Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

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

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Electronic Supplementary Information

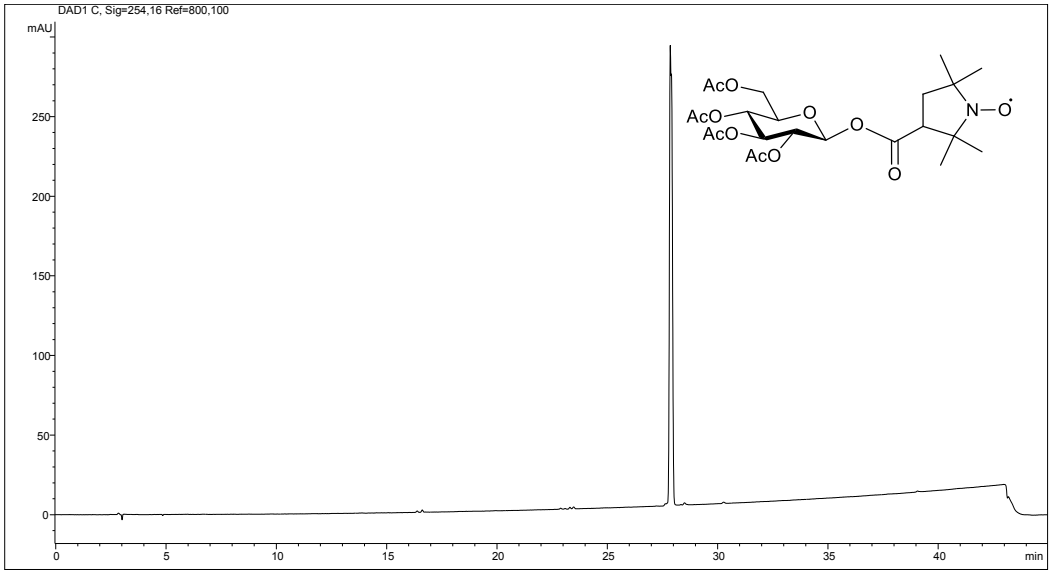
General synthetic information; HPLC traces and EPR spectra for compounds **29, 30, 15, 16** and **20**; ^{1}H and ^{13}C NMR spectra of compounds **29a, 30a** and **20a**; Biological materials and methods (12 pages).

Synthesis

General synthetic information

Anhydrous reactions were performed in oven-dried or flame-dried glassware under inert argon atmosphere. Anhydrous Et_2O, CH_2Cl_2 and tetrahydrofuran (THF) were dispensed from a glass contour dry solvent dispensing system and other anhydrous solvents were purified as per published procedures.[1] All other chemicals were obtained from commercial suppliers and were used without further purification. Analytical TLC was performed on Merck aluminium-backed 2 μm thick silica gel plates (Kieselgel 60 GF254). Compounds were visualized under a 254 nm or 365 nm UV lamp or by staining with phosphomolybdic acid (PMA) (30 mM in EtOH), $KMnO_4$ (0.063 M $KMnO_4$, 0.48 M K_2CO_3 and 0.021 M NaOH in H_2O), or 0.5 M H_2SO_4 in EtOH. Scharlau silica gel 60 (particle size 0.04 x 0.06 mm) was used for flash column chromatography and Analtech silica gel G TLC plates (20 cm x 20 cm x 2000 μm) were used for preparatory TLC in conjunction with the indicated solvent systems. Melting points (MP) were measured on an Electrothermal Melting Point Apparatus (Mark II) and are uncorrected. Low resolution ESI-MS were recorded on an Agilent 6220 ESI-TOF mass spectrometer. Parent ions are denoted by $[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$, $[MH + H]^+$, $[M]^{\bullet-}$ or $[M]^+$. IR spectra were recorded neat on a Perkin-Elmer Spectrum One FT-TR spectrometer, equipped with a zinc selenide/diamond universal ATR sampling accessory. Optical rotations were measured on a Jasco DIP-1000 digital polarimeter. NMR spectra were recorded on an Agilent MR400 (^{1}H NMR: 400 MHz, ^{13}C NMR: 100 MHz) or Agilent DD2 (^{1}H NMR: 500 MHz, ^{13}C NMR: 126 MHz) NMR spectrometer in deuterated chloroform ($CDCl_3$) or methanol (CD_3OD) as indicated. Chemical shifts (δ) are reported in parts per million (ppm) relative to either $CDCl_3$ (^{1}H NMR: 7.26 ppm; ^{13}C NMR: 77.0 ppm) or CD_3OD (^{1}H NMR: 3.31 ppm; ^{13}C NMR: 49.0 ppm). ^{1}H NMR spectral data are reported as follows: chemical shift (δ), relative integral, multiplicity, and coupling constant/s (*J*) in hertz (Hz). EPR spectra were recorded on a Bruker Elexsys E-500 CW-EPR and data are reported as follows: peak shape, g-factor, and coupling constant (a_N) in Gauss (G). High resolution mass spectroscopy (HRMS) was conducted on a Finnigan hydbrid linear triple-quadrupole (LTQ) Fourier Transform ion cyclotron resonance (FTICR) mass spectrometer.

A



Area Percent Report

Signal 1 : DAD1 C, Sig = 254, 16 Ref = 800, 100

Peak #	RetTime (min)	Type	Width (min)	Area (mAu*s)	Height (mAu)	Area (%)
1	27.856	MM	0.1509	2621.80664	289.55630	100.0000
Totals :				2621.80664	289.55630	

B

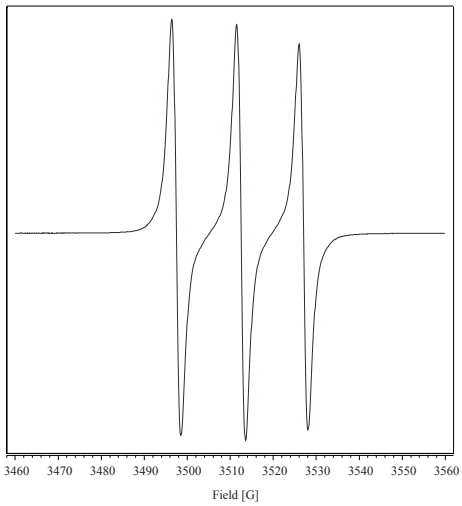
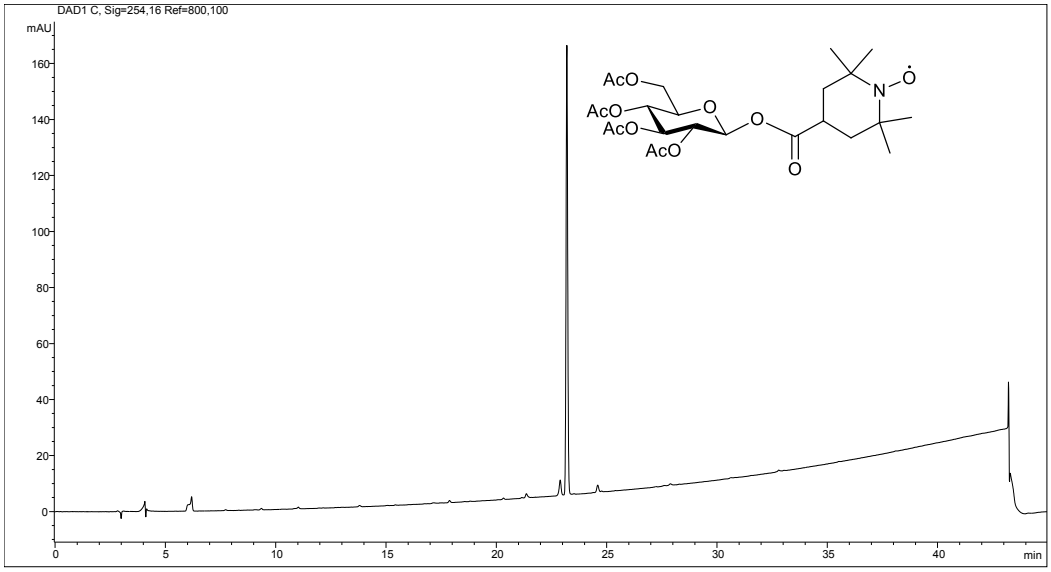


Figure S1. HPLC trace (**A**) and EPR spectrum (**B**) for nitroxide **29**.

A



Area Percent Report

Signal 1 : DAD1 C, Sig = 254, 16 Ref = 800, 100

Peak #	RetTime (min)	Type	Width (min)	Area (mAu*s)	Height (mAu)	Area (%)
1	22.892	BV	0.0943	33.97796	5.48486	3.8467
2	23.193	VB	0.0805	832.54419	160.64217	94.2528
3	24.593	BB	0.1005	16.78761	2.56168	1.9005
Totals :				883.30977	168.68871	

B

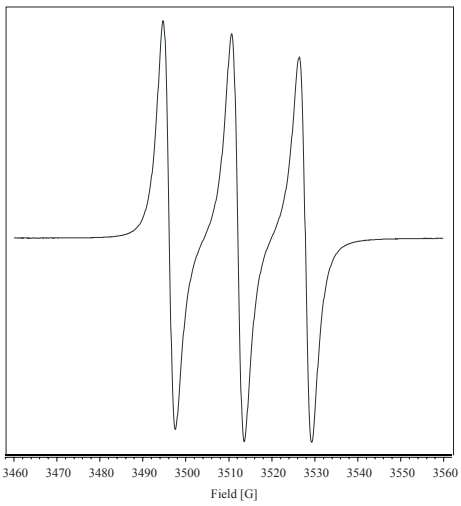
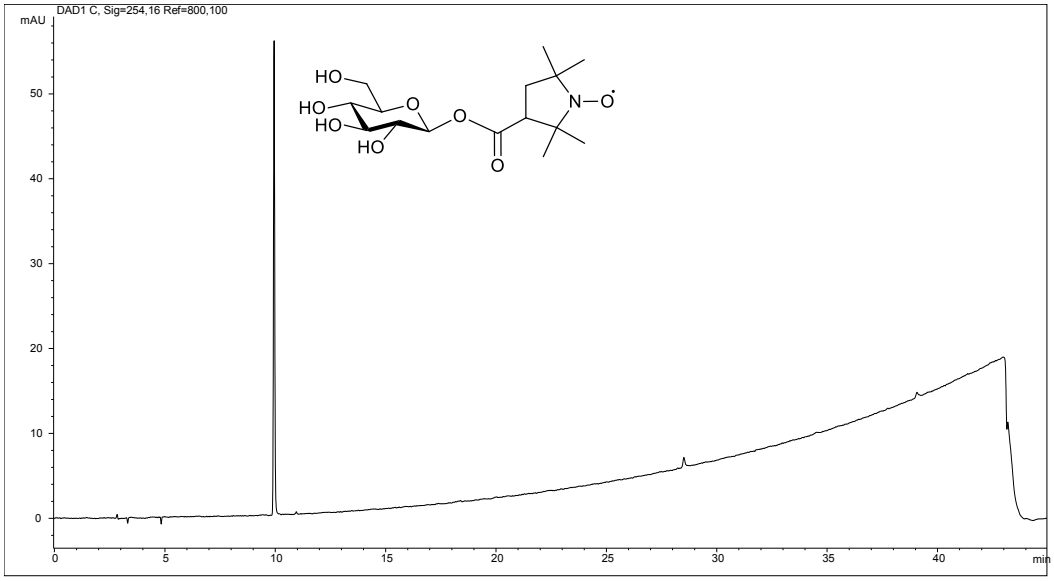


Figure S2. HPLC trace (**A**) and EPR spectrum (**B**) for nitroxide **30**.

A



Area Percent Report

Signal 1 : DAD1 C, Sig = 254, 16 Ref = 800, 100

Peak #	RetTime (min)	Type	Width (min)	Area (mAu*s)	Height (mAu)	Area (%)
1	9.945	BB	0.0607	223.66263	55.84998	96.5900
2	28.508	BB	0.1068	7.14077	1.14077	3.4100
Totals :				231.55885	56.99075	

B

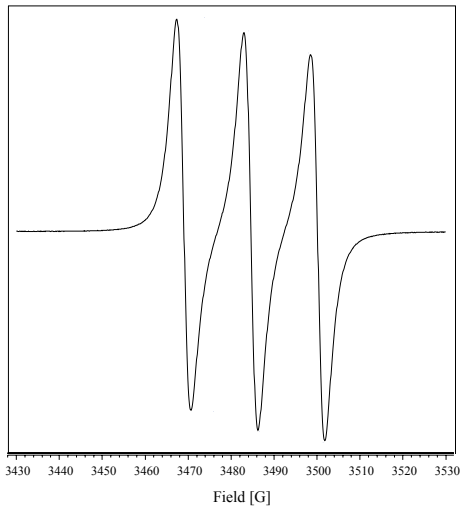
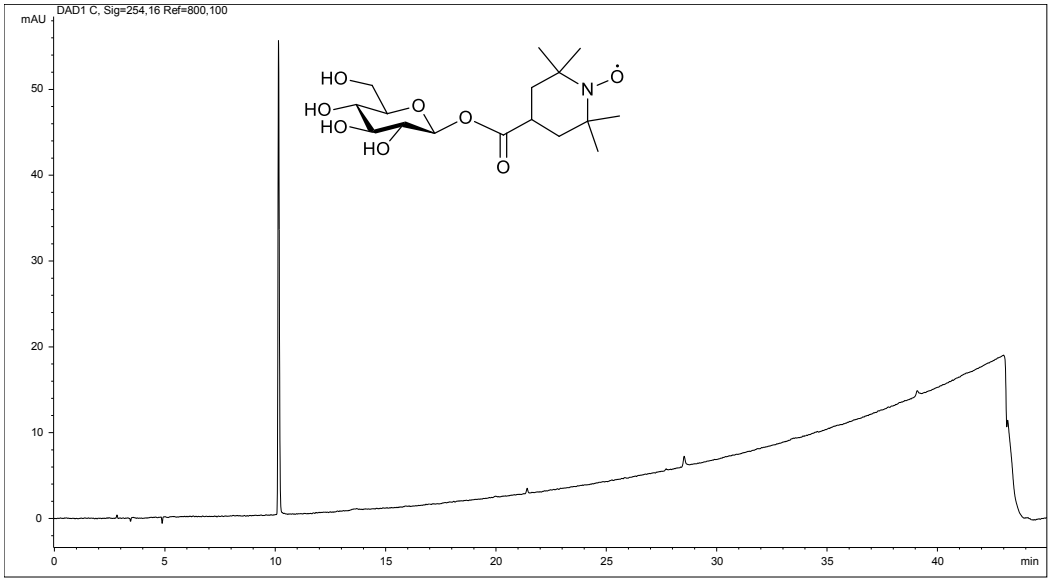


Figure S3. HPLC trace (**A**) and EPR spectrum (**B**) for nitroxide **15**.

A



Area Percent Report

Signal 1 : DAD1 C, Sig = 254, 16 Ref = 800, 100

Peak #	RetTime (min)	Type	Width (min)	Area (mAu*s)	Height (mAu)	Area (%)
1	10.148	BB	0.0653	234.25749	55.45144	96.6155
2	28.254	BB	0.1023	8.20617	1.19296	3.3845
Totals :				242.46366	56.64440	

B

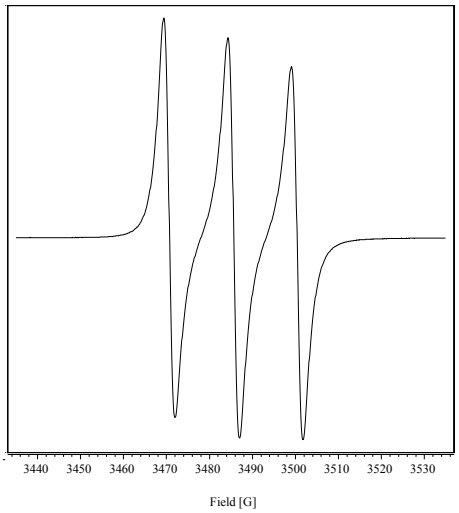
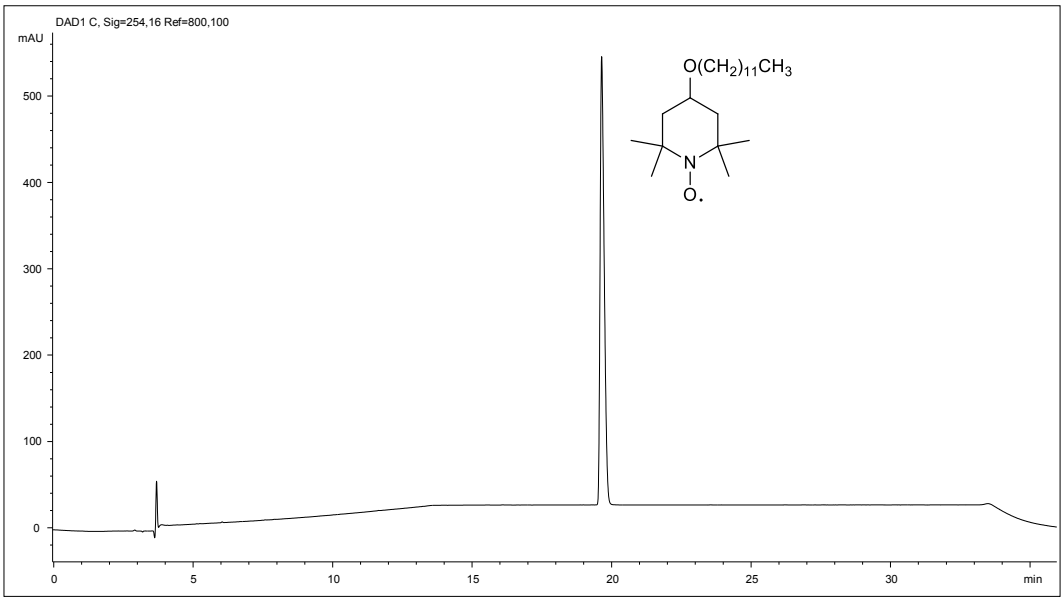


Figure S4. HPLC trace (**A**) and EPR spectrum (**B**) for nitroxide **16**.

A



Area Percent Report

Signal 1 : DAD1 C, Sig = 254, 16 Ref = 800, 100

Peak #	RetTime (min)	Type	Width (min)	Area (mAu*s)	Height (mAu)	Area (%)
1	19.641	BB	0.1544	5271.68652	519.46625	100.0000
Totals :				5271.68652	519.46625	

B

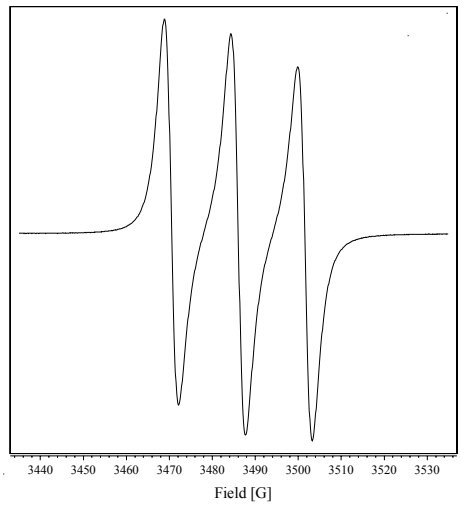


Figure S5. HPLC trace (**A**) and EPR spectrum (**B**) for nitroxide **20**.

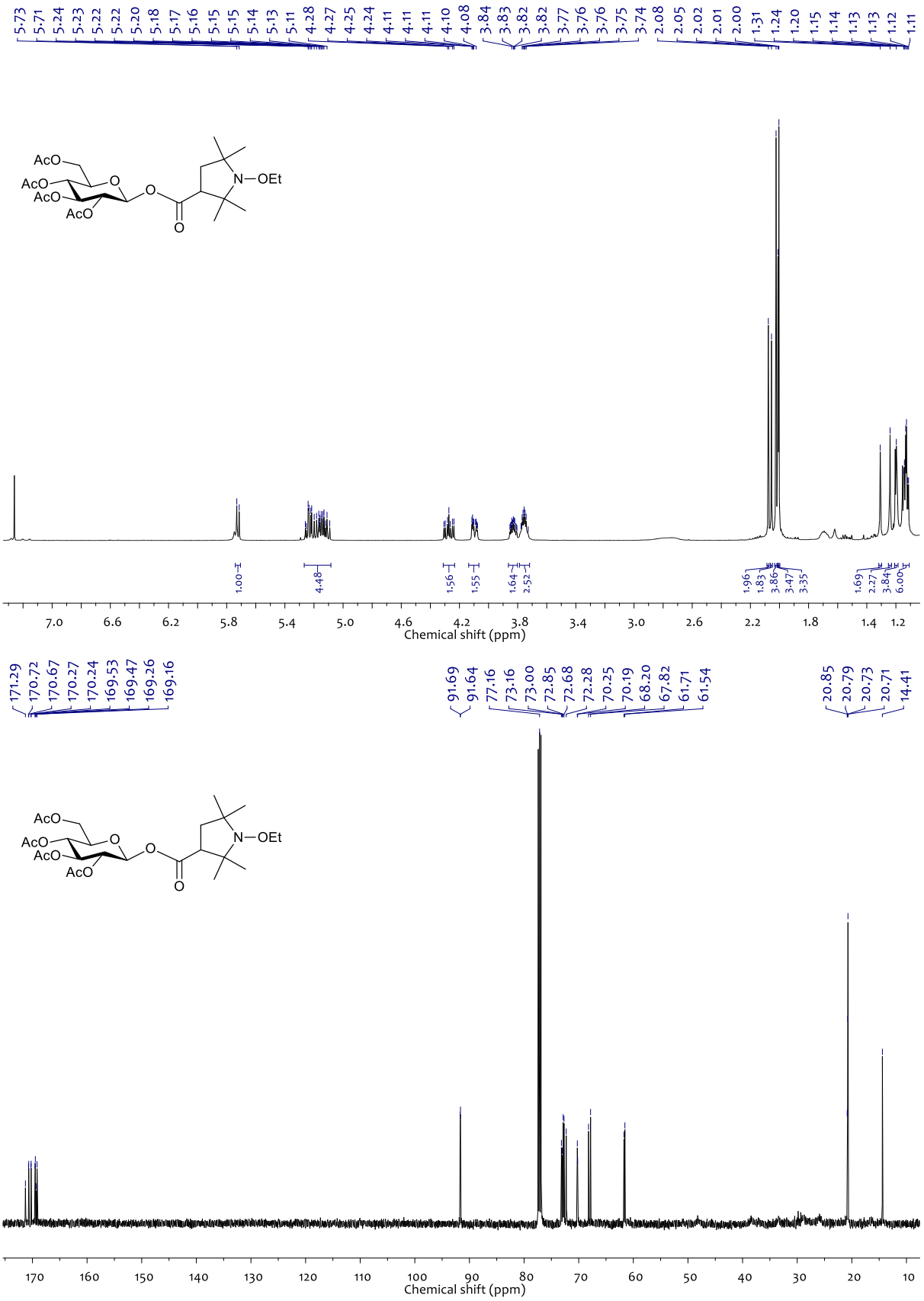


Figure S6. ^{1}H (top) and ^{13}C (bottom) NMR spectra for ethoxyamine **29a**.

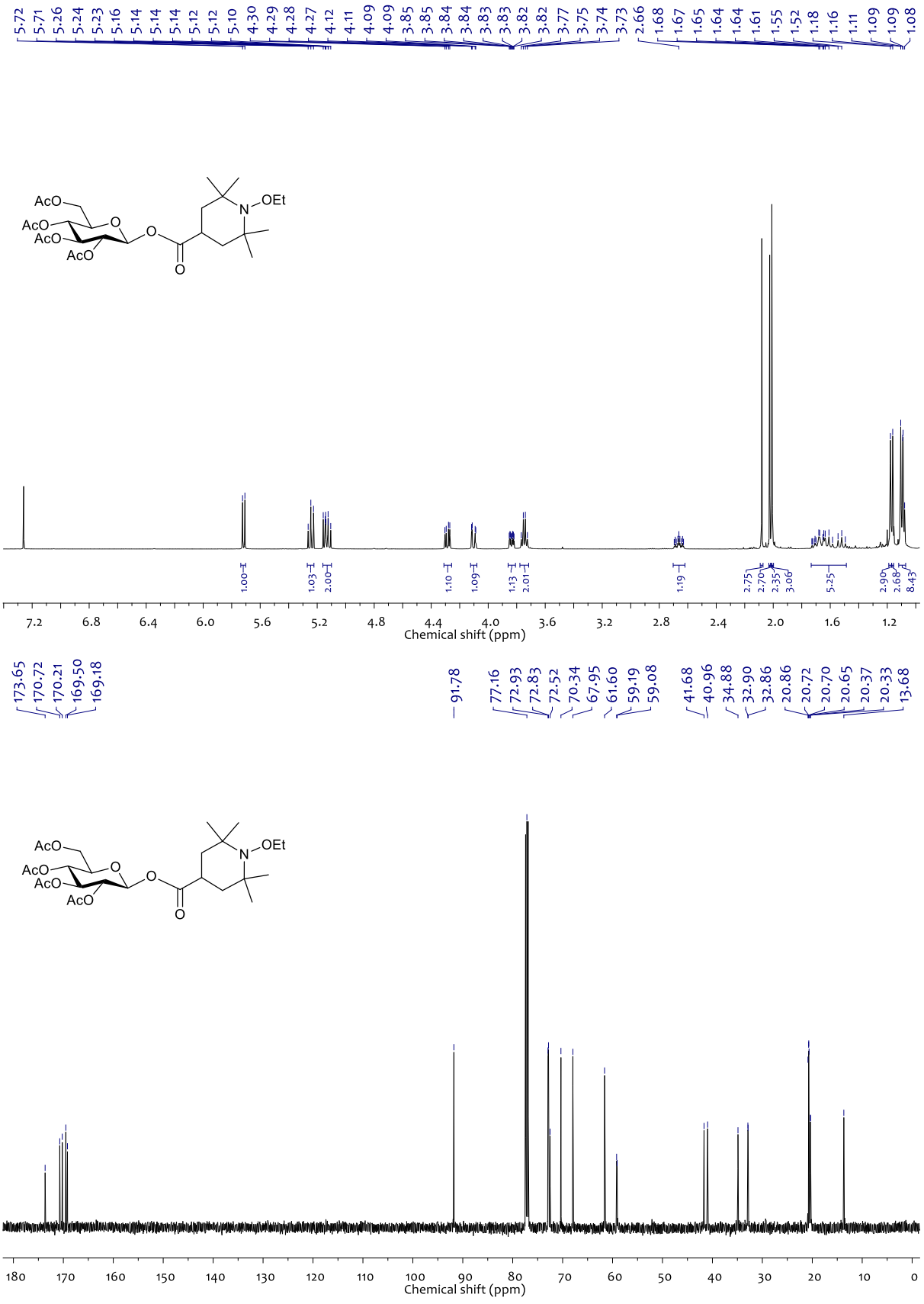


Figure S7. ^{1}H (top) and ^{13}C (bottom) NMR spectra for ethoxyamine **30a**

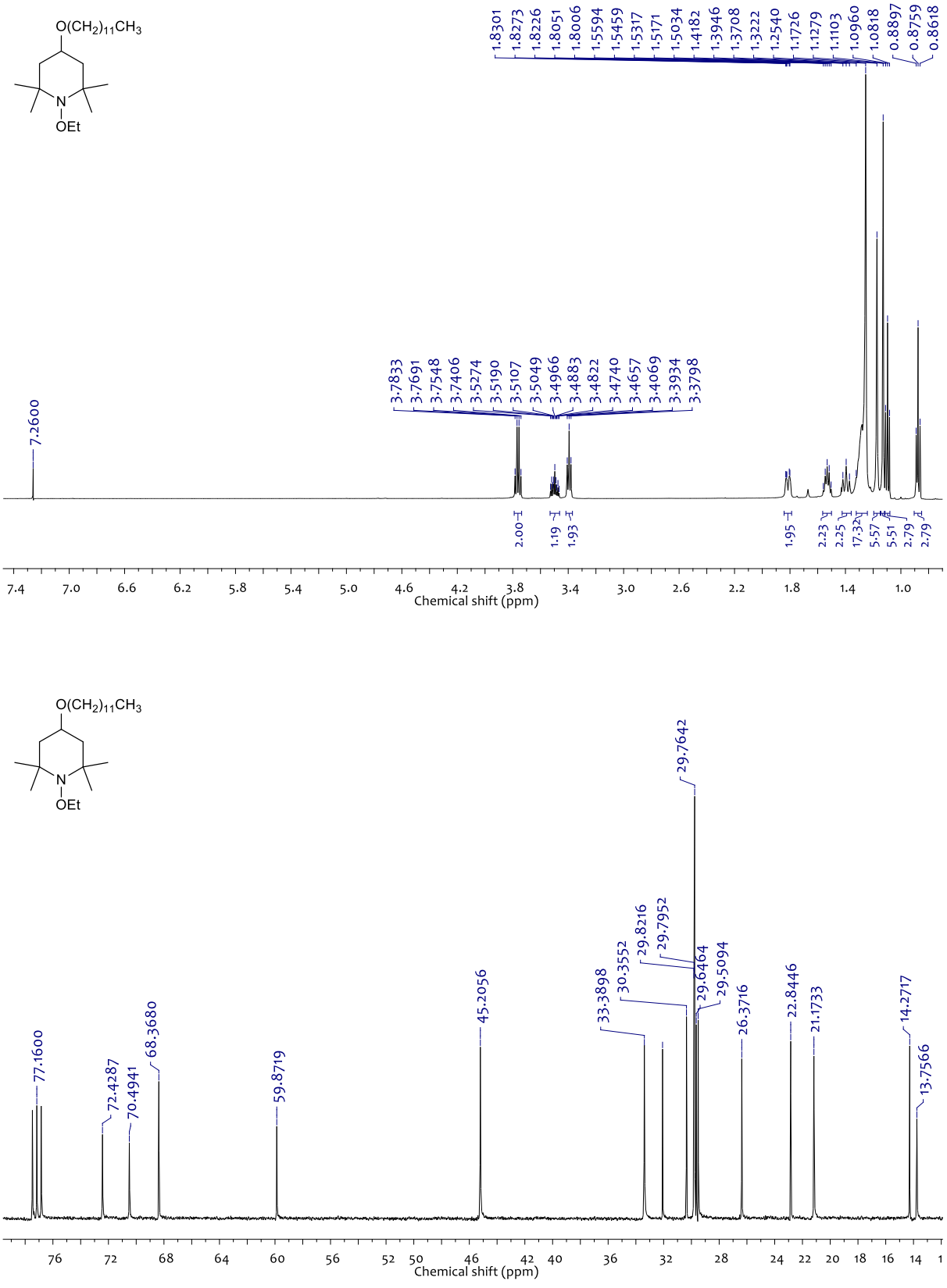


Figure S8. ^{1}H (top) and ^{13}C (bottom) NMR spectra for ethoxyamine **20a**.

Biological materials and methods

Bacterial strains and overnight cultures

Pseudomonas aeruginosa PAO1 was sourced from Microbiologics KWIKSTIK™ Plus Pseudomonas aeruginosa ATCC®. The cultural material organism (CMO) broth was kindly supplied by Caroline Paula Kyi. Briefly, following a literature modified procedure,[2] samples (0.4 g) of cultural materials (metal, canvas, and paper) were sonicated (Bendelin Sonorex Super RK255H) in PBS (137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4 in Milli-Q H_2O) solution for 5 minutes at ambient temperature. An aliquot (0.1 ml) of each of the PBS solutions was transferred to three different nutrient medium types – nutrient broth (NB) (3 g l^{-1} beef extract and 5 g l^{-1} peptone in Milli-Q H_2O), M9 minimal medium (48 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 19 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 22 mM (0.4 % w/v) glucose in Milli-Q H_2O), and Mueller Hinton (3 g l^{-1} beef extract, 17.5 g l^{-1} acid hydrolysate of Casein, and 1.5 g l^{-1} of starch in Milli-Q H_2O) - and incubated for 16 hours at 37°C. After incubation, aliquots of each medium type were added together and stored in a 10% glycerol solution at -80°C. Overnight cultures of PAO1 and CMO were grown routinely in 10 ml of luria-Bertani (LB) medium (0.1 g ml^{-1} (w/v) Tryptone, 5 mg ml^{-1} (w/v) yeast extract and 171 mM NaCl in Milli-Q H_2O) with shaking (225 rpm) at 37 °C for 24 hours.

Crystal violet biofilm assay

Using an adaptation of the traditional crystal violet technique,[3-5] overnight cultures of *P. aeruginosa* and CMO 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 500 nM to 5 mM (added as 10 µl aliquots in DMSO or Milli-Q H_2O) 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 OD measurements at 600 nm (OD_{600}) 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% CH_3OH (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 OD measurements at 590 nm (OD_{590}). DMSO control wells comprised 1%

(v/v) DMSO (**5-14**, **19-23**) or Milli-Q H_2O (**15-18**, **24-27**) depending on nitroxide treatment. Eight replicate wells were used for each nitroxide treatment and each biofilm assay was repeated at least twice. Statistical analysis was performed using GraphPad Prism (GraphPad Software). Statistical differences between the DMSO 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.

Cell motility assays

Swimming, swarming and twitching motilities were evaluated on M9 minimal media plates containing 1% (v/v) 2,3,5-triphenyl tetrazolium chloride (TTC) and solidified with agar (swimming: 0.3% (w/v); swarming: 0.5% (w/v); twitching: 1% (w/v)). Nitroxide **22** in DMSO was added to the media prior to plate pouring to final concentrations of 500 μM and 5 mM (control plates contained 1% (v/v) DMSO). Plates were dried for 120 minutes under ambient conditions and either *P. aeruginosa* or CMO were inoculated in the center of the agar plate (as 5 μl aliquots on the agar surface for swimming and swarming and stab inoculated to the plate-agar interface for twitching) from overnight cultures. Swimming plates were incubated for 16 hours at 30°C, twitching plates were incubated for 16 hours at 30°C then 10 days at ambient temperature, and swarming plates were incubated for 16 hours at 30°C then 5 days at ambient temperature. The zone of spread (diameter in mm) for each motility type was then measured.[6]

References

1. Armarego, W. L. F.; Perrin, D. D. *Purification of laboratory chemicals*. Butterworth-Heineman: Oxford, 1996.
2. Bjerkan, G.; Witso, E.; Bergh, K. *Acta Orthopaedica* **2009**, *80*, 245-250.
3. O'Toole, G. A.; Kolter, R. *Mol. Microbiol.* **1998**, *28*, 449-461.
4. Furukawa, S.; Akiyoshi, Y.; O'Toole, G. A.; Ogihara, H.; Morinaga, Y. *Int. J. Food Microbiol.* **2010**, *138*, 176-180.
5. Jackson, D. W.; Suzuki, K.; Oakford, L.; Simecka, J. W.; Hart, M. E.; Romeo, T. *J. Bacteriol.* **2002**, *184*, 290-301.
6. Chow, S.; Gu, K.; Jiang, L.; Nassour, A. *JEMI* **2011**, *15*, 22-29.