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Supplemental Information

Polyketide Quinones Are Alternate Intermediate Electron Carriers during Mycobacterial Respiration in Oxygen-Deficient Niches

Amitesh Anand, Priyanka Verma, Anil Kumar Singh, Sandeep Kaushik, Rajesh Pandey, Ce Shi, Harneet Kaur, Manbeena Chawla, Chandra Kumar Elechalawar, Dhirendra Kumar, Yong Yang, Neel S. Bhavesh, Rajkumar Banerjee, Debasis Dash, Amit Singh, Vivek T. Natarajan, Anil K. Ojha, Courtney C. Aldrich, and Rajesh S. Gokhale

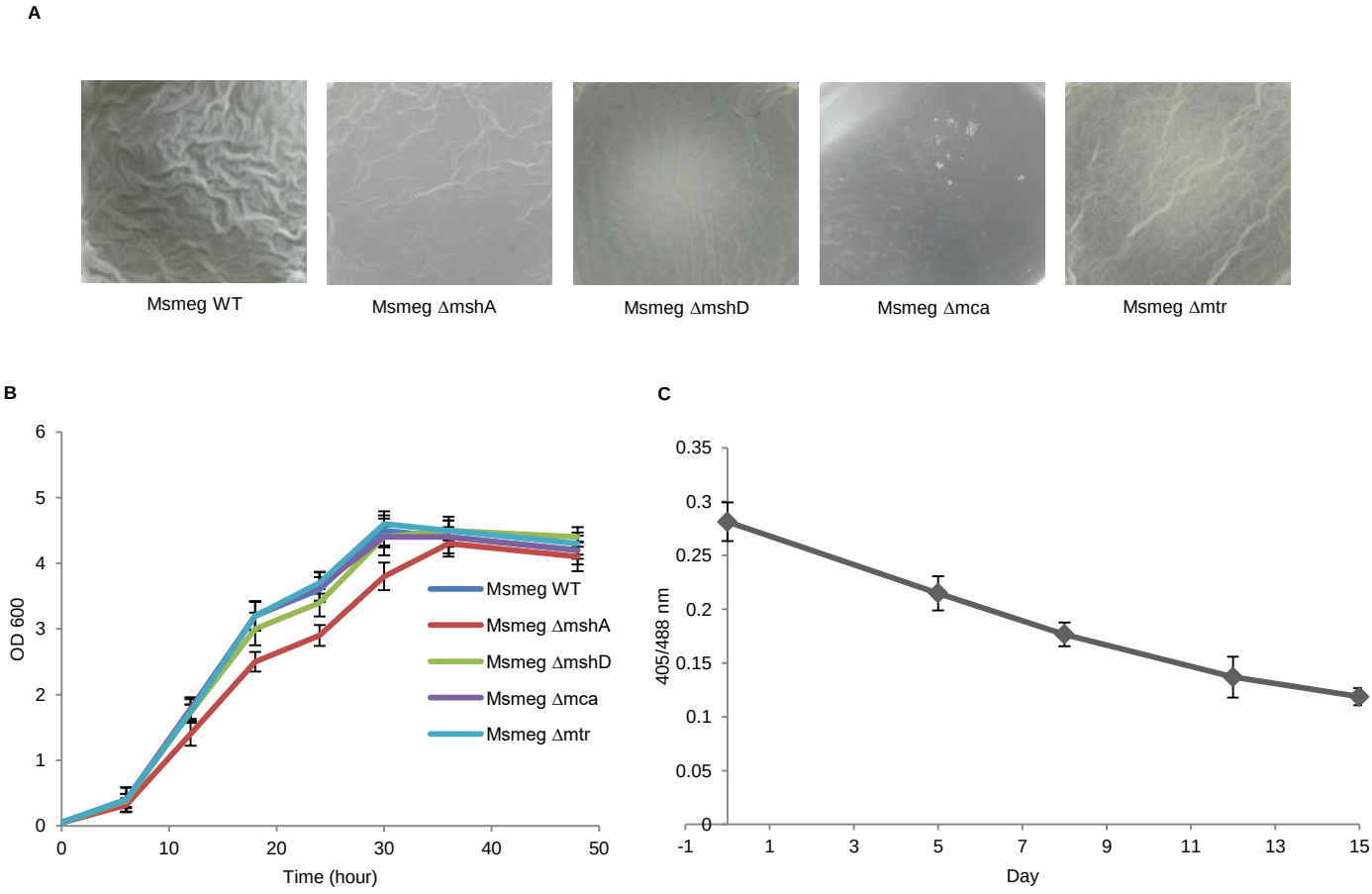

Figure S1: Growth profiles of mutants of mycothiol pathway and mycothiol redox potential in hypoxic condition

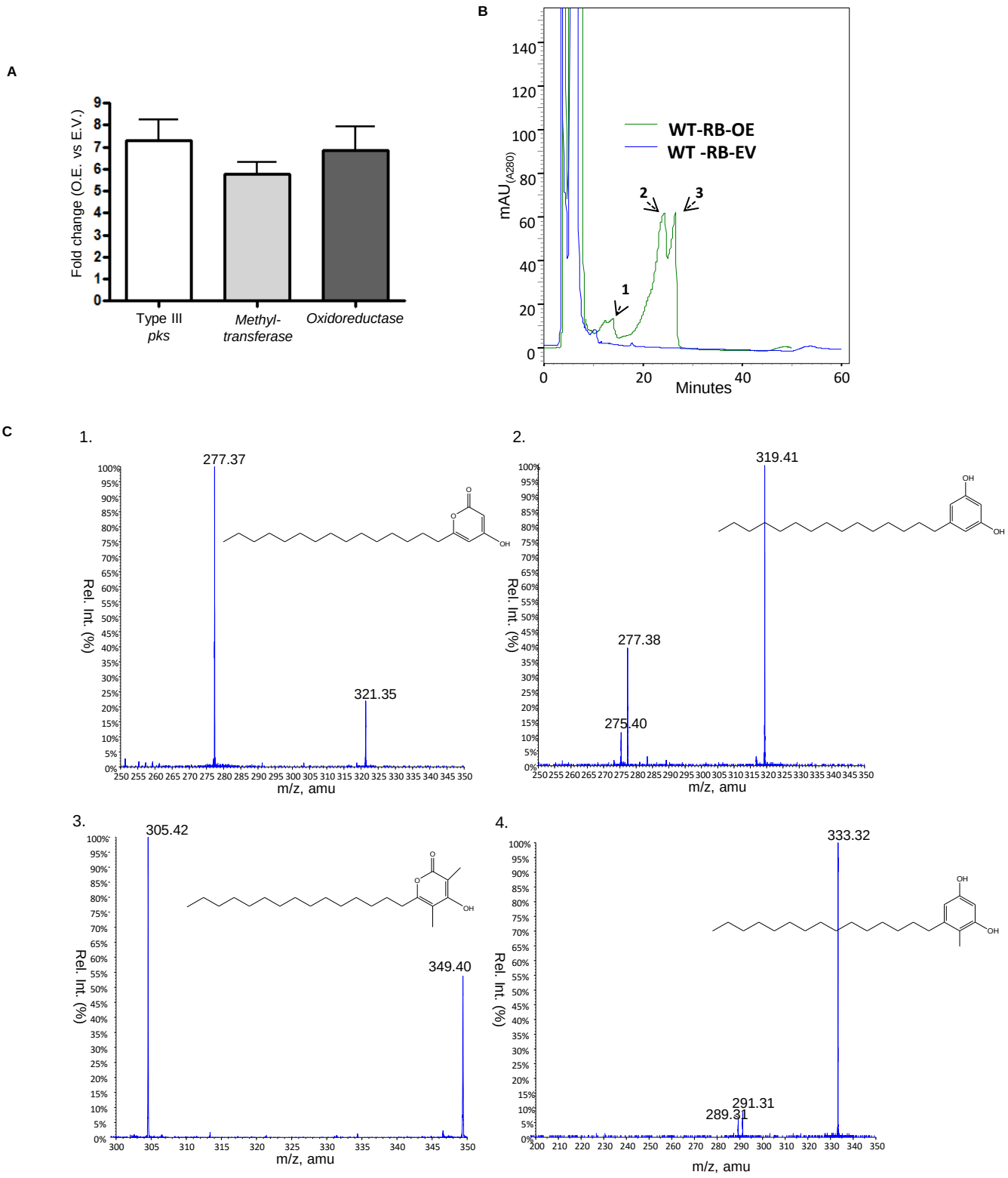

Figure S2: Palmitate feeding to Msmeg over-expressing the type III pks operon in planktonic culture

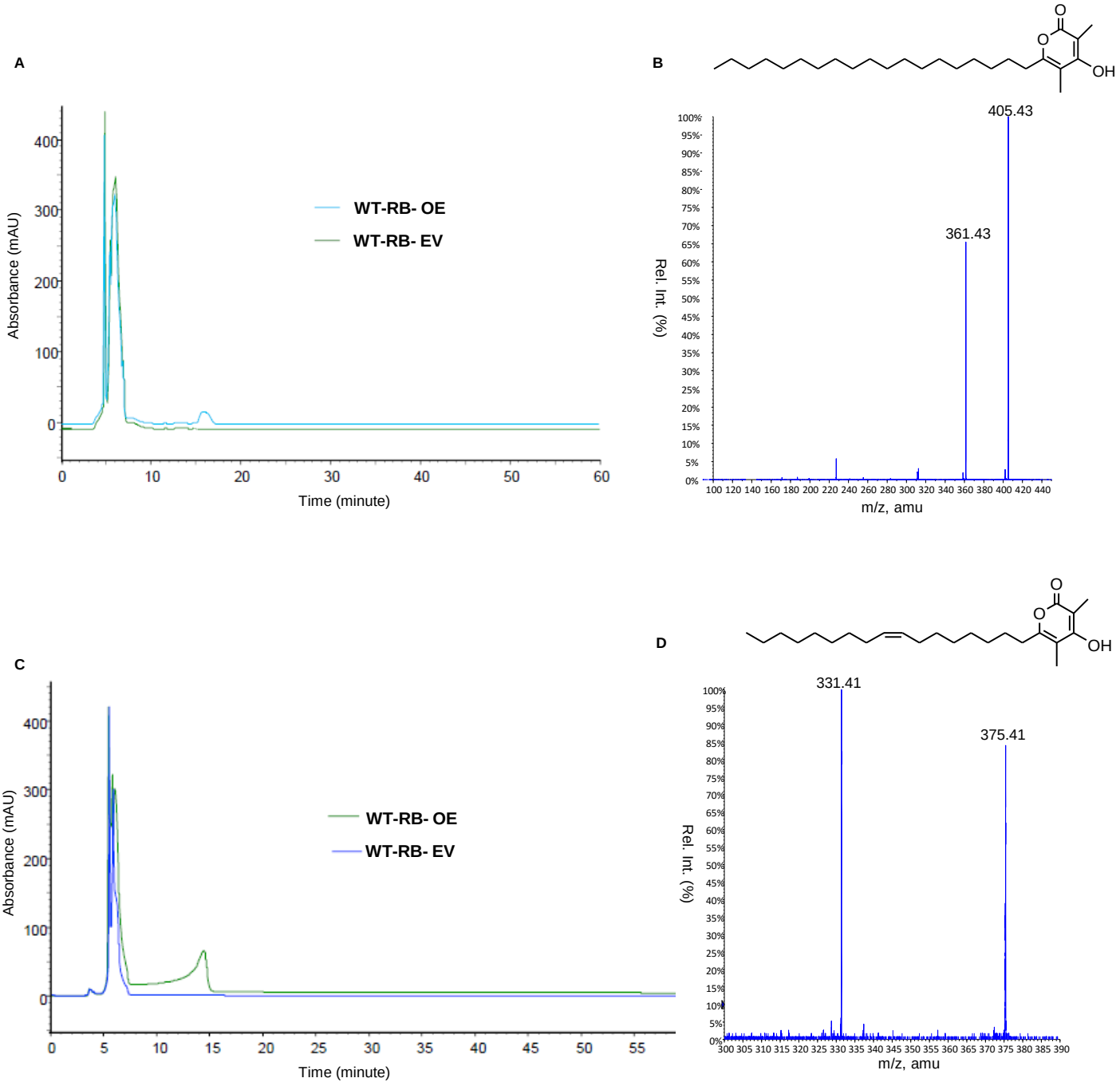

Figure S3: Analysis of cytosolic fraction of arachidonate and oleate fed culture

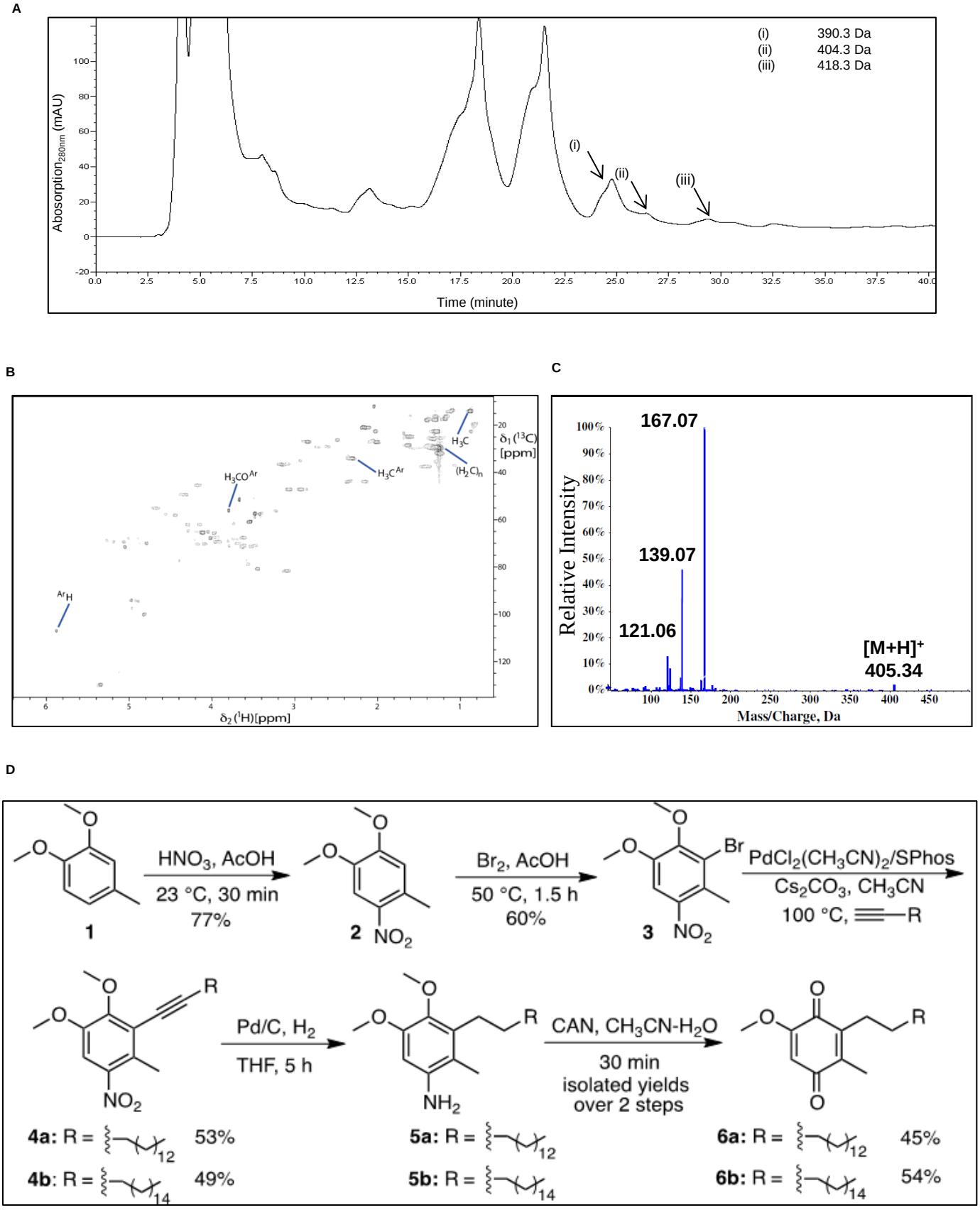

Figure S4: Characterization of alkyl benzoquinone isolated from mycobacteria

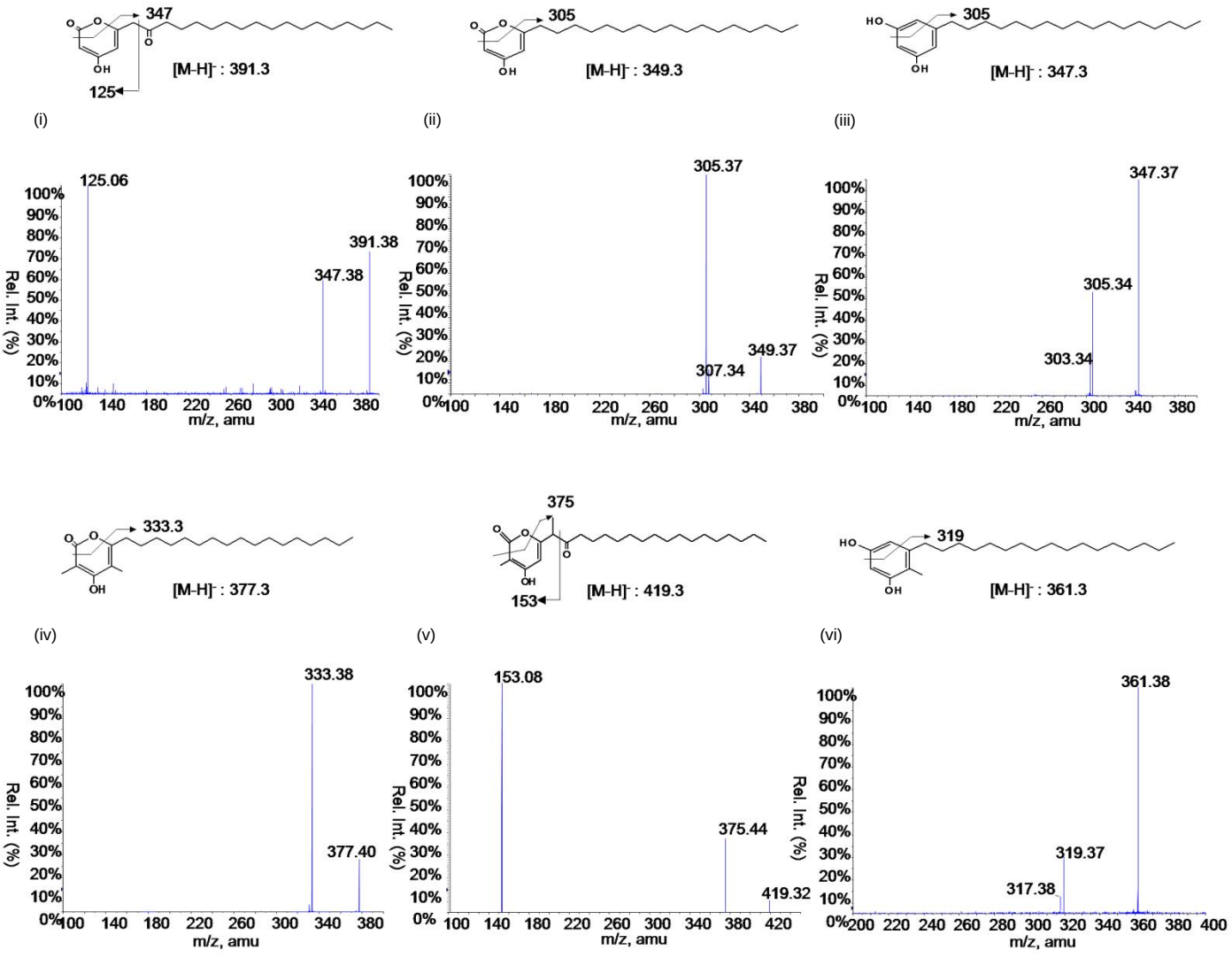

Figure S5: Tandem-MS analysis of *in vitro* products of PKS10 protein

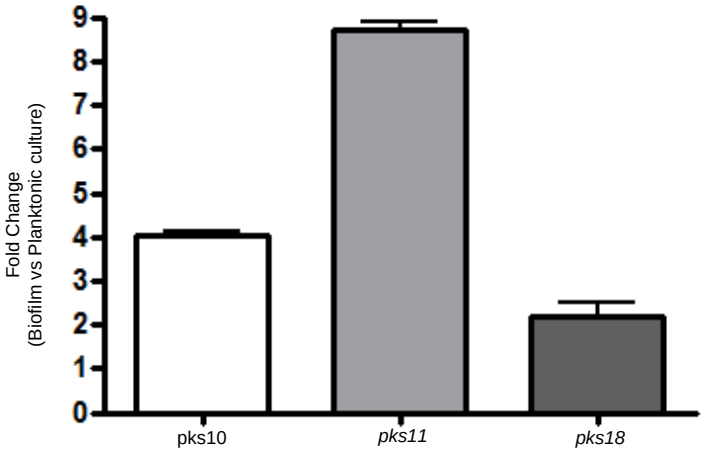

Figure S6: Expression analysis of type III pks genes of Mtb

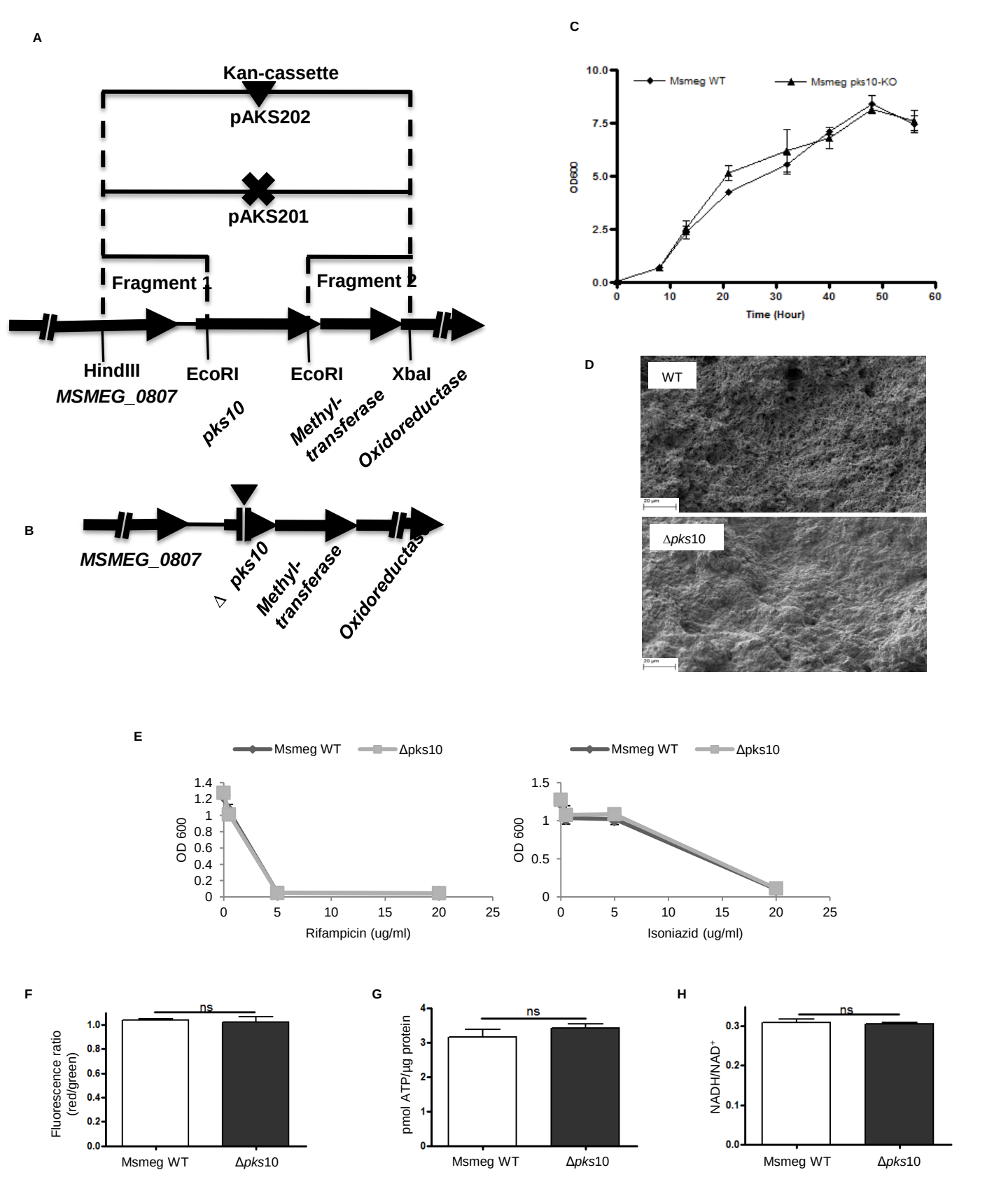

Figure S7: Generation and comparison of Δpks10 mutant with wild type strain

SUPPLEMENTARY FIGURE LEGEND

Figure S1: Growth profiles of mutants of mycothiol pathway and mycothiol redox potential in hypoxic condition (Related to Figure 1)

(A) Phenotype of the pellicle formed by the indicated mutants of mycothiol pathway. (B) Planktonic growth curve of the indicated mutants of mycothiol pathway. (C) Change in mycothiol redox potential with the progression of hypoxia in planktonic condition.

Figure S2: Palmitate feeding to Msmeg over-expressing the type III *pks* operon in planktonic culture (Related to Figure 2)

(A) Real time expression analysis for the validation of over-expression. (B) Overlay of HPLC chromatogram for ethyl acetate extract of palmitate fed RB-OE (over-expression) and RB-EV (empty vector) cultures. (B) Tandem MS profile of 321.5, 319.4, 349.4 and 333.3 the major molecular ion peak differentially present in OE culture fed with palmitate.

Figure S3: Analysis of cytosolic fraction of arachidonate and oleate fed culture of Msmeg over-expressing the type III *pks* operon (Related to Figure 2)

(A) Overlay of HPLC chromatogram for ethyl acetate extract of arachidonate fed RB-OE and RB-EV culture. (B) Tandem MS profile for 405.42, the major molecular ion peaks differently present in OE culture fed with arachidonate. (C) Overlay of HPLC chromatogram for ethyl acetate extract of oleate fed RB-OE and RB-EV culture. (D) Tandem MS profile for 375.41, the major molecular ion peaks differently present in OE culture fed with oleate.

Figure S4: Characterization of alkyl benzoquinones isolated from Msmeg (Related to Figure 2)

(A) UPLC chromatogram for alkyl benzoquinones isolated from Msmeg biofilm. (B) 2D [^{13}C, ^{1}H] HSQC spectrum showing chemical fingerprint of alkyl benzoquinone isolated from Msmeg biofilm (m/z 391.3199). (C) Tandem mass spectrum of the standard with C18 alkyl chain showing fragmentation pattern of alkyl benzoquinone. (D) Scheme for the chemical syntheses of alkyl benzoquinones.

Figure S5: *In vitro* characterization of Msmeg PKS10 product (Related to Figure 2)

HR-ESI (-ve) tandem MS characterization of products formed by Msmeg PKS10 with stearoyl CoA as starter unit and different combination of MCoA and MMCoA as extender units. (i-iii) with MCoA only, (iv) with MMCoA only and (v, vi) new products obtained when both MCoA and MMCoA were used as extender unit.

Figure S6: Real time expression analysis of Mtb type III *pks* genes (Related to Figure 3)

qRT-PCR expression analysis of *pks10*, *pks11* and *pks18* in biofilm and planktonic cultures of Mtb.

Figure S7: Generation of Msmeg Δ*pks*10 strain and comparison with wild type strain (Related to Figure 4)

(A) Genetic location and schematic representation of the plasmid DNA construct for Msmeg *pks10* mutant generation. Plasmid pAKS201 include fragment 1 and 2 without 1071 bp (from amino acid 4 to 360, indicated as "×") region of Msmeg*pks10* gene and plasmid pAKS202 includes the kanamycin cassette (indicated as ▼). All the generated restriction sites are also indicated. (B) Genetic organization of Msmeg *Δpks10* strain in Msmeg. (C) Planktonic growth profile of Msmeg WT and Δ*pks*10 strains. (D) SEM characterization of the biofilm surface

formed by the WT and the Δ*pks*10 strain at 1.66 kX magnification. (E) Antibiotic sensitivity profiling pf Msmeg WT and Δ*pks*10 strains in planktonic condition. (F) Membrane potential of cells of Msmeg WT and Δ*pks*10 strains in planktonic condition. (G) Level of intracellular ATP in Msmeg WT and Δ*pks*10 strains in planktonic condition. (H) Estimation of intracellular NADH/NAD^+ ratio in Msmeg WT and Δ*pks*10 strains in planktonic condition.

Table S1: Primers used in the study

For cloning MSMEG_0808	RSG-1769: 5' TT CAT ATG GAA AAC ACC GCC TTT ACG CCG TCG 3' RSG-1771:5' AA AAG CTT A CG CCA CCG CAG CAG GAC 3'
For cloning MSMEG_0809	RSG-2908: 5' TTG AAT TCA TGA AGG CAT TCA CAG C 3' RSG-2909: 5' TTA AGC TTT CAT GTC AGG CTA CGT AG 3'
For cloning MSMEG_0811	RSG-2910: 5' AA CAT ATG ATC GAT CTG CTC G TGG 3' RSG-2911: 5' AA TCT AGA CTA CTC CCC CAA CGC 3'
RT primer for Msmeg 16S rRNA	RSG-2603(a): 5'GCCGTAAACGGTGGGTACTA 3' RSG-2604(b): 5'TGCATGTCAAACCCAGGTAA 3'
RT primer for Msmeg sigA	RSG-6469 : 5' CCAAGGGCTACAAGTTCTCG 3' RSG-6470 : 5' TGGATCTCCAGCACCTTCTC 3'
RT primer for MSMEG_0808	RSG-2944: 5'AAG GGA TTC GAG ATC GTC CT 3' RSG-2945: 5'GAA GGC AGG TCC AGT GTC TC 3'
RT primer for MSMEG_0809	RSG-2946: 5'GCT GGT GGT GCA TCA AAA C 3' RSG-2947: 5'GTT GGC GAT CGT GAA TCC 3'
RT primer for MSMEG_0811	RSG-2948: 5' AGG TGG ACA CCA TCA TCC AG 3' RSG-2949:5'AGT ACA CCT CGA CGC AGT CC 3'
Primer used for KO generation (for fragment 1)	1F-5'-ACCCAAGCTTTCTAGAAAACCGCGCTGCAACTCGGCG 3' 1R-5'-ATGAATTCGTTTTCCATACCCCTAACACG 3'
Primer used for KO Generation (for fragment 2)	2F-5'- TTCTAGAAGGCGGTCGAGTTGCCGCACG 3' 2R-5'-ATGAATTCTGGCGTTGATGAAGGCATTCA 3'

Primer used for KO Confirmation	3F-5'-CGGCATAACCCGTGTTAGGGGTATG 3' 3R-5'-GACCAGGGCTGTGAATGCCTTCATC3'
RT primer for Mtb 16S rRNA	RSG-2603: 5' GTG CAA TAT TCC CCA CTG 3' RSG-2604: 5' ATG CAT GTC TTG TGG TGG 3'
RT primer for Mtb pks10	RSG-7439: 5' GACCCAAGATCATCAACGCC 3' RSG-7440 : 5' ATCATCAACCCGGGACTTCC 3'
RT primer for Mtb pks11	RSG-7441 : 5' GATCGAACGGTACCTAGCCA 3' RSG-7442 : 5' GACGAAAGGTTGCCGATCTC 3'
RT primer for Mtb pks18	RSG-7443 : 5' GTTGCGATCGTCAAAGAGCT 3' RSG-7444 : 5' CAAGCTGTGAATGACGACGT 3'
RT primer for Mtb Rv1138c	RSG-7445 : 5' GGGGTGAGGCTTATGTGACT 3' RSG-7446 : 5' TTAGGGCATCTTCGTACCCG 3'
RT primer for Mtb Rv1139c	RSG-7447 : 5' AACACCCGGGTAATCGTGTT 3' RSG-7448 : 5' CATTGGCCAGGGTGAAAACC 3'
For cloning Msmeg pks10 promoter	RSG-7628 : 5' GCATCGGGTACGGCTACTTCACCTACGACGG 3' RSG-7629 : 5' GCATCGCATATGACCCCTAACACGGGTTATGC 3'

SUPPLEMENTARY EXPERIMENTAL PROCEDURE

Bacterial strain and materials

E. coli XL-1 blue and *E. coli* BL21 (DE3) were used as cloning and expression strains, respectively. *Mycobacterium tuberculosis* H37Rv and *Mycobacterium smegmatis* mc^2155 was used as wild type strains for all experiments except for the metabolite isolation from Mtb which was performed using *M. tuberculosis* mc^27000 and the over-expression analysis was performed using glycopeptidolipid null (GPL-null) strain of *M. smegmatis* (Msmeg RB strain) to avoid interference by abundant GPLs. Non-radioactive alkyl-CoA starter substrates were purchased from Sigma. Radiolabeled [2-^{14}C]malonyl-CoA (MCoA) and [2-^{14}C]methylmalonyl-CoA (MMCoA) were obtained from American Radiolabeled Chemicals. Middlebrook 7H9 and 7H11

media were procured from Difco. Sauton's Fluid Media Base was obtained from Himedia. Sodium palmitate was bought from Sigma. TRIzol and Superscript III RT-PCR kit were bought from Invitrogen. RNase-Free DNase Set and RNeasy Mini Kit were purchased from QIAGEN. SYBR green PCR master mix was procured from Fermentas. All other chemicals used were of analytical grade and purchased from Sigma, New England Biolabs and Merck. pCHERRY3 was a gift from Tanya Parish (Addgene plasmid # 24659) (Carroll et al., 2010).

Cloning, expression and purification of Msmeg PKS10, Mtb PKS11 and Mtb PKS18

Msmeg *pks10* was amplified using gene specific primers and insert was ligated into pET21c expression vector system. The protein was expressed with C-terminal hexa-histidine tag in the BL21 DE-3 strain of *E. coli*. The recombinant *E. coli* cells harboring the expression plasmid were grown in LB broth at 30°C until an OD_{600} of 0.8 units was reached. For Msmeg PKS10, the culture was induced with 0.025 mM of IPTG and further incubated in shaker at 22°C for 18-20 h. After harvesting, the cells were re-suspended in lysis buffer (50 mM Tris pH 8.0, 10% glycerol, 0.15 M NaCl) and disrupted using French Press at 1100 psi pressure. Cell debris was removed by centrifugation at 16,000 rpm for 30 min at 4°C. 0.75 ml of Ni^{2+}-NTA slurry per liter of culture was added to the supernatant and was incubated at 4°C for 1 h. The mixture was loaded onto a column using gravity flow. The resin was washed with wash buffer (50 mM Tris pH 8.0, 10% glycerol) till the unbound proteins were removed. The protein was eluted using elution buffers containing increasing concentration of imidazole. Fractions containing the proteins of interest were pooled and 1 mM TCEP was added and stored at -70°C. Expression and purification of Mtb proteins were performed same as protocol mentioned in earlier literature (Saxena et al., 2003).

Radiolabeled enzymatic assay and product characterization using mass spectrometry

The standard reaction conditions involved 100 μM starter CoA and 50 μM MCoA [inclusive of 9.12μM of [2-^{14}C]-MCoA (58.40 mCi/mmol)] and/or 50 μM MMCoA [inclusive of 9.12 μM of [2-^{14}C]-MMCoA (58.40 mCi/mmol)]. Reactions were carried out with 45 μg of protein at 30°C for 120 min and quenched with 5% acetic acid. Products were extracted with 2x300 μl ethyl acetate and dried under vacuum. Radiolabeled products were resolved on silica gel 60 F_{254} TLC plates (Merck) in ethyl acetate: hexane: acetic acid (63:27:5 v/v/v). Resolved radiolabeled products were read using Fuji FLA-5000 phosphor imager.

Multiple reactions were set up as mentioned above (except no radiolabeled substrate was used). Products from at-least 10 reactions were pooled after vacuum drying by dissolving in 100 μl of methanol and then diluted 1:1 with 90% CH_3CN and 0.1% HCOOH. Products were characterized using Nanospray ESI-MS (API QSTAR Pulsar i MS/MS, Applied Biosystems) in negative ion mode.

Construction of *pks10* mutant strains

The marked mutation construct of *pks10* was generated by PCR, using genomic DNA of *M. smegmatis* mc^{2}155 as a template (Figure S7). Forward primer 1F containing HindIII-XbaI restriction sites and reverse primer 1R containing EcoRI restriction site, were used to amplify fragment1 encoding N-terminal amino acids 1 to 3, along with a 676 bp sequence upstream of the Msmeg *pks10*. Similarly, the forward primer 2F and reverse primer 2R containing the EcoRI and XbaI sites respectively, were used to amplify fragment 2 encoding amino acids 361 to 363, along with a 666 bp downstream of the Msmeg *pks10* gene. Fragment 1 and fragment 2 were digested by either HindIII/EcoRI or EcoRI/XbaI (Biolabs) and cloned in a HindIII-XbaI digested pBluescript II SK (+) (Stratagene) to generate pAKS201. The kanamycin cassette was extracted from the pUC4K vector (Pharmacia biotech) by EcoRI digestion, purified, and cloned into the

EcoRI restriction site of the pAKS201 to generate the pAKS202. This allelic-exchange substrate was used to perform mutagenesis in both *M. smegmatis* mc^2155 and *M. smegmatis*-RB strains as described previously (van Kessel and Hatfull, 2007). Genomic DNA isolated from the WT and Δ*pks*10 strains were used for PCR amplification, using primers 3F and 3R to identify the clones harboring the deletion in Msmeg *pks10*. PCR products were confirmed by sequencing.

Construction of Msmeg *pks10* promoter reporter strains and quantification

Hsp60 promoter of mycobacteria replicating vector pMV261 was replaced by 500 bp upstream sequence of *pks10* coding sequence to place expression of GFP under control of *pks10* promoter. The GFP expressing population was quantified using Amnis ImageStream Mark II Imaging Flow Cytometer and analysis by done using IDEAS 2.0.

Biofilm development

Msmeg strains were routinely cultured in 7H9-ADC media containing 0.2% glycerol and 0.05% Tween 80 and grown at 37°C at 180 rpm. Biofilm culture for Msmeg was grown in Sauton's media supplemented with 2% glucose. The plates were sealed with parafilm and incubated at 37°C in humidified incubator for 7 day.

For complementation of alkyl benzoquinone, chemically synthesized alkyl benzoquinone dissolved in methanol was added during biofilm set-up.

In KNO_3 feeding experiments, KNO_3 was added to the biofilm media in following three concentrations 0.5%, 1% and 2%.

Biofilm of Mtb was grown in Sauton's medium (without Tween-80) by incubating without shaking at 37°C for 5 weeks in humidified conditions. The dishes were wrapped with parafilm during incubation.

Hypoxic culture set-up

In order to generate low oxygen condition in planktonic condition, cultures were grown in 4:1 culture to head space ratio. 1.5 µg/ml methylene blue was added to monitor establishment of hypoxia. Cultures were maintained in Anaerobic System Mark III jar (HIMedia) to avoid oxygen seepage.

RNA isolation and qRT-PCR

Bacteria were grown in Sauton's media either as biofilm or planktonic culture to the desired time or density, harvested and immediately processed for RNA isolation using QIAGEN RNeasy Mini Kit. RNA was quantified using a NanoDrop spectrophotometer before being frozen at -80°C for later use. qRT-PCR was performed using SuperScript III reverse transcriptase using random hexamer primer, according to manufacturer's protocol. Reverse transcriptase PCR was performed with Superscript III (Invitrogen) according to manufacturer's instruction. Quantitative real time PCR was carried out with maxima SYBR green/ROX qPCR mix (Fermentas) in MasterCycler RealPlex real time PCR system (Eppendorf).

Scanning electron microscopy

Scanning electron microscopy was performed for the biofilm samples using standard protocols. Briefly, the biofilm was fixed with fixative containing 4% paraformaldehyde and 2.5% glutaraldehyde in sodium cacodylate buffer. The biofilm was then osmicated with 1% OsO_4 for 1 hr at room temperature and gradually dehydrated with increasing concentrations of ethanol followed by 15 min incubation in dark with hexamethyldisilazane (HMDS). The sample was mounted on stubs, sputter coated with gold particles for 300 s, and imaged on a Zeiss-EVO LS15 Scanning Electron Microscope.

Extraction and analysis of type III PKS products from planktonic and biofilm cultures

Msmeg cells were inoculated in 7H9 media supplemented with 0.5% Glycerol and ADC and incubated at 37°C with shaking at 180 rpm. Wherever required, emulsion of 200 μM sodium salt of fatty acid conjugated with BSA was fed into the cultures. Late stationary phase cultures OD_{600} of 2.5-3.0 were harvested by centrifugation and suspended in appropriate volume of 100 mM Tris-Cl, pH 8.0 and disrupted by bead beater using 10 pulses of 1 min each. For extraction of lipids from biofilms, 7 day old biofilm cultures were taken and were sonicated to prepare the whole cell lysate. The resultant whole cell lysate was acidified by addition of 6 M HCl.

Low molecular weight molecules were then extracted by adding double volume of ethyl acetate and left overnight on a stirrer. The organic layer was separated, evaporated to dryness, and the residual material was dissolved in a minimal volume of methanol and resolved on HPLC using C5-reverse phase column (Phenomenex). The compounds were eluted in varying percent of 0.1% trifluoroacetic acid containing CH_3CN and water. Each peak tip was analyzed using Nanospray ESI-MS (API QSTAR Pulsar i MS/MS, Applied Biosystems). Ions were introduced into the ion source via direct injection at 5 μl/min and spectra were acquired and analyzed using Xcalibur, version 2.0.7 (Thermo). UPLC-HR-ESI-MS analysis was carried out on AB SCIEX TripleTOF 5600 System mass spectrometer, in positive ion mode, coupled to a Dionex UltiMate 3000 system equipped with a C5-reverse phase column (Phenomenex) using varying percent of 0.1% trifluoroacetic acid containing CH_3CN and water at a flow rate of 0.6 mL/min.

***hspX* promoter activity analysis in biofilm**

Mixed culture biofilm of two recombinant strains of Msmeg was grown as regular biofilm set-up. One strain was electroporated with pCHERRY3 and other strain was electroporated with

phspX. Imaging was done using Leica (Germany) TCS-SP8 and analysis was done with LAX 1.8.0. To quantify the image (tiff format), we have used custom written R-script (R version 3.2.1). Image was imported using tiff-package (version 0.1-5). Images were extracted and then each image was segmented/sectioned into 7 sections based on z-axis (e.g., 1-5 µm, 6-10 µm, ... 31-35 µm). Complete y-axis covers 699 pixels per section. RGB color proportion (range 0 to 1) was extracted from red channel (pCHERRY3) and green channel (phspX). Section-wise median RGB color proportion were computed per pixel of y-axis for both images and merged during figure generation.

Mycothiol redox potential estimation

The mycothiol redox potential was estimated using previously characterized MSH-specific intracellular probe, Mrx1-roGFP2 (Bhaskar et al., 2014). Msmeg Mrx1-roGFP2 planktonic culture was grown till an OD of 0.4. Simultaneously, the mature biofilm samples were washed and suspended in 1X PBS. Both planktonic and biofilm samples were separated into control and test sets. The control sets were treated with 2 mM cumene hydroperoxide (for 100% oxidation) and 20 mM dithiothreitol (for 100% reduction). The test and control sets were treated with 10 mM N-ethylmaleimide for fixing the redox state of roGFP2 (this precludes any oxidation artifacts during processing) and further treated with 4% PFA. The samples were finally washed with 1X PBS and then analyzed by flow cytometry. The ratio of emission at 510 nm after excitation at 405 and 488 nm was calculated. Data were analyzed using the FACSuite software. Using automatic and manual gating options, biofilm population was categorized into three subpopulations: E_{MSH}-oxidized, E_{MSH}-reduced and E_{MSH}-basal. Number of events per subpopulation was counted and representative percentage of each subpopulation was estimated.

Antibiotic sensitivity assay

Desired dose of antibiotic was added to mature biofilm and was incubated for 24 hour. At the end of the incubation, 0.1% Tween-80 was added and plate was gently swirled to disperse the detergent. After 15 minute of incubation, pellicles were collected and washed thrice with PBS containing 10% glycerol and 0.05% Tween-80. Pellicles were kept on rocker at 4˚C for 12 hour in the same buffer for dispersal of extracellular matrix. Suspension was then homogenized by vortexing and serially diluted. The serial dilution was plated on 7H11-OADC plates. Colony forming units (CFUs) were counted and percent decrease in CFU was estimated by comparing the antibiotic treated culture with its respective untreated control culture. Planktonic cultures were treated with different concentrations of antibiotics for 24 hour and OD_{600} was measured to estimate growth inhibition.

Phylogenetic analysis

Presence or absence of gene cluster was determined by sequence similarity searches using ncbi web tblastn. Protein sequences of *Mycobacterium smegmatis* type III PKS cluster were queried against a database of all available bacterial genomes (RefSeq genomes, 1,192,124 sequence entries). Orthologs were only considered if 60% of query protein was covered in alignment and e-value was less than 10^{-5}. Gene cluster were determined if orthologs of queried gene members were present in same genomic strand and in close proximity (<200bp gap between genes).

Chemical syntheses and characterization of alkyl benzoquinones

All chemical reactions were performed under an inert atmosphere of dry Ar or N_2 in oven-dried (150 °C) glassware. ^{1}H and ^{13}C NMR spectra were recorded on Varian 400 or 600 MHz spectrometers. Proton chemical shifts are reported in ppm from an internal standard of residual

chloroform (7.26 ppm) and carbon chemical shifts are reported using an internal standard of residual chloroform (77.0 ppm). Proton chemical data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad), integration, coupling constant. High resolution mass spectra were obtained on an Agilent TOF II TOF/MS instrument equipped with an APCI interface. TLC analyses were performed on TLC silica gel 60F254 from EMD Chemical Inc., and were visualized with UV light, iodine chamber, 10% sulfuric acid or 10% PMA solution. Purifications were performed by flash chromatography on silica gel (Dynamic Adsorbents, 60A).

1,2-Dimethoxy-4-methyl-5-nitrobenzene (2). 3,4-Dimethoxytoluene (500 mg, 3.3 mmol, 1.0 equiv) was dissolved in acetic acid (1 mL) followed by the addition of nitric acid (0.23 ml, 3.6 mmol, 1.1 equiv). The reaction mixture was stirred at 23 °C for 30 min and then poured into H_2O (20 mL) which resulted in the formation of a light orange precipitate. The precipitate was collected and dried to afford the title compound (496 mg, 77 %): ^{1}H NMR (600 MHz, $CDCl_3$) 2.63 (s, 3H), 3.94 (s, 3H), 3.97 (s, 3H), 6.72 (s, 1H), 7.66(s, 1H).

3-Bromo-1,2-dimethoxy-4-methyl-5-nitrobenzene (3).4,5-Dimethoxy-2-nitrotoluene (500 mg, 2.5 mmol, 1.0 equiv) was dissolved in acetic acid (3.0 mL) and stirred at 23 °C. Bromine (0.39 mL, 7.6 mmol, 3.0 equiv) was dissolved in acetic acid (1.0 mL) and added to the reaction flask. The reaction mixture was heated at 50 °C for 1.5 h then cooled to 23 °C and poured intoH_2O (20 mL) which resulted in the formation of an orange precipitate. Purification by silica gel flash chromatography with 10% EtOAc–hexanes afforded the title compound (423 mg, 60%) as a white solid: R_f0.19 (10% EtOAc–hexane); mp 95–97 °C;^{1}H NMR (600 MHz, $CDCl_3$) 2.57 (s, 3H), 3.92 (s, 3H), 3.93 (s, 3H), 7.42 (s, 1H); ^{13}C NMR (150 MHz, $CDCl_3$)19.6, 56.5, 60.8, 107.9, 122.9, 126.6, 146.2, 150.4, 151.1.

3-(Hexadec-1-yn-1-yl)-1,2-dimethoxy-4-methyl-5-nitrobenzene (4a). To a flask flushed with Ar, was added $PdCl_2(CH_3CN)_2$ (2.6 mg, 0.01 mmol, 5 mol%) and SPhos (12 mg, 0.03 mmol, 15 mol%). The flask was purged with Ar for 10 min then CH_3CN (2.0 mL) was injected and the reaction was stirred for an additional 15 min. Next, **3** (55 mg, 0.2 mmol, 1.0 equiv) was added and the resulting solution incubated at 23 °C for 30 min. To the mixture, was added Cs_2CO_3 (293 mg, 0.9 mmol, 4.5 equiv) and 1-hexadecyne (66.6 mg, 0.3 mmol, 1.5 equiv) sequentially. The reaction was heated at 90 °C and stirred for 16 h. The mixture was partitioned between CH_2Cl_2 (10 mL) and saturated aqueous NH_4Cl (10 mL). The organic layer was separated and the aqueous layer extracted with CH_2Cl_2 (2 × 10 mL). The combined organic extracts were washed with saturated aqueous NaCl (20 mL), dried ($MgSO_4$) and concentrated. Purification by silica gel flash chromatography with 10% EtOAc–hexanes afforded the title compound (44 mg, 53%) as a light brown solid: R_f 0.23 (10% EtOAc–hexane); mp 52–55 °C; ^{1}H NMR (600 MHz, $CDCl_3$) 0.88 (t, *J*= 7.2 Hz, 3H), 1.26–1.34 (m, 20H), 1.48–1.49 (m, 2H), 1.64–1.67 (m, 2H), 2.53 (t, *J*=7.2 Hz, 2H), 2.59 (s, 3H), 3.90 (s, 3H), 3.97 (s, 3H), 7.44 (s, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) 14.3, 17.7, 20.0, 22.9, 28.8, 29.1, 29.4, 29.6, 29.77, 29.83, 29.86, 29.88, 32.1, 56.4, 61.1, 74.2, 101.8, 108.2, 121.4, 129.4, 145.3, 150.5, 154.4; HRMS (APCI+) calcd for $C_{25}H_{40}NO_4[M+H]^+$418.2952, found 418.2949 (error 0.7 ppm).

1,2-Dimethoxy-4-methyl-5-nitro-3-(octadec-1-yn-1-yl)benzene (4b). The title compound was prepared analogously to **4a** employing 1-octadecyne and obtained in 49% isolated yield as a light brown solid: R_f 0.33 (10% EtOAc–hexane); mp 54–56 °C; ^{1}H NMR (600 MHz, $CDCl_3$) 0.88 (t, *J*= 6.0 Hz, 3H), 1.26–1.34 (m, 24H), 1.47–1.52 (m, 2H), 1.63–1.68 (m, 2H), 2.52 (t, *J*=6.0 Hz, 2H), 2.59 (s, 3H), 3.89 (s, 3H), 3.96 (s, 3H), 7.44 (s, 1H);^{13}C NMR (150 MHz, $CDCl_3$)14.3, 17.7, 20.0, 22.9, 28.8, 29.12, 29.36, 29.56, 29.78, 29.84, 29.86, 29.90, 32.1, 56.5,

61.1, 74.2, 101.8, 108.2, 121.4, 129.4, 145.3, 150.5, 154.5; HRMS (APCI+) calcd for $C_{27}H_{44}NO_4[M+H]^+$446.3265, found 446.3294 (error 6.3 ppm).

3-Hexadecyl-4,5-dimethoxy-2-methylaniline (5a). To a solution of **4a** (38 mg, 0.09 mmol, 1.0 equiv) in THF(3.0mL) at 23 °C was added 10% w/w Pd/C (9.6 mg, 0.009 mmol, 10 mol%) and the mixture was stirred under a hydrogen atmosphere using a hydrogen balloon for 6h. Upon completion of the reaction, the mixture was filtered over Celite and washed with CH_2Cl_2. The solution was concentrated *in vacuo* to afford the crude product, which was used in the following step without further purification: R_f 0.29 (30% EtOAc–hexane); ^{1}H NMR (600 MHz, $CDCl_3$) 0.89 (t, *J*= 6.0 Hz, 3H), 1.27–1.32 (m, 24H), 1.38–1.47 (m, 4H), 2.06 (s, 3H), 2.63 (t, *J*=6.0 Hz, 2H), 3.74 (s, 3H), 3.81 (s, 3H), 6.25 (s, 1H); ^{13}C NMR (150 MHz, $CDCl_3$)12.7, 14.3, 22.9, 27.3, 29.56, 29.73, 29.83, 29.86, 29.90, 30.24, 30.68, 32.1, 55.8, 61.2, 98.5, 112.9, 136.1, 140.2, 140.7, 151.2; HRMS (APCI+) calcd for $C_{25}H_{46}NO_2[M+H]^+$ 392.3523, found 392.3507 (error 5.3 ppm).

4,5-Dimethoxy-2-methyl-3-octadecylaniline (5b). The title compound was prepared analogously to **5a**, and was obtained as a white solid: R_f 0.21 (30% EtOAc–hexane); ^{1}H NMR (600 MHz, $CDCl_3$)0.90 (t, *J*= 6.0 Hz, 3H), 1.28–1.33 (m, 30H), 1.38–1.43 (m, 2H), 1.45–1.50 (m, 2H), 2.05 (s, 3H), 2.64 (t, *J*=6.0 Hz, 2H), 3.47 (brs, 2H), 3.75 (s, 3H), 3.81 (s, 3H), 6.20 (s, 1H); ^{13}C NMR (150 MHz, $CDCl_3$)12.7, 14.3, 22.9, 27.3, 29.57, 29.74, 29.83, 29.86, 29.91, 30.25, 30.69, 32.1, 55.8, 61.2, 98.5, 112.8, 136.0, 140.19, 140.8, 151.2; HRMS (APCI+) calcd for $C_{27}H_{50}NO_2[M+H]^+$ 420.3836, found 420.3849 (error 3.0 ppm).

3-Hexadecyl-5-methoxy-2-methylcyclohexa-2, 5-diene-1, 4-dione (6a). A solution of crude **5a** from the previous step (0.09 mmol, 1.0 equiv) in 1:1 MeCN–CH_2Cl_2 (1.2 mL) was added dropwise to a solution of ceric ammonium nitrate (197 mg, 0.36 mmol, 4.0 equiv) in1:1 H_2O–

MeCN (2.6 mL) at 23 °C. The reaction mixture was stirred for 30 min and then diluted with CH_2Cl_2. The aqueous layer was separated and extracted with CH_2Cl_2 (2 × 10 mL). The combined organic extracts were washed with saturated aqueous NaCl (20 mL), dried ($MgSO_4$) and concentrated. Purification by silica gel flash chromatography afforded the title compound (18.3 mg, 45%) as a yellow solid: R_f0.34 (30% EtOAc–hexane); ^{1}H NMR (600 MHz, $CDCl_3$)0.88 (t, *J*=7.2 Hz, 3H), 1.42–1.26 (m, 28H), 2.04 (s, 3H), 2.49 (t, *J*=7.8 Hz, 2H), 3.80 (s, 3H), 5.88 (s, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) 12.4, 14.3, 22.9, 26.5, 28.8, 29.58, 29.62, 29.74, 29.86, 29.88, 29.89, 29.92, 30.01, 32.2, 56.3, 107.2, 141.4, 143.4, 158.6, 182.3, 188.0; HRMS (APCI+) calcd for $C_{24}H_{41}O_3$ $[M + H]^+$ 377.3050, found 377.3044(error1.5ppm).

5-Methoxy-2-methyl-3-octadecylcyclohexa-2,5-diene-1,4-dione (6b). The title compound was prepared analogously to **6a**, and obtained in 54% isolated yield over 2 steps as a light brown solid: R_f 0.13 (30% EtOAc–hexane); ^{1}H NMR (600 MHz, $CDCl_3$)0.88 (t, *J*= 6.0 Hz, 3H), 1.25–1.39 (m, 32H), 2.23 (s, 3H), 2.53 (t, *J*=6.0 Hz, 2H), 3.78 (s, 3H), 6.03 (s, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) 13.5, 14.3, 22.9, 26.7, 28.9, 29.57, 29.66, 29.77, 29.87, 29.89, 29.91, 30.01, 32.1, 55.8, 108.6, 128.5, 141.4, 154.3, 167.4, 181.5; HRMS (APCI+) calcd for $C_{26}H_{45}O_3$ $[M + H]^+$ 405.3369, found 405.3379 (error 2.5 ppm).

SUPPLEMENTARY INFORMATION REFERENCES

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