Supporting Information

Elucidating Substrate Promiscuity within the FabI Enzyme Family.

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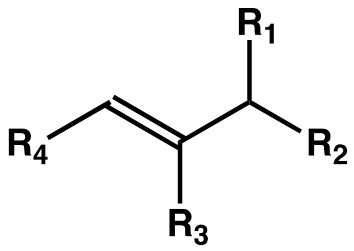
Figure S1: Substrates and enzyme selection.



R_1 = OH, =O

R_2 = H, OCH_3, OH, SCH_3, CH_3

R_3 = H, Ethyl

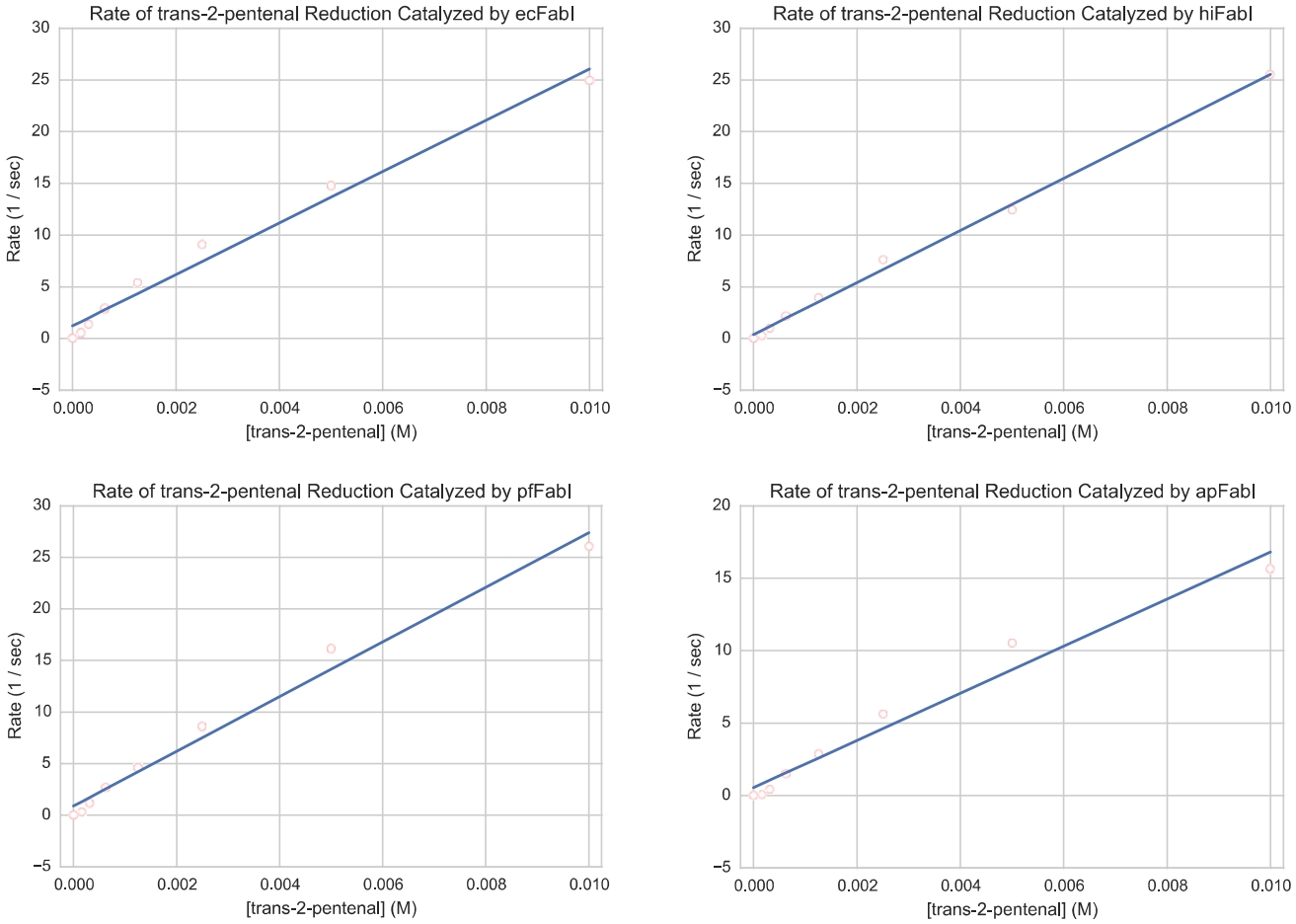
R4 = Ethyl, Butyl, Hexyl, Butenyl, Phenyl

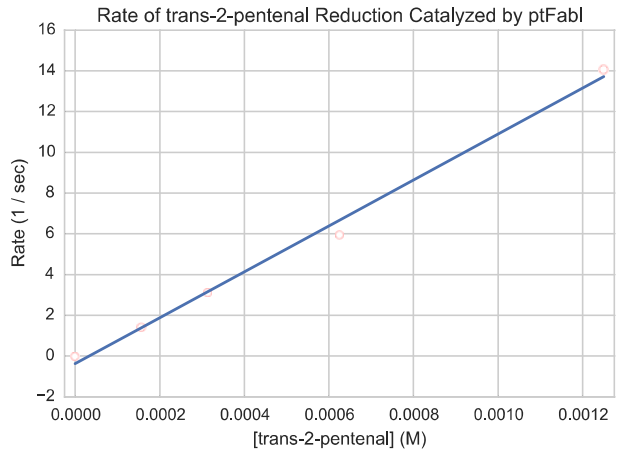
For the substrate there are four key regions that were explored. R1 is the carbonyl in the native substrate. For the majority of the substrates examined the carbonyl was maintained, but in order to test catalytic promiscuity we also examined the α,β - unsaturated alcohol. R2 is the position that tests the different +3 oxidation and +2 oxidation states. At R3 we examined how much steric demand the enzyme can accommodate. R4 tested the effect of conjugation, steric demand and chain length on activity.

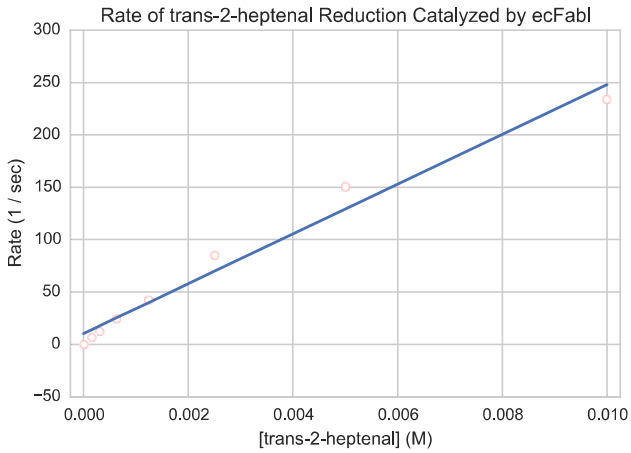
FabI Ortholog	ecFabI	pfFabI	hiFabI	ptFabI	apFabI
ecFabI		19.2	73.5	23.8	27
pfFabI	19.2		20.5	45.9	41.8
hiFabI	73.5	20.5		26.5	27.7
ptFabI	23.8	45.9	26.5		60.1
apFabI	27	41.8	27.7	60.1	

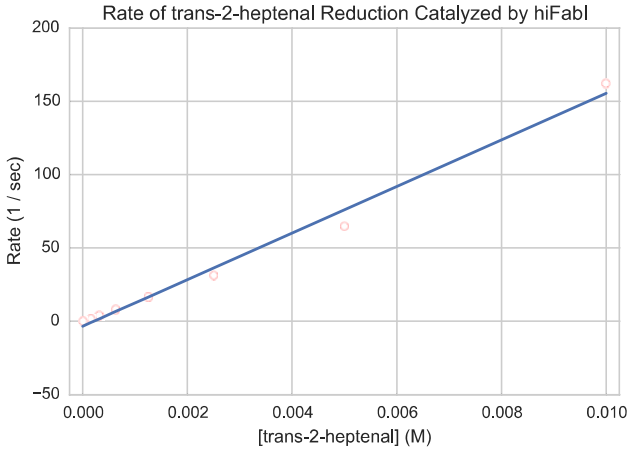
Pairwise percent sequence identities from the amino acid sequences for each of the FabI orthologs. The identity varies from 19.2 to 73.5%, with an average value of 36.6% identity.

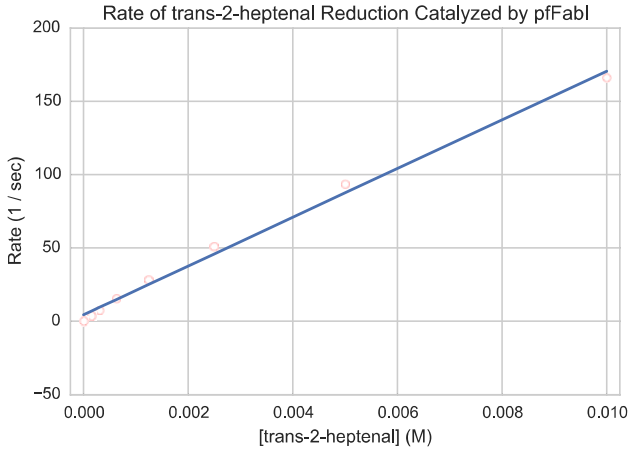
Figure S2: Substrate versus velocity plots

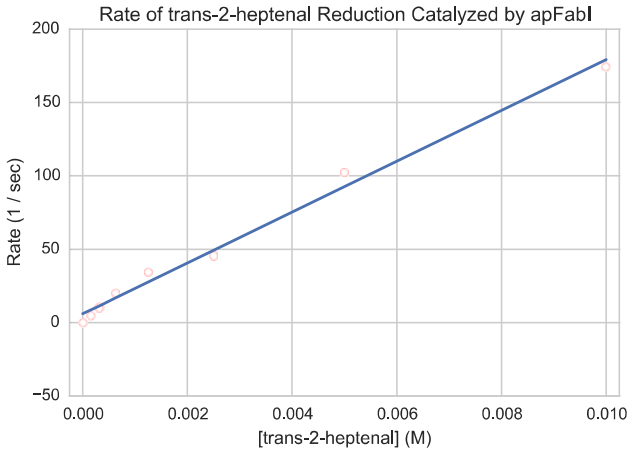


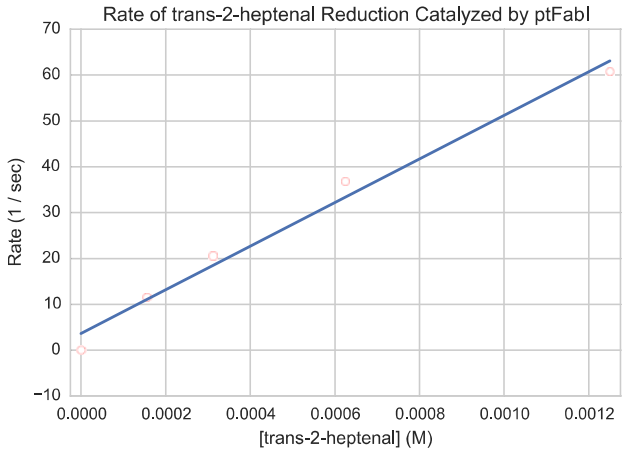

Rate of trans-2-pentenal Reduction Catalyzed by ptFabI
Rate (1 / sec)
[trans-2-pentenal] (M)

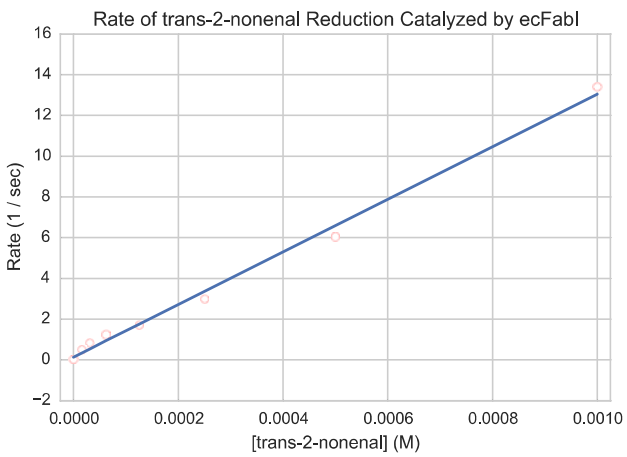

Rate of trans-2-heptenal Reduction Catalyzed by ecFabI
Rate (1 / sec)
[trans-2-heptenal] (M)

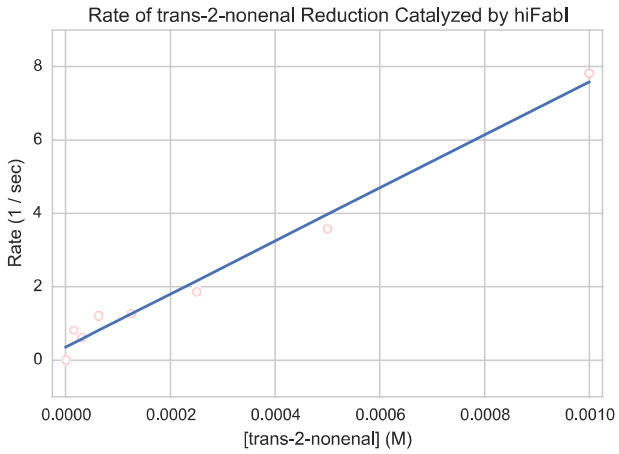

Rate of trans-2-heptenal Reduction Catalyzed by hiFabI
Rate (1 / sec)
[trans-2-heptenal] (M)


Rate of trans-2-heptenal Reduction Catalyzed by pfFabI
Rate (1 / sec)
[trans-2-heptenal] (M)


Rate of trans-2-heptenal Reduction Catalyzed by apFabI
Rate (1 / sec)
[trans-2-heptenal] (M)


Rate of trans-2-heptenal Reduction Catalyzed by ptFabI
Rate (1 / sec)
[trans-2-heptenal] (M)


Rate of trans-2-nonenal Reduction Catalyzed by ecFabI
Rate (1 / sec)
[trans-2-nonenal] (M)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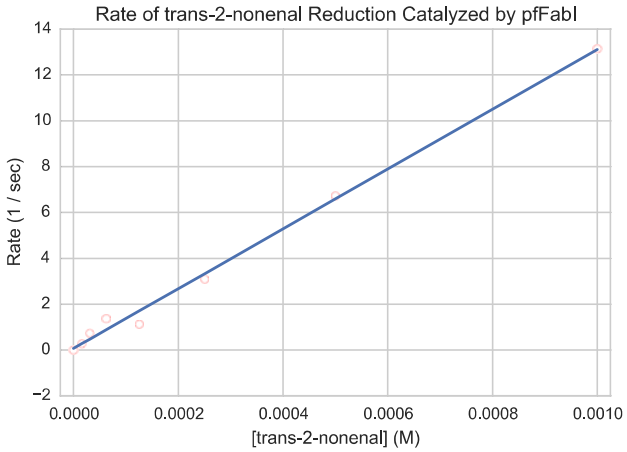
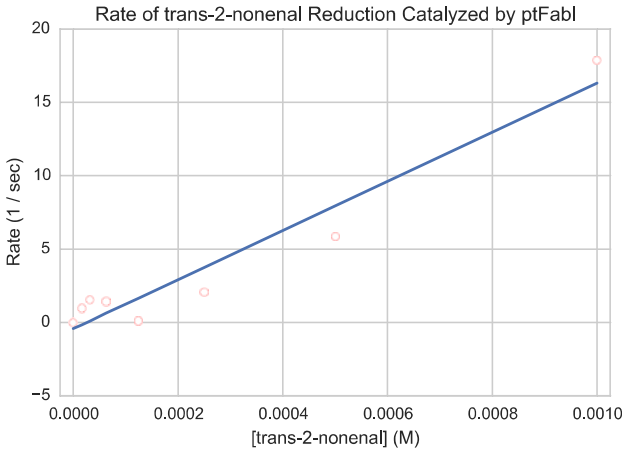
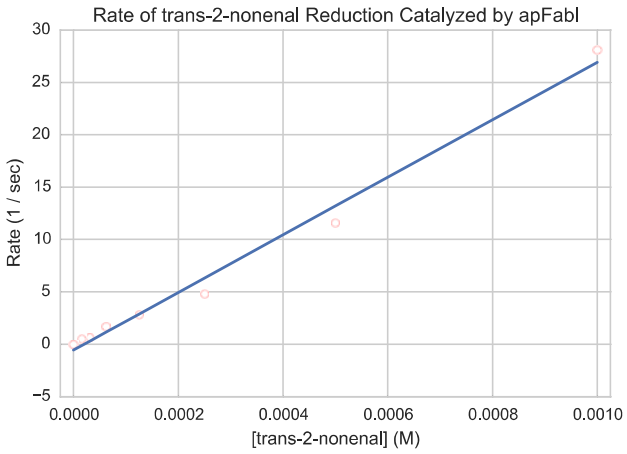
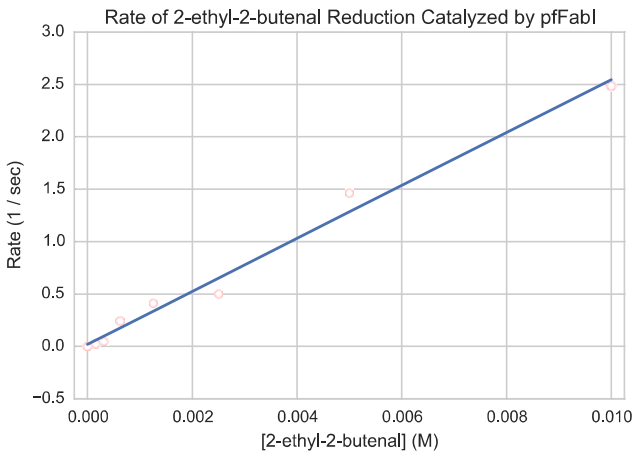
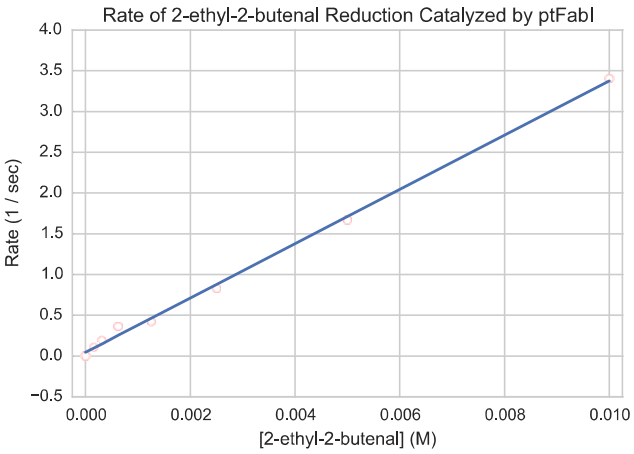
Rate (1 / sec)
[trans-2-nonenal] (M)

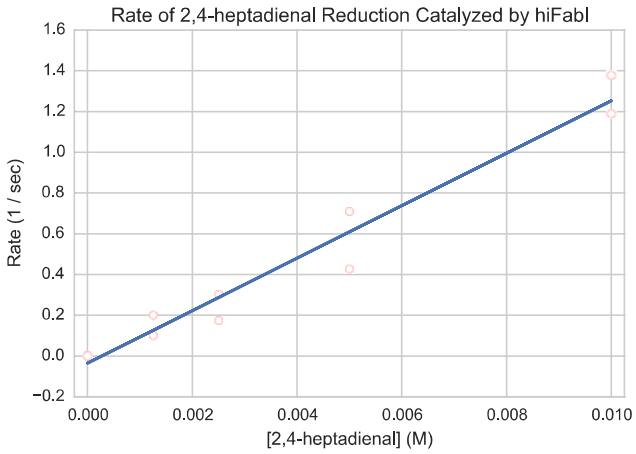

Rate of trans-2-nonenal Reduction Catalyzed by pfFabI
Rate (1 / sec)
[trans-2-nonenal] (M)

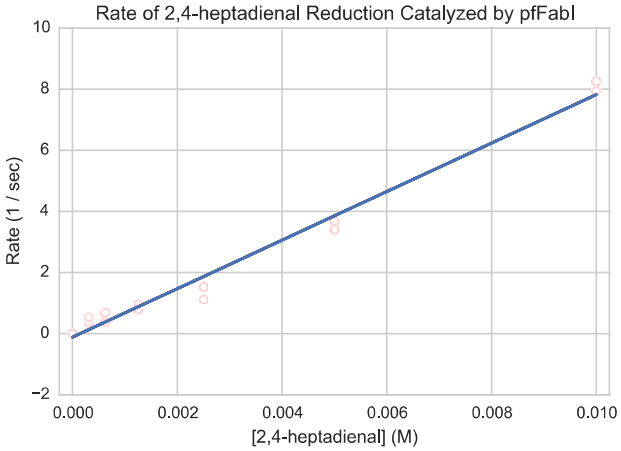

Rate of trans-2-nonenal Reduction Catalyzed by ptFabI
Rate (1 / sec)
[trans-2-nonenal] (M)

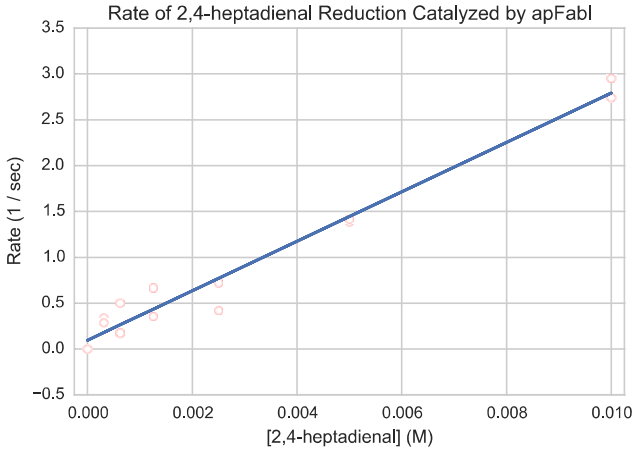

Rate of trans-2-nonenal Reduction Catalyzed by apFabI
Rate (1 / sec)
[trans-2-nonenal] (M)

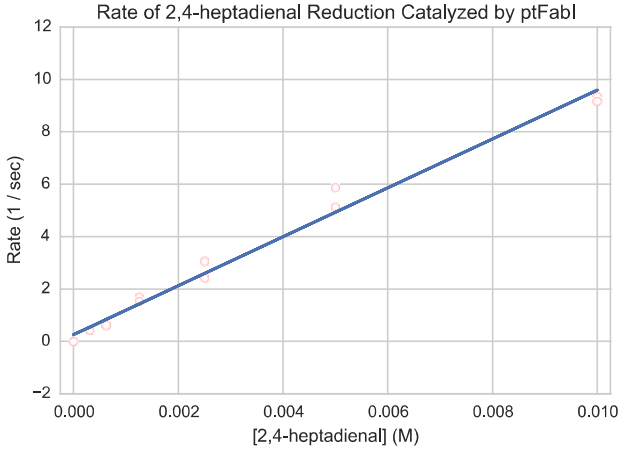

Rate of 2-ethyl-2-butenal Reduction Catalyzed by pfFabI
Rate (1 / sec)
[2-ethyl-2-butenal] (M)

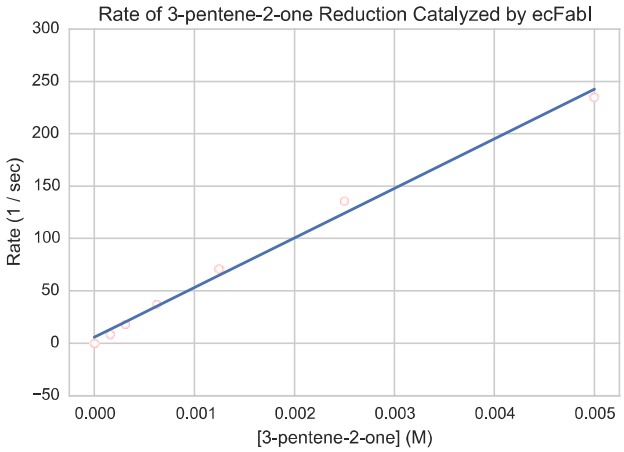

Rate of 2-ethyl-2-butenal Reduction Catalyzed by ptFabI
Rate (1 / sec)
[2-ethyl-2-butenal] (M)

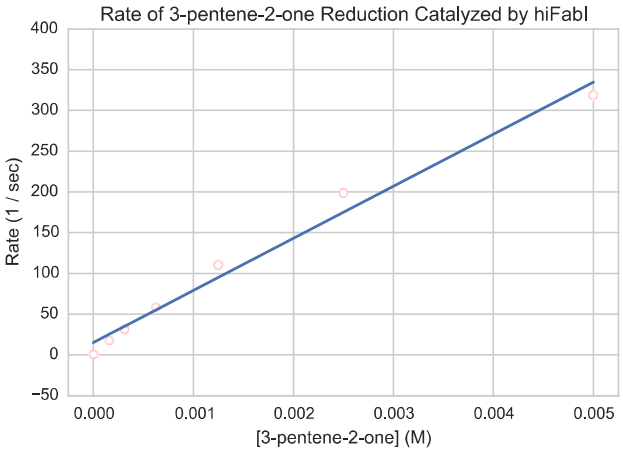

Rate of 2,4-heptadienal Reduction Catalyzed by hiFabI
Rate (1 / sec)
[2,4-heptadienal] (M)


Rate of 2,4-heptadienal Reduction Catalyzed by pfFabI
Rate (1 / sec)
[2,4-heptadienal] (M)


Rate of 2,4-heptadienal Reduction Catalyzed by apFabI
Rate (1 / sec)
[2,4-heptadienal] (M)


Rate of 2,4-heptadienal Reduction Catalyzed by ptFabI
Rate (1 / sec)
[2,4-heptadienal] (M)


Rate of 3-pentene-2-one Reduction Catalyzed by ecFabI
Rate (1 / sec)
[3-pentene-2-one] (M)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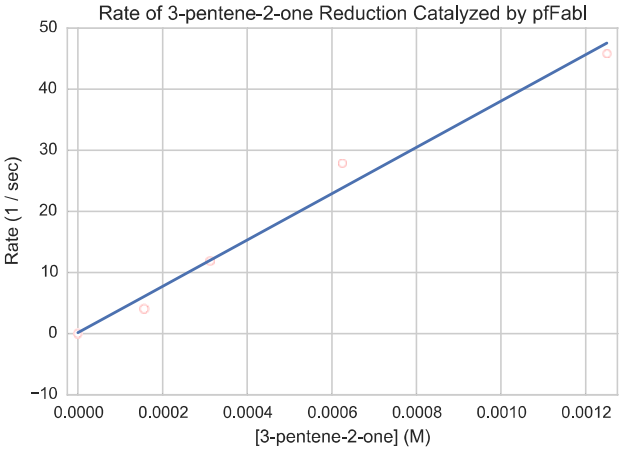
Rate (1 / sec)
[3-pentene-2-one] (M)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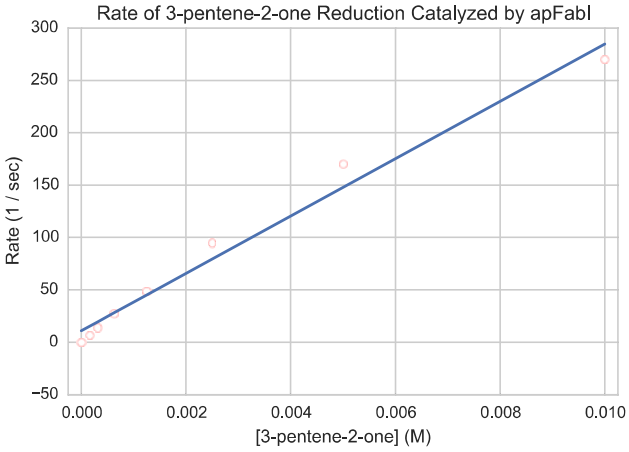
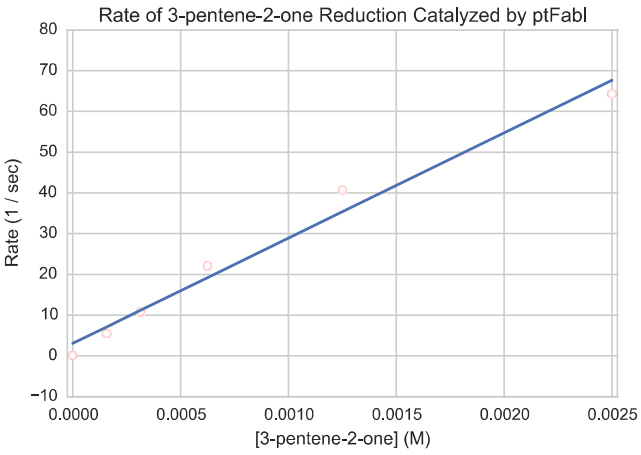
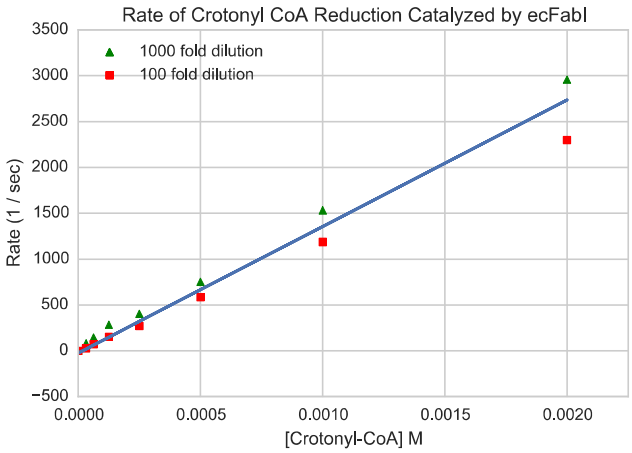
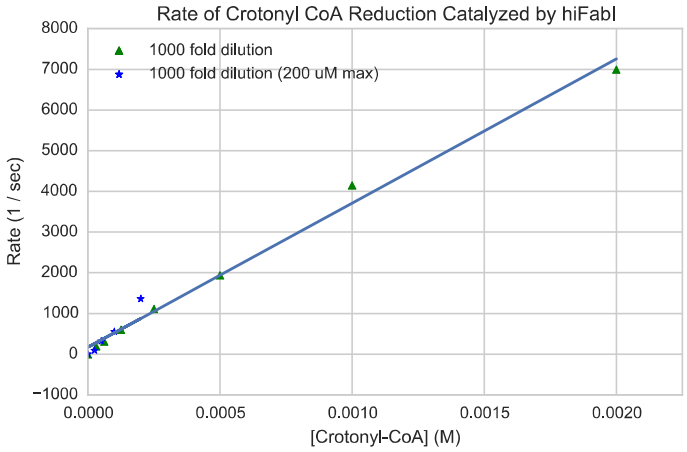
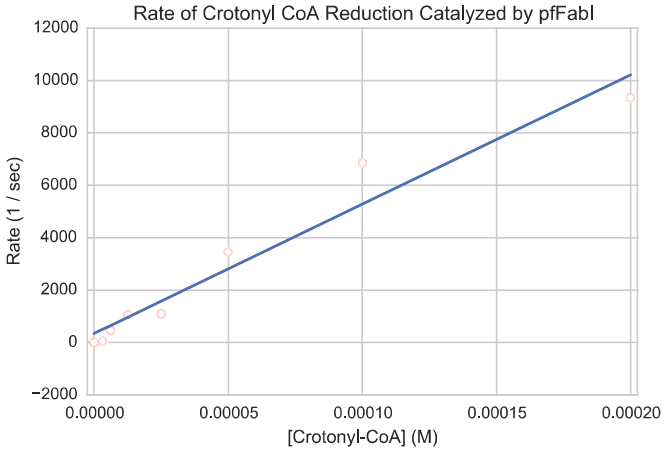
Rate (1 / sec)
[3-pentene-2-one] (M)

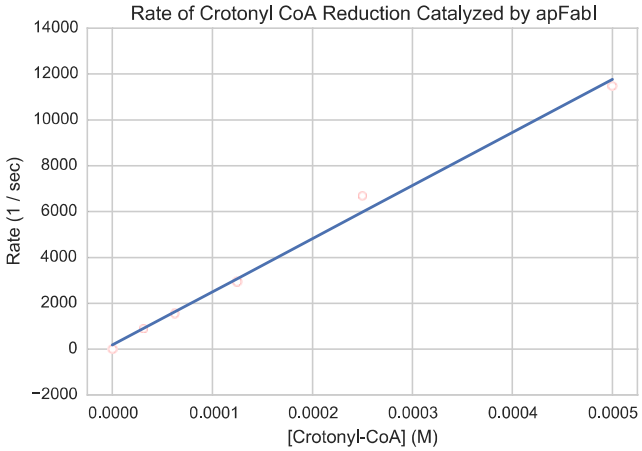

Rate of 3-pentene-2-one Reduction Catalyzed by apFabI
Rate (1 / sec)
[3-pentene-2-one] (M)


Rate of 3-pentene-2-one Reduction Catalyzed by ptFabI
Rate (1 / sec)
[3-pentene-2-one] (M)









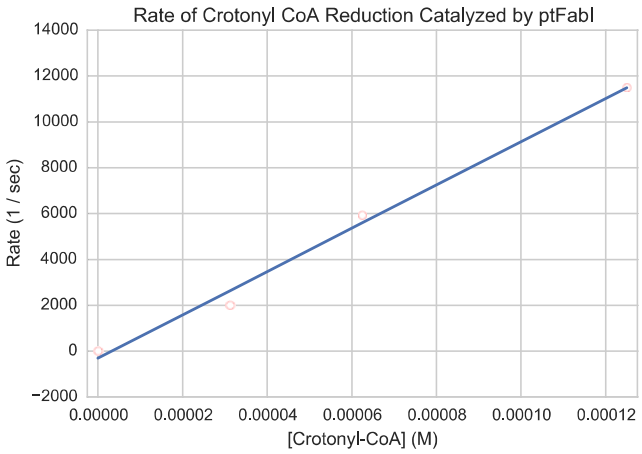
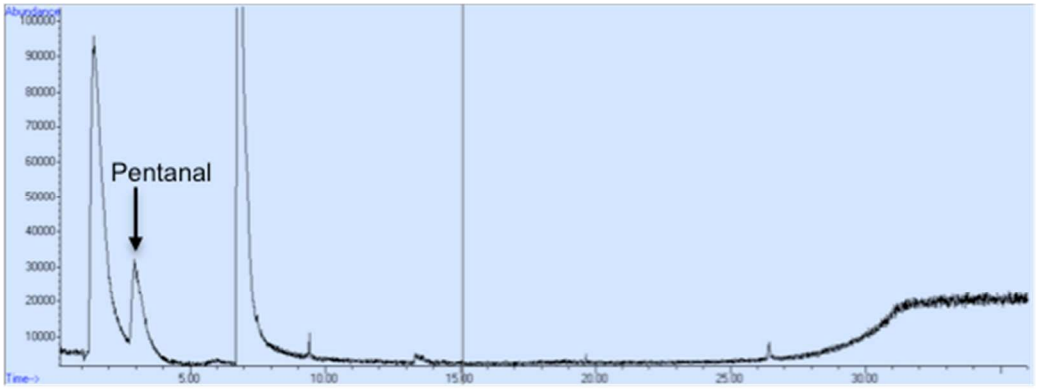


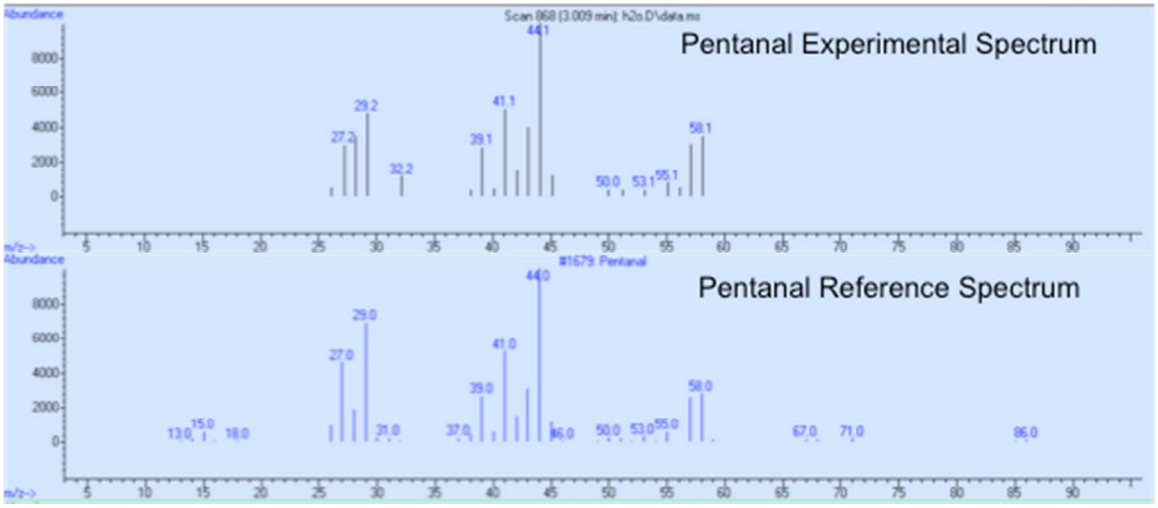
Figure S2: Substrate versus velocity plots

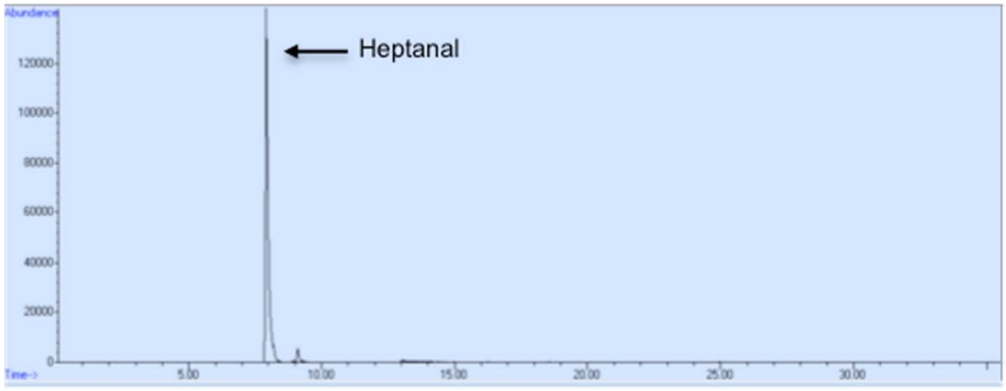
Table S3: Kinetic constants for each enzyme on each substrate

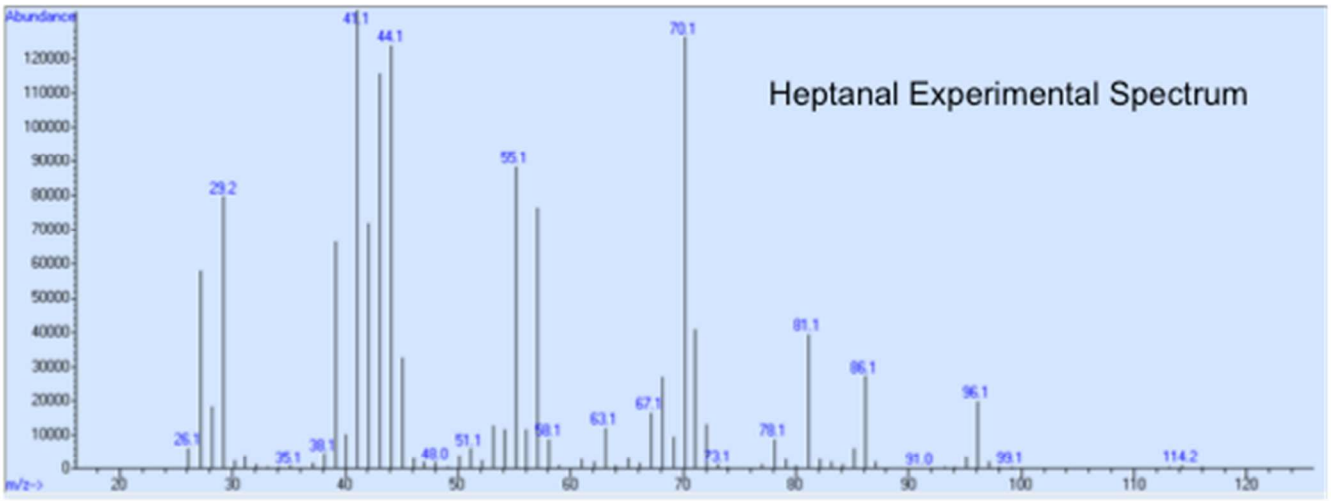
Fabi Ortholog	Substrate	k_{cat}/K_M (M^{-1} s^{-1})
ecFabI	*trans*-2-pentenal	2.48E+03±138
	trans-2-heptenal	2.38E+04±1.46E+03
	trans-2-nonenal	1.29E+04±393
	2-ethyl-2-butenal	N.D
	2,4-heptadienal	N.D
	3-pentene-2-one	4.74E+04±1.73E+03
	Crotonyl CoA	1.38E+06±7.90E+04
hiFabI	*trans*-2-pentenal	2.52E+03±60.2
	trans-2-heptenal	1.59E+04±654
	trans-2-nonenal	7.23E+03±377
	2-ethyl-2-butenal	N.D
	2,4-heptadienal	1.29E+02±9.20
	3-pentene-2-one	6.39E+04±3.76E+03
	Crotonyl CoA	3.54E+06±1.17E+05
pfFabI	*trans*-2-pentenal	2.65E+03±134
	trans-2-heptenal	1.66E+04±491
	trans-2-nonenal	1.30E+04±370
	2-ethyl-2-butenal	2.53E+02±12.0
	2,4-heptadienal	7.94E+02±28.2
	3-pentene-2-one	3.79E+04±2.82E+03
	Crotonyl CoA	4.94E+07±4.56E+06
apFabI	*trans*-2-pentenal	1.63E+03±118
	trans-2-heptenal	1.73E+04±7.00E+02
	trans-2-nonenal	2.75E+04±1204
	2-ethyl-2-butenal	N.D
	2,4-heptadienal	2.70E+02±13.4
	3-pentene-2-one	2.74E+04±1.52E+03
	Crotonyl CoA	2.32E+07±9.47E+05
ptFabI	*trans*-2-pentenal	1.13E+04±519
	trans-2-heptenal	4.75E+04±3.45E+03
	trans-2-nonenal	1.67E+04±1.80E+03
	2-ethyl-2-butenal	3.33E+02±6.80
	2,4-heptadienal	9.33E+02±30.4
	3-pentene-2-one	2.58E+04±1.86E+03
	Crotonyl CoA	9.44E+07±6.03E+06

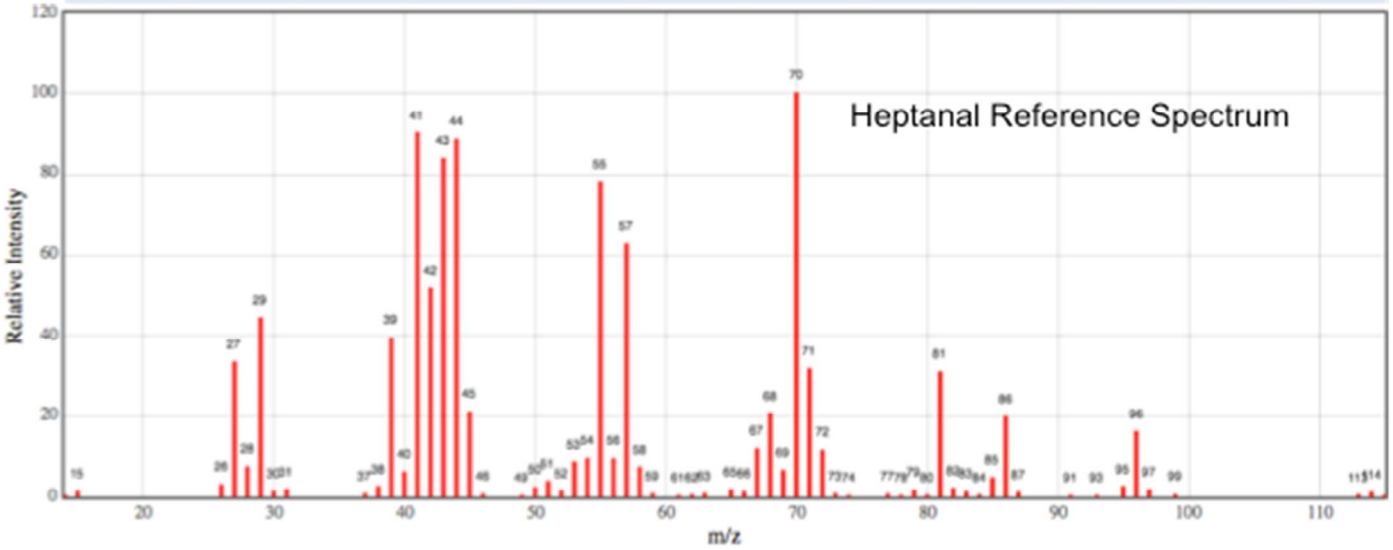
Figure S3: Gas Chromatography Mass Spectrometry results

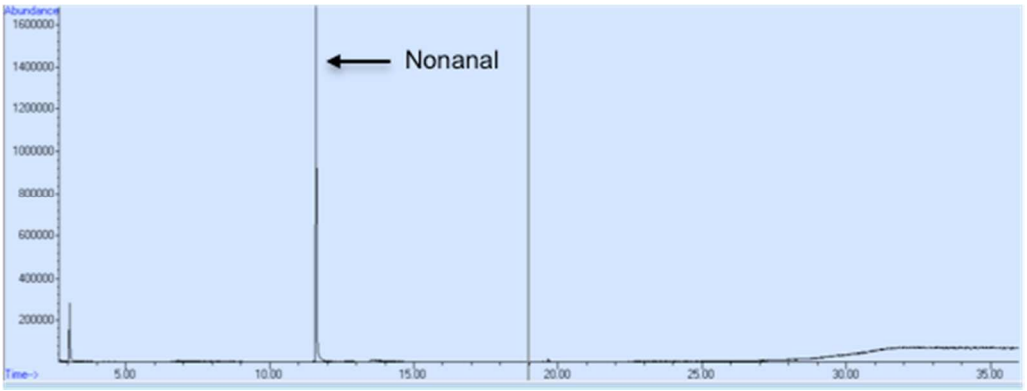







Abundance
m/z->
Heptanal Experimental Spectrum


Heptanal Reference Spectrum
Relative Intensity
m/z


Abundance
Time->
Nonanal

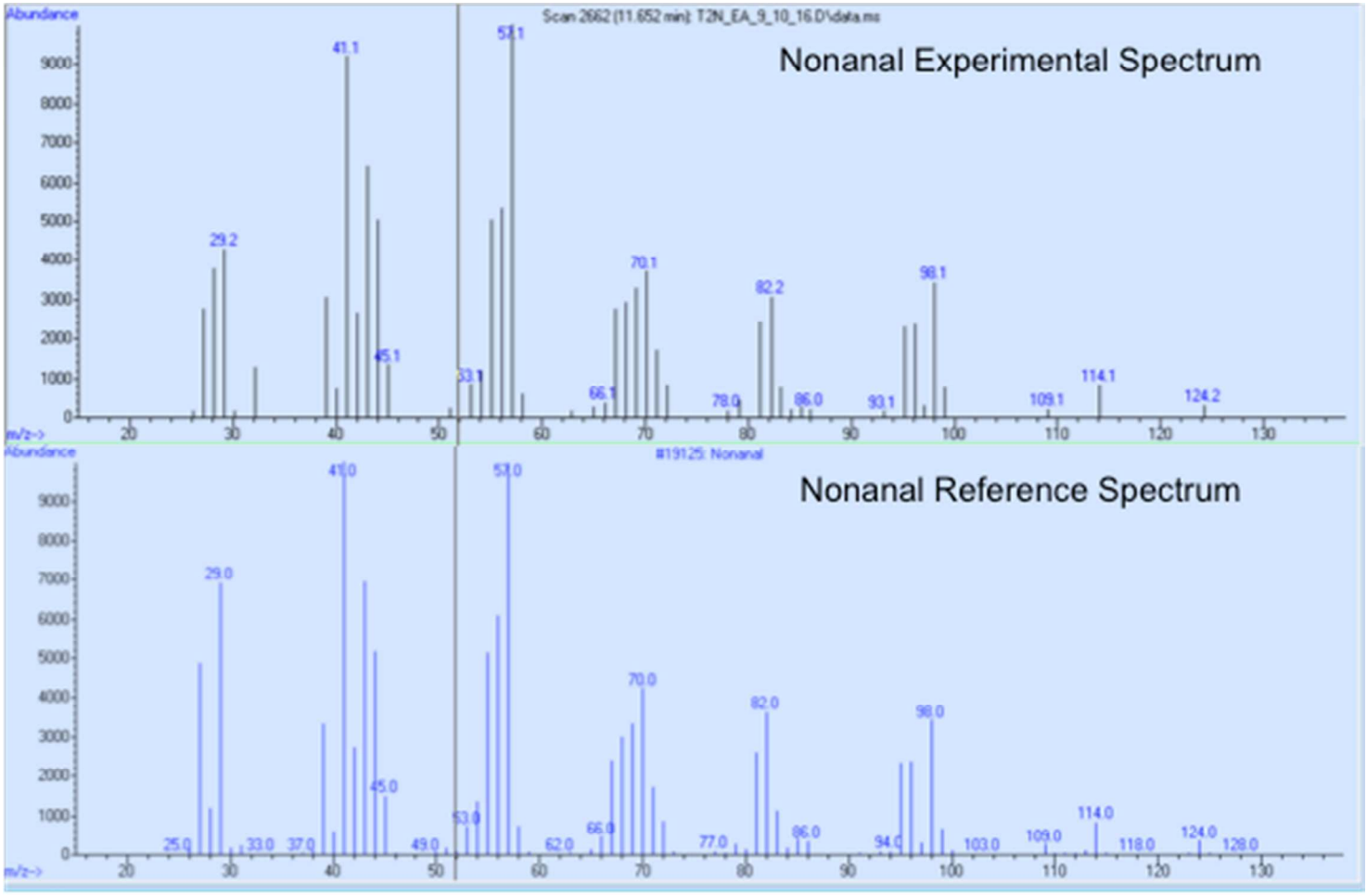


Figure S3: Gas Chromatography Mass Spectrometry results

Trans-2-pentenal and *trans-2*-heptenal substrates loaded onto column by direct injection, and trans-2-nonenal from an ethyl acetate extraction. Reactions were allowed to run overnight. For *trans-2-nonenal*, equal volume of ethyl acetate was added, and the organic phase was removed and loaded on the column. Figures correspond to the saturated aldehyde products.

Direct injection parameters

(*trans*-2-pentenal and *trans*-2-heptenal)

ALS
Syringe size 10.0 µL
Injection Volume 1.0 µL

Inlet
Heater 250°C
Pressure: 4.6 psi
Total Flow: {He} 13.9 mL/min
Mode: Splitless
Purge Flow to Split Vent: 10.0 mL/min at 2.00 min

Column
VF-5MS CP-8944 30 m X 0.25 mm X 0.25 µM
Constant flow of 1 mL/min
Oven
30°C from 0-4 minutes then ramp at 10C/min to 300°C then hold for 5 minutes at 300°C

MS scan parameters
Start scanning at 20 -300 (amu)
Solvent delay: 0 min

Ethyl acetate extraction parameters (*trans*-2-nonenal)

ALS
Syringe size 10.0 µL
Injection Volume: 2.0 µL

Inlet
Heater: 250°C
Pressure: 4.6 psi
Total Flow:{He} 13.9 mL/min
Mode: Split
Split Ratio: 10.0:1 10.0 mL/min

Column
VF-5MS CP-8944 30 m X 0.25 mm X 0.25 µM
Constant flow of 1 mL/min

Oven

30°C from 0-4 minutes then ramp at 10°C /min to 300°C then hold for 5 minutes at 300°C

MS scan parameters

Used Single Ion Mode (SIM) searching for m/z of 29.00, 41.00, 43.00, 44.00, 57.00, 58.00, 71.00, 85.00, and 86.00

Solvent delay: 0 min

Figures S5 & S6 Docking Results Additional Information

TRANS

Substrate	relative affinity	constraint	ligand score	hb sidechain	total score
tCoA	0.00	1.91	-1.19	0	-524
Carboxylate	23.92	0.09	-1.39	0	-531.24
Aldehyde	-5.43	0.22	-1.19	0	-535.7
Keto	-0.16	0.13	-2.17	0	-535.73
Ester	6.07	2.88	-0.51	0	-529.8
Branched Aldehyde	-2.95	0.09	-1.86	0	-529.98
Cyclopentenone	-4.70	0.04	-1.85	0	-535.23
Cinnamaldehyde	-4.15	0.13	-3.03	0	-528.79
Alcohol	36.84	0.04	-2.06	0	-557.83
Heptadienal	0.60	0.07	-2.14	0	-518.99

Figure S5. Relative hydride affinity (kcal/mol), constraint, ligand and hydrogen bond to sidechain scores and total (protein) score (Rosetta energy units) for the substrates in the s-*trans* conformation.

CIS

Substrate	relative affinity	constraint	ligand score	hb sidechain	total score
cCoA	0.00	0.04	-3.5	-0.52	-569.45
Carboxylate	23.92	0.04	-2.82	-0.7	-557.97
Aldehyde	-5.43	0.02	-3.14	-0.66	-565.57
Keto	-0.16	0.02	-3.98	-0.74	-558.73
Ester	6.07	0.02	-3.23	-0.57	-567.33
Branched Aldehyde	-2.95	0.02	-3.81	-0.79	-567.1
Cyclopentenone	-4.70	0.03	-3.54	-0.6	-555.31
Cinnamaldehyde	-4.15	0.07	-3.22	0	-538.38
Alcohol	36.84	0.05	-3.54	-1.23	-565.75
Heptadienal	0.60	0.04	-2.87	-0.44	-540.14

Figure S6. Relative hydride affinity (kcal/mol), constraint, ligand and hydrogen bond to sidechain scores and total (protein) score (Rosetta energy units) for the substrates in the s-*cis* conformation.

Amongst the s-*trans* conformer the ligand score (**Figure S5**) differ from each other by only ~2 units difference. This difference is within the noise of force-field based scoring and consequently we didn't feel that we could conclude that one substrate was better or worse than another. This same problem exists in the s-*cis* conformation as well (**Figure S6**), but the scores vary by an even smaller degree of ~0.5 energy units. Comparing the ligand scores for the s-*trans* conformers to the s-*cis*, the s-*trans* were generally not as good as the s-*cis* conformation. Again, the difference is quite small (~1 energy unit). Ligand Score (docking score) clearly doesn't discriminate between substrates in the same conformation or between the two possible conformations.

Figure S7.

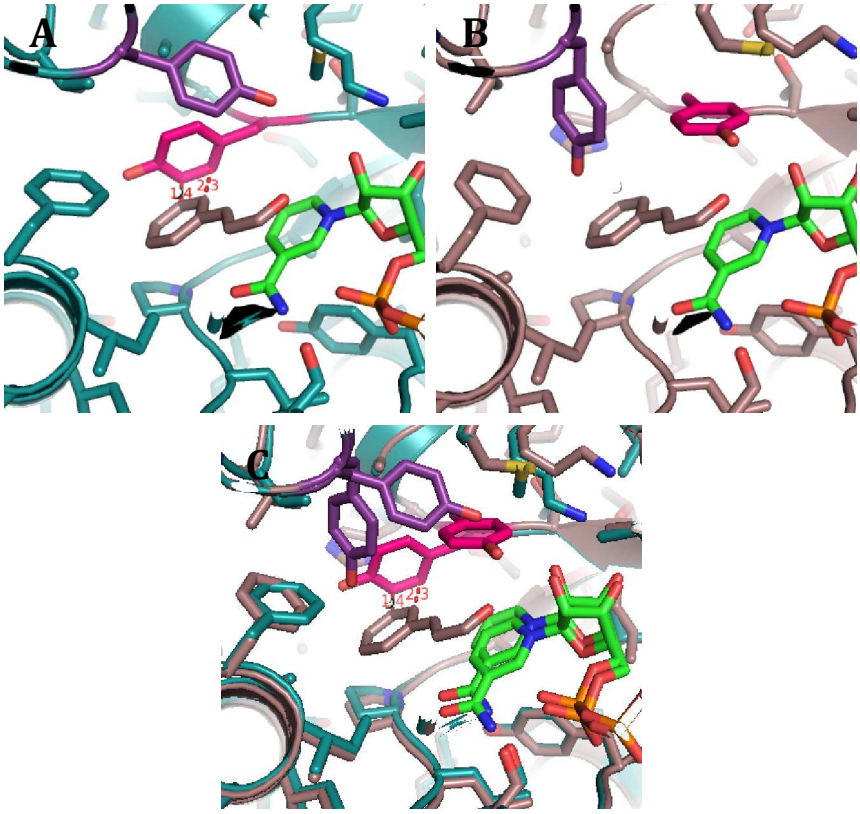
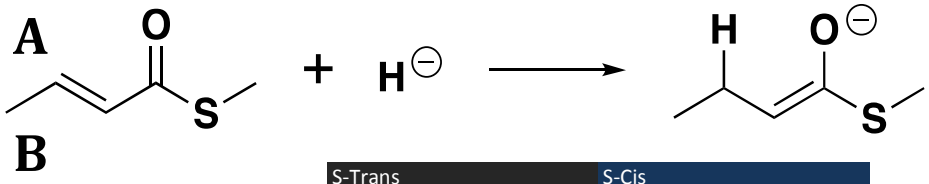


Figure S7. Key catalytic tyrosine shown in purple. Tyrosine potentially clashing with the substrate shown in magenta (TYR267). The NAD cofactor is shown in green. (**A**) Crystal structure of FabI (1NNU) drawn in teal with cinnamaldehyde placed in the active site. The substrate appears to be clashing with TYR267, with distances of 1.4 between nearest carbons. (**B**) Low energy output from docking the substrate cinnamaldehyde into the pocket, drawn in taupe. Not the rearrangement of the two tyrosines required to accommodate the substrate. (**C**) Overlap of both the crystal structure and low energy docking run.

While cinnamaldehyde would be predicted to be active by the QM calculations, no activity is observed in the assay conducted. The docking study conducted provides a rationale for the observed experimental result – that there is a rearrangement of the active site residues, including the catalytically required tyrosine, in order to accommodate cinnamaldehyde.

Table S8. Additional details of the quantum mechanics calculations.



B

Name	S-Trans		S-Cis	
	energy (hartrees)	energy (kcal/mol)	energy (hartrees)	energy (kcal/mol)
Hydride	-0.6481	-406.71	-	-
CoA Analog	-668.7012	-419616.71	-668.7012	-419616.71
Carboxylate	-306.0118	-192025.45	-306.0118	-192025.45
Aldehyde	-231.2032	-145082.31	-231.1990	-145079.68
Linear Ketone	-270.5041	-169744.03	-270.5037	-169743.78
Ester	-345.7325	-216950.58	-345.7334	-216951.16
Branched Aldehyde	-270.4982	-169740.30	-270.4925	-169736.78
Cyclopentenone	-269.3132	-168996.71	-308.5952	-193646.57
Cinnamaldehyde	-422.9094	-265379.89	-422.9051	-265377.17
Alcohol	-232.3856	-145824.27	-232.3840	-145823.26
Heptadienaldehyde	-347.8786	-218297.30	-347.8740	-218294.42
reduced CoA Analog	-669.4107	-420061.94	-669.4152	-420064.75
reduced Carboxylate	-306.6877	-192449.58	-306.6876	-192449.56
reduced Aldehyde	-231.9207	-145532.56	-231.9216	-145533.14
reduced Linear Ketone	-271.2151	-170190.16	-271.2179	-170191.98
reduced Ester	-346.4360	-217392.04	-346.4377	-217393.13
reduced Branched Aldehyde	-271.2125	-170188.57	-271.2112	-170187.77
reduced Cyclopentenone	-270.0213	-169441.07	-309.3167	-194099.30
reducedCinnamaldehyde	-423.6258	-265829.41	-423.6257	-265829.36
reduced Alcohol	-233.0409	-146235.48	-233.0393	-146234.46
reduced Heptadienaldehyde	-348.5876	-218742.20	-348.5870	-218741.86

Table S8. (**A**) Equation used to calculate the hydride affinity for all substrates in the manuscript. (**B**) The absolute energies of the substrates in both conformations. Calculations were run at the SMD(H_2O)-B3LYP/6-31+G(d,p) level of theory. Energies listed are sum of electronic and thermal free energy.

Table of the energies used in the determination of hydride affinity for the various substrates and conformations examined in the main text. All structures are also provided as a *.mol2 file which is attached separately.

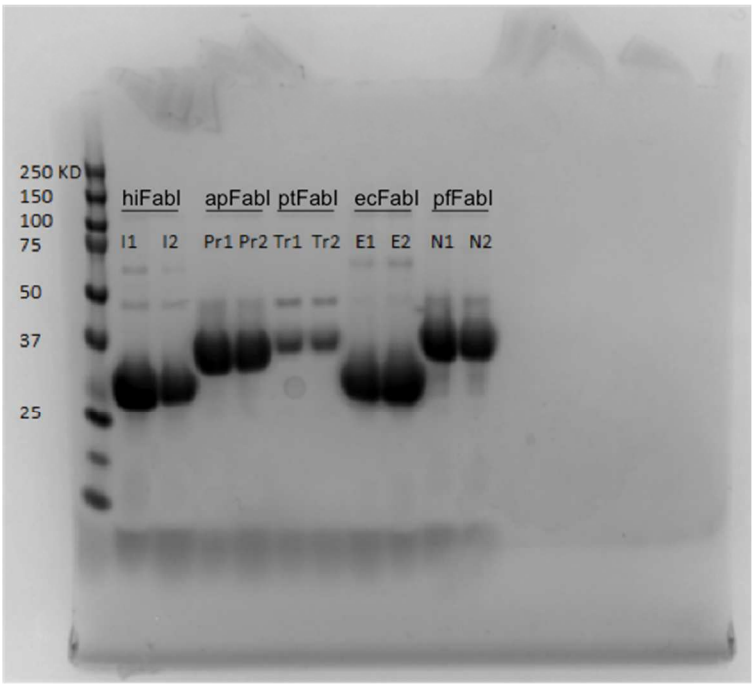
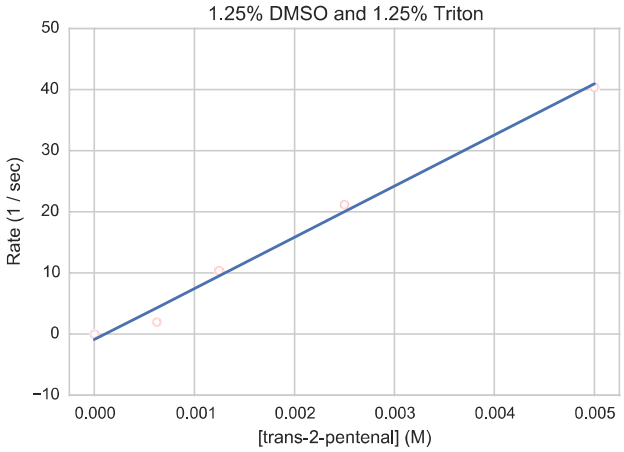
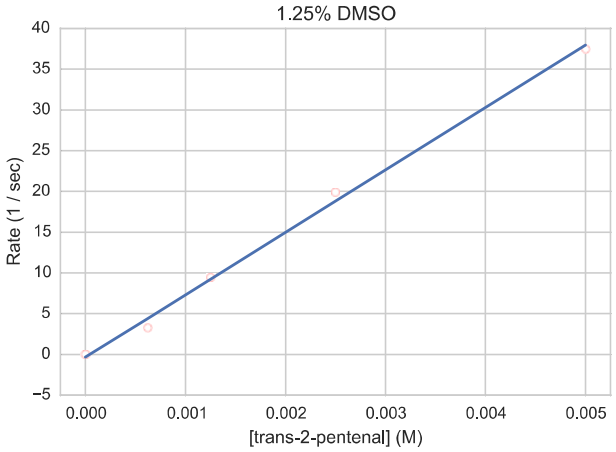


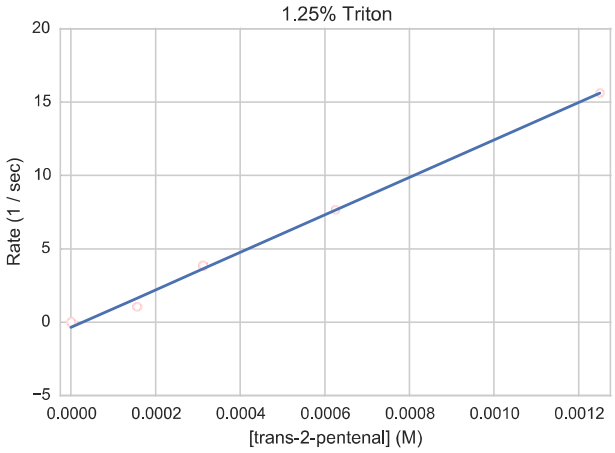
Figure S9. Protein purity assessed by SDS-PAGE. Ladder is in the left lane. Each ortholog is in the labeled lane. For purification conditions please see the main text.

Figure S10: Effect of solvents on enzymatic activity

Fabi Ortholog	Substrate	Solvent Condition	k_{cat}/K_M (M^{-1} s^{-1})
ptFabI	*trans*-2-pentenal	1.25% DMSO 1.25% triton	8.36E+03±430
ptFabI	*trans*-2-pentenal	1.25% DMSO	7.66E+03±251
ptFabI	*trans*-2-pentenal	1.25% Triton	1.28E+04±421
ptFabI	*trans*-2-pentenal	No DMSO No Triton	1.29E+04±370







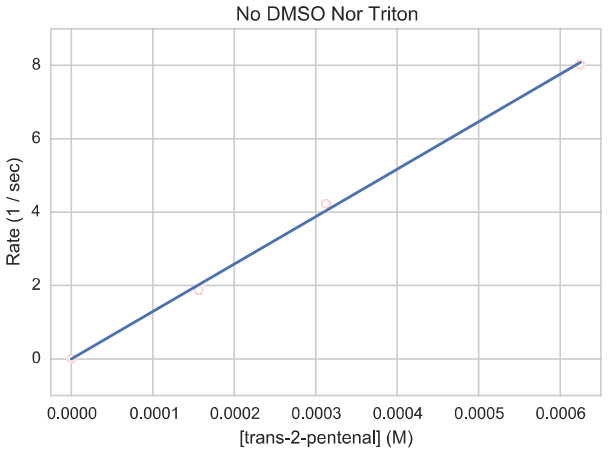


Figure S11: Triclosan inhibition assay

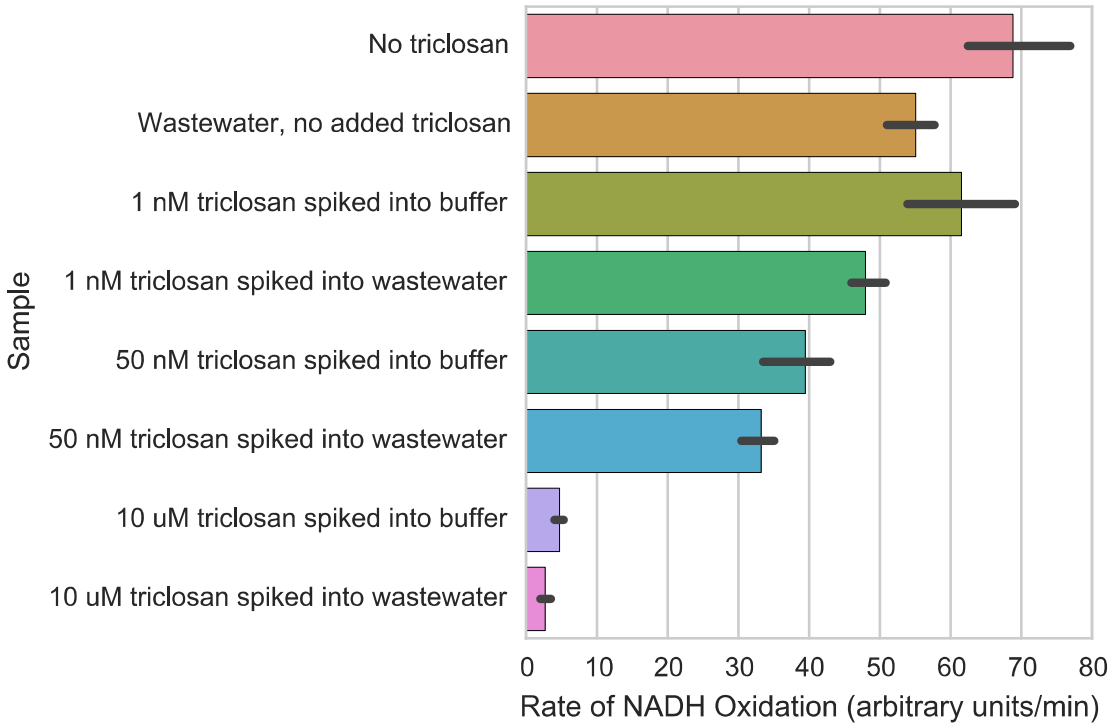


Figure S11: Triclosan inhibition assay using pfFabI and trans-2-pentenal as an alternative substrate to crotonyl-CoA. Triclosan was spiked in to buffer at 10 µM, 50 nM, and 1 nM

Figure S12: fabI DNA sequences

>ecfabI
ATGGGGTTCCTTAGCGGAAAACGGATATTAGTTACAGGTGTAGCCAGCAAGTTATCAAT
AGCCTATGGGATTGCACAGGCTATGCATCGCGAAGGGGCGGAACTTGCTTTTACGTATCA
GAATGATAAACTGAAAGGCCGTGTCGAAGAGTTTGCAGCTCAATTGGGTAGCGACATTG
TACTGCAATGCGACGTCGCTGAGGATGCTTCAATAGACACTATGTTCGCTGAACTGGGAA
AAGTATGGCCAAAATTTGACGGTTTTGTCCATTCGATCGGATTTGCCCCCGGTGACCAAC
TTGATGGCGATTATGTAAACGCTGTAACGCGCGAGGGGTTCAAGATTGCCCATGACATT
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GCCCTGCTTACATTGTCGTATTTGGGTGCGGAACGCGCAATCCCTAACTACAATGTGATG
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CGTTACCATTGAAGACGTGGGTAATAGCGCCGCGTTTTTATGTTCGGATTTGAGTGCTGG
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ACCACCACTGA

>hifabI
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CCACCACTGA

>pffabI
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>apfabI
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>ptfabI
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CTGGTCTCGACGGTTACACGATTTCGGAGGTTGCGAAAGCCGTTGAAGCAGACTATGGTA
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CGGCCGCACTCGAGCACCACCACCACCACCACTGA

Figure S13: FabI amino acid sequences

>ecFabI
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QCDVAEDASIDTMFAELGKVWPKFDGFVHSIGFAPGDQLDGDYVNAVTREGFKIAHDISS
YSFVAMAKACRSMLNPGSALLTLSYLGAERAIPNYNVMGLAKASLEANVRYMANAMGPE
GVRVNAISAGPIRTLAASGIKDFRKMLAHCEAVTPIRRTVTIEDVGNSAAFLCSDLSAGISG
EVVHVDGGFSIAAMNELELKNSSSVDKLAAALEHHHHHH

>hiFabI
MGFLTGKRILVTGLASNRSIAYGIAKSMKEQGAELAFTYLNDKLQPRVEEFAKEFGSDIVLP
LDVATDESIQNCFAELSKRWDKFDGFIHAIAFAPGDQLDGDYVNAATREGYRIAHDISAYS
FVAMAQAARPYLNPNAALLTLSYLGAERAIPNYNVMCLAKASLEAATRVMAADLGKEGIR
VNAISAGPIRTLAASGIKNFKKMLSTFEKTAALRRTVTIEDVGNSAAFLCSDLASGITGEIVH
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>pfFabI
MEDICFIAGIGDTNGYGWGIAKELSKRNVKIIFGIWPPVYNIFMKNYKNGKFDNDMIIDKD
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LANAKEVQKDLLNTSRKGYLDALSKSSYSLISLCKYFVNIMKPQSSIISLTYHASQKVVPGYG
GGMSSAKAALESDTRVLAYHLGRNYNIRINTISAGPLKSRAATAINKLNNTYENNTNQNK
NRNSHDVHNIMNNSGEKEEKKNSASQNYTFIDYAIEYSEKYAPLRQKLLSTDIGSVASFLLS
RESRAITGQTIYVDNGLNIMFLPDDLEHHHHHH

>apFabI
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ALNIFESSYRRGKFDANRKLANGSLLEFAHIYPMDAVFDTPEDVPPEIAENKRYAGNEGYT
VSEAAEKLARDVGKIDILVHSLANGPEVQKPLLETSRRGYLAALSASSYSLISMLQRFGPLL
NPGGAVISLTYLAGARVIPGYGGGMSSAKAALESDTKVLAFEAGRKWNVRVNTISAGPLGS
RAAKAIGFIDDMIKYSYENAPIQKELSAYEIGSVAAFLCSPLASAVTGHVMYVDNGLNTMG
LALDSKVLEREPVDASANSSSVDKLAAALEHHHHHH

>ptFabI
MAAQVDLKGKVAFVAGVADSTGYGWAIAKALAEAGATIIVGTWPPVLKIFQMGLKKGQF
NEDSTLADGSLMTIEKVYPLDAVFDAPDDVPDEIKENKRYAGLDGYTISEVAKAVEADYG
KIDILVHSLANGPEVTKPLLETTRKGYLAASSASAYSAVSLLQKFGPIMNEGGAMLSLTYIA
SEKVIPGYGGGMSSAKAQLESDTRTLAYEAGRKWGIRVNTISAGPLKSRAASAIGKEPGQK
TFIEYAIDYSKANAPLEQDLYSDDVGNASLFLTSPMARTVTGVTLYVDNGLHSMGMALDS
KAMEGSRENSSSVDKLAAALEHHHHHH

Supplemental Figure S17

In order to directly compare the very different scales of hydride affinity and hydrogen bond score, they were standardized using Equation 1. The log of the relative rate was standardized similarly and then compared to the normalized hydride affinity (Figure S14A) and the normalized hydrogen bond score (Figure S14B). While we did not have a sufficiently large data set to train a function to weight each metric (hydride affinity and hydrogen bond score) without overfitting, we used a simple approach of averaging the scores while down-weighting hydrogen bonding score by 2-fold (Eq 2). This was done as the hydride affinity alone was almost a perfect predictor for reactivity with the exception of cinnamaldehyde, while the hydrogen bonding score only predicted roughly half of the substrates correctly, which isn't a very strong predictor. Using the Combined Standardized Score (CSS) from the simple QM and docking calculations we are able to accurately categorize if FabI can utilize the compound as a substrate (Figure S14C). Ultimately, the graphs gave an impression of a quantitative analysis for which we didn't have sufficient data, but the data did allow for the prediction of active substrates. This lead to the development of the decision tree, which portrays what the combined method can predict.

$$Standardized\ Value = \frac{Value - Mean_{Values}}{Standard\ Deviation_{Values}} \qquad \textbf{(1)}$$

$$CSS = \frac{(Standardized\ Hydride\ Affinity) + \frac{1}{2}(Standardized\ Hydrogen\ Bond\ Score)}{2} \qquad \textbf{(2)}$$

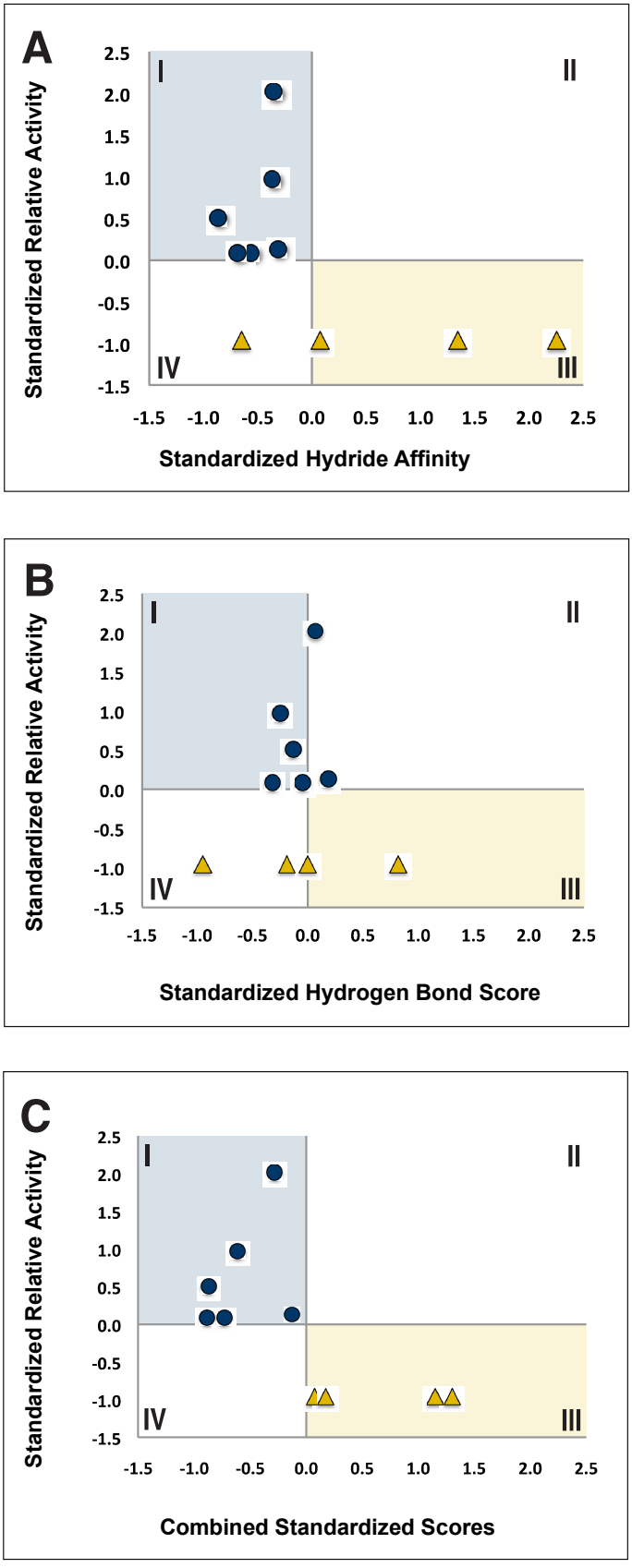
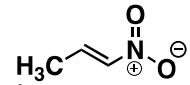
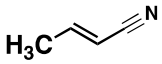


Figure S14. Predicting activity from QM and docking calculations. Yellow triangles represent *inactive* compounds and blue circles active compounds. The graphs are broken into four quadrants (I-IV) for illustration proposes. (**A**) Standardized log of activity versus the standardized hydride affinity. The yellow triangle in quadrant IV is cinnamaldehyde. (**B**) Standardized log of activity versus standardized hydrogen bond score. Cinnamaldehyde is the yellow triangle in quadrant III with the highest hydrogen bond score (with a low value being more favorable). (**C**) Standardized log of activity versus the sum of the standardized hydride affinity plus half the standardized hydrogen bond score, the total was then divided by two. All active compounds cluster in quadrant I and inactive in quadrant III.

Supplemental Figure S18

Two potential substrates were examined computationally – an α,β-unsaturated nitropropene and a α,β-unsaturated nitrile. Both compounds have a relative hydride affinities that would lead us to predict them to be active, but only one of the two substrates, the nitropropene, when docked can find a pose for which it can interact with

we

Substrate	Relative Hydride Affinity (kcal/mol)	Hydrogen Bond Score (Rosetta energy units)
 Nitro	-72.90	-0.94
 Nitrile	-52.90	0.00