Supporting Information

Structural insights into the ene-reductase synthesis of Profens

J. Waller,[a‡] H. S. Toogood,[a ‡] V. Karuppiah,[a] N. Rattray,[a] D. J. Mansell, [a,c] D. Leys,[a] J. M. Gardiner,[a] A. Fryszkowska,[b] S. T. Ahmed,[a] R. Bandichhor,[c] G. P. Reddy[c] and N. S. Scrutton[a*]

[a]*Manchester Institute of Biotechnology, University of Manchester, Manchester M1 7DN, UK.*

[b]*Dr Reddy's Laboratories, Chirotech Technology Centre, 410 Cambridge Science Park Milton Rd, Cambridge CB4 0PE U.K.*

[c]*Innovation Plaza, IPDO Bachupally, Dr. Reddy's Laboratories, Hyderabad-500090, India*

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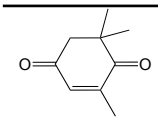
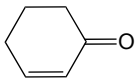
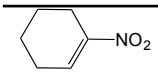
1. Supplementary Table

Table 1. Comparative steady state kinetics of ene-reductases with substrates **6**-**8**.

Substrate	Enzyme	Specific Activity (μmol/min/mg)	Literature values (μmol/min/mg)	Ref
	PETNR	5.26 ± 0.01	5.51 ± 0.10	[1]
	OYE2	0.20 ± 0.19	N/A	
	OYE3	0.31 ± 0.20	N/A	
	XenA	0.95 ± 0.01	1.34	[2]
	GYE	2.77 ± 0.02	N/A	
	LeOPR1	0.67 ± 0.06	N/A	
	TOYE	1.37 ± 0.04	2.29 ± 0.01	[3]
	NerA	0.31 ± 0.01	N/A	
	NtDBR	1.69 ± 0.13	1.48 ± 0.05	[4]

Reactions (1 mL) were performed in in buffer (K_2HPO_4/KH_2PO_4 pH 7.5) containing the alkene (1 mM; stock in 100 % ethanol) and NADPH (100 μM) at 25°C. [a] Reactions carried out at 50°C. [b] Reactions carried out with 200 μM NADPH. [c] Reactions were performed in citrate buffer pH 5.4. N/A = published kinetic data not available for comparison. OYEs tested were PETNR = pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2;[1] LeOPR1 = 12-oxophytodienoate reductase 1 from *Solanum lycopersicum*;[5] NerA = GTN reductase from *Agrobacterium radiobacter*;[6] OYE2 from *Saccharomyces cerevisiae*;[7] OYE3 from *Saccharomyces cerevisiae*;[7] GYE from *Gluconobacter oxydans*;[8] XenA = xenobiotic reductase from *Pseudomonas putida*;[2] and TOYE = thermophilic Old Yellow Enzyme from *Thermoanaerobacter pseudethanolicus* E39.[3]

2. Supplementary Figures

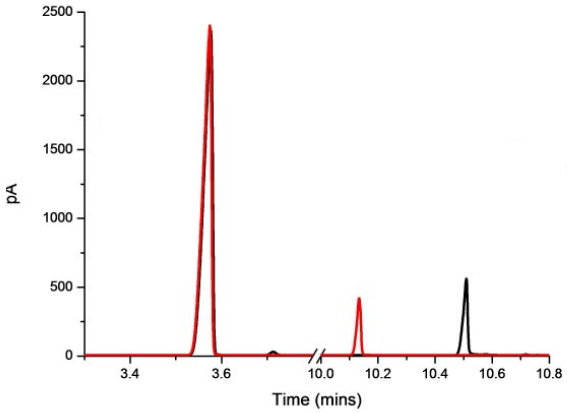


Figure S1. Achiral GC analysis of methyl ester-derivatised **5a** and **1a** to **3a** and **4b**, respectively. Samples were run on an Agilent Technologies 7890A GC system with FID detector and a ZB-semi volatiles column (30 m; 0.25 mm; 0.25 µm film thickness). Red (10.12 min) and black (10.5 min) peaks show the separation of **1a** and **5a**, respectively. The large peak at 3.55 min is the limonene internal standard.

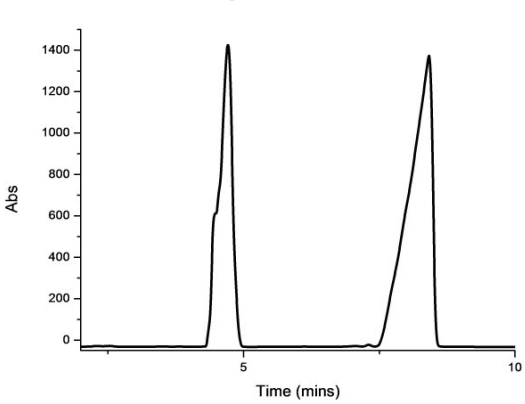


Figure S2. Achiral preparative HPLC separation of **1a** and **5a**. Samples were run on an Agilent 1260 Infinity preparative HPLC system with a diode array detector, using a Phenomenex Luna C18 column (5 □m x 21.2 mm x 250 mm). The flow rate was 15 mL/min with a gradient of acetonitrile:H_2O 10:90 to 50:50 over 15 minutes. Peaks at 9 and 13 minutes are **5a** and **1a**, respectively.

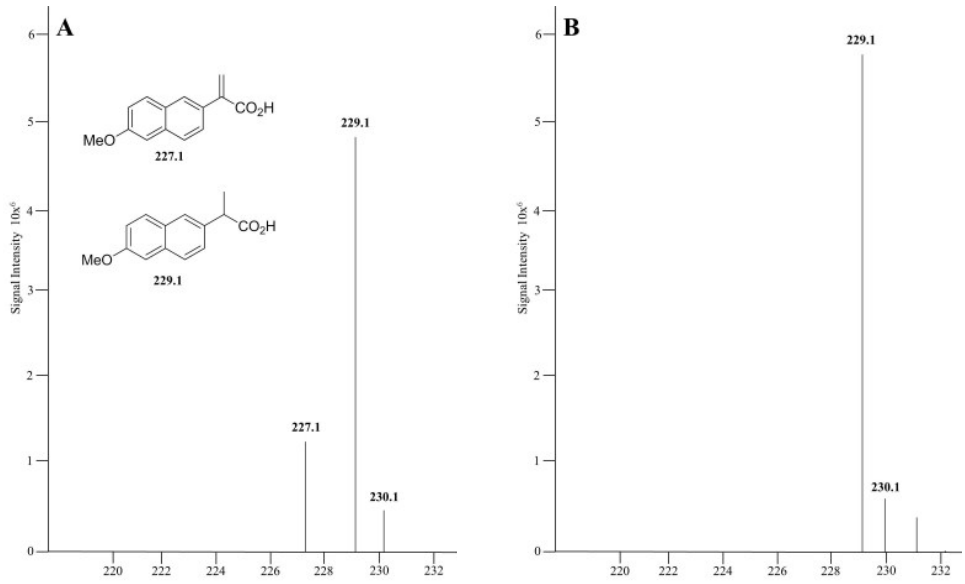


Figure S3. MS spectra of **1a** generated in the biotransformation of **5a** by A) XenA and B) GYE. Samples were run on an Agilent 1100 LC/MSD Ion Trap System.

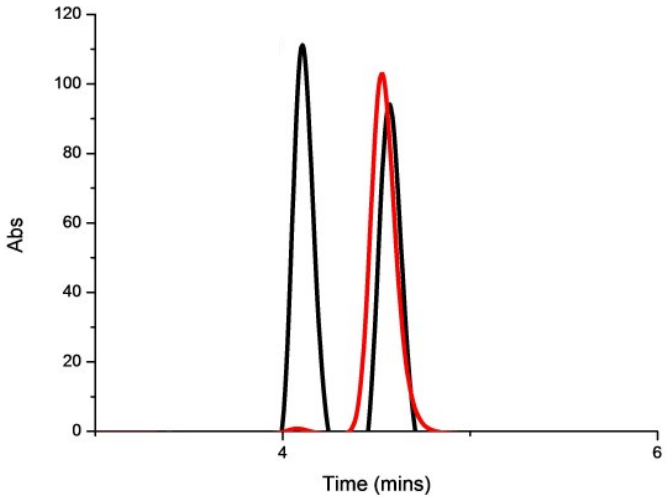


Figure S4. Chiral HPLC analysis of enantiomers of **1a**. Samples were run on an Agilent 1100 HPLC system with a Chiralpak AS-RH reverse phase column (150 x 4.6 mm ID) using a diode array detector (220 nm). Mobile phase was composed of acetonitrile/H_2O 40:60 and with a flow rate of 0.8 mL/min at 20 °C. Black and red peaks show the racemic and (*S*)-**1a** products, respectively.

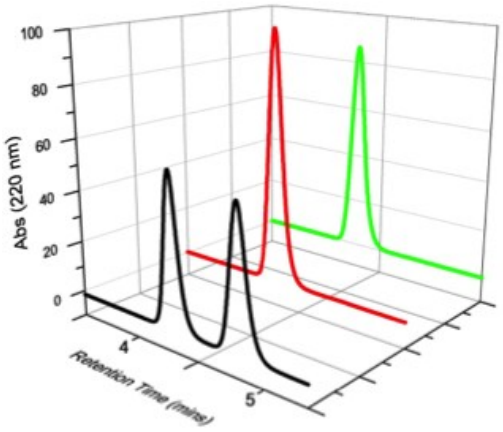


Figure S5. Chiral HPLC analysis of enantiomers of **1a** generated by reactions with GYE and XenA. Samples were run on an Agilent 1100 HPLC system with a Chiralpak AS-RH reverse phase column (150 x 4.6 mm ID) using a diode array detector (220 nm). Mobile phase was composed of acetonitrile/H_2O 40:60 and with a flow rate of 0.8 mL/min run at 20 °C. Black, red and green traces show the racemic standard and the GYE and XenA reaction products, respectively.

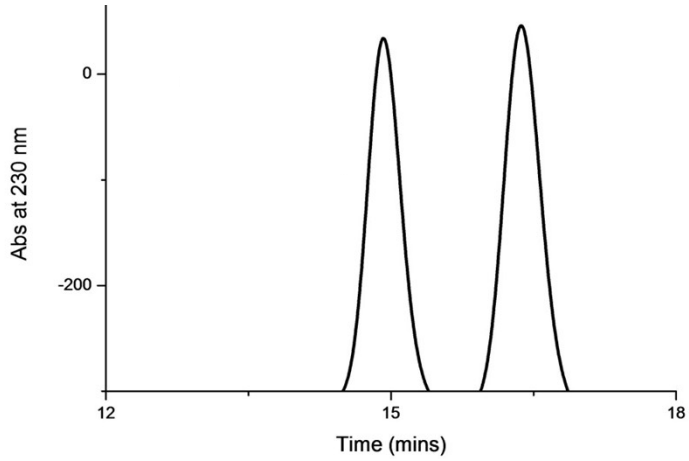


Figure S6. Chiral HPLC analysis of enantiomers of **4a**. Samples were run on an Agilent 1100 HPLC system with a Chiralpak AS-RH reverse phase column (150 x 4.6 mm ID) using a diode array detector (220 nm). Mobile phase was composed of acetonitrile/H_2O 40:60 and with a flow rate of 0.8 mL/min at 20 °C. Peaks at 15 and 17.5 minutes are (*R*)-**4a** and (*S*)-**4a**, respectively.

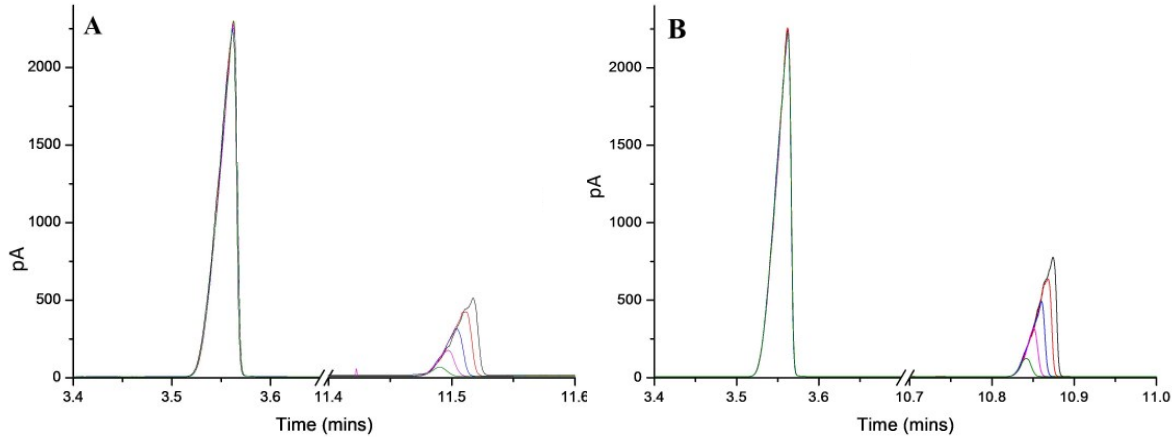


Figure S7. Achiral analysis and standard curves of A) **5b** and B) **1b** by GC. Samples were run on an Agilent Technologies 7890A GC system with FID detector and a ZB-semi volatiles column (30 m; 0.25 mm; 0.25 µm film thickness). The large peak at 3.55 min is the limonene internal standard. calibration curve; —10 mM; — 7.5 mM; — 5 mM; — 2.5 mM; — 1 mM.

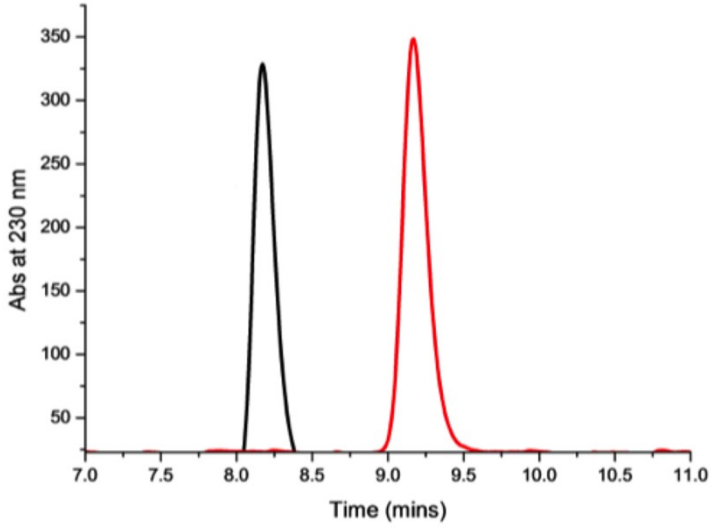


Figure S8. Chiral HPLC analysis of enantiomers of **1b**. Samples were run on an Agilent 1100 HPLC system with a Chiralpak AD-H column (150 x 4.6 mm ID) using a diode array detector (220 nm). Mobile phase was composed of hexane:isopropanol (95:5) at 20 °C with a flow rate of 1 mL/min at 20 °C. Black and red peaks show the (*R*)-**1b** and (*S*)-**1b** products, respectively.

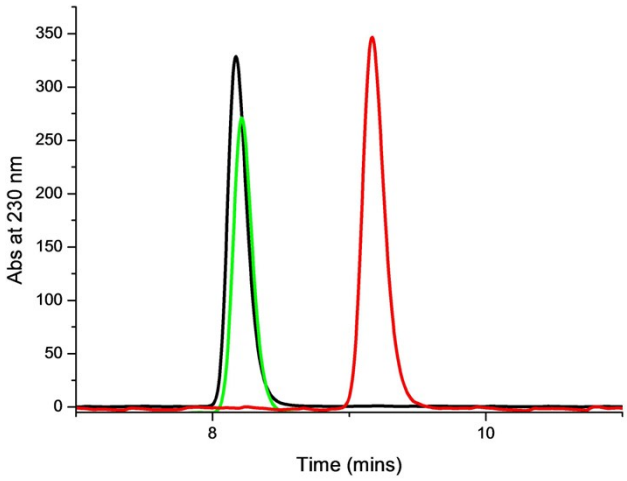


Figure S9. Chiral HPLC analysis of enantiomers of **1b** generated by the reaction of **5b** with XenA. Samples were run on an Agilent 1100 HPLC system with a Chiralpak AS-RH reverse phase column (150 x 4.6 mm ID) using a diode array detector (220 nm). Mobile phase was composed of acetonitrile/H_2O 40:60 and with a flow rate of 0.8 mL/min run at 20 °C. Black, red and green traces show the (*R*)-**1b** and (*S*)-**1b** standards and XenA reaction products, respectively.

3. LCMS Identification of products of the biotransformation of 5b to 1b

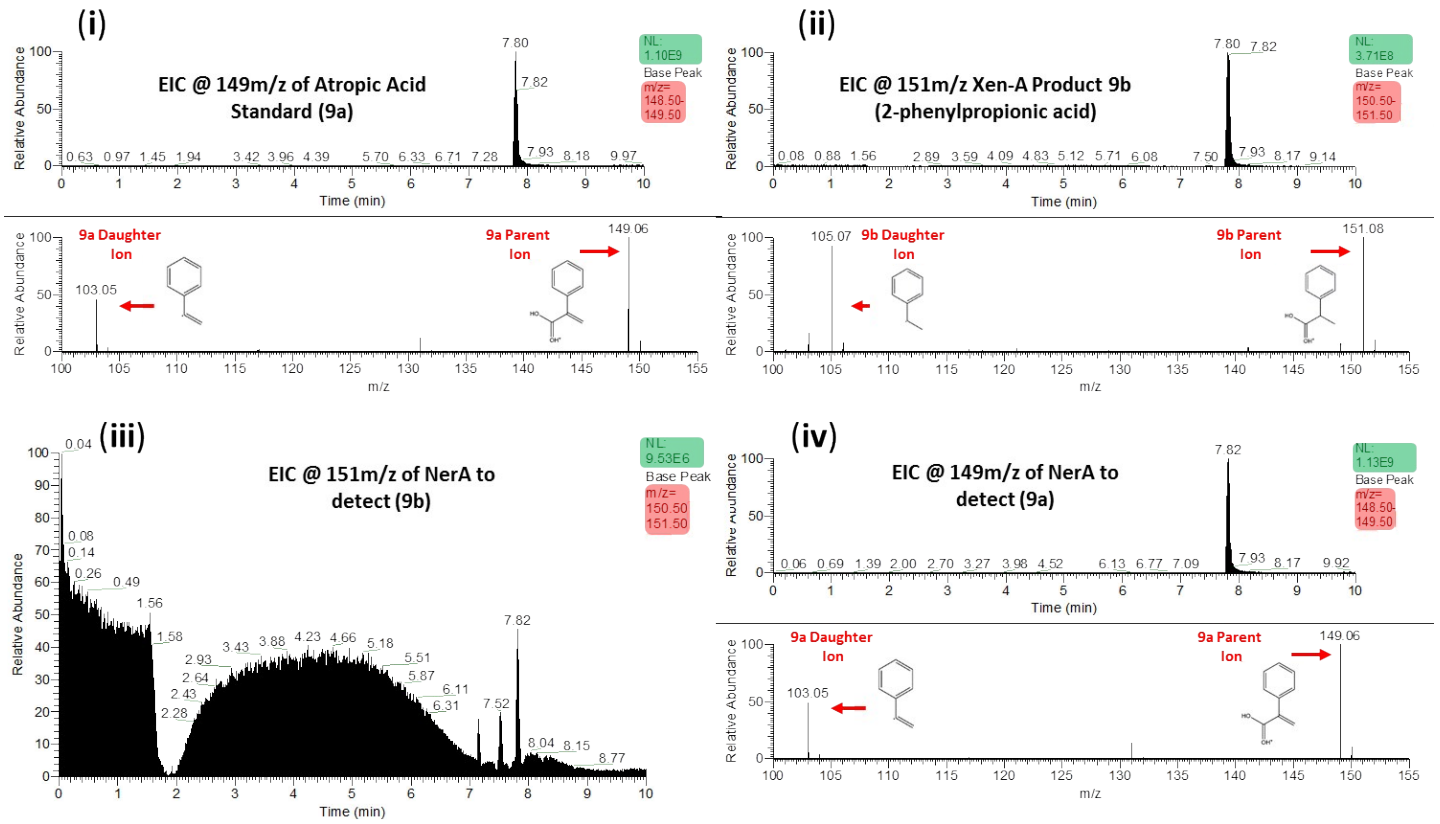


Figure S10. UHPLC-MS analysis of the XenA and NerA reactions. Red boxes indicate extracted ion chromatogram (EIC) mass target, green box indicates ion count. i) **Top** - EIC at 149m/z from the atropic acid standard indicating the presence of a peak at 7.8 mins. **Bottom** - Subsequent summation of all mass spectra within peak at 7.8 indicates a parent-daughter pairing that represent the [Atropic Acid]$^{H+}$ and [decarboxylated form]$^{H+}$ as a result of fragmentation. **ii) Top** - Extracted ion chromatogram at 151m/z indicating the presence of a peak at 7.8 mins. **Bottom** - Subsequent summation of all mass spectra within peak at 7.8 indicates a parent-daughter pairing that represent the [Atropic Acid]$^{H+}$ and [decarboxylated form]$^{H+}$ as a result of fragmentation. **iii**) Extracted ion chromatogram of mass 151 indicating the lack of any major peak at 7.8 mins. Intensity of 9.53×10^6 indicating a background signal (green box) **iv**) **Top** - Extracted ion chromatogram of mass 149 indicating the presence of a peak at 7.8 mins. **Bottom** - Subsequent summation of all mass spectra within peak at 7.8 indicates a parent-daughter pairing that represent the [Atropic Acid]$^{H+}$ and [decarboxylated form]$^{H+}$ as a result of fragmentation.

An initial full scan MS over 50-750m/z of a pure standard of 2-phenylacrylic acid **5b** revealed a parent ion peak centred at 7.80 min with a primary mass of 149.05977 m/z. MS^2 analysis indicates a decarboxylated daughter fragment. An identical analysis on the XenA enzyme system indicates the presence of a peak at 7.8 mins with a primary mass of 151.0754 m/z. MS^2 analysis indicates a decarboxylated daughter fragment. The extra 2Da increase in product of the XenA system is representative of a mass gain from two protons by the reduction of the methylene carbons. Figure S10 iii/iv indicate the lack of the reduced 151m/z form but the presence of the unreacted 2-phenylacrylic acid **5b**.

4. NMR Traces

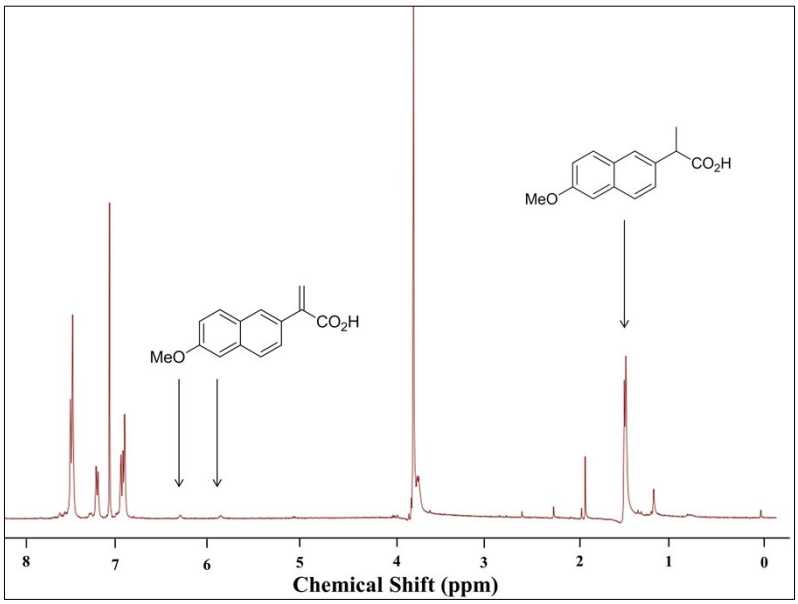


Figure S10. ^{1}H NMR spectrum of compound **1a** generated by large-scale biotransformation of **5a** by GYE. Spectrum was recorded on a 400 MHz spectrometer in $CDCl_3$.

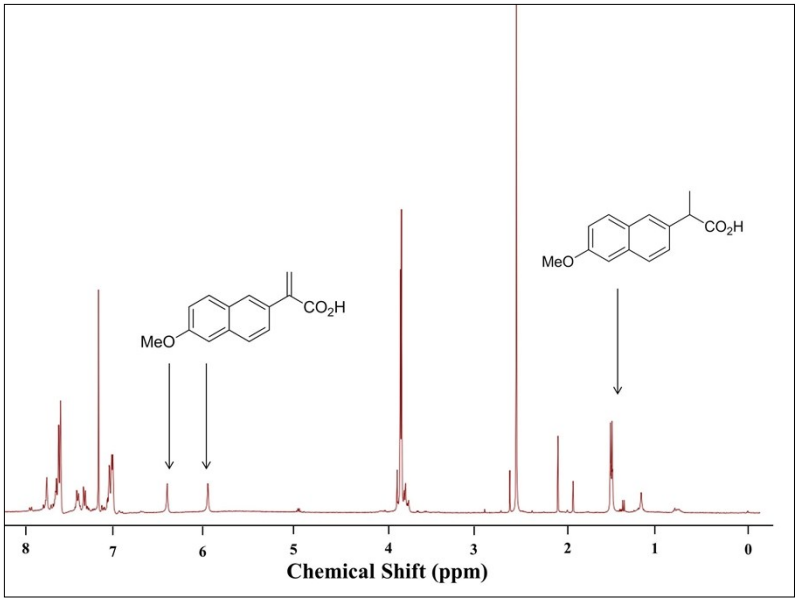


Figure S11. ^{1}H NMR spectrum of compound **1a** generated by preparative biotransformation of **5a** by XenA. Spectrum was recorded on a 400 MHz spectrometer in $CDCl_3$.

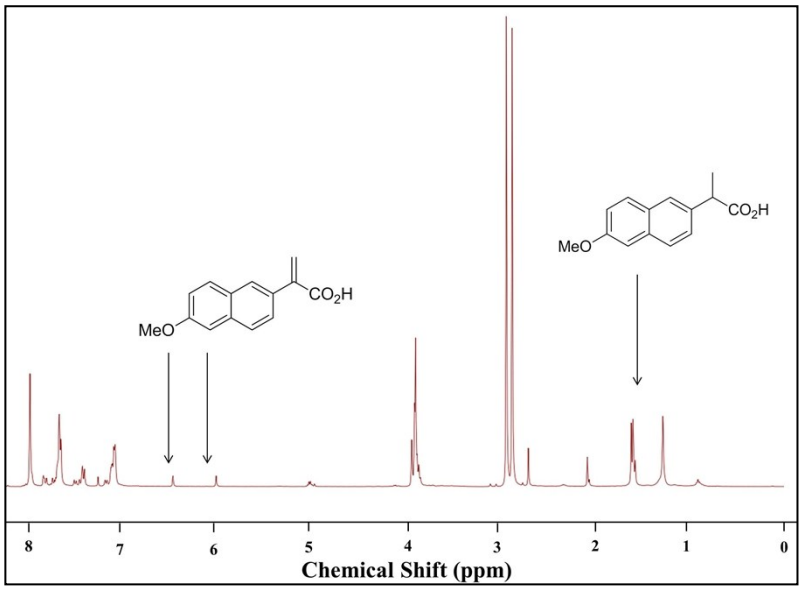


Figure S12. ^{1}H NMR spectrum of compound **1a** generated by large-scale biotransformation of **5a** by XenA after 72 hours. Spectrum was recorded on a 400 MHz spectrometer in $CDCl_3$.

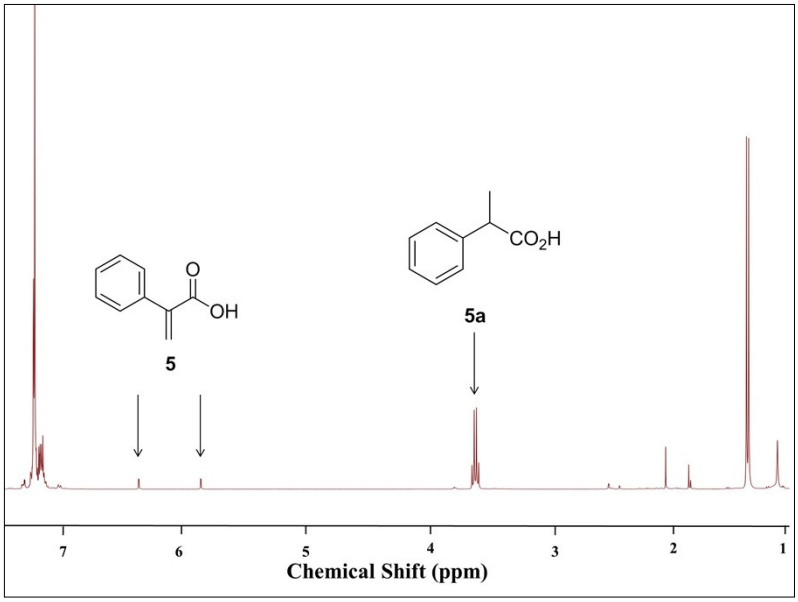


Figure S13. ^{1}H NMR spectrum of compound **7b** generated by large-scale biotransformation of **7a** by XenA. Spectrum was recorded on a 400 MHz spectrometer in $CDCl_3$.

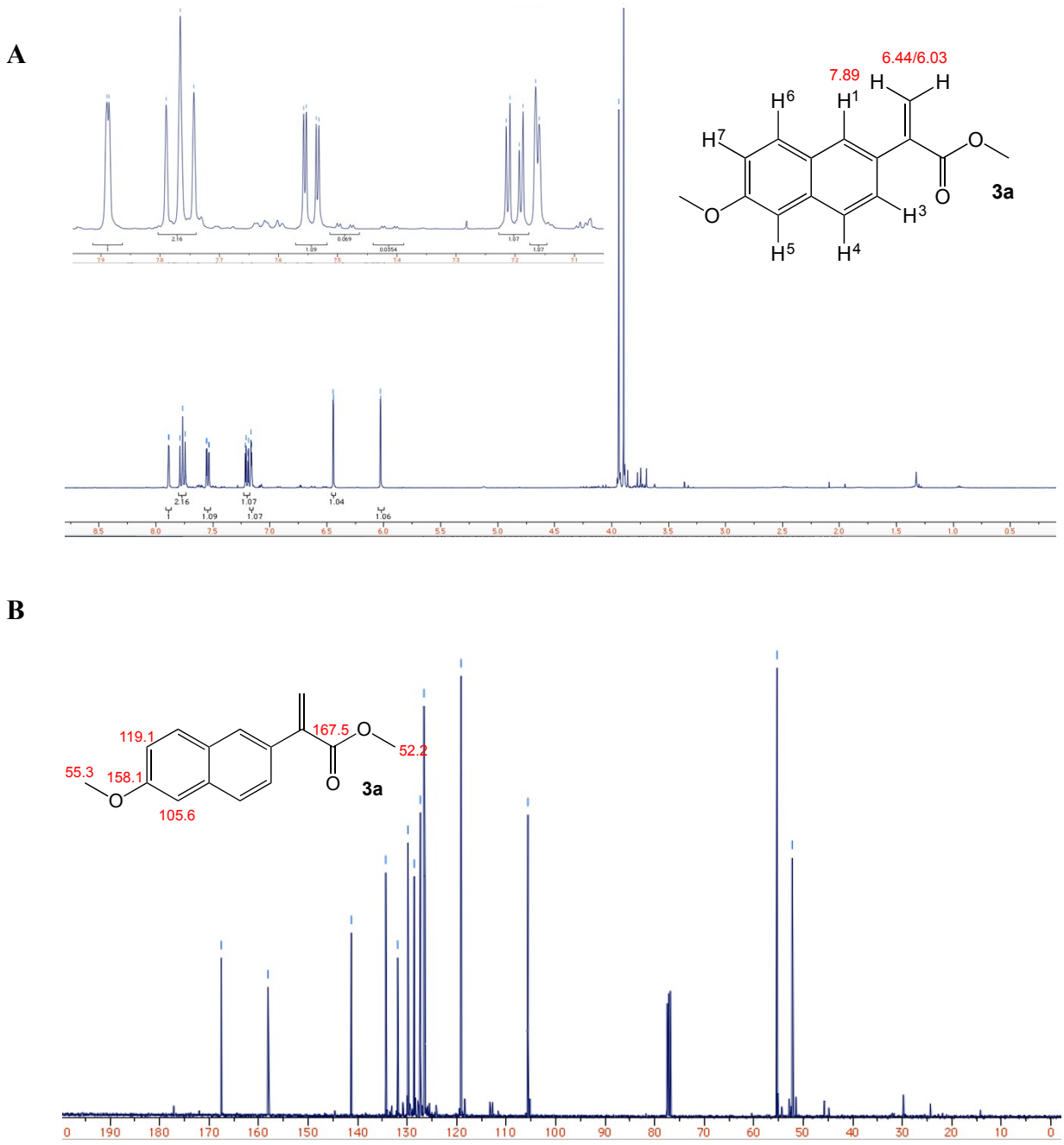


Figure S14. A)^{1}H NMR and B)^{13}C NMR spectra of compound **3a**. Spectra were recorded on a 400 MHz spectrometer in $CDCl_3$. The inset in A shows the aromatic region in more detail.

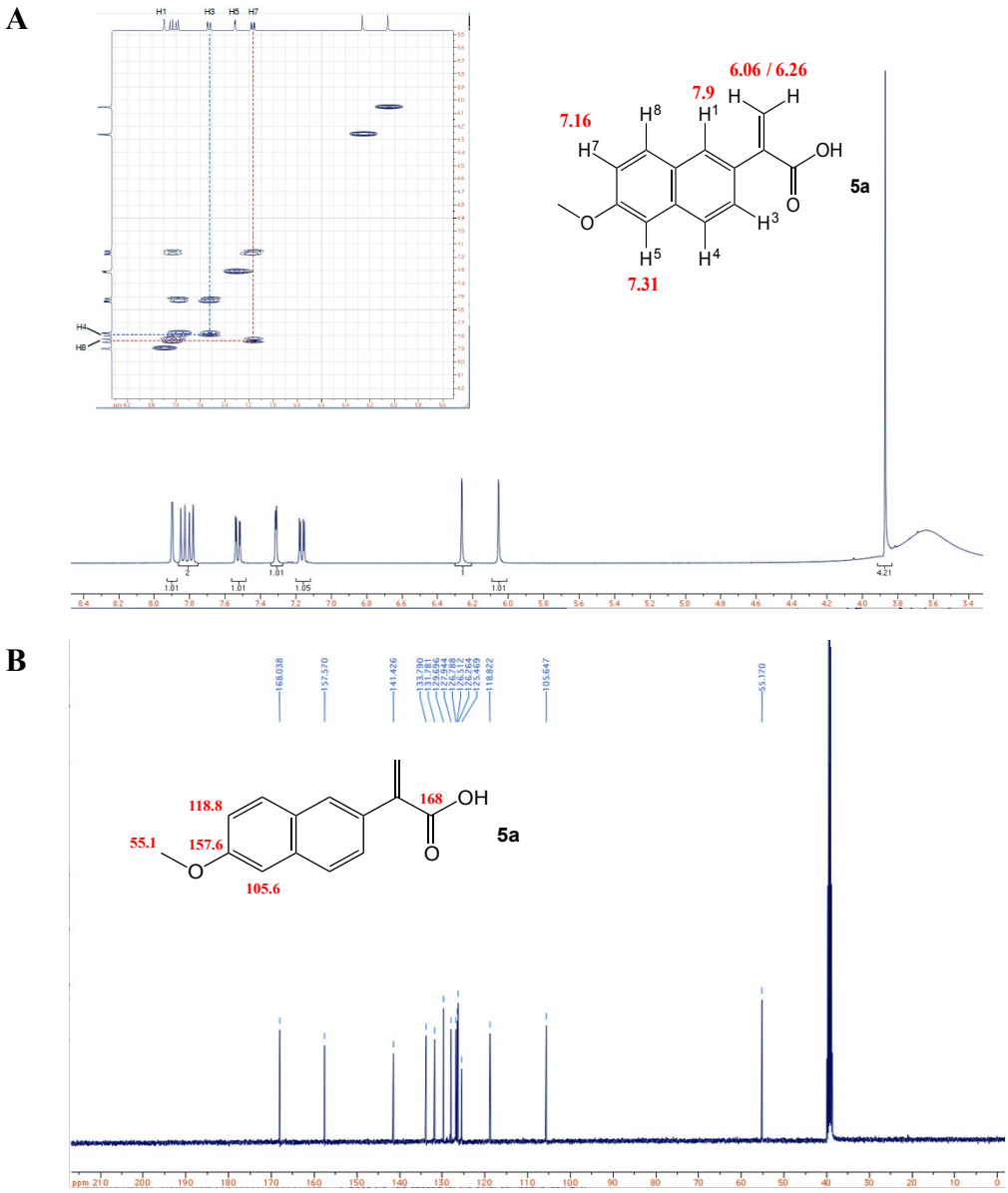


Figure S15. A)^{1}H NMR and B)^{13}C NMR spectra of compound **5a**. Inset: COSY spectrum of compound **5a**. Spectra were recorded on a 400 MHz spectrometer in d_6-DMSO.

References

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