Electronic Supplementary Information

Funneling aromatic products of chemically depolymerized lignin into 2-pyrone-4-6- dicarboxylic acid with *Novosphingobium aromaticivorans*

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Supplemental Figures and Tables

Table S1: Bacterial strains and plasmids used in this study

Strains	Details	Reference
Novosphingobium aromaticivorans strains		
12444Δ1879	DSM12444 (WT) ΔSaro1879	(1)
12444ΔligI	12444Δ1879 ΔSaro2819	This study
12444ΔdesC/D	12444Δ1879 ΔSaro2864/5	This study
12444ΔligIΔdesC/D	12444Δ1879 ΔSaro2819 ΔSaro2864/5	This study
Escherichia coli strains		
DH 5α	F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1	Bethesda Research Laboratories (2)
S17-1	recA pro hsdR RP4-2-Tc::Mu-Km::Tn7	(3)
Plasmids		
pK18*mobsacB*	pMB1ori sacB kanR mobT oriT(RP4) lacZα	(4)
pK18msB/ΔSaro2819	pK18*mobsacB* containing genomic regions flanking Saro2819	This study
pK18msB/ΔSaro2864/5	pK18*mobsacB* containing genomic regions flanking Saro2864/5	This study

Table S2: Primers used in this study

Name	**Sequence**	**Obs**
Saro2819_Del-R	5'-GCGCCAATCCATACCACGGATTATGCGAATACTACTCCATCCATCAGCTTG-3'	
Saro2819-pK18_Amp-F	5'-**CGATTCATTAATGCAGCTGGCACGACAG**GAGCGAATGGCATGAGTTCACATTCAGC-3'	Region in bold matches sequence in pK18msB
Saro2819_Del-F	5'-GCTGATGGATGGAGTAGTATTCGCATAATCCGTGGTATGGATTGGCGCATG-3'	
Saro2819-pK18_Amp-R	5'-**GTTTCTGCGGACTGGCTTTCTAGATGTTC**CTGCATGGTCTGGTCCTGTTCAAGCAG-3'	Region in bold matches sequence in pK18msB
Saro2864-5_Del_R	5'-GGGTAGTCTGGATCATTCAGACTCGCATGGTGCCGAG-3'	
Saro2864-5-pK18_Amp_F	5'-**CGATTCATTAATGCAGCTGGCACGACAG**CAGGTCGGCTTCAAGGAGGAAGTTCTG-3'	Region in bold matches sequence in pK18msB
Saro2864-5_Del_F	5'-CCATGCGAGTCTGAATGATCCAGACTACCCGCCGTTATC-3'	
Saro2864-5-pK18_Amp_R	5'-**GTTTCTGCGGACTGGCTTTCTAGATGTTC**GACCACTATGCAATGGAATGGAACCTGC-3'	Region in bold matches sequence in pK18msB
Saro2865_Start-SNP_F	5'-GGCATGCTCGGCACCATGCG-3'	
Saro2865_Start-SNP_R2	5'-GCCGTCGACCGCGAGAGCTTG-3'	

Table S3: Multiple reaction module (MRM) conditions for HPLC-MS quantification of compounds used in this study

Compound	MW (g/mol)	Parent (-) m/z	Transition 1	Transition 2	Transition 3
PDC	184.103	183	183 -> 139.05 CE11	183 -> 111 CE14	183 -> 94.95 CE12
Protocatechuic acid	154.12	153	153 -> 108.95 CE14	153 -> 107.95 CE25	153 -> 90.95 CE27
p -hydroxybenzoic acid	138.12	137	137 -> 93 CE15	137 -> 65 CE30	
Vanillic acid	168.15	167	167 -> 123.05 CE15	167 -> 108 CE21	167 -> 152.05 CE18
p -hydroxybenzaldehyde	122.12	121.2	121.2 -> 92.05 CE26	121.2 -> 93.10 CE22	121.2 -> 41 CE49
Syringic acid	198.17	197	197 -> 121.05 CE18	197 -> 153.10 CE15	197 -> 182.10 CE15
Vanillin	152.15	151	151 -> 136 CE17	151 -> 92 CE22	151 -> 108 CE24
p -Coumaric acid	164.16	163	163 -> 119.05 CE15	163 -> 93 CE31	163 -> 116.95 CE33
Syringaldehyde	182.18	181	181 -> 166.10 CE16	181 -> 151 CE22	181 -> 123 CE28
Ferulic acid	194.19	193	193 -> 149 CE13	193 -> 134 CE16	193 -> 133 CE27
G-diketone	194.19	193	193 -> 178.10 CE20	193 -> 136 CE21	193 -> 107 CE31
S-diketone	224.21	223	223 -> 208.10 CE19	223 -> 193.10 CE20	223 -> 165.10 CE27

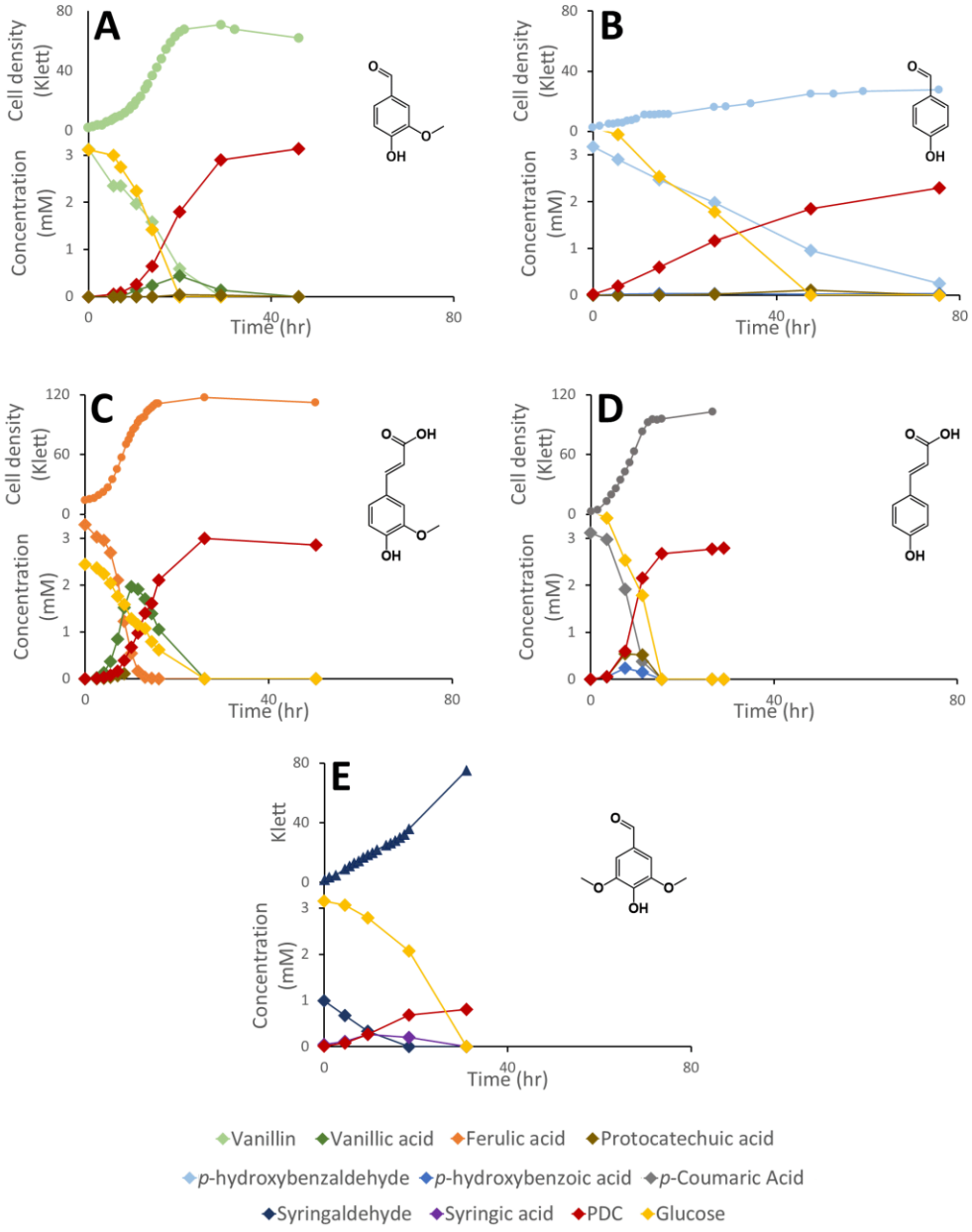


Figure S1. Cell density and extracellular metabolite concentrations of *N. aromaticivorans* strains 12444*ΔligI* (solid circles) or 12444*ΔligIΔdesCD* (solid triangles) grown on a combination of glucose and vanillin (A), *p*-hydroxybenzaldehyde (B), ferulic acid (C), p-coumaric acid (D), and syringaldehyde (E).

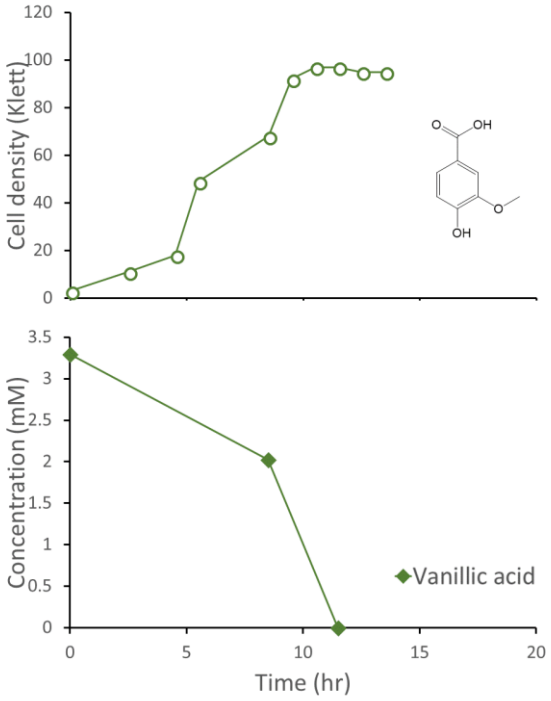


Figure S2. Cell density and extracellular metabolite concentrations of representative *N. aromaticivorans* strain 12444*ΔdesCD* cultured on 3 mM vanillic acid.

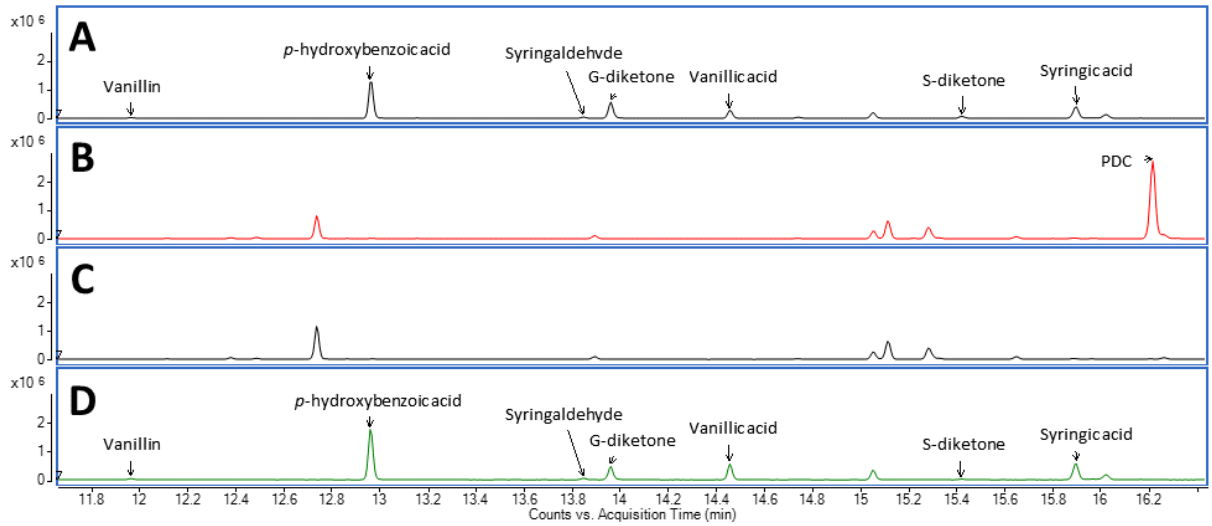


Figure S3. GC-MS peaks of compounds identified in media containing glucose plus the products of formic-acid-induced depolymerization of oxidized poplar lignin; before inoculation (A), after growth of *N. aromaticivorans* strain 12444*ΔligIΔdesCD* (B), after growth of *N. aromaticivorans* strain 12444*Δ1879* (C). Only strain 12444*ΔligIΔdesCD* accumulates PDC in the growth medium. Panel D shows the absence of additional peaks in an abiotic control experiment.

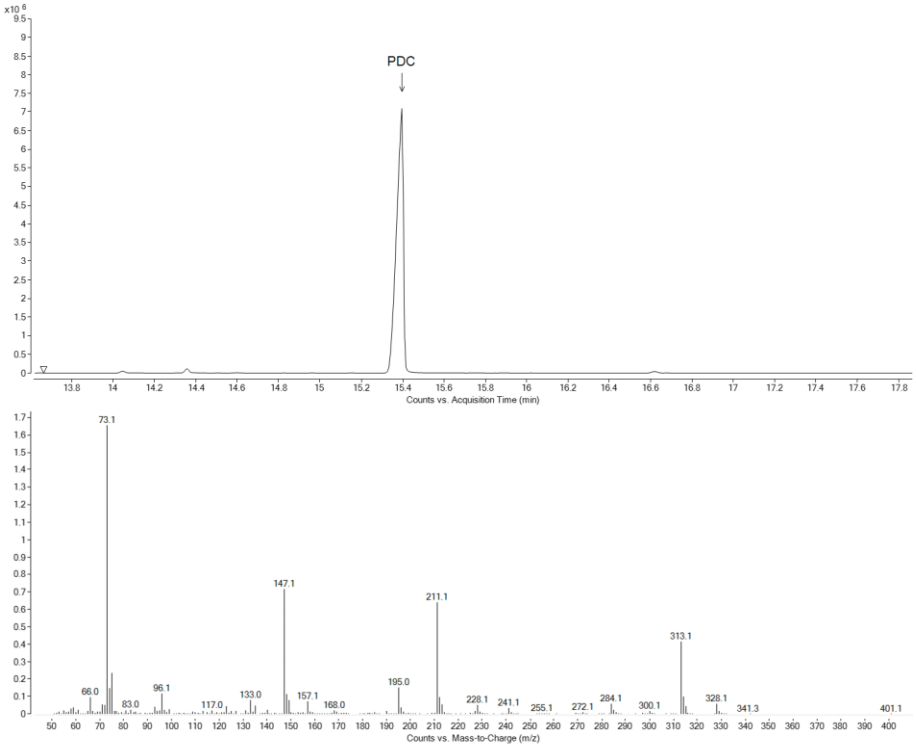


Figure S4. GC-MS chromatogram (A) and MS spectrum (B) of PDC isolated from a culture of PDC-producing *N. aromaticivorans* strain 12444*ΔligI* grown on a mixture of 3 mM vanillic acid and 3 mM glucose.

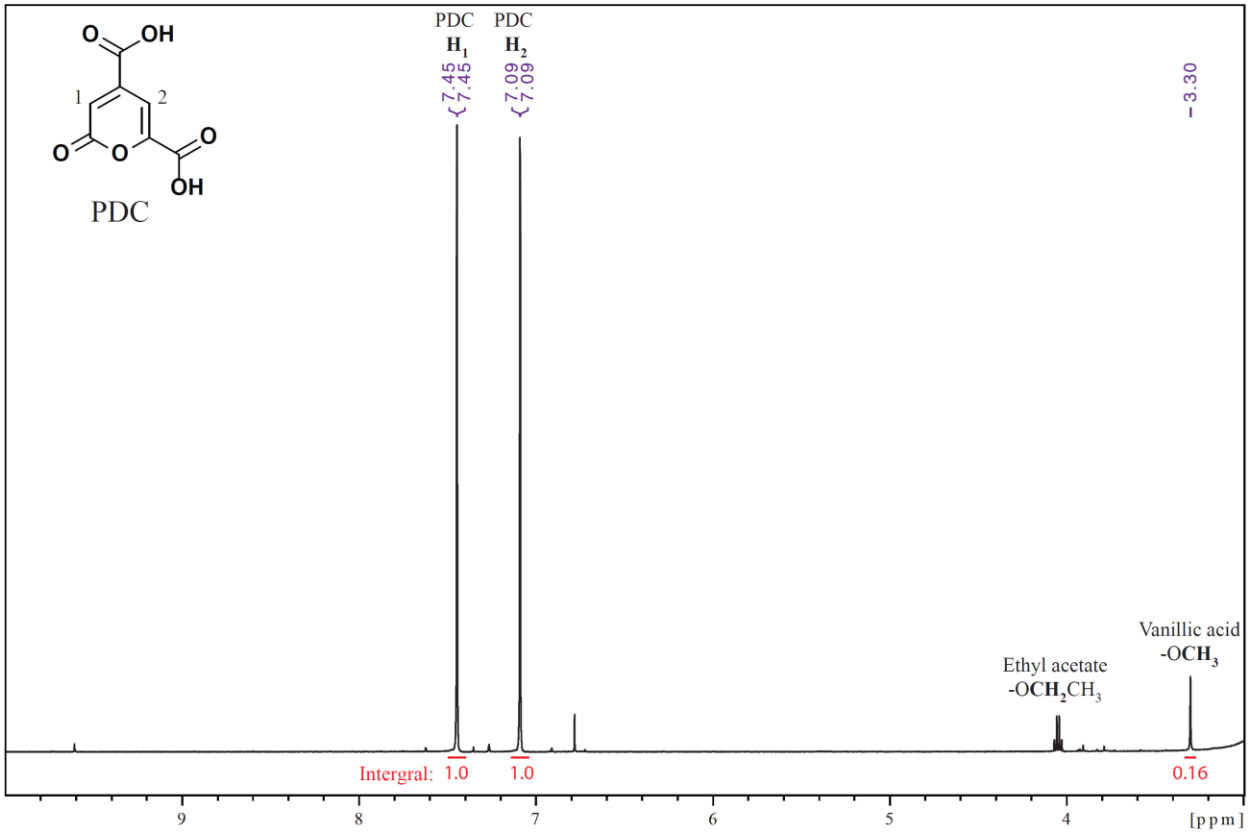


Figure S5. ^{1}H NMR spectra in acetone-d_6 of PDC isolated from a culture of PDC-producing *N. aromaticivorans* strain 12444*ΔligI* grown on a mixture of 3mM vanillic acid and 3 mM glucose.

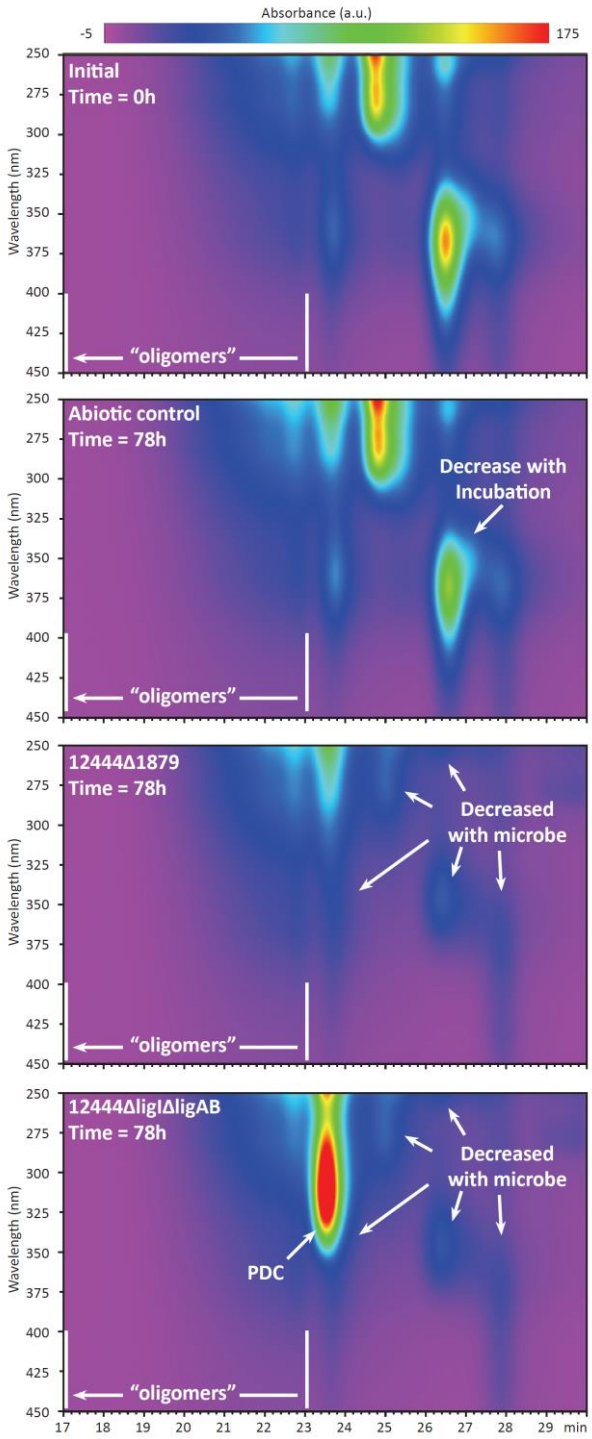


Figure S6. GPC chromatogram of media containing glucose plus the products of formic-acid-induced depolymerization of oxidized poplar lignin; before inoculation (A), abiotic control after 78 hours of incubation (B), after growth of *N. aromaticivorans* strain 12444*Δ1879* (C), after growth of *N. aromaticivorans* strain 12444*ΔligIΔdesCD* (D).

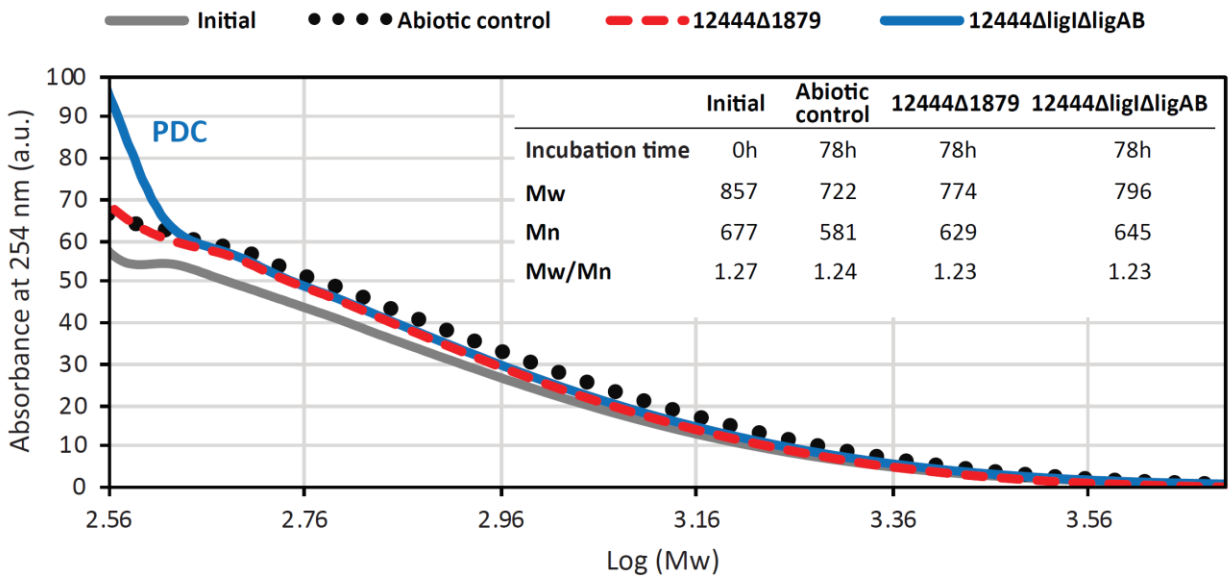


Figure S7. GPC chromatogram of the "oligomers" range at λ=254 of media containing glucose plus the products of formic-acid-induced depolymerization of oxidized poplar lignin. Mw: weight average molecular weight; Mn: number average molecular weight; Mw/Mn: dispersity index.

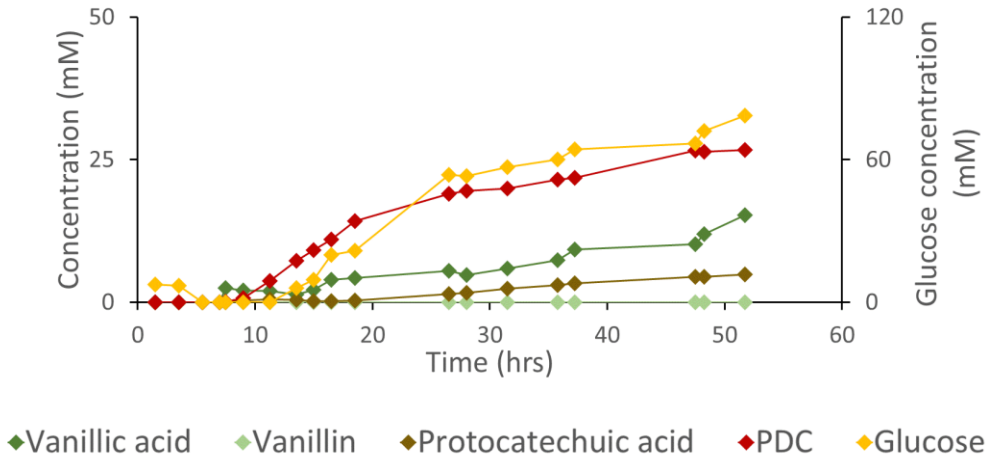


Figure S8. Extracellular metabolite concentrations of a *N. aromaticivorans* strain 12444*ΔligIΔdesCD* culture fed with a concentrated mixture of vanillic acid, vanillin, and glucose. A maximum PDC concentration of 26.7 mM was observed after 48 hours of cultivation.

Supplemental Methods

Construction of deletion mutants of *N. aromaticivorans*

Construction of plasmids for deleting genes Saro_2819 or Saro_2864/5. Regions of *N. aromaticivorans* genomic DNA containing ~1100 bp upstream and downstream of Saro_2819 or Saro_2864/5 were PCR amplified separately using the pairs of primers Saro2819_Del-R / Saro2819-pK18_Amp-F and Saro2819_Del-F / Saro2819-pK18_Amp-R for Saro_2819, and Saro2864-5_Del_R / Saro2864-5-pK18_Amp_F and Saro2864-5_Del_F / Saro2864-5-pK18_Amp_R for Saro_2864/5 (Table S2). The pairs of DNA amplified flanking regions for each gene were combined with linearized pK18msB using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA) to produce the plasmids pK18msB/ΔSaro2819 and pK18msB/ΔSaro2864/5, respectively. A 32 bp region of Saro_2865 (including the start codon) is predicted to overlap with Saro_2866. To prevent transcription of this region of Saro_2865, this putative start codon of Saro_2865 was mutated by replacing a T by a C at position 3088561 in the genome (in addition to deleting the sequence of Saro_2865 downstream of the Saro_2866 stop codon). To mutate the Saro_2865 start site, PCR was performed on plasmid pK18msB/ΔSaro2864/5 using the primers Saro2865_Start-SNP_F and Saro2865_Start-SNP_R2, which were previously phosphorylated with polynucleotide kinase from Promega (Madison, WI). The amplified product was circularized with T4 DNA ligase from New England Biolabs to obtain the circular plasmid pK18msB/ΔSaro2864/5. The plasmids were then transformed into NEB 5-alpha competent *E. coli* (New England Biolabs). The transformed *E. coli* cells were then

cultured in LB media + kanamycin and the plasmids purified using a Quiagen® Plasmid Maxi Kit (Quiagen, Germany).

Deletion of genes Saro_2819 and Saro_2864/5. The purified plasmids were then transformed into competent *E. coli* S17-1 and subsequently mobilized into *N. aromaticivorans* strain 12444*Δ1879* or 12444*ΔligI* cells via conjugation. Transconjugant cells of *N. aromaticivorans* (single cross overs) were isolated on SISnc-V0 plates containing 1g/L glucose and 50ug/mL kanamycin. To select for cells that eliminated the plasmid via a second instance of homologous recombination (double crossovers), single crossover cells were cultured on SISnc-V0 media containing 1g/L glucose and 10% sucrose. Double crossover cells were isolated on SISnc-V0 plates containing 1g/L glucose and 10% sucrose. PCR amplified regions of the target genes were sequenced to verify the deletions.

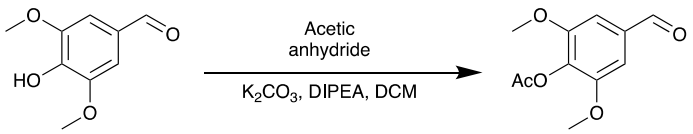
Purification of PDC

PDC was biologically produced by culturing *Novosphingobium aromaticivorans* strain 12444*ΔligI* in SISnc-V0 media supplemented with 3 mM vanillic acid and 3 mM glucose. Cells were grown to stationary phase and the culture media spun at 5000 RPM for 10 minutes and then filtered using a 500 ml Rapid – Flow bottle top filter with 0.2 μM SFCA membrane (Thermo Scientific). The filtrate (~900 mL) was transferred to a large 2 L separatory funnel and prepared for extraction of the acidic PDC by dilution with 50 mL brine (saturated sodium chloride) and 20 mL concentrated hydrogen chloride. The acidified PDC was extracted with ethyl acetate (4×100 mL). The combined ethyl acetate fraction (~400 mL) was extracted with 0.1 M sodium hydroxide (4×50 mL). The combined sodium hydroxide fraction was acidified with 2 M

hydrogen chloride (20 mL) and brine (50 mL), then extracted with ethyl acetate (3×100 mL). The combined ethyl acetate fraction was dried using anhydrous sodium sulfate, filtered through a qualitative cellulose filter (VWR 28320-100), and the solvent removed on a rotatory evaporator giving 297 mg of PDC as a light orange solid. A TMS derivatized sample of the isolated PDC was characterized by GC-MS (method described in materials and methods section), which showed that PDC was the only peak, indicating a fairly high purity. The identity and purity of the PDC was confirmed by comparison of the ^{1}H NMR data to previously published values. The NMR and GC-MS spectra indicated the purity of PDC to be approximate 97%.

Steps in the synthesis of S diketone

Synthesis of 4-acetyl syringaldehyde:



To a 100 mL round bottom flask with stir bar were added syringaldehyde (3.296 g, 18.09 mmol), acetic anhydride (3.2 mL, 33.85 mmol), diisopropyl ethyl amine (1 mL, 5.74 mmol), potassium carbonate (793 mg, 5.74 mmol), and dichloromethane (50 mL). The solution was allowed to stir at room temperature. After 24 hours, the reaction was added to a separatory funnel, washed with saturated sodium bicarbonate (3 x 100 mL), and concentrated in vacuo to yield 4-acetyl syringaldehyde as an off-white solid (3.812 g, 17.00 mmol, 94% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.91 (s, 1H), 7.16 (s, 2H), 3.91 (s, 6H), 2.37 (s, 3H).

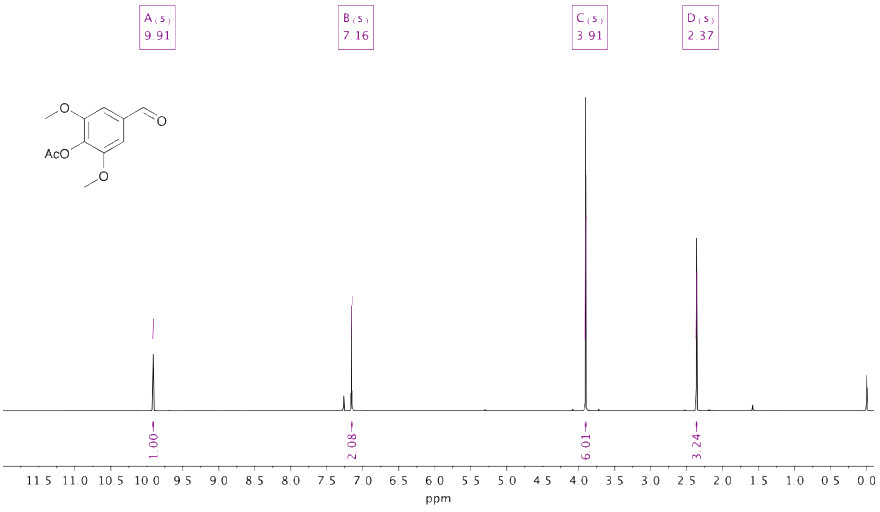
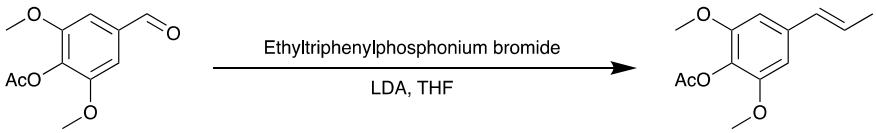


Figure S6. ^{1}H NMR spectra of synthesized 4-acetyl syringaldehyde.

Synthesis of 1-(4-acetoxy-3,4-dimethoxyphenyl)-1-propene:



An oven dried, 100 mL round bottom flask with stir bar was charged with ethyltriphenylphosphonium bromide (7.0 g, 18.85 mmol), outfitted with a rubber septum, and the atmosphere within it purged with nitrogen. Freshly distilled THF (50 mL) was added via syringe and cooled to -78° C. While stirring, a solution of 2.0 M lithium diisopropyl amide (9.5 mL, 19 mmol) was added to generate ethenyltriphenylphosphonium bromide. While this solution stirred for 30 minutes, an oven dried, 250 mL round bottom flask with stir bar was charged with 4-acetyl-syringaldehyde (3.812 g, 17.0 mmol), sealed with a rubber septum, and purged with nitrogen. Freshly distilled THF (50 mL) was added via syringe and cooled to -78° C. Once the aldehyde was fully dissolved, the ethenyltriphenylphosphonium bromide solution was

transferred by cannula and positive pressure to the 4-acetyl-syringaldehyde solution in a dropwise manner over the course of 45 minutes. Upon completion, the reaction was allowed to stir at -78° C for an hour. The reaction was then brought to room temperature and stirred for two hours. The solution was quenched with saturated aqueous ammonium chloride and concentrated under reduced pressure. The remaining solution was diluted with water and extracted with ethyl acetate (3 x 100 mL). The organic layer was then evaporated leaving behind a pale yellow solid. The crude was purified by flash silica chromatography (5:1 hexanes/ethyl acetate). Fractions corresponding to the desired product were combined and evaporated, leaving behind 1-(4-acetoxy-3,4-dimethoxyphenyl)-1-propene as a white powder (1.2 g, 5.36 mmol, 32 % yield, 1.08:1 cis/trans). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.54 (s, 2H), 6.35 (dq, J = 11.6, 1.9, 1H), 5.79 (dq, *J* = 11.6, 7.2 Hz, 1H), 3.82 (s, 6H), 2.34 (s, 3H), 1.92 (dd, *J* = 7.2, 1.9 Hz, 3H).

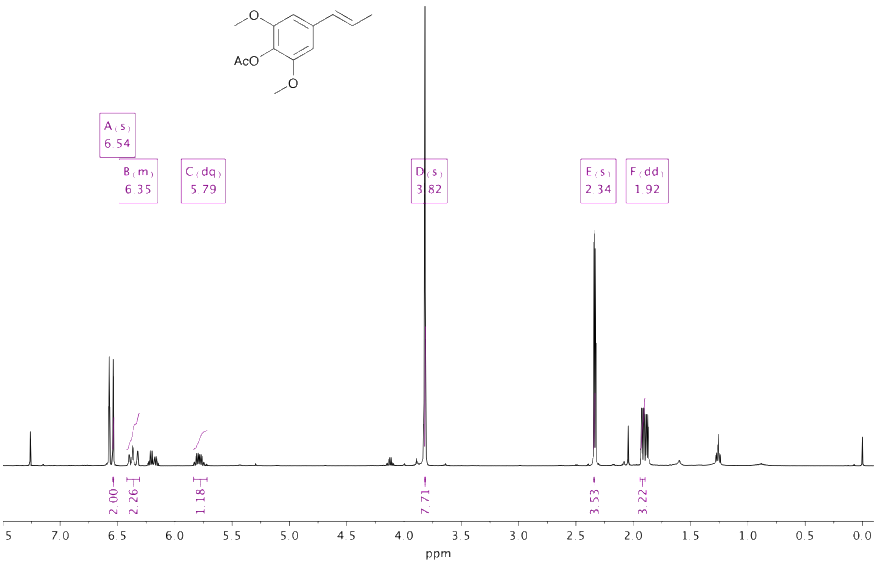
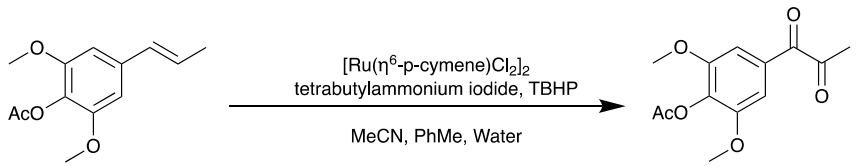


Figure S7.^{1}H NMR spectra of synthesized 1-(4-acetoxy-3,4-dimethoxyphenyl)-1-propene.

Synthesis of 1-(4-acetoxy-3,4-dimethoxyphenyl)-1,2-propane dione:



To a 100 mL round bottom flask with stir bar were added 1-(4-acetoxy-3,4-dimethoxyphenyl)-1-propene (720 mg, 3.05 mmol), dichloro(*p*-cymene)Ru(II) dimer (69.2 mg, 0.042 mmol), tetrabutylammonium iodide (336.4 mg, 0.91 mmol), tert-butyl hydroperoxide (70% solution in water, 3.6 mL), toluene (20 mL), acetonitrile (20 mL), and water (2.2 mL). The solution was allowed to stir at room temperature for 30 minutes then quenched with an excess of saturated aqueous sodium thiosulfate. The organic layer was isolated, concentrated in vacuo to a thick residue, and then purified by flash silica chromatography (4:1 hexanes/ethyl acetate). The resulting bright yellow fractions corresponding to the product were combined and evaporated to yield 1-(4-acetoxy-3,4-dimethoxyphenyl)-1,2-propane dione as a bright yellow solid (445 mg, 1.67 mmol, 55% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.33 (s, 2H), 3.88 (s, 6H), 2.53 (s, 3H), 2.36 (s, 3H).

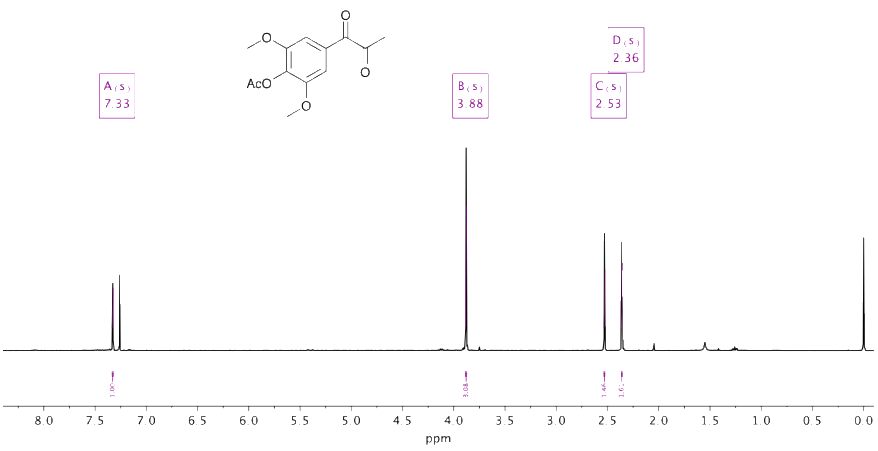
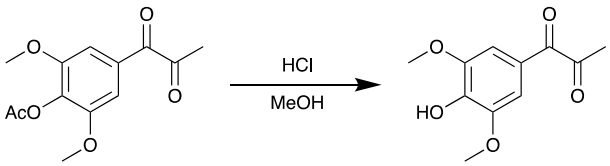


Figure S8. ^{1}H NMR spectra of synthesized 1-(4-acetoxy-3,4-dimethoxyphenyl)-1,2-propane dione.

Synthesis of 1-(4-hydroxy-3,4-dimethoxyphenyl)-1,2-propane dione (S-diketone):



To a 250 mL round bottom flask were added 1-(4-acetoxy-3,4-dimethoxyphenyl)-1,2-propane dione (445 mg, 1.67 mmol), 3 M HCl (35 mL), and methanol (75 mL). The solution stirred at room temperature and reaction progress was monitored by TLC. Upon completion, the reaction was concentrated, diluted with saturated sodium bicarbonate, and washed with ethyl acetate. The aqueous layer was acidified with dilute ammonium chloride and extracted with ethyl acetate (3 x 50 mL). The resulting organic layer was concentrated and purified by flash silica chromatography (4:1 hexanes/ethyl acetate). The desired fractions were combined and evaporated to yield 1-(4-hydroxy-3,4-dimethoxyphenyl)-1,2-propane dione (S-diketone) as a

bright yellow solid (259 mg, 1.16 mmol, 69% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.34 (s, 2H), 6.11 (s, 1H), 3.95 (s, 3H), 2.53 (s, 3H).

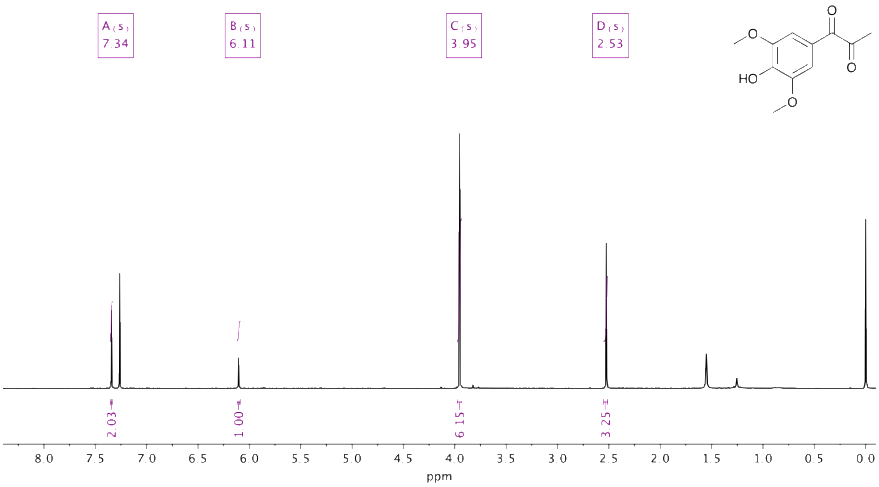
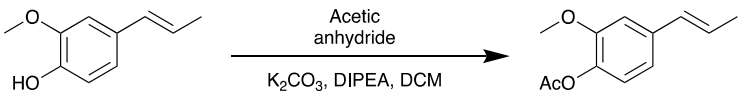


Figure S9.^{1}H NMR spectra of synthesized 1-(4-hydroxy-3,4-dimethoxyphenyl)-1,2-propane dione (S-diketone).

Steps in the synthesis of G diketone

Synthesis of isoeugenyl acetate:



To a 100 mL round bottom flask with stir bar were added isoeugenol (2.6 mL, 17.10 mmol), acetic anhydride (3.00 mL, 31.73 mmol), diisopropyl ethyl amine (1 mL, 5.74 mmol), potassium carbonate (793 mg, 5.74 mmol), and dichloromethane (500 mL). The solution was allowed to stir at room temperature. After 24 hours, the reaction was added to a separatory funnel, washed with saturated sodium bicarbonate (3 x 100 mL), and concentrated in vacuo. The

resulting off white powder was recrystallized from hot acetone to yield isoeugenyl acetate as white crystals (2.292 g, 11.11 mmol, 65% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.95 (d, *J* = 8.1 Hz, 1H), 6.92 (d, *J* = 1.8 Hz, 1H), 6.89 (dd, *J* = 8.1, 1.9 Hz, 1H), 6.36 (dq, *J* = 15.6, 1.7 Hz, 1H), 6.18 (dq, *J* = 15.7, 6.6 Hz, 1H), 3.84 (s, 3H), 2.30 (s, 3H), 1.88 (dd, *J* = 6.6, 1.6 Hz, 3H).

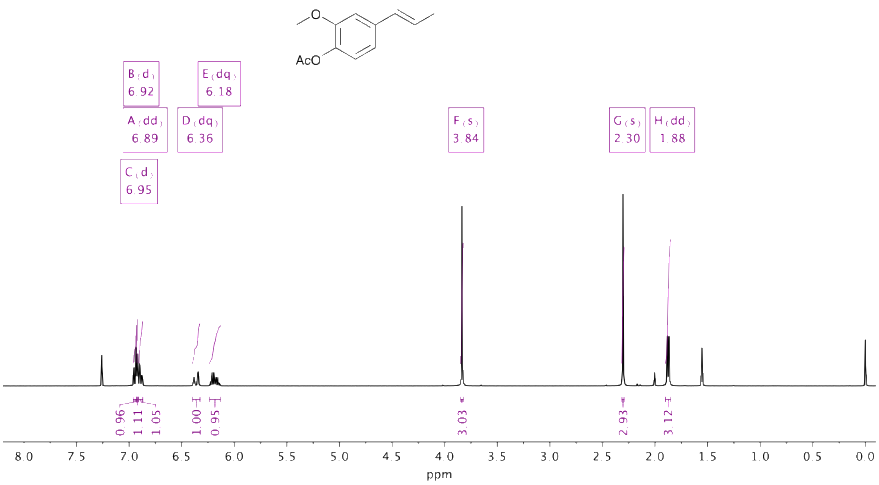
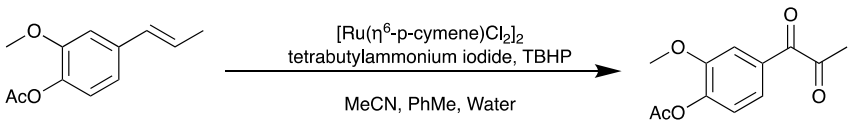


Figure S10. ^{1}H NMR spectra of synthesized isoeugenyl acetate

Synthesis of 1-(4-acetoxy-3-methoxyphenyl)-1,2-propane dione:



To a 250 mL round bottom flask with stir bar were added isoeugenyl acetate (2.060 g, 9.99 mmol), dichloro(*p*-cymene)Ru(II) dimer (69.2 mg, 0.11 mmol), tetrabutylammonium iodide (1.12 g, 3.03 mmol), tert-butyl hydroperoxide (70% solution in water, 10 mL), toluene (30 mL), acetonitrile (30 mL), and water (7 mL). The solution was allowed to stir at room temperature for 45 minutes then quenched with an excess of saturated aqueous sodium thiosulfate. The organic

layer was isolated, concentrated in vacuo to a thick residue, and then purified by flash silica chromatography (4:1 hexanes/ethyl acetate). The resulting bright yellow fractions corresponding to the product were combined and evaporated to yield 1-(4-acetoxy-3-methoxyphenyl)-1,2-propane dione as a bright yellow solid (1.28 g, 5.42 mmol, 54% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.66 (d, *J* = 1.9 Hz, 1H), 7.64 (dd, *J* = 8.1, 1.9 Hz, 1H), 7.16 (d, *J* = 8.2 Hz, 1H), 3.90 (s, 3H), 2.52 (s, 3H), 2.34 (s, 3H).

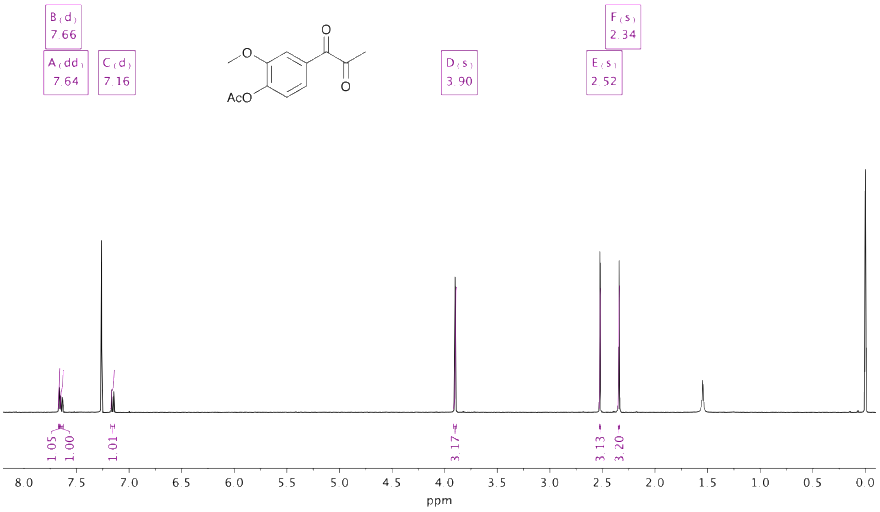
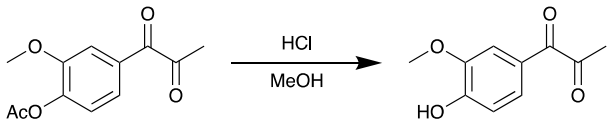


Figure S11. ^{1}H NMR spectra of synthesized 1-(4-acetoxy-3-methoxyphenyl)-1,2-propane dione.

Synthesis of 1-(4-hydroxy-3-methoxyphenyl)-1,2-propane dione (G-diketone):



To a 500 mL round bottom flask were added 1-(4-acetoxy-3-methoxyphenyl)-1,2-propane dione (1.00 g, 4.23 mmol), 3 M HCl (90 mL), and methanol (190 mL). The solution was stirred at room

temperature and reaction progress was monitored by TLC. Upon completion, the reaction was concentrated, diluted with saturated sodium bicarbonate, and washed with ethyl acetate. The aqueous layer was acidified with dilute ammonium chloride and extracted with ethyl acetate (3 x 100 mL). The resulting organic layer was concentrated and purified by flash silica chromatography (4:1 hexanes/ethyl acetate). The desired fractions were combined and evaporated to yield 1-(4-hydroxy-3-methoxyphenyl)-1,2-propane dione as a bright yellow, viscous oil (526 mg, 2.71 mmol, 64% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.61 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.58 (d, *J* = 1.9 Hz, 1H), 6.98 (d, *J* = 8.3 Hz, 1H), 6.21 (s, 1H), 3.97 (s, 3H), 2.51 (s, 3H).

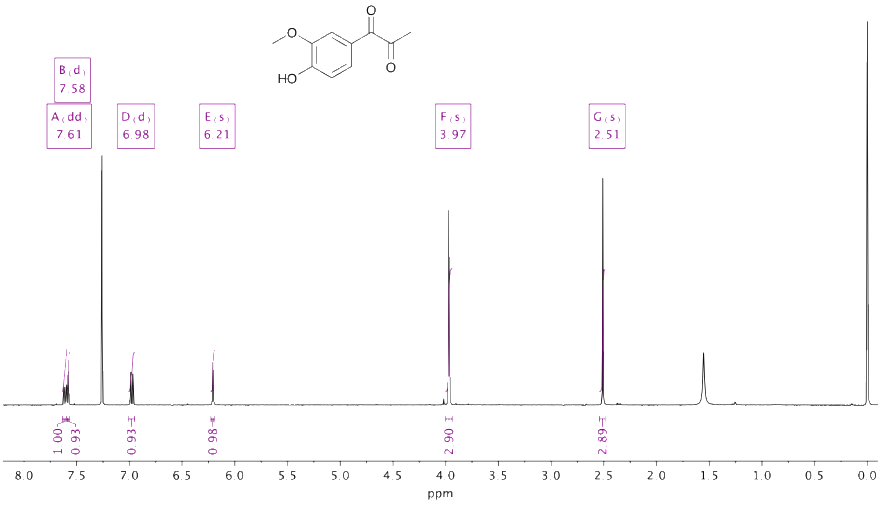


Figure S12. ^{1}H NMR spectra of synthesized 1-(4-hydroxy-3-methoxyphenyl)-1,2-propane dione (G-diketone).

Supporting information references

1. W. S. Kontur, C. A. Bingman, C. N. Olmsted, D. R. Wassarman, A. Ulbrich, D. L. Gall, R. W. Smith, L. M. Yusko, B. G. Fox, D. R. Noguera, J. J. Coon and T. J. Donohue, *J. Biol. Chem.*, 2018, **293**, 4955-4968.
2. R. G. Taylor, D. C. Walker and R. R. McLnnes, *Nucleic Acids Res.*, 1993, **21**, 1677-1678.
3. R. Simon, U. Priefer and A. Pühler, *Bio/Technology*, 1983, **1**, 784.
4. A. Schäfer, A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach and A. Pühler, *Gene*, 1994, **145**, 69-73.