pages).



3-(Dodecane-1-thiyl)-4-formyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl (4)

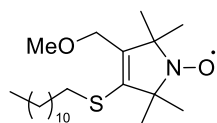
To a cooled (0°C) mixture of 3-bromo-4-formyl-2,2,5,5-tetramethyl pyrrolidine-1-oxyl (**3**)¹⁻⁴ (500 mg, 2.0 mmol) and 1-dodecanethiol (480 μ l, 2.0 mmol) in anhydrous CH₃CN (6 ml) was added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (330 μ l, 2.2 mmol) drop-wise. The reaction mixture was left without stirring for 1 hour. The solvent was removed *in vacuo* and the resulting residue was dissolved in EtOAc (10 ml) and washed with H₂O (2 x 5 ml) and sat. aq. NaCl (1 x 5 ml). The organic phase was separated, dried (MgSO₄), and concentrated *in vacuo*. Flash column chromatography (EtOAc in hexane gradient, 0 %, 5 %, 10%) furnished the title compound **4** as an orange solid (611 mg, 1.64 mmol, 82%). R_f 0.57 (hexane/EtOAc, 8:1); MP 32-35 °C; MS (ESI⁺) m/z 369 [M + H]⁺; IR (neat): ν_{\max} 2912, 2849, 1665 cm⁻¹; EPR (CH₂Cl₂): triplet, g = 2.0056, a_N = 14.55 G; HRMS (ESI⁺) calc. For C₂₁H₃₈NO₂SNa [M + Na]⁺ 391.25155, found 391.25152; HPLC purity analysis: >99% pure, retention time: 18.18 min.



3-(Dodecane-1-thiyl)-4-hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl (5)

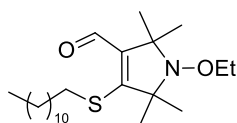
To a cooled (0°C) solution of **4** (570 mg, 1.5 mmol) in anhydrous EtOH (6 ml) was added NaBH₄ (18 mg, 0.5 mmol) portion-wise. After 30 minutes stirring at 0°C, the reaction mixture was diluted with CH₂Cl₂ (6 ml) and the phases separated. The aqueous phase was further extracted with CH₂Cl₂ (2 x 3 ml) and the combined organic phases were dried (MgSO₄) and concentrated *in vacuo* to give the title compound **5** as a yellow solid (542 mg, 1.4 mmol, 91%). R_f 0.22 (10% EtOAc in hexane); MP 45-46 °C; MS (ESI⁺) m/z 371 [M + H]⁺; IR (neat): ν_{\max} 3408, 2919, 2851, 1471, 1045, 719 cm⁻¹; EPR (CH₂Cl₂): triplet, g = 2.0061, a_N = 14.45 G; HRMS (ESI⁺) calc. For C₂₁H₄₂NO₂S [MH+H]⁺ 372.29308, found 372.29303; HPLC purity analysis: >99% pure, retention time: 15.38 min; Crystal data: C₂₁H₄₀NO₂S, *M* = 157.17, *T* = 130.0(2) K, λ = 1.5418 Å, Triclinic, space group P-1 *a* = 5.8562(3), *b* = 8.0977(4), *c* = 23.150(1) Å, α = 84.648(4)°, β = 86.401(4)°, γ = 84.429(4)° *V* = 1086.27(9) Å³, *Z* = 2, D_c = 1.133 Mg M⁻³ μ (Cu-K α) 1.411 mm⁻¹, F(000) = 410, crystal size 0.5 x 0.15 x 0.09 mm. θ_{\max} = 76.84°, 7334 reflections measured, 4408 independent reflections (R_{int} = 0.0314) the final R was 0.0472 [*I* > 2 σ (*I*)] and wR(*F*²) was 0.1266 (all

data) GOOF = 1.053.



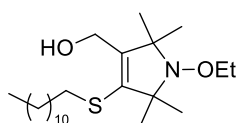
3-(Dodecane-1-thiyl)-4-methoxymethyl-2,2,5,5-tetramethyl-1-pyrrolinoxyl (6)

To **5** (0.26 mmol, 100 mg) in anhydrous THF (3 ml) was added NaH (60% mineral oil) (21 mg, 0.52 mmol). The reaction mixture was refluxed for 30 minutes, and MeI (24 μ l, 0.39 mmol) was subsequently added. The mixture was refluxed for a further 48 hours and the solvent was removed under reduced pressure. The resultant residue was dissolved in CHCl_3 , washed with H_2O , dried (Na_2SO_4). Removal of the solvent *in vacuo* and purification by flash column chromatography (hexane/EtOAc gradient, 1:0, 9:1, 8:1) furnished the title compound **6** as an orange syrup (97.1 mg, 0.24 mmol, 94 %). R_f 0.71 (EtOAc/hexane, 1:4); MS (ESI^+) m/z 385 $[\text{M} + \text{H}]^+$; IR (neat): ν_{max} 2924, 2854, 1465, 1355, 1094 cm^{-1} ; EPR (CH_2Cl_2): triplet, $g = 2.0056$, $a_N = 14.73$ G; HRMS (ESI^+) calc. for $\text{C}_{22}\text{H}_{44}\text{NO}_2\text{S}$ $[\text{MH} + \text{H}]^+$ 386.30873, found 386.30911; HPLC purity analysis: >91% pure, retention time: 23.94 min.



3-(Dodecane-1-thiyl)-1-ethoxy-4-formyl-2,2,5,5-tetramethylpyrroline (7)

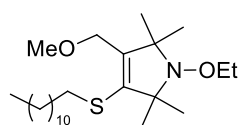
To a solution of nitroxide **4** (20 mg, 54 μ mol) in anhydrous THF (2 ml) was added triethylborane (1.0 M in THF, 163 μ l, 163 μ mol). The solution was allowed to stir under a saturated oxygen atmosphere for 15 minutes. The reaction mixture was concentrated *in vacuo*, and purified by preparatory TLC (EtOAc/hexane, 1:9) to give the title compound **7** as a colourless oil (11 mg, 28 μ mol, 51%). R_f 0.66 (EtOAc/hexane, 1:9); MS (ESI^+) m/z 398 $[\text{M} + \text{H}]^+$; IR (neat): ν_{max} 2925, 2854, 1673, 1045, 704 cm^{-1} ; ^1H NMR (CDCl_3 , 500 MHz): δ_{H} 10.03 (1H, s), 3.87 (2H, q, $J = 7.1, 14.2$ Hz), 2.89 (2H, t, $J = 7.4$ Hz), 1.61-1.58 (2H, m), 1.37-1.35 (18H, m), 1.26 (12H, s), 1.19 (3H, t, $J = 7.1$ Hz), 0.88 (3H, t, $J = 6.8$ Hz); ^{13}C NMR (CDCl_3 , 125 MHz): δ_{C} 188.72, 161.18, 143.53, 72.93, 72.79, 68.87, 37.40, 32.05, 29.88, 29.75, 29.67, 29.57, 29.48, 29.22, 28.71, 22.83, 14.54, 14.26; HRMS (ESI^+) calc. for $\text{C}_{23}\text{H}_{44}\text{NO}_2\text{S}$ $[\text{M} + \text{H}]^+$ 398.30873, found 398.30850.



3-(Dodecane-1-thiyl)-1-ethoxy-4-hydroxymethyl-2,2,5,5-tetramethylpyrroline (8)

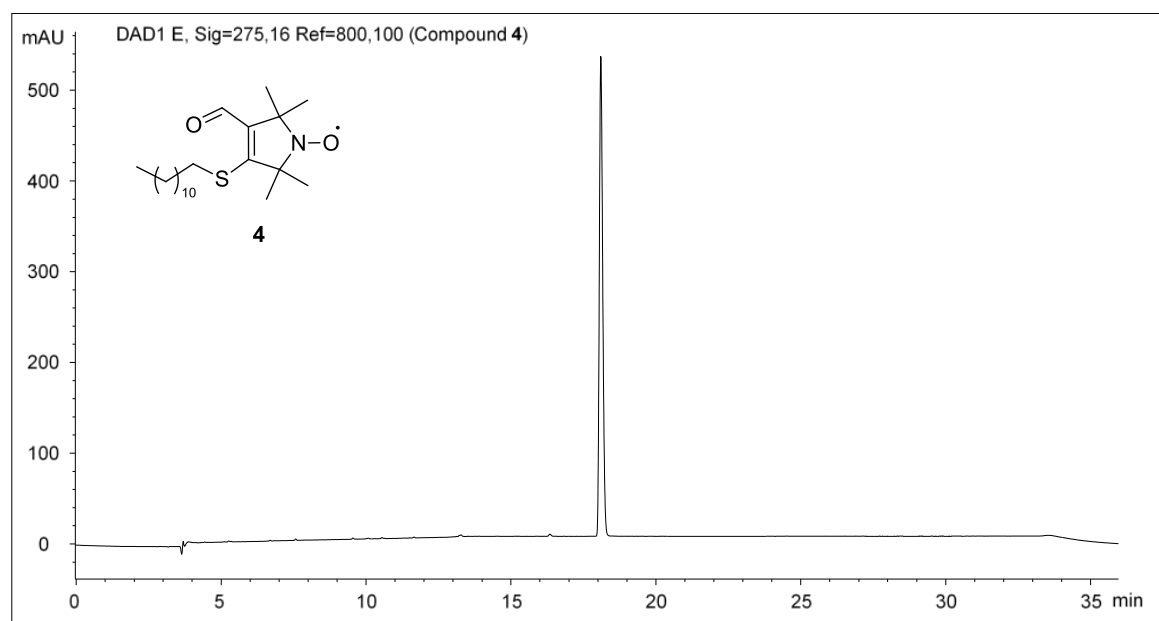
Compound **8** was prepared by using the procedure described for **7** above. Nitroxide **5** (22

mg, 59 μmol) and triethylborane (1.0 M in THF, 170 μl , 178 μmol) in THF (2 ml) followed by preparatory TLC (EtOAc/hexane, 1:9) afforded the title compound **8** as a colourless oil (15 mg, 37 μmol , 63%). R_f 0.40 (EtOAc/hexane, 1:9); MS (ESI⁺) m/z 400 [M + H]⁺; IR (neat): ν_{max} 3426, 2924, 2854, 1461, 1358, 1046, 721 cm^{-1} ; ¹H NMR (CDCl₃, 500 MHz): δ_{H} 4.32 (2H, s), 3.86 (2H, q, J = 7.1, 14.2 Hz), 2.68 (2H, t, J = 7.5 Hz), 1.60-1.54 (2H, m), 1.39-1.34 (2H, m), 1.27-1.26 (28H, m), 1.19 (3H, t, J = 7.1 Hz), 0.88 (3H, t, J = 6.9 Hz); ¹³C NMR (CDCl₃, 125 MHz): δ_{C} 149.83, 135.21, 72.52, 71.69, 69.62, 58.52, 36.23, 32.05, 29.95, 29.78, 29.76, 29.71, 29.63, 29.45, 29.33, 28.92, 22.82, 14.62, 14.26; HRMS (ESI⁺) calc. for C₂₃H₄₆NO₂S [M + H]⁺ 400.32438, found 400.32417.



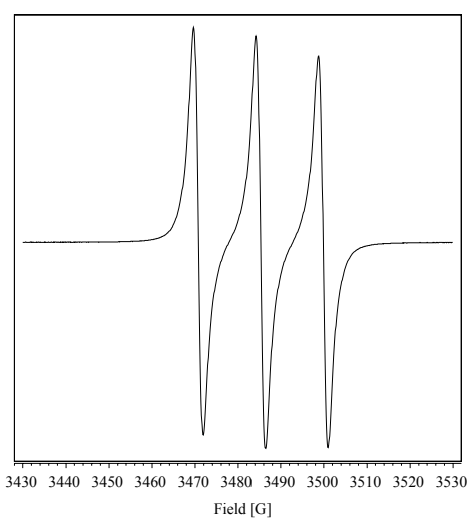
3-(Dodecane-1-thiyl)-1-ethoxy-4-methoxymethyl-2,2,5,5-tetramethylpyrroline (9)

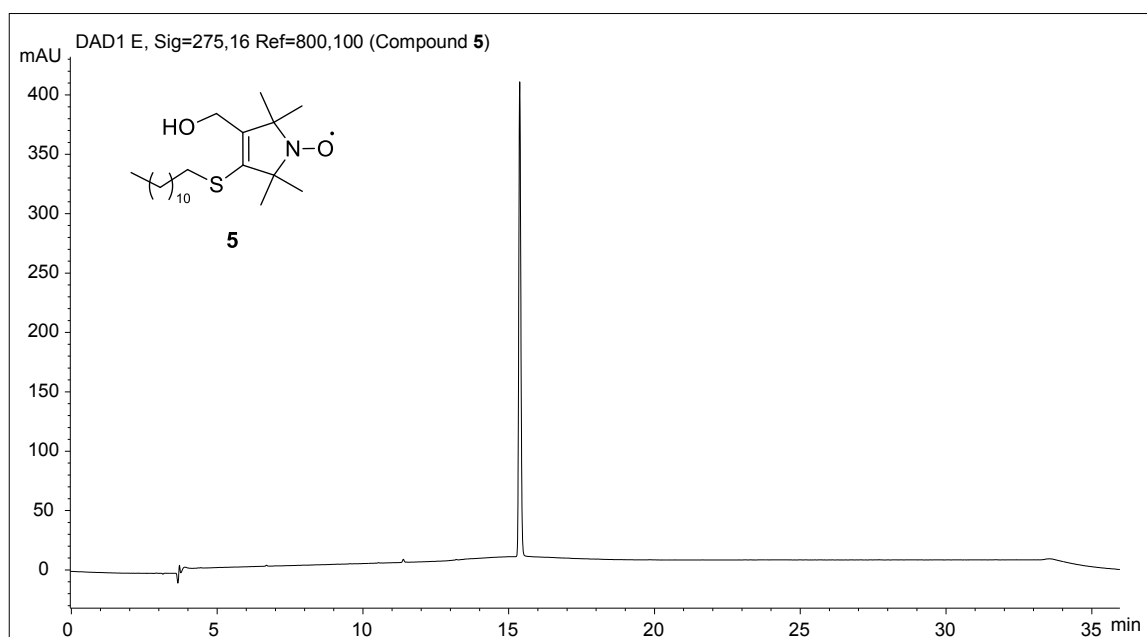
Compound **9** was prepared by using the procedure described for **7** above. Nitroxide **6** (90 mg, 231 μmol) and triethylborane (1.0 M in THF, 225 μl , 694 μmol) in THF (2 ml) followed by preparatory TLC (EtOAc/hexane, 1:9) furnished the title compound **9** as a colourless oil (52 mg, 134 μmol , 58%). R_f 0.71 (EtOAc/hexane, 1:9); MS (ESI⁺) m/z 414 [M + H]⁺; IR (neat): ν_{max} 2924, 2854, 1458, 1357, 1094, 1046, 985 cm^{-1} ; ¹H NMR (CDCl₃, 500 MHz): δ_{H} 4.14 (2H, s), 3.85 (2H, q, J = 7.1, 14.2 Hz), 3.31 (3H, s), 2.64 (2H, t, J = 7.4 Hz), 1.58-1.52 (2H, m), 1.37-1.34 (2H, m), 1.27-1.25 (28H, m), 1.19 (3H, t, J = 7.1 Hz), 0.88 (3H, t, J = 6.9 Hz); ¹³C NMR (CDCl₃, 125 MHz): δ_{C} 146.74, 138.40, 72.47, 71.64, 69.75, 67.30, 58.24, 36.40, 32.05, 29.94, 29.79, 29.76, 29.73, 29.65, 29.48, 29.34, 28.91, 22.82, 14.64, 14.25; HRMS (ESI⁺) calc. for C₂₄H₄₈NO₂S [M + H]⁺ 414.34003, found 414.33980.

A**Area Percent Report**

Signal 1 : DAD1 E, Sig = 275, 16 Ref = 800, 100

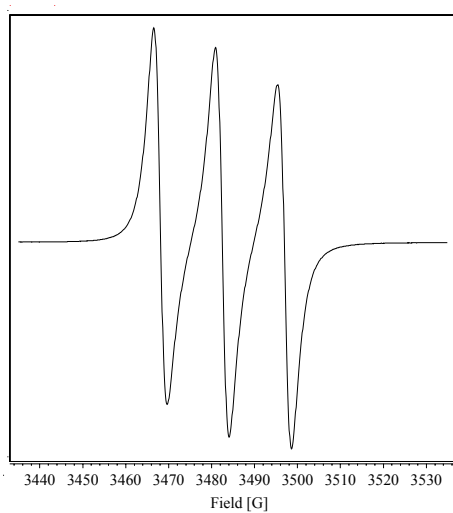
Peak #	RetTime (min)	Type	Width (min)	Area (mAu*s)	Height (mAu)	Area (%)
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Totals :				4332.3	530.5	

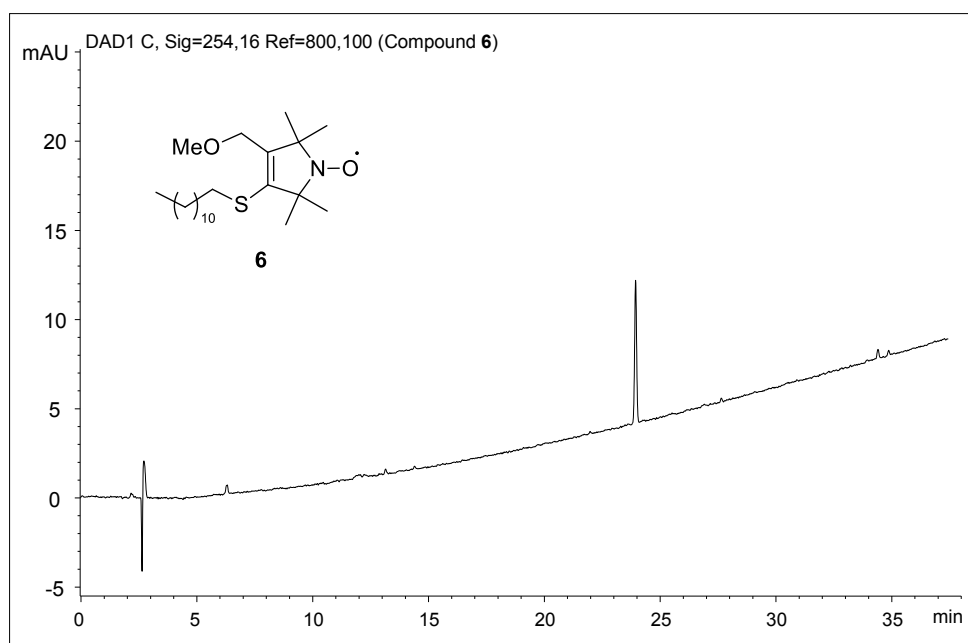
B**Figure S1.** HPLC trace (**A**) and EPR spectrum (**B**) for nitroxide **4**.

A**Area Percent Report**

Signal 1 : DAD1 E, Sig = 275, 16 Ref = 800, 100

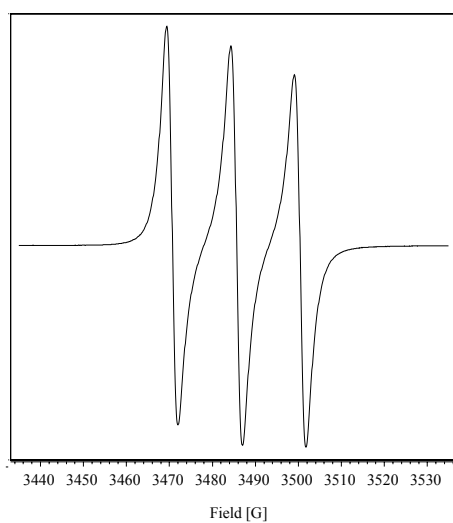
Peak #	RetTime (min)	Type	Width (min)	Area (mAu*s)	Height (mAu)	Area (%)
1	15.376	MM	0.0822	1977.7	401	100.000
Totals :				1977.7	401	

B**Figure S2.** HPLC trace (**A**) and EPR spectrum (**B**) for nitroxide **5**.

A**Area Percent Report**

Signal 1 : DAD1 E, Sig = 275, 16 Ref = 800, 100

Peak #	RetTime (min)	Type	Width (min)	Area (mAu*s)	Height (mAu)	Area (%)
1	23.937	MM	0.1046	50.5	8	91.654
2	34.402	MM	0.0853	2.4	4.7E-1	4.365
3	34.864	MM	0.1248	2.2	2.9E-1	3.981
Totals :				55.1	8.76	

B**Figure S3.** HPLC trace (**A**) and EPR spectrum (**B**) for nitroxide **6**.

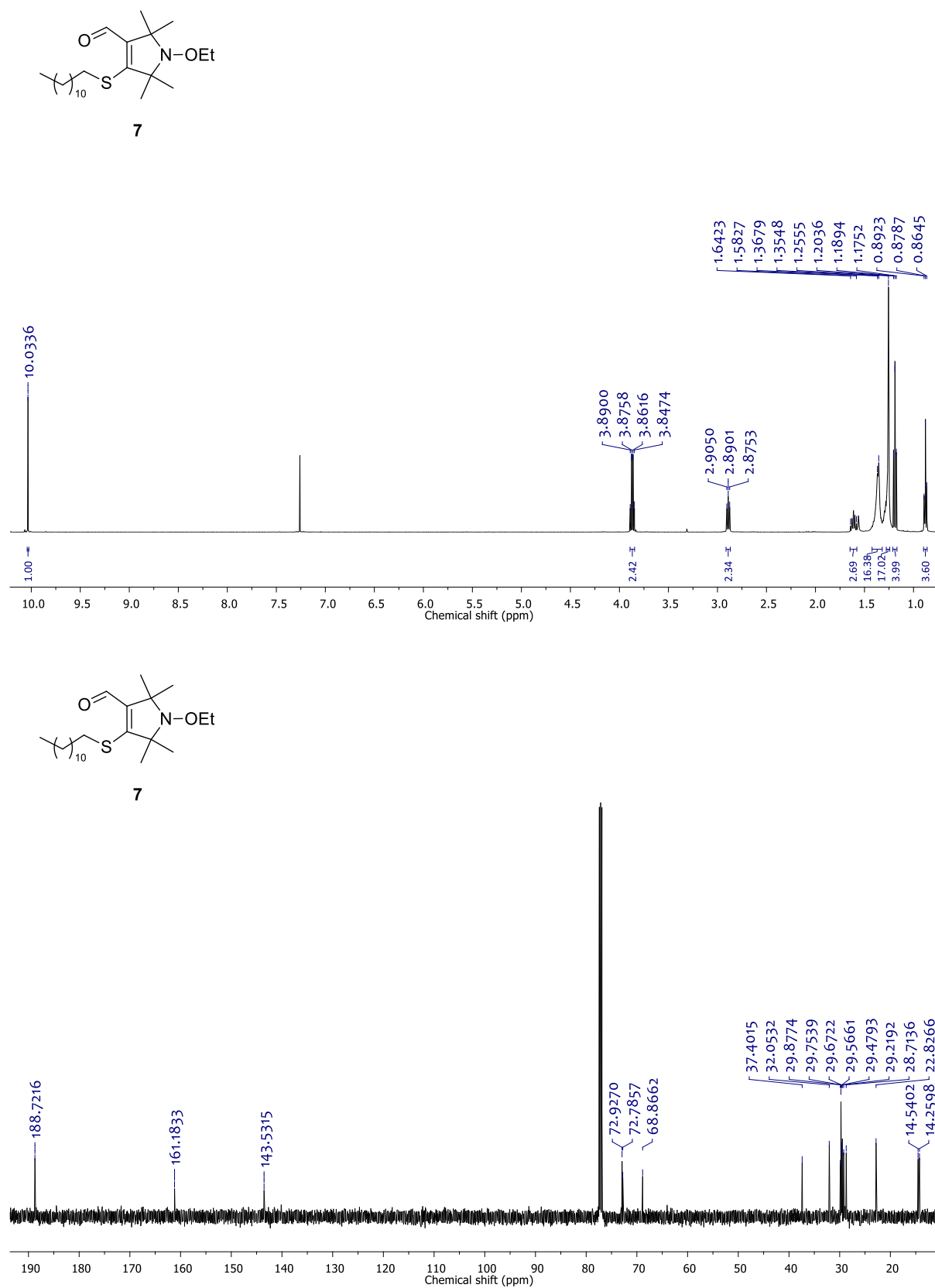


Figure S4. ¹H (top) and ¹³C (bottom) NMR spectra for ethoxyamine **7**.

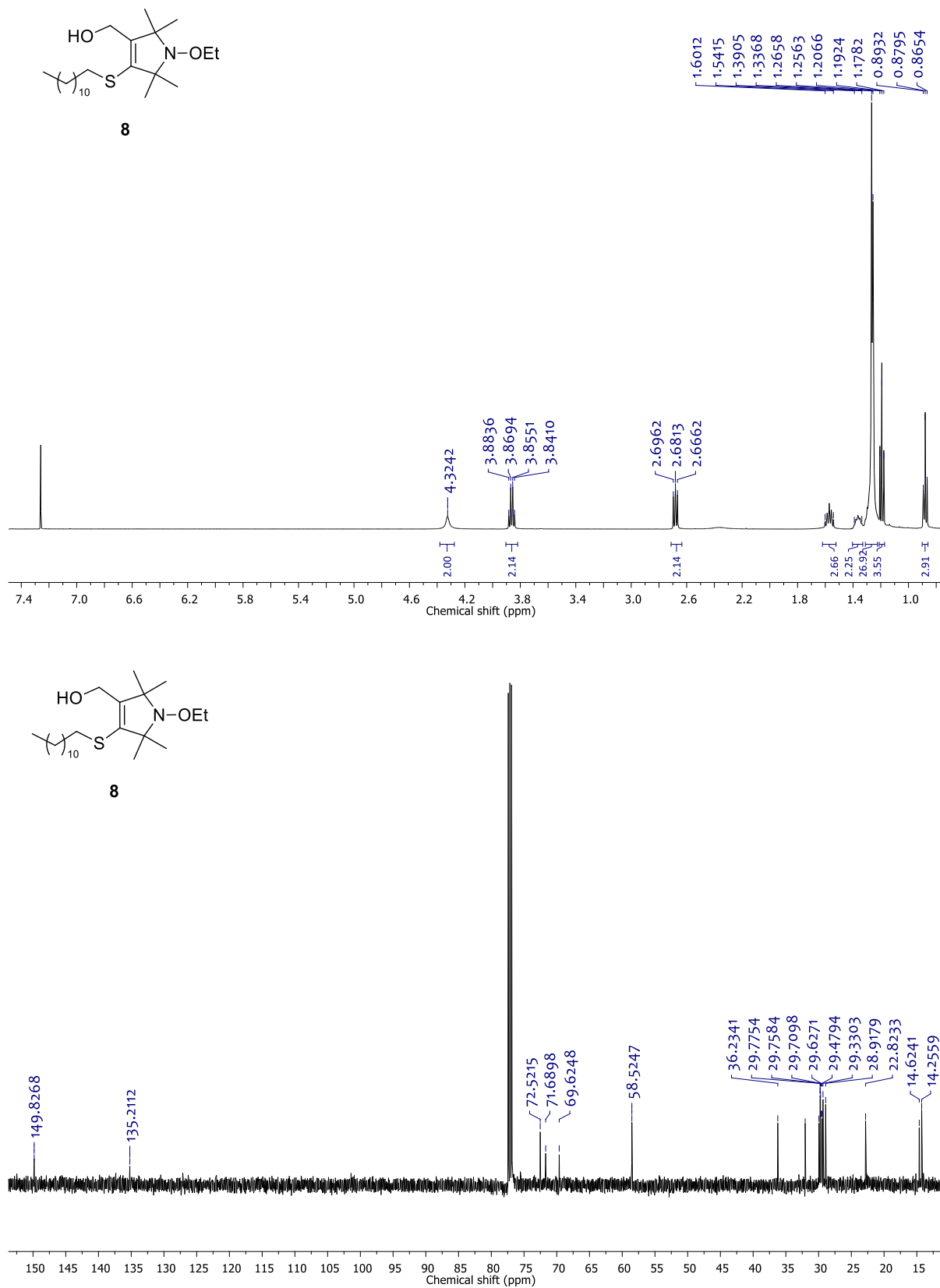


Figure S5. ¹H (top) and ¹³C (bottom) NMR spectra for ethoxyamine **8**.

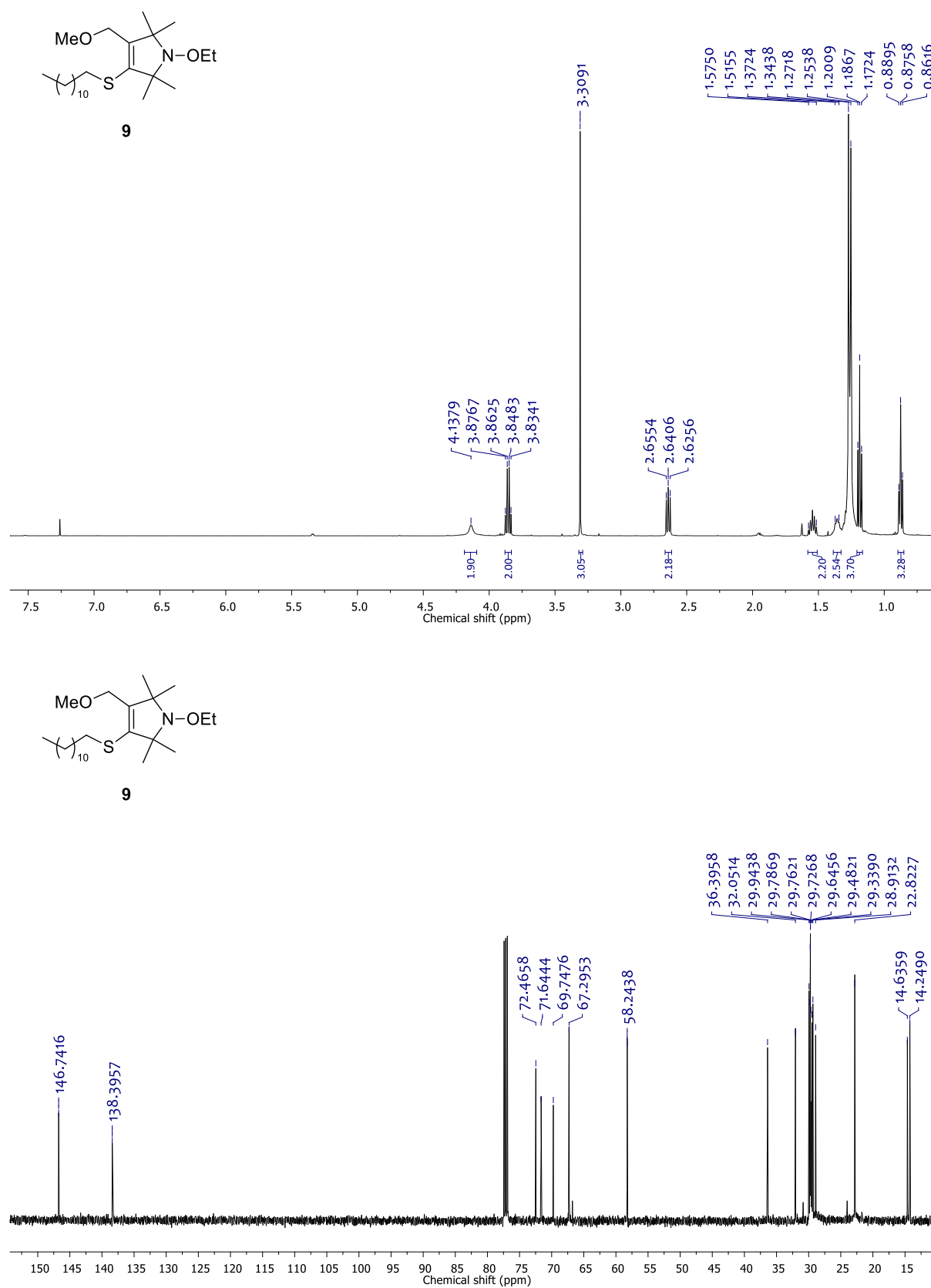


Figure S6. ¹H (top) and ¹³C (bottom) NMR spectra for ethoxyamine **9**.

Biological materials

Media, buffers and stock solutions

Table S1. Media and buffer recipes

Medium or buffer	Reagents
Luria-Bertani (LB)	0.1 g ml ⁻¹ (w/v) Tryptone, 5 mg ml ⁻¹ (w/v) yeast extract and 171 mM NaCl in Milli-Q H ₂ O.
M9 minimal medium	48 mM Na ₂ HPO ₄ , 22 mM KH ₂ PO ₄ , 8.6 mM NaCl, 19 mM NH ₄ Cl, 2 mM MgSO ₄ , 0.1 mM CaCl ₂ , and 22 mM (0.4 % w/v) glucose in Milli-Q H ₂ O.
Phosphate buffered saline (PBS)	137 mM NaCl, 3 mM KCl, 10 mM Na ₂ HPO ₄ , and 2 mM KH ₂ PO ₄ in Milli-Q H ₂ O.

* All media and buffer solutions was prepared in bulk and sterilized using an autoclave (Steroclave E08) prior to use.

Bacterial strains and overnight cultures

Pseudomonas aeruginosa PAO1 was sourced from Microbiologics KWIKSTIK™ Plus *Pseudomonas aeruginosa* ATCC®. A mixed population of organisms derived from a wooden sculpture were collected using sterile swabs and incubated overnight in LB medium. Both *P. aeruginosa* and organisms derived from a wooden sculpture were stored in a 10% glycerol solution at -80°C. Overnight cultures of *P. aeruginosa* and organisms derived from a wooden sculpture were grown routinely in 10 ml of LB medium with shaking (225 rpm) at 37 °C for 24 hours.

Biological methods

Batch-culture biofilm assay

Using a method adopted from Barraud *et al.*,⁵ overnight cultures of *P. aeruginosa* or organisms derived from a wooden sculpture were diluted 100-fold in M9 minimal media and inoculated in 24 well plates. Plates were incubated for 24 hours at ambient temperatures (19–22°C). Nitroxide candidates were added to the wells to final concentrations in the range of 50 nM to 5 mM (added as 10 µl aliquots in DMSO) either before incubation (preventive treatment model), or as a 30 minute treatment after the 24 hour incubation period (reactive treatment model). The liquid planktonic phase was then transferred to a new plate and planktonic biomass was quantified by optical density measurements at 600 nm (OD₆₀₀) using a Microplate fluorometer (Thermo Scientific Varioskan with the SkanIt RE for Varioskan

2.4.3 software). Wells were washed PBS (1 x 1 ml), fixed with 99% CH₃OH (1 ml) for 15 minutes, dried and stained for 15 minutes with 0.2 % (w/v) crystal violet. Wells were washed with PBS (2 x 1 ml) and the remaining crystal violet dissolved in 33% AcOH. Biofilm biomass was quantified by optical density measurements at 590 nm (OD₅₉₀). Untreated control wells comprised 1% (v/v) DMSO. Eight replicate wells were used for each nitroxide treatment and each batch-culture assay was repeated at least twice. Statistical analysis was performed using GraphPad Prism (GraphPad Software). Statistical differences between the untreated control data and the nitroxide-treated data were identified by the two-tailed Mann-Whitney t-test, and *p* values of less than 0.05 were deemed statistically significant.

Swarming motility assay

Swarming motility was evaluated on M9 minimal media plates containing 0.5% (w/v) agar and 1% (v/v) 2,3,5-triphenyl tetrazolium chloride (TTC). Nitroxide **5** in DMSO was added to the media prior to plate pouring to final concentrations of 500 µM and 5 mM ('untreated' control plates contained 1% (v/v) DMSO). Plates were dried for 120 minutes under ambient conditions and *P. aeruginosa* was inoculated in the center of the agar plate as 5 µl aliquots from overnight cultures. Plates were incubated for 16 hours at 30°C, then 10 days at ambient temperature and the swarming zone (diameter spread in mm) of each plate was measured.⁶⁻⁸ Three replicate plates were used for each nitroxide treatment and each motility assay was repeated at least twice. An average diameter spread for each plate was calculated based on diameter measurements taken at three different cross sections. Statistical analysis was performed using GraphPad Prism (GraphPad Software). Statistical differences between the untreated control data and **5**-treated data were identified by the two-tailed Mann-Whitney t-test, and *p* values of less than 0.05 were deemed statistically significant.

Cell viability assay

Overnight cultures of organisms derived from a wooden sculpture were diluted 100-fold in M9 minimal media and inoculated in 24 well plates. To each well was added nitroxide **5** as 10 µl aliquots in DMSO to final concentrations 50 µM and 5 mM. Plates were incubated for 24 hours at ambient temperatures (19–22°C). Benzalkonium chloride (BAC) (0.001% w/v) was then added to each well and incubated for a further 2 hours at ambient temperature. In a 96 well plate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 µl, 0.01% w/v) was added to 45 µl of liquid planktonic phase and incubated for 2 hours at

37°C.⁹⁻¹¹ DMSO (50 µl) was then added to each well and the number of viable cells were quantified by optical density measurements at 550 nm (OD₅₅₀) using a Microplate fluorometer. Three replicate wells were used for each treatment. Statistical analysis was performed using GraphPad Prism (GraphPad Software). Statistical differences between the untreated control data and nitroxide **5** and/or BAC-treated data were identified by the two-tailed Mann-Whitney t-test, and *p* values of less than 0.05 were deemed statistically significant.

Biofilm batch-culture assay results for nitroxides 4 – 6, 8 and 10 showing the full concentration range tested

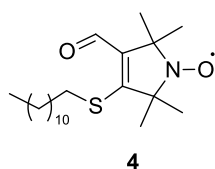
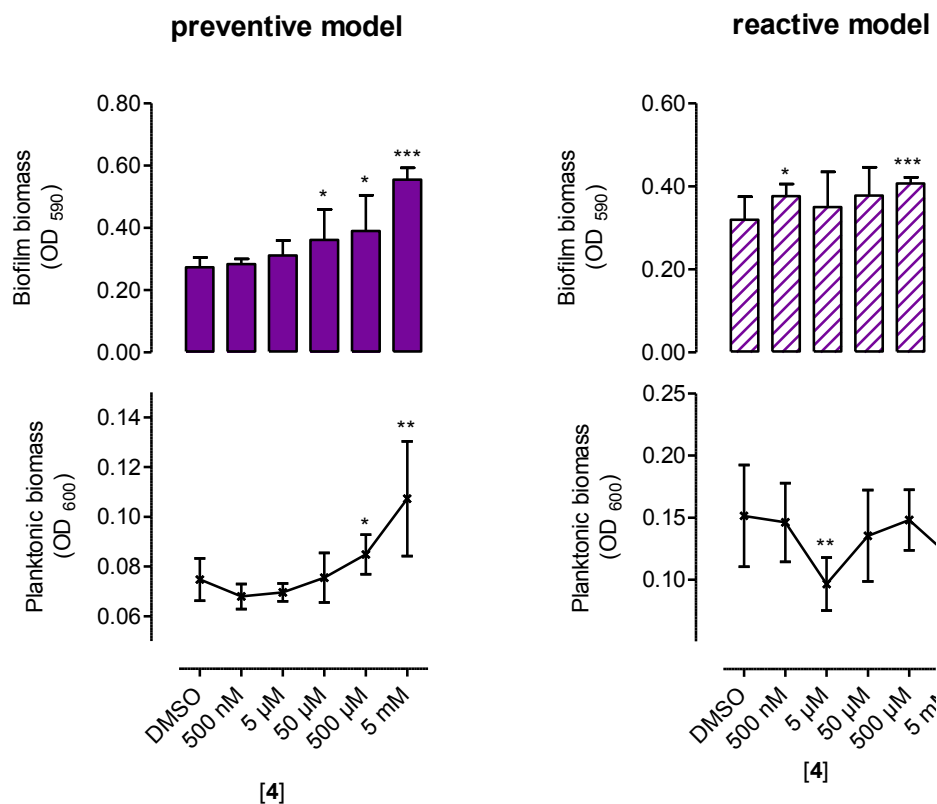


Figure S7. Nitroxide 4 does not inhibit biofilm formation or induce dispersal of *P. aeruginosa* biofilms.

P. aeruginosa was grown in 24 well plates in the presence of **4** for 24 hr in a preventive treatment model (left) or **4** was added as a 30 min treatment to 24 hr old biofilms in a reactive treatment model (right). Planktonic biomass was quantified by OD₆₀₀ measurements and biofilm biomass by crystal violet staining and subsequent OD₅₉₀. *, **, *** denote statistically significant differences compared to untreated 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.

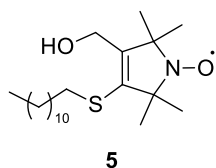
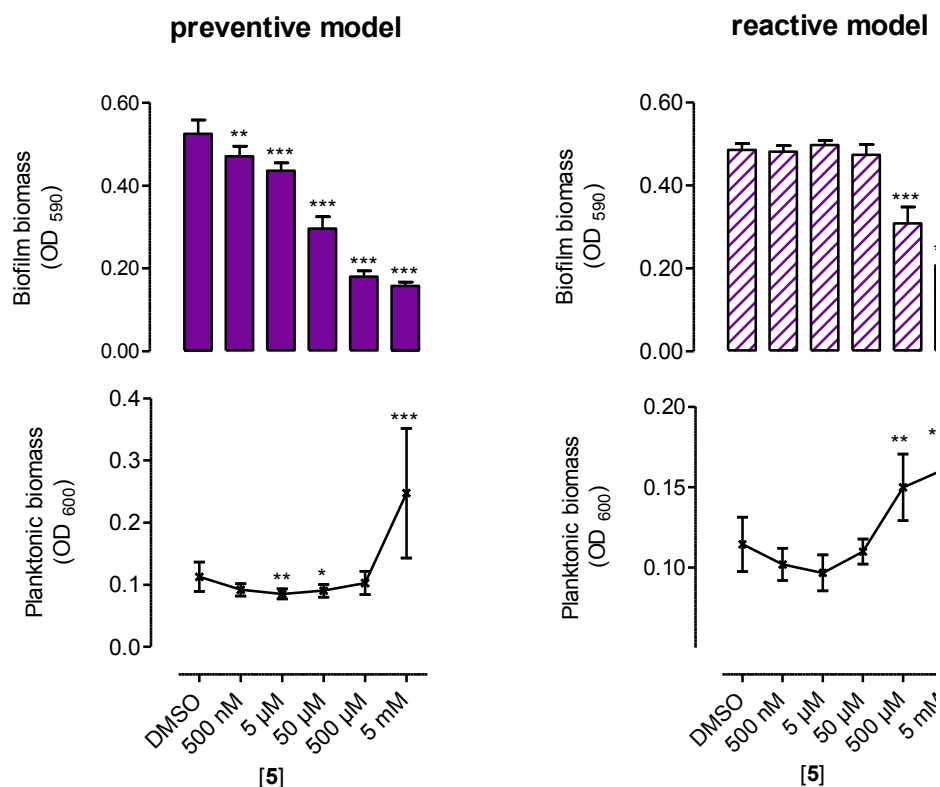


Figure S8. Compound 5 has anti-biofilm activity, inhibiting biofilm formation and inducing dispersal of *P. aeruginosa*.

P. aeruginosa was grown in 24 well plates in the presence of **5** for 24 hr in a preventive treatment model (left) or **5** was added as a 30 min treatment to 24 hr old biofilms in a reactive treatment model (right). Planktonic biomass was quantified by OD₆₀₀ measurements and biofilm biomass by crystal violet staining and subsequent OD₅₉₀. *, **, *** denote statistically significant differences compared to untreated 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 three times.

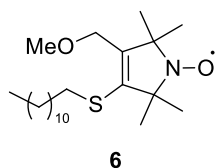
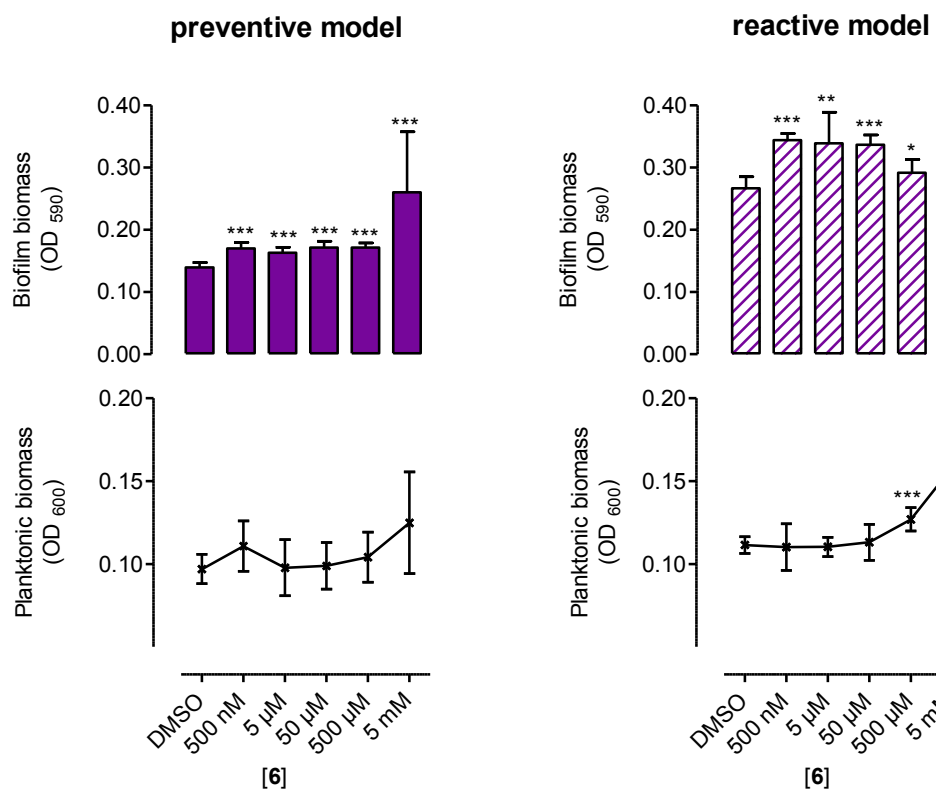


Figure S9. Compound 6 does not inhibit biofilm formation or induce dispersal of *P. aeruginosa*.

P. aeruginosa was grown in 24 well plates in the presence of **6** for 24 hr in a preventive treatment model (left) or **6** was added as a 30 min treatment to 24 hr old biofilms in a reactive treatment model (right). Planktonic biomass was quantified by OD₆₀₀ measurements and biofilm biomass by crystal violet staining and subsequent OD₅₉₀. *, **, *** denote statistically significant differences compared to untreated 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.

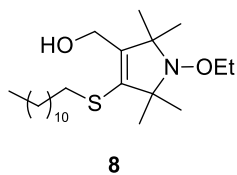
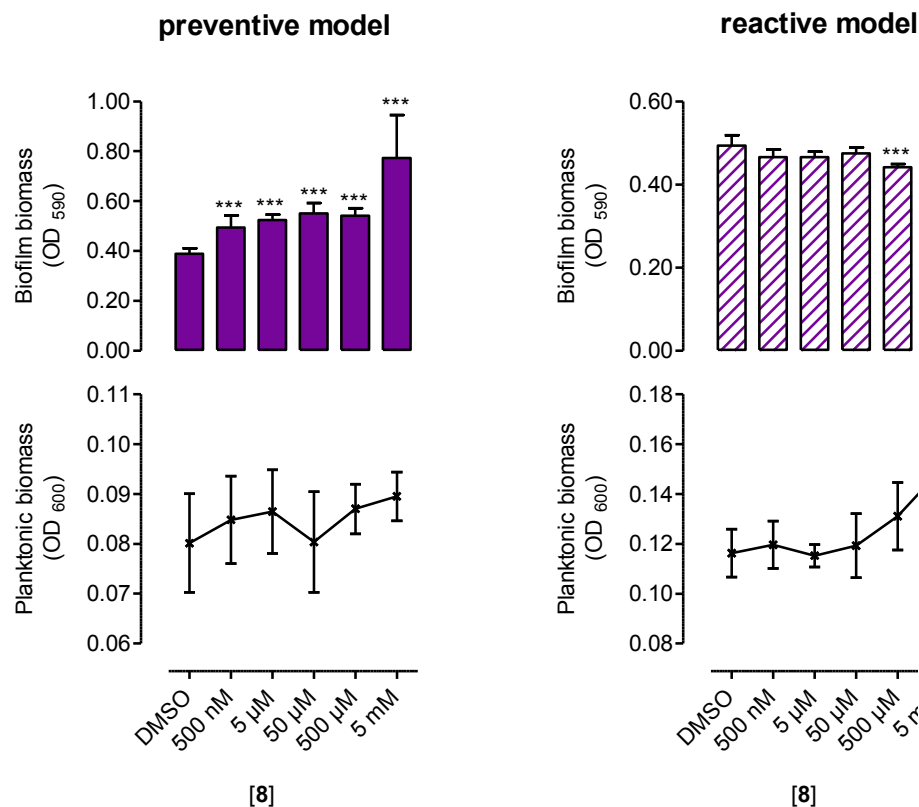
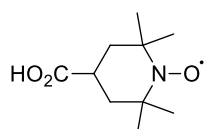
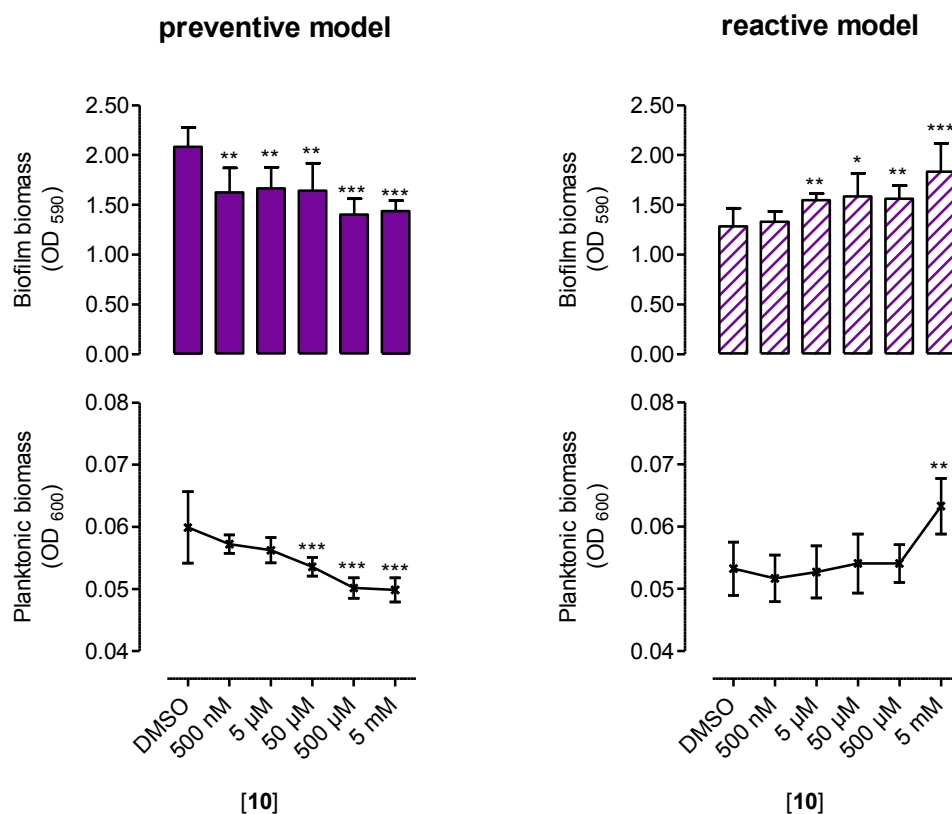


Figure S10. Compound 8 induces dispersal of *P. aeruginosa* biofilms at 5 mM.

P. aeruginosa was grown in 24 well plates in the presence of 7 for 24 hr in a preventive treatment model (left) or 8 was added as a 30 min treatment to 24 hr old biofilms in a reactive treatment model (right). Planktonic biomass was quantified by OD₆₀₀ measurements and biofilm biomass by crystal violet staining and subsequent OD₅₉₀. *** denote statistically significant differences compared to untreated wells where p < 0.001 (Mann-Whitney t-test: two-tailed, unpaired, equal variance, n = 8); the results of one representative experiment are presented, each was repeated twice.



10

Figure S11. Compound 10 does not inhibit biofilm formation or induce dispersal of *P. aeruginosa* biofilms.

P. aeruginosa was grown in 24 well plates in the presence of **10** for 24 hr in a preventive treatment model (left) or **10** was added as a 30 min treatment to 24 hr old biofilms in a reactive treatment model (right). Planktonic biomass was quantified by OD₆₀₀ measurements and biofilm biomass by crystal violet staining and subsequent OD₅₉₀. *, **, *** denote statistically significant differences compared to untreated 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.

References

1. Powell, J.; Johnson, E.; Gannett, P. *Molecules* **2000**, *5*, 1244-1250.
2. Zhdanov, R. I. *Bioactive Spin Labels*. Springer-Verlag: New York, 1992.
3. Chudinov, A. V.; Rozantsev, É. G.; Rozynov, B. V. *B. Acad. Sci. USSR Ch.* **1983**, *32*, 370-375.
4. Chudinov, A. V.; Rozantsev, É. G.; Tarasov, V. F.; Sholle, V. D. *B. Acad. Sci. USSR Ch.* **1983**, *2*, 394.
5. Barraud, N.; Hassett, D. J.; Hwang, S.; Rice, S. A.; Kjelleberg, S.; Webb, J. S. *J. Bacteriol.* **2006**, *188*, 7344-7353.
6. Köhler, T.; Curty, L. K.; Barja, F.; van Delden, C.; Pechère, J.-C. *J. Bacteriol.* **2000**, *182*, 5990-5996.
7. Tremblay, J.; Déziel, E. *J. Basic Microb.* **2008**, *48*, 509-515.
8. Shrout, J. D.; Chopp, D. L.; Just, C. L.; Hentzer, M.; Givskov, M.; Parsek, M. R. *Mol. Microbiol.* **2006**, *62*, 1264-1277.
9. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55-63.
10. Wang, H.; Cheng, H.; Wang, F.; Wei, D.; Wang, X. *J. Microbiol. Meth.* **2010**, *82*, 330-333.
11. Gabrielson, J.; Hart, M.; Jarelov, A.; Kuhn, I.; McKenzie, D.; Mollby, R. *J. Microbiol. Meth.* **2002**, *50*, 63-73.