Supporting Information for:

Substrate channel evolution of an esterase for the synthesis of Cilastatin

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Table S1. Primers used for site-directed mutagenesis.

Library	Primers	Oligonucleotide sequences (5'-3')[a]
Library A		
	I128-F	TGGTCGGGGCGNDTACCAGCNDTGGGCGCG
	I128-R	CGCGCCCAHNGCTGGTAHNCGCCCCGACCA
	I131-F	TGGTCGGGGCGNDTACCAGCNDTGGGCGCG
	I131-R	CGCGCCCAHNGCTGGTAHNCGCCCCGACCA
	G138-F	GCGAAAAAGGTNNKAAGGTCGGCTCGGCGA
	G138-R	TCGCCGAGCCGACCTTMNNACCTTTTTCGC
	V140-F	GCGAAAAAGGTGGCAAGNNKGGCTCGGCGA
	V140-R	GCGCTACGCATCGCCGAGCCMNNCTTGCCACCTTTTT
	M144-F	TGGCAAGGTCGGCTCGGCGNNKCGTAGCGCAGTTCCCGGC
	M144-R	GCCGGGAACTGCGCTACGMNNCGCCGAGCCGACCTTGCCA
	A147/V148-F	TGCGTAGCNDTNDTCCCGGCGCGATGTCCG
	A147/V148-R	GCCGGGAHNAHNGCTACGCATCGCCGAGCC
	F200-F	GGGTGCGCGCAGCACTGNNKAACCGTGCTGTCGG
	F200-R	TCGTGACCGACAGCACGGTTMNNCAGTGCTGCGCG
	V227/V228-F	CACGGAACCGACGACTCCNDTNDTGACGTG
	V227/V228-R	TACCTGCGCTCACGTCAHNAHNGGAGTCGT
Library B		
	W33/A34-F	GGTGTTGCTGCACGGCTGGNDTNDTTCCTCGCAG
	W33/A34-R	CCAGCACTGCGAGGAAHNAHNCCAGCCGTG
	W100-F	ACGCGATCCTGCTCGGCNNKTCGTACGGCG
	W100-R	ATCACCAGACCGCCGTACGAMNNGCCGAGC
	F166-F	CGTGCACTCGGCGCTNNKGGCAATGCTCTCACC
	F166-R	GGTGAGAGCATTGCCMNNAGCGCCGAGTGCACG
	L170-F	GCTTTCGGCAATGCTNNKACCGGCCCACCCGA
	L170-R	TCGGGTGGGCCGGTMNNAGCATTGCCGAAAGC
	L184-F	GGGTGCAGCGTCGCAGGCCNNKTTCGGGTA
	L184-R	CGAGAGGCTGTACCCGAAMNNGGCCTGCGA

[a] The nucleotide sequences corresponding to the mutated amino acids are underline.

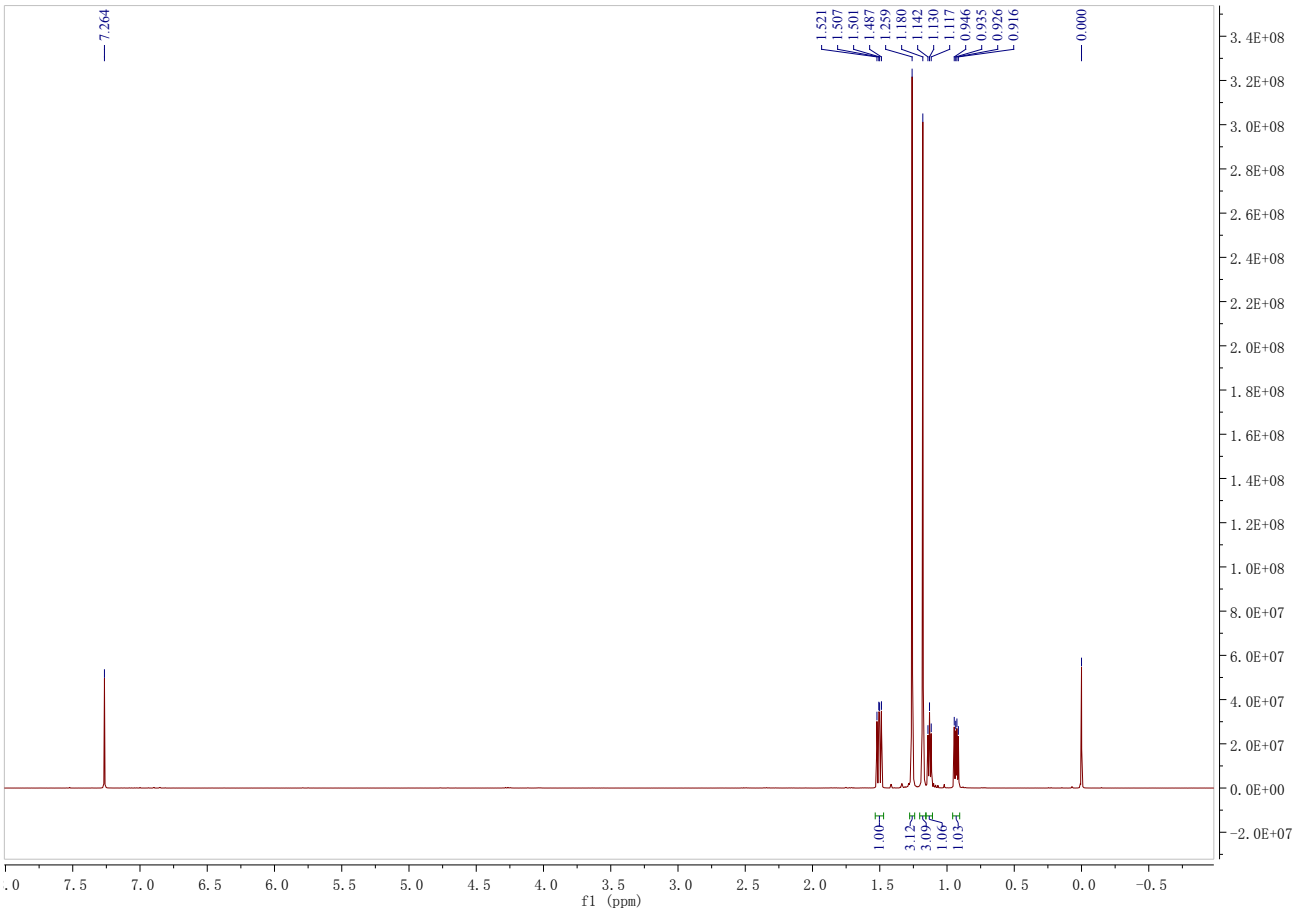
Table S2. Data collection and refinement statistics of *Rh*Est1.

Data collection	***Rh*Est1-native**	***Se-Rh*Est1**
Wavelength (Å)	1.5418	0.98
Space group	C_2	P_{3121}
a (Å)	213	96.0
b (Å)	45.4	96.0
c (Å)	77.4	206
α, β, γ (°)	90, 106, 90	90, 90, 120
Resolution rang (Å)[a]	44.3-1.95 (2.02-1.95)	35.1-2.40 (2.49-2.40)
Total/Unique reflections	52476 / 13119	43537 / 3721
Redundancy[a]	4 (3.8)	11.7 (11.9)
Average (I/δ)[a]	15.33 (2.3)	46.27 (13.4)
Completeness (%)[a]	100 (100)	99.9 (100)
Rmerge (%)[b]	0.043 (0.594)	0.082 (0.514)
Refinement	***Rh*Est1-native**	***Se-Rh*Est1**
Resolution range (Å)	44.35-1.95	35.09-2.40
No. of reflections	49674	42836
R-factor[c], R_{free} (%)[c]	0.1650, 0.2155	0.2504, 0.2861
No. of amino acid residues	830	539
No. of water	592	291
Rmsd bond lengths (Å)	0.007	0.010
Rmsd bond angles (°)	1.059	1.303
Favored (%)	95.9	96.61
Allowed (%)	3.5	2.64
Disallowed (%)	0.6	0.75

[a] Numbers in parentheses are values for the highest-resolution shell.

[b] $R_{merge} = \sum_{hkl}|I_i - I_m|/\sum_{hkl} I_m$, where I_i and I_m are the observed intensity and the mean intensity of related reflections, respectively. Values in parentheses indicate high-resolution shell.

[c] R-factor = $\sum||F_o - F_c||/\sum|F_o|$. $R_{free} = \sum_T||F_o - F_c||/\sum_T|F_o|$, where T is a test data set of 5% of the total reflections randomly chosen and set aside prior to refinement.



^{1}H NMR (400 MHz, $CDCl_3$), δ/ppm: 0.92-0.95 (m, 1H, CH), 1.11-1.14 (t, 1H, CH, J = 5.1 Hz), 1.18 (s, 3H, CH3), 1.26 (s, 3H, CH3), 1.48-1.52 (m, 1H, CH).

Fig. S1 ^{1}H NMR spectrum of product (*S*)-(+)-DmCpCa.