

Supplementary Information

A Designed Photoenzyme Promotes Enantioselective [2+2]-Cycloadditions *via* Triplet Energy Transfer

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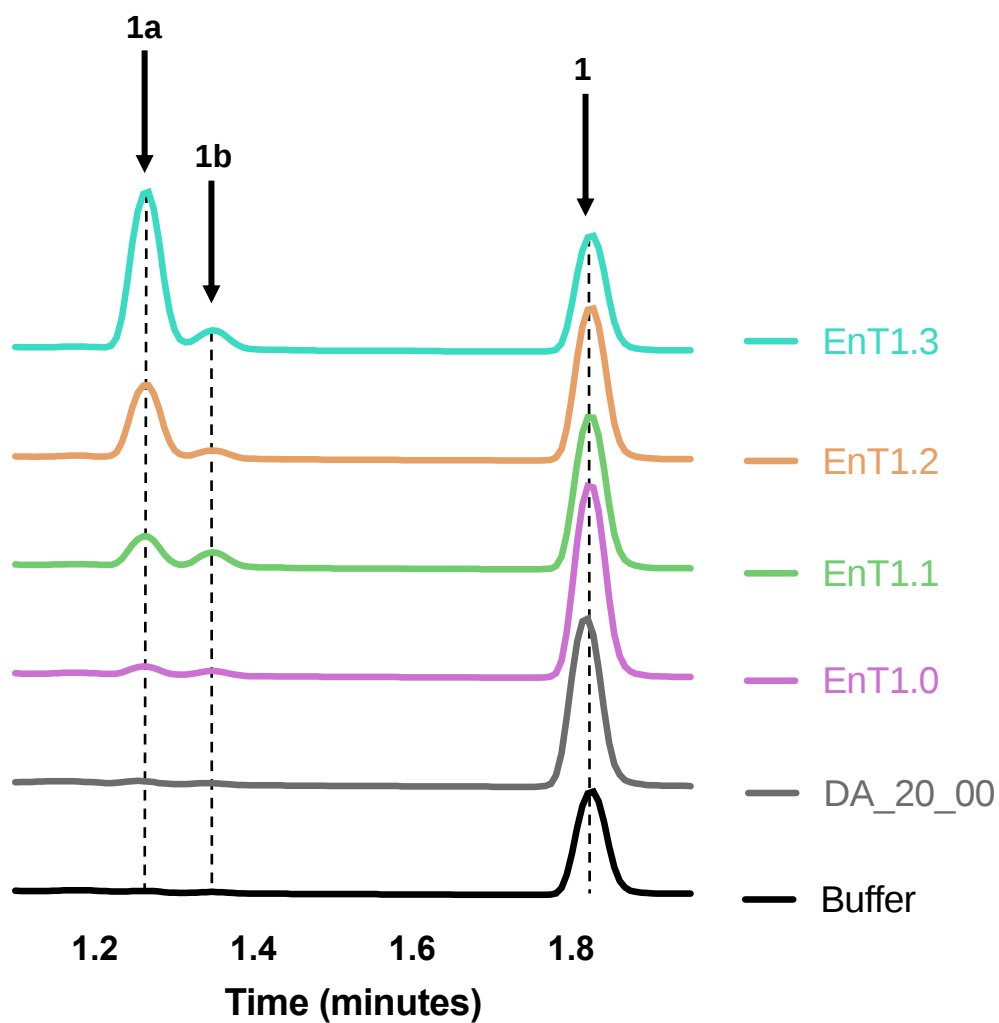
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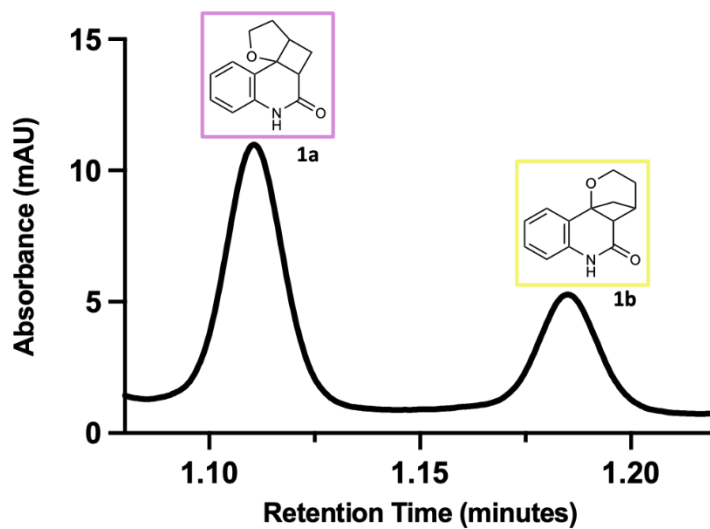
5.213	5.304	5.477	5.330	5.346	5.299	5.326	5.019	5.006	5.040	4.708	4.637
5.309	5.529	5.448	5.233	5.519	5.336	5.217	5.171	5.202	5.134	5.168	5.130
5.488	5.459	5.294	5.555	5.542	5.325	5.290	5.457	5.322	5.124	4.643	4.631
5.354	5.454	5.435	5.306	5.475	5.462	5.452	5.289	5.226	5.185	4.821	4.686
5.366	5.526	5.580	5.456	5.521	5.380	5.420	5.393	5.156	5.178	4.785	4.924
5.397	5.333	5.410	5.451	5.483	5.422	5.431	5.416	5.135	5.121	5.023	5.003
5.250	5.251	5.491	5.467	5.183	5.626	5.428	5.310	5.132	5.175	5.030	4.788
5.213	5.266	5.403	5.405	5.501	5.444	5.230	5.179	5.258	4.863	5.194	5.250

Supplementary Figure S1: Uniformity of light source. Uniformity of light irradiation of a UV curing LED oven chamber (NovaChem) was investigated by monitoring conversions across a 96-well plate. Reaction conditions: 7.5 μ M EnT1.0, 400 μ M **1**, 30 minutes irradiation (10 seconds on/off pulse) at 365 nm, at 4 °C, PBS (pH = 7.4) with 5% DMSO as a cosolvent. Conversions (**1a** and **1b** combined) are shown as a matrix, correlating to their respective plate positions. The relative coefficient of variance is 4.3%.

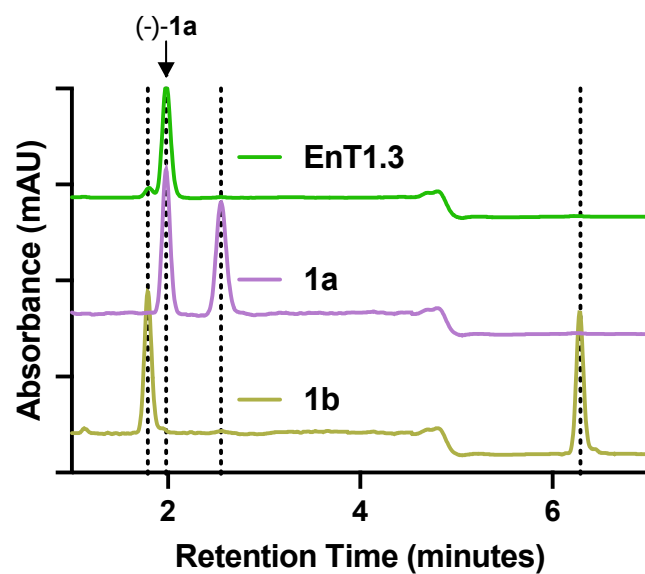


Supplementary Figure S2. UPLC chromatograms of evolution trajectory. UPLC absorbance chromatograms at 260 nm for reactions containing no enzyme, DA_20_00, EnT1.0, EnT1.1, EnT1.2 or EnT1.3. Reaction conditions: 3 μ M catalyst, 400 μ M **1**, 60 minutes irradiation (10 seconds on/off pulse) at 365 nm, at 4 $^{\circ}$ C, PBS (pH = 7.4) with 5% DMSO as a cosolvent.

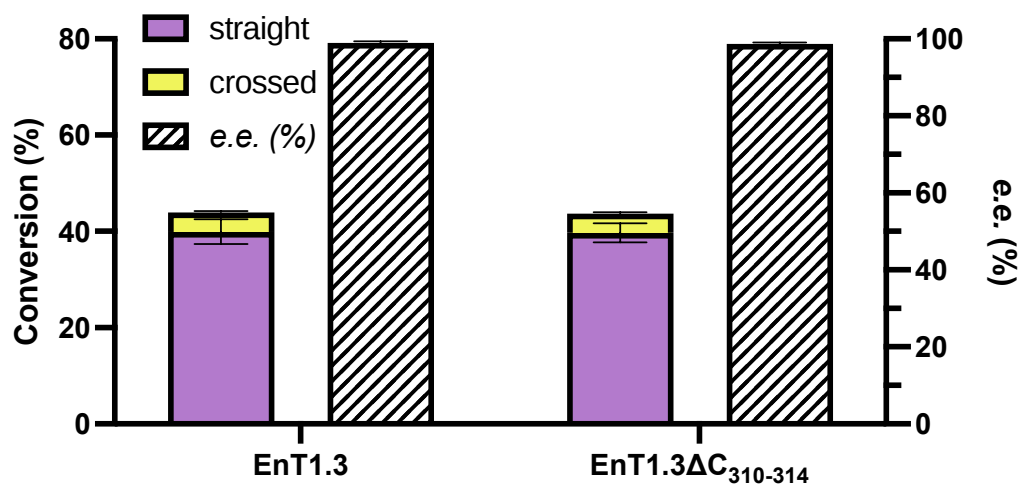
**UPLC trace of EnT1.0 derived
[2+2]-cycloaddition products**



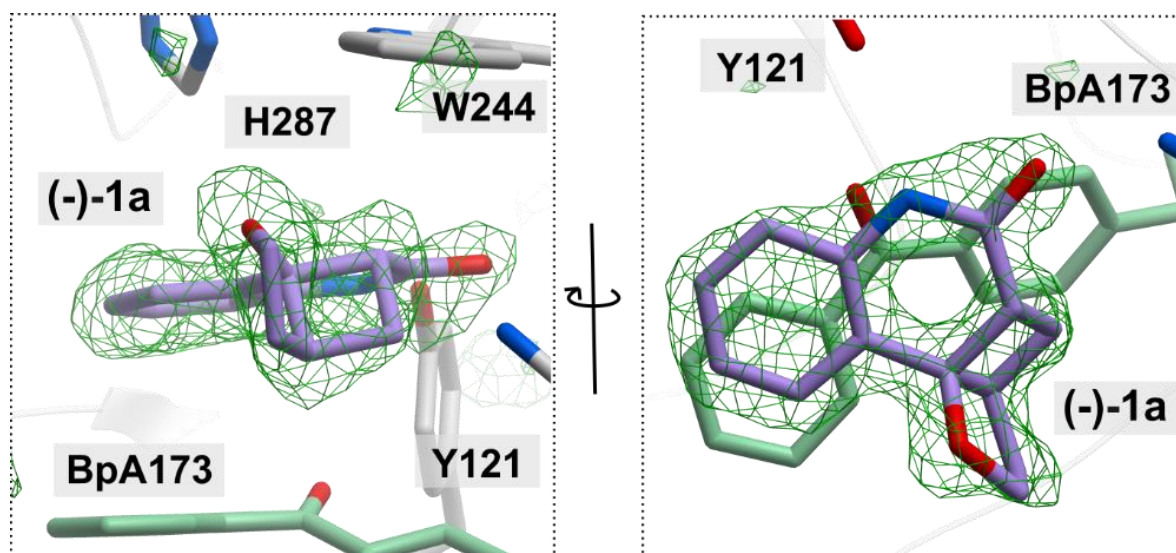
Supplementary Figure S3: UPLC chromatogram showing products derived from reactions with EnT1.0. UPLC chromatogram at 260 nm showing product **1a** and product **1b** (2:1 ratio). Reaction conditions: 7.5 μ M EnT1.0, 400 μ M **1**, 30 minutes irradiation (10 seconds on/off pulse) at 365 nm, at 4 $^{\circ}$ C, PBS (pH = 7.4) with 5% DMSO as a cosolvent.



Supplementary Figure S4: SFC chromatograms of racemic product standards and the product of EnT1.3-catalysed reaction using substrate 1. Reaction conditions: 10 μ M EnT1.3, 400 μ M **1**, 60 minutes irradiation (10 seconds on/off pulse) at 365 nm, at 4 $^{\circ}$ C, PBS (pH = 7.4) with 5% DMSO as a cosolvent.



Supplementary Figure S5: A comparison of the activity and selectivity of EnT1.3 and EnT1.3ΔC₃₁₀₋₃₁₄. Reaction conditions: 10 μM catalyst, 400 μM **1**, 15 minutes irradiation (10 seconds on/off pulse) at 365 nm, at 4 °C, PBS (pH = 7.4) with 5% DMSO as a cosolvent. The error bars represent the standard deviation of measurements made in triplicate.



Supplementary Figure 6: The active site of EnT1.3ΔC₃₁₀₋₃₁₄ in complex with the product (-)-1a. The $F_o - F_c$ omit density corresponding to (-)-1a is contoured at 3σ (green mesh). The product, BpA173 and active site residues are shown as atom-coloured sticks (with carbons shown in purple, green, and grey, respectively).

Supplementary Information, Table S1: Data summary of conversion and selectivity values of EnT1.0. Reaction conditions: 15 μ M catalyst, 400 μ M **1**, 30 minutes irradiation (10 seconds on/off pulse) at 365 nm, at 4 °C, PBS (pH = 7.4) with 5% DMSO as a cosolvent. Standard deviations are given for measurements in triplicate.

Catalyst	Average Conversion Straight (%)	S.D.	Average Conversion Crossed (%)	S.D.	<i>e.e.</i>	S.D.
-	0.33	0.031	0.19	0.079	-	-
Benzophenone	4.5	0.40	3.1	0.29	-	-
DA_20_00	0.55	0.21	0.32	0.044	-	-
EnT1.0	9.0	0.75	4.4	0.19	46	1.53
EnT1.0 ^Ψ	0.0	-	0.0	-	-	-

[Ψ] Reaction was performed without exposure to 365nm light.

Supplementary Information, Table S2: Data summary of conversion values of EnT1.0 and EnT1.3 for Extended Data Figure 4. Reaction conditions: 15 μ M catalyst, 400 μ M **1**, 10 seconds on/off pulse at 365 nm, 4 °C, PBS (pH 7.4) with 5% DMSO as a cosolvent. At each time point, 100 μ L of reaction mix was quenched with 1 volume MeCN and analysed by UPLC. Standard deviations are given for measurements in triplicate.

	EnT1.3		EnT1.0	
Time (minutes)	Average Conversion (%)	S.D.	Average Conversion (%)	S.D.
0.0	0.0	-	0.0	-
1.0	4.35	0.07	1.34	0.05
2.0	8.77	0.42	2.38	0.11
3.0	12.76	0.45	3.54	0.17
4.0	17.13	0.55	4.55	0.07
5.0	21.34	0.74	5.49	0.43

Supplementary Information, Table S3: Substrate scope of EnT1.2, EnT1.3 and EnT1.3 Y121F. Reaction conditions for the synthesis of **1a-12a** and **1b, 2b** and **12b**, conversion is displayed as a total of major and minor product where appropriate. All reactions were performed using PBS buffer pH 7.4 and 5% DMSO in triplicate. Standard deviations are given for measurements in triplicate.

Substrate	Product	Variant	Catalyst loading (mol%)	Substrate loading (mM)	Time (hours)	Conversion (%)	S.D.	e.e.
1	1a, 1b	EnT1.3	1.5	0.4	2 ^[1]	94	0.70	99
1	1a, 1b	EnT1.3	2.5	0.4	1 ^[1]	100	0.07	99
2	2a, 2b	EnT1.3	10	0.3	4 ^[1]	99	0.29	90
3	3a	EnT1.2	7.5	0.2	3 ^[1]	76	0.95	80
4	4a	EnT1.3	5	0.5	2 ^[1]	98	0.39	92
5	5a	EnT1.3	5	0.4	3 ^[2]	100	N.D.	95
6	6a	EnT1.3	10	0.4	3 ^[2]	95	0.31	94
7	7a	EnT1.2	7.5	0.3	4 ^[2]	98	0.5	26
8	8a	EnT1.3	5	0.4	3 ^[2]	100	N.D.	97
9	9a	EnT1.3	7.5	0.5	3 ^{[2][†]}	89	0.46	87
10	10a	EnT1.3	7.5	0.5	3 ^{[2][†]}	92	0.71	81
11	11a	EnT1.2	7.5	0.5	4 ^{[2][†]}	89	0.9	48
12	12a, 12b	EnT1.3	7.5	1	3 ^{[1][†]}	69	0.57	36
12	12a, 12b	EnT1.3 Y121F	7.5	1	3 ^{[1][†]}	94	0.32	78

[1] 365nm irradiation wavelength

[2] 395nm irradiation wavelength

[†] LED intensity was set to 50% to minimise background reactions.

Supplementary Information, Table S4. Protein crystallization conditions.

Variant	Mother liquor composition
EnT1.0	0.2 M Ammonium sulfate, 0.1 M BIS-Tris, pH = 5.5, 25% w/v PEG 3350 JCSG condition H7
EnT1.3	0.2 M Ammonium chloride, 0.1 M HEPES, pH = 7.0, 20% w/v PEG 6000 PACT premier Eco condition C8
EnT1.3 Δ C ₃₁₀₋₃₁₄	0.2 M Magnesium formate dihydrate, 20% w/v PEG 3350 JCSG condition A5

Supplementary Information, Table S5. Data collection and refinement statistics

	EnT1.0	EnT1.3	EnT1.3ΔC:product
PDB ascension number	7ZP5	7ZP6	7ZP7
Wavelength (Å)	0.9763	0.9762	0.9763
Resolution range	81.57 - 1.54 (1.595 - 1.54) ^a	54.66 - 1.9 (1.968 - 1.9) ^a	74.59 - 1.7 (1.761 - 1.7) ^a
Space group	C 1 2 1	P 2 ₁ 2 ₁ 2 ₁	P 1 2 ₁ 1
Unit cell dimensions a, b, c, (Å) α, β, γ (°)	173.847, 43.397, 81.7644, 90, 93.9843, 90	50.894, 75.5961, 78.5996 90, 90, 90	50.7517, 73.9581, 74.7216 90, 93.4574, 90
Total reflections	585059 (35012)	329486 (33095)	417082 (35910)
Unique reflections	89549 (8084)	24554 (2405)	60455 (5861)
Multiplicity	6.5 (4.3)	13.4 (13.8)	6.9 (6.1)
Completeness (%)	98.84 (89.62)	99.96 (99.92)	99.64 (97.25)
Mean I/sigma(I)	17.22 (0.67)	8.95 (1.81)	15.85 (2.08)
Wilson B-factor	23.72	18.41	16.81
R-merge	0.05761 (0.8321)	0.2025 (0.873)	0.08916 (0.4921)
R-meas	0.06245 (0.9503)	0.2105 (0.9067)	0.09642 (0.5379)
R-pim	0.02381 (0.448)	0.05688 (0.2423)	0.03641 (0.2143)
CC _{1/2}	0.998 (0.801)	0.996 (0.872)	0.997 (0.876)
CC*	1 (0.943)	0.999 (0.965)	0.999 (0.966)
Reflections used in refinement	89457 (8058)	24547 (2403)	60431 (5860)
Reflections used for R-free	4542 (449)	1239 (123)	3069 (264)
R-work	0.1959 (0.3637)	0.1690 (0.2104)	0.1508 (0.1964)
R-free ^b	0.2231 (0.3768)	0.2133 (0.2776)	0.1807 (0.2400)
CC (work)	0.965 (0.844)	0.962 (0.928)	0.974 (0.922)
CC (free)	0.962 (0.840)	0.942 (0.896)	0.961 (0.874)
Number of non-hydrogen atoms	5359	2840	5621
macromolecules	4901	2566	4883
ligands	10	0	68
solvent	448	274	702
Protein residues	617	321	614
RMS (bonds)	0.010	0.005	0.020
RMS (angles)	1.00	0.90	0.87
Ramachandran favoured (%)	96.71	97.15	96.69
Ramachandran allowed (%)	3.29	2.85	3.31
Ramachandran outliers (%)	0.00	0.00	0.00
Rotamer outliers (%)	0.00	0.37	0.58
Clashscore	2.26	4.93	3.71
Average B-factor	31.98	21.37	21.07
macromolecules	31.42	20.56	19.77
ligands	48.84	-	30.61
Solvent	37.75	28.96	29.67
Number of TLS groups	1	1	1

^aValues in parentheses are for highest resolution shell. ^bR-free was calculated using ~5% of the data separate from the rest

Supplementary Information, Table S6: Cosolvent tolerance of EnT1.3. Reaction conditions: 7.5 μ M EnT1.3, 400 μ M **1**, 10 seconds on/off pulse at 365 nm, 4 °C, PBS (pH 7.4) with the stated cosolvents. Standard deviations are given for measurements in triplicate.

Co-solvent	Average Conversion Straight (%)	S.D.	Average Conversion Crossed (%)	S.D.	<i>e.e.</i>
5% DMSO	33.6	0.5	3.6	0.0	99
10% DMSO	31.1	1.7	3.4	0.2	98
15% DMSO	27.8	0.5	3.1	0.1	98
20% DMSO	24.0	0.3	2.9	0.2	98
25% DMSO	17.8	0.3	2.3	0.0	93
30% DMSO	13.1	0.5	1.8	0.1	90
5% MeCN	28.4	0.8	3.0	0.0	99
10% MeCN	27.8	0.3	3.0	0.1	98
15% MeCN	25.3	0.5	3.0	0.2	94
20% MeCN	16.4	1.8	2.1	0.2	91
25% MeCN	6.3	1.3	1.1	0.1	N/A
30% MeCN	1.4	0.4	0.4	0.1	N/A

Supplementary Information, Table S7: Mass spectrometry data for DA_20_00 and EnT1.0 variants.

Variant	Expected Mass	Observed Mass
DA_20_00	36012.98	36012.6
DA_20_00 G29BpA	36207.2	36206.4
DA_20_00 D30BpA	36149.17	36148.4
DA_20_00 L148BpA	36151.07	36150.8
DA_20_00 Q195BpA	36136.17	36135.8
EnT1.0	36193.19	36193.2
EnT1.0 Y37A	36101.08	36101.3
EnT1.0 I72A	36151.08	36153.05
EnT1.0 M90A	36133.08	36133.2
EnT1.0 P135A	36167.1	36167.0
EnT1.0 I148A	36151.08	36152.94
EnT1.0 Q195A	36136.18	36137.69
EnT1.0 W244A	36078.08	36080.19
EnT1.0 H287A	36127.08	36128.97
EnT1.2	36136.08	36135.3
EnT1.3	36196.08	36195.8
EnT1.3 Y37F	36180.08	36178.89
EnT1.3 Y121F	36180.08	36179.17
EnT1.3 Q195A	36139.07	36137.99
EnT1.3 S229A	36180.08	36178.99
EnT1.3 W244A	36080.97	36079.32
EnT1.3 H287A	36129.97	36127.63

Supplementary Information, Table S8: Analytical UPLC methods for products 1a-12a and 1b, 2b 12b.

Substrate	Flow (mL.min ⁻¹)	Mobile Phase (% MeCN in MQ H ₂ O with 0.1% TFA) ^[1]	Run Time (min)	Extinction Coefficient (mM ⁻¹ cm ⁻¹) ^[2]	
				substrate	product(s)
1	1.1	5-40% (1.8 min) 5% (1.81 – 2.2 min)	2.2	747 (1)	718 (1a) 693 (1b)
2	1	20% (2 min) 20-40% (2.1 min) 40% (3 min) 40-20% (3.1 min) 20% (4 min)	4	800 (2)	883 (2a) 491 (2b)
3	1	30%	4	306 (3)	628 (3a)
4	1	17%	5.5	812 (4)	935 (4a)
5	1.5	17%	3	804 (5)	1241 (5a)
6	1.5	20%	3.5	297 (6)	929 (6a)
7	1	25%	3.5	592 (7)	1195 (7a)
8	1.8	25%	2.5	522 (8)	963 (8a)
9	1.5	27%	4	452 (9)	671 (9a)
10	1	37%	2.5	796 (10)	1039 (10a)
11	1	30%	3	651 (11)	502 (11a)
12	1	25% (2 min) 25-50% (2.05 min) 50% (3 min) 50-25% (3.01 min) 25% (4 min)	4	283 (12)	496 (12a) 488 (12b)

^[1] Column temperature was set to 20 °C

^[2] Detector wavelength was set to 260nm

Supplementary Information, Table S9: SFC chiral analytical methods for products 1a-12a and 1b, 2b 12b.

Substrate	Flow (mL.min⁻¹)	Mobile Phase (% MeOH in CO₂)	Run Time (min)	Product(s)	Column Temperature (°C)
1	1	25% (4 min) 25-50% (4.01 min) 50% (8 min) 50-25% (8.01 min) 25% (10 min)	10	1a 1b	20
2	0.8	50%	5	2a 2b	20
3	0.8	50%	4	3a	20
4	0.8	25%	6	4a	30
5	1	15%	7	5a	30
6	1	30%	5	6a	30
7	0.4	35%	6	7a	30
8	0.5	30%	6	8a	20
9	1	20%	6	9a	20
10	1	30%	5	10a	30
11	1	30%	5	11a	30
12	1	3-15% (15 min) 15-3% (15.1 min) 3% (16 min)	16	12a 12b	10

Supplementary Information, Table S10: Primers for the alanine scan, rounds 1 & 2.

Flanking Primers	
XHO_R	ATGCATGCCTCGAGCGATCCGA
NDE_F	CATGCATGCATATGGAGATCCCTGTCA
Alanine Scan	
Y37A_F	TATATTGTGGCTCCA GCA GTGGAGGTAAACGGTAAAC
I72A_F	AACGGCTATGGAGGC GCA CCAGCCGGGTG
M90A_F	TTATTTGTAGCTGAT GCA CGTCTGGGCCTTCT
P135A_F	TTATGGATTACGGCC GCA GCGGGCGAGGT
I146A_F	CCTGCCGATTTTACC GCA TCGTTACAAGAAAAATTCGGG
Q195A_F	CTGATTGTGGCCGAG GCA CCAATAAAAAAGTTATGG
W244A_F	TTGTTAGTTGCCAAT GCA GGCTCCTCGCATATC
H287A_F	ATTTTCGTGACGGAG GCA GAAAACAATGCAGTCTGG
Y37A_R	TGGAGCCACAATATAAAAGTC
I72A_R	GCCTCCATAGCCGT
M90A_R	ATCAGCTACAAATAACTGGTTG
P135A_R	GGCCGTAATCCATAAGTT
I146A_R	GGTAAAATCGGCAGGG
Q195A_R	CTCGCCACAATCAG
W244A_R	ATTGGCAACTAACAAATTGTTG
H287A_R	CTCCGTCACGAAAATGG
Round 1	
A74x_F	TATGGAGGCATACC NNK GGGTGCCAGTGC
M90x_F	TTATTTGTAGCTGAT NNK CGTCTGGGCCTTCTT
A120x_F	CGTATGCAGGGTTGC NNK TACTGCGCTTTTGATTATGA
Y121x_F	ATGCAGGGTTGCGCC NNK TGCGCTTTTGATTATGAAG
P135x_F	TTATGGATTACGGCC NNK GCGGGCGAGG
I146x_F	CCTGCCGATTTTACC NNK TCGTTACAAGAAAAATTCGGG
L148x_F	GATTTTACCATCTCG NNK CAAGAAAAATTCGGGTCA
Q149x_F	TTTACCATCTCGTT NNK GAAAAATTCGGGTCAATCTATTG
A175x_F	GCATTTCTAGTAGCC NNK GGTATTGCCGTGCG
Q195x_F	CTGATTGTGGCCGAG NNK CCAATAAAAAAGTTATGGTCC
K225x_F	ATCCCGGGCACTCAC NNK GGTGGAGCCGCC
A229x_F	CACAAAGGTGGAGCC NNK GGTATGGATTTTCGACG
W244x_F	TTGTTAGTTGCCAAT NNK GGCTCCTCGCATATC
E268x_F	ATCCGCTGCCC GTT NNK AAACCTTCTGCTTTACACTTT
K269x_F	CGCTGCCCGTTTCA NNK CCTTCTGCTTTACACTTTAAGC
S271x_F	CCGTTTCAAAAACT NNK GCTTTACACTTTAAGCCACAG
H287x_F	ATTTTCGTGACGGAG NNK GAAAACAATGCAGTCTGGA
A74x_R	TGGTATGCCTCCATAGC
M90x_R	ATCAGCTACAAATAACTGGT
A120x_R	GCAACCCTGCATACG

Y121x_R	GGCGCAACCCTG
P135x_R	GGCCGTAATCCATAAGTT
I146x_R	GGTAAAATCGGCAGGG
L148x_R	CGAGATGGTAAAATCGGC
Q149x_R	TAACGAGATGGTAAAATCGG
A175x_R	TGGCTACTGAAATGCGG
Q195x_R	CTCGGCCACAATCAG
K225x_R	GTGAGTGCCCCG
A229x_R	GGCTCCACCTTTGTG
W244x_R	ATTGGCAACTAACAAATTGTT
E268x_R	GAACGGGCAGCG
K269x_R	TTCGAACGGGCAGC
S271x_R	AGGTTTTTCGAACGGG
H287x_R	CTCCGTCACGAAAATGG
Round 2	
G19x_F	ACCGAAGATATTCCTNNKGCTGCTGGTCCAGT
A21x_F	GATATTCCTGGTGCTNNKGGTCCAGTGTTTCGAC
Y37x_F	TATATTGTGGCTCCANNKGTGGAGGTAAACGGTAAAC
I72x_F	AACGGCTATGGAGGCNNKCCAGCCGGGTG
Y121x_F	ATGCAGGGTTGCGCCNNKTGCGCTTTTGATTATGAAG
T133x_F	GGAAACTTATGGATTNNKGCCCCAGCGG
A136x_F	TGGATTACGGCCCCANNKGGCGAGGTGGCCCCCT
F144x_F	GTGGCCCCTGCCGATNNKACCATCTCGTTAGATGAAAAATT
S147x_F	GCCGATTTTACCATCNNKTTAGATGAAAAATTCGGGTCA
G153x_F	TTAGATGAAAAATTCNNKTCAATCTATTGTTTTACCACCG
Q172x_F	GTGGATACCGCATTTNNKTAGCCAGCTGGTATTGC
P174x_F	ACCGCATTTTCAGTAGNNKGCTGGTATTGCCGTG
G176x_F	TTTCAGTAGCCAGCTNNKATTGCCGTGCGC
Q195x_F	CTGATTGTGGCCGAGNNKCCAACTAAAAAGTTATGGTCCT
P196x_F	ATTGTGGCCGAGCAGNNKACTAAAAAGTTATGGTCCTATGAC
K225x_F	ATCCCGGGCACTCACNNKGGTGGAGCCTCG
G226x_F	CCGGGCACTCACAAANNKGGAGCCTCGGGTAT
G227x_F	GGCACTCACAAAGGTNNKGCCTCGGGTATGGAT
A242x_F	AACAATTTGTTAGTTNNKAATTGGGGCTCCTCG
W244x_F	TTGTTAGTTGCCAATNNKGGCTCCTCGCATATC
S271x_F	CCGTTCGAAAAACCTNNKGCTTTACACTTTAAGCCACA
A272x_F	TTCGAAAAACCTTCTNNKTTACACTTTAAGCCACAGAC
G19x_R	AGGAATATCTTCGGT
A21x_R	AGCACCAGGAATATCTTC
Y37x_R	TGGAGCCACAATATAAAAGT
I72x_R	GCCTCCATAGCCGT
Y121x_R	GGCGCAACCCTG

T133x_R	AATCCATAAGTTTCCTTCATAATCA
A136x_R	TGGGGCCGTAATCC
F144x_R	ATCGGCAGGGGC
S147x_R	GATGGTAAAATCGGCAGG
G153x_R	GAATTTTTCATCTAACGAGATGGT
Q172x_R	AAATGCGGTATCCACTTG
P174x_R	CTACTGAAATGCGGTATCC
G176x_R	AGCTGGCTACTGAAATGC
Q195x_R	CTCGGCCACAATCAG
P196x_R	CTGCTCGGCCACA
K225x_R	GTGAGTGCCCCG
G226x_R	TTTGTGAGTGCCCCG
G227x_R	ACCTTTGTGAGTGCC
A242x_R	AACTAACAAATTGTTGTCTTCGT
W244x_R	ATTGGCAACTAACAAATTGTTG
S271x_R	AGGTTTTTCGAACGGG
A272x_R	AGAAGGTTTTTCGAACGG