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Kinetic Resolution of Nearly Symmetric 3-Cyclohexene-1-carboxylate Esters Using a Bacterial Carboxylesterase Identified by Genome Mining

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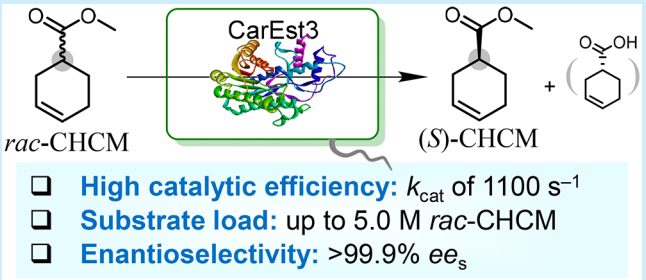
ABSTRACT: A new bacterial carboxylesterase (CarEst3) was identified by genome mining and found to efficiently hydrolyze racemic methyl 3-cyclohexene-1-carboxylate (*rac*-CHCM) with a nearly symmetric structure for the synthesis of (*S*)-CHCM. CarEst3 displayed a high substrate tolerance and a stable catalytic performance. The enantioselective hydrolysis of 4.0 M (560 g·L^{-1}) *rac*-CHCM was accomplished, yielding (*S*)-CHCM with a >99% *ee*, a substrate to catalyst ratio of 1400 g·g^{-1}, and a space-time yield of 538 g·L^{-1}·d^{-1}.



The optically active molecule methyl 3-cyclohexene-1-carboxylate (CHCM) is an important building block for the synthesis of a wide range of pharmaceuticals and high-value products that confer significant biological activities. For example, (*S*)-CHCM has been applied to the synthesis of FK-506,[1] the aglycone of the antitumor drug (+)-phyllanthocin,[2] the sex pheromone components periplanone A and B,[3] the toxin pumiliotoxin C,[4] the repellent SS220,[5] the unnatural amino acid *β*-homoglutamic acid,[6] carbacyclic-(1 → 3)-glucan mimetics,[7] and the inhibitor of coagulant factor Xa (fXa), which is commercially known as Edoxaban and used for the treatment of cancer-associated venous thromboembolism due to its superior oral adsorption and lower bleeding risk compared to those of other anticoagulants (Scheme 1A).[8] (*R*)-CHCM has also been applied to the synthesis of the immunosuppressant FK-506 (Tacrulimus),[9] the anti-influenza drug oseltamivir phosphate (Tamiflu),[10] the natural products leustroducsin B[11] and phyllanthocin,[12] potential antibacterials,[13] and E-selectin antagonists.[14]

Chiral CHCM can be synthesized through either asymmetric Diels–Alder reactions[15a,b] or the optical resolution of the corresponding racemic 3-cyclohexene-1-carboxylic acid (*rac*-CHCA) at the expense of stoichiometric chiral resolution reagents such as (*S*)-*α*-methylbenzylamine and (*R*)-*α*-phenethylamine.[15c,16] The drawback of these synthesis routes is that these processes are often cumbersome, requiring expensive chiral reagents and excessive hazardous organic solvents. Recently, developing biocatalysts for the preparation of chiral building blocks has drawn special attention for "green" enantioselective chemical conversions. Biocatalytic approaches are generally acceptable as highly enantioselective and environmentally benign alternatives, but few enzymes display desirable activities and enantioselectivities toward CHCM

Scheme 1. (A) Pharmaceutical or Natural Products Containing a Chiral CHCA Moiety As a Key Building Block and (B) Enantioselective Resolution by Lipases or Esterases

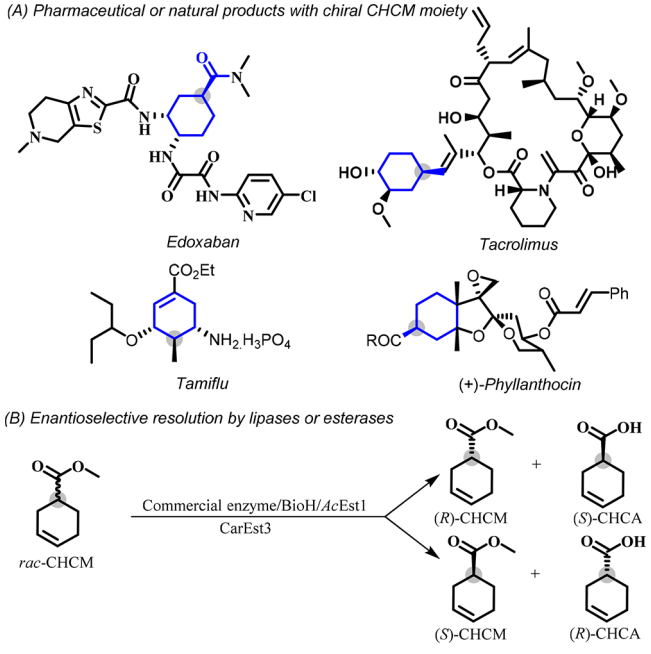


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Table 1. Genome Mining of Carboxylesterases for the Enantioselective Resolution of *rac*-CHCM

enzyme	accession no.	ΔG_R[a] (kcal·mol^{-1})	ΔG_S[a] (kcal·mol^{-1})	$\Delta\Delta G$[b] (kcal·mol^{-1})	act. to pNPS[c] (U·mg^{-1})	act. to CHCM[c] (U·mg^{-1})	ee_p/config.[d] (%/*R* or *S*)	*E* value[e]
CarEst1	ANF82944	−19.2 ± 1.1	−17.0 ± 0.8	−2.2**	11.7 ± 0.1	230 ± 3	77.2 ± 0.3/*S*	13.1
CarEst2	CAG70279	−21.6 ± 0.9	−18.9 ± 0.8	−2.7**	14.0 ± 0.1	367 ± 5	88.0 ± 0.3/*S*	17.6
CarEst3	AXY61874	−20.5 ± 1.2	−17.6 ± 1.0	−2.9**	12.7 ± 0.1	376 ± 5	90.1 ± 0.4/*S*	22.8
CarEst4	AMW77467	−18.9 ± 0.8	−16.3 ± 0.9	−2.6**	4.70 ± 0.01	114 ± 3	72.0 ± 0./*S*	7.1
CarEst5	AWL30387	−18.3 ± 0.9	−16.5 ± 1.0	−1.8**	6.10 ± 0.06	317 ± 6	72.2 ± 0.4/*S*	7.1
CarEst6	OUY06386	−16.3 ± 0.8	−15.4 ± 0.6	−0.9**	3.10 ± 0.08	42.5 ± 1.2	43.1 ± 0.3/*S*	2.7
CarEst7	ENV84364	−17.5 ± 1.0	−15.8 ± 0.7	−1.7**	5.70 ± 0.10	405 ± 8	67.3 ± 0.5/*S*	6.7
CarEst8	KEZ78936	−21.9 ± 1.1	−20.8 ± 0.8	−1.1*	22.5 ± 0.7	1.5 ± 0.1	13.5 ± 0.8/*S*	1.4
CarEst9	BAB65028	−19.7 ± 0.5	−19.1 ± 1.1	−0.6	11.2 ± 0.8	2.1 ± 0.1	17.2 ± 0.6/*R*	0.7
CarEst10	AOA56957	−22.3 ± 1.0	−19.8 ± 1.3	−2.5**	26.8 ± 1.0	90.5 ± 0.8	83.0 ± 0.4/*S*	13.8

[a]ΔG_R, binding free energy to (*R*)−CHCM; ΔG_S, binding free energy to (*S*)-CHCM. [b]$\Delta\Delta G = \Delta G_R - \Delta G_S$. Student's *t* test was performed with $p < 0.1$ (denoted as *) and $p < 0.05$ (denoted as **). [c]One unit (U) of activity was defined as the amount of enzyme required to catalyze the hydrolysis of 1 μmol CHCM per minute at 30°C; act., specific activity toward either *p*-nitrophenyl 3-cyclohexene-1-carboxylate or *rac*-CHCM. [d]ee_p, *ee* value on *rac*-CHCM; config., configuration. [e]The *E* value was calculated according to Chen et al.[24]

Table 2. Optimization of Biocatalytic Conditions for the Synthesis of (*S*)-CHCM[a]

	substrate		biocatalyst		S/C	temperature	time	conversion	ee_s	space-time yield
entry	(g·L^{-1})	(mol·L^{-1})	(g·L^{-1})	(kU·L^{-1})	(g·g^{-1})	(°C)	(h)	(%)	(%)	(g·L^{-1}·d^{-1})
1	140	1.0	0.08	4	1750	30	3.3	63.3	99.4	275
2	140	1.0	0.08	4	1750	20	6.0	60.3	99.0	222
3	140	1.0	0.08	4	1750	10	6.0	58.8	98.7	231
4	280	2.0	0.08	4	3500	30	8.0	64.2	99.7	300
5	420	3.0	0.2	10	2100	30	10.0	64.3	99.4	363
6	560	4.0	0.4	20	1400	30	9.0	64.3	99.3	538
7	700	5.0	1.4	70	500	30	12.0	64.3	99.6	500

[a]Reactions were performed in 100 mL of PBS buffer (100 mM, pH 8.0) with 1.0−5.0 M *rac*-CHCM and 0.08−1.4 g·L^{-1} lyophilized cell powder.

because of its nearly symmetric cyclohexene structure (Scheme 1B).

Commercial pig liver esterase (PLE) and horse liver esterase (HLE) have been reported to catalyze the enantioselective hydrolysis of racemic CHCM (*rac*-CHCM). About 10 g·L^{-1} *rac*-CHCM could be converted to (*S*)-CHCA by either 2 mL·L^{-1} PLE at a 43% isolation yield and a >99% enantiomeric excess (*ee*) or 0.2 g·L^{-1} HLE at a 41% isolation yield and a 97% *ee*.[17] However, commercial enzymes are generally expensive, and the reproducibility of their outcomes depends on their commercial availability. Recently, the carboxylesterase BioH, which is involved in biotin synthesis in *Escherichia coli*, was reported to hydrolyze *rac*-CHCM; however, it had an *ee* of only 32.3% and an enantioselectivity value (*E* value) of 2.1.[18] Through the combinatorial modulation of steric and aromatic interactions, the best-performing variant of BioH, Mu3 (L24A/W81A/L209A), reached a 70.9% *ee* and an *E* value of 7.1 at 40 mM *rac*-CHCM. However, high substrate loads resulted in a decreased enantioselectivity. Therefore, the challenges of a low substrate tolerance, a low enantioselectivity, and a low volumetric productivity together limit the industrial biosynthesis of enantiomerically enriched CHCA. Moreover, as most of the commercial esterases or lipases are from yeast or animal sources, the identification of bacterial esterases is of special interest due to the low cost of feasible and scalable prokaryotic expression systems.[19]

In previous work, we have isolated the esterase-producing strain *Acinetobacter* sp. JNU9335 with the ability to enantioselectively hydrolyze *rac*-CHCM.[20a] Four putative esterases were identified from the genome of JNU9335 that demonstrated activity toward *rac*-CHCM, and one esterase designated as *Ac*Est1 exhibited the highest enantioselectivity.[20b] Genome mining is a relatively new, inexpensive, and readily available bioinformatic tool that can aid in the identification of new enzymes and natural products based on the homologies of preidentified genomic information.[21] Herein, we performed genome mining to obtain robust carboxylesterases (CarEsts)[22] and established an efficient biocatalytic process for the synthesis of chiral (*S*)-CHCM.

Ten putative carboxylesterases originating from different microorganisms, including *Acinetobacter*, *Serratia*, *Salinisphaera*, and *Sulfolobus* species (Table S1), were identified by BLASTp searches to have a 35−70% sequence identity of *Ac*Est1 and were ligated into pET28a with an N-terminal His-tag and heterologously expressed in *E. coli* BL21(DE3) (Figure S1). The binding free energies (ΔG) of CarEst1−10 toward (*R*)-CHCM (ΔG_R) and (*S*)-CHCM (ΔG_S) were calculated using the molecular mechanics Poisson−Boltzmann surface area (MM-PBSA) method.[23] The preference for the generation of (*R*)- or (*S*)-CHCM can be indicated by the difference between the ΔG_R and ΔG_S values ($\Delta\Delta G = \Delta G_R - \Delta G_S$). As shown in Table 1, these recombinant carboxylesterases displayed variable ΔG values toward (*R*)- or (*S*)-CHCM. The lowest $\Delta\Delta G$ was found in CarEst3, with a $\Delta\Delta G$ of −2.9 kcal·mol^{-1}. Further screening was carried out to evaluate the ability of these CarEsts to generate enantioselective products from *rac*-CHCM. All of them showed hydrolytic activity toward 3-cyclohexene-1-carboxylate esters (Table 1 and Figure S2). Recombinant CarEst10 exhibited the highest specific activity of 26.8 U·mg^{-1} toward *p*-nitrophenyl 3-cyclohexene-1-carboxylate, which was 5.74-fold higher than that of *Ac*Est1. However, the ee_p value of CarEst10 was 83.0% lower than the value of

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Table 3. Kinetic Parameters of Recombinant CarEst3 Toward CHCM

substrate	K_M (mM)	V_{max} (μmol·(min·mg)$^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1}·mM^{-1})
rac-CHCM	12.4 ± 1.4	1650 ± 120	1100 ± 81	89.0 ± 6.5
(*R*)−CHCM	13.8 ± 2.3	1502 ± 97	1001 ± 64	72.3 ± 4.6
(*S*)−CHCM	33.0 ± 8.6	679 ± 99	452 ± 66	13.7 ± 2.0

89.1% from *Ac*Est1. CarEst3 displayed the highest enantioselectivity and specific activity at 90.1% ee_p and 12.7 U·mg^{-1}, respectively, the latter of which is significantly higher than the value of 4.67 U·mg^{-1} from *Ac*Est1. The high ee_p value of CarEst3 was consistent with its low $\Delta\Delta G$ value, demonstrating the utility of binding affinity analyses when mining enantioselective biocatalysts from genomic databases. Considering that it had the highest enantioselectivity and a relatively high activity, CarEst3 was selected as the best-identified biocatalyst for the enantioselective resolution of *rac*-CHCM for the synthesis of (*S*)-CHCM.

To establish an efficient biocatalytic process, reaction parameters, including temperature, substrate load, and biocatalyst dosage, were assessed. Initial experiments were carried out in 100 mL of PBS buffer (100 mM, pH 8.0) with 0.1 mol (140 g·L^{-1}) substrate *rac*-CHCM and 480 U (0.08 g·L^{-1}) of CarEst3 derived from lyophilized cells of *E. coli* BL21 at 30°C (Table 2, entry 1). The reaction pH was maintained at 8.0 by titrating 2.0 M Na_2CO_3. After merely 3.3 h, a 99.4% ee_s was achieved at a conversion ratio of 63.3%, with a substrate to biocatalyst ratio (S/C) of 1750 g·g^{-1} and a space-time yield of 275 g·L^{-1}·d^{-1}. Furthermore, lower temperatures, including 20 and 10°C, were tested (Table 2, entries 2 and 3; Figure S3) since they might be favorable for the enzymatic enantioselective reaction. However, the temperature had little influence on the enantioselectivity of recombinant CarEst3. Longer reaction times were required to achieve ee_s values similar to those at 30°C because of the decreased activity at lower temperatures. Therefore, lower space-time yields were achieved at 20 and 10°C compared to those at 30°C.

Based on the high performance of CarEst3 at 1.0 M, the substrate load was increased to 2.0 M (280 g·L^{-1}) while the other conditions were kept consistent with those in entry 1 of Table 2. The ee_s value reached 99.7% at 8.0 h. The S/C was determined to be as high as 3500 g·g^{-1}, and the space-time yield was 300 g·L^{-1}·d^{-1}. This newly identified CarEst3 is thus highly efficient in the enantioselective hydrolysis of *rac*-CHCM. A high S/C (>100 g·g^{-1}) value is especially important for industrial applications due to the lower emulsion, better mass transfer, and lower downstream costs.[25] Furthermore, to explore the substrate or product tolerance of recombinant CarEst3, the substrate loads were increased to 3.0−5.0 M (Table 2, entries 5−7; Figure S4). Using merely 0.2 g·L^{-1} recombinant CarEst3, 3.0 M (420 g·L^{-1}) *rac*-CHCM was enantioselectively hydrolyzed, yielding (*S*)-CHCM with an ee_s of 99.4%, an S/C of 2100 g·g^{-1}, and a space-time yield of 363 g·L^{-1}·d^{-1} by 10.0 h. When the substrate load was further increased to 4.0 M, the biocatalyst dosage was elevated to complete the reaction within 12 h. Surprisingly, 4.0 M *rac*-CHCM (560 g·L^{-1}) could be easily hydrolyzed, producing (*S*)-CHCM with an ee_s of 99.3%, an S/C of 1400 g·g^{-1}, and a space-time yield of 538 g·L^{-1}·d^{-1} within 9.0 h. Although a lower S/C should be sacrificed to achieve a higher space-time yield, the S/C of >100 g·g^{-1} is permissible for scale-up synthesis. As a result, the substrate load was further increased to 5.0 M *rac*-CHCM (700 g·L^{-1}) at a biocatalyst dosage of 1.4 g·L^{-1}. Within 12.0 h, the ee_s reached 99.6%, with an S/C of 500 g·g^{-1} and a space-time yield of 500 g·L^{-1}·d^{-1}. Recombinant CarEst3 tolerated a load as high as 700 g·L^{-1} *rac*-CHCM. For feasible biocatalytic manufacturing processing standards, the essential requirements of a ≥100 g·L^{-1} substrate load, a ≤5 g·L^{-1} biocatalyst dosage, a ≥20 g·g^{-1} S/C, a ≤24 h reaction time, a ≥99% *ee*, and a ≥100 g·L^{-1}·d^{-1} space-time yield should be satisfied.[25] This recombinant CarEst3 exhibits a great potential for industrial applications.

To explore the intrinsic properties of recombinant CarEst3, it was purified by nickel-affinity chromatography. The purified CarEst3 migrated as a single band at 43 kDa according to the SDS-PAGE analysis (Figure S5). The specific activity of the purified CarEst3 was 74.3 U·mg^{-1}, representing a 5.85-fold increase compared to a value of 12.7 U·mg^{-1} for the crude extract. The apparent kinetic parameters of purified CarEst3 toward CHCM were analyzed (Table S2). The V_{max} and k_{cat} values were calculated to be 1649 μmol·min^{-1}·mg^{-1} and 1100 s^{-1}, respectively, providing evidence for its prominent performance, especially at high substrate loads. The K_M value of CarEst3 was 12.4 mM, and the k_{cat}/K_M value was calculated to be 89.0 s^{-1}·mM^{-1}. The kinetic parameters of CarEst3 toward (*R*)- and (*S*)-CHCM were also determined to understand its enantioselectivity (Table 3). The K_M and V_{max} values toward (*R*)-CHCM were 13.8 mM and 1502 μmol·min^{-1}·mg^{-1}, respectively, while values of 33.0 mM and 679 μmol·min^{-1}·mg^{-1} were determined for (*S*)-CHCM, indicating CarEst3 displays a higher affinity and catalytic efficiency toward (*R*)-CHCM. As a result, the k_{cat}/K_M value of 72.3 s^{-1}·mM^{-1} toward (*R*)-CHCM was much higher than that of 13.7 s^{-1}·mM^{-1} for (*S*)-CHCM, agreeing with the (*R*)-selectivity of CarEst3.

Substrate profiling of the purified CarEst3 was also done by determining its activity toward 3-cyclohexene-1-carboxylate esters with different alkoxy groups, such as methoxy (CHCM), ethoxy (CHCE), isopropoxy (CHCP), and butoxy (CHCB) groups. The specific activities toward CHCM, CHCE, CHCP, and CHCB were 376, 388, 159, and 182 U·mg^{-1}, respectively (Figures 1 and S6). Recombinant CarEst3 displayed a higher

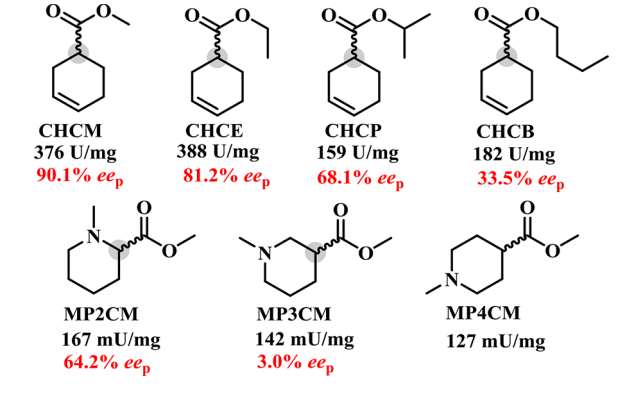

Figure 1. Substrate profiles of recombinant CarEst3 toward various cyclohexene and piperidine carboxylate esters.

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activity toward esters with short chain lengths, such as CHCM and CHCE. Interestingly, CarEst3 also exhibited a higher enantioselectivity toward CHCM and CHCE, with ee_p values of 90.1% and 81.2%, respectively, that are much higher than those of 68.1% and 33.5% for CHCP and CHCB, respectively. The increased chain length of the alcohol group might further decrease the difference between the cyclohexyl group and the alcohol group in regard to the steric hindrance and hydrophobicity, which lead to a poor enantioselective recognition of substrates. *N*-Heterocycle piperidine carboxylate esters were also investigated, including *N*-methylpiperidine-2-carboxylate methyl ester (MP2CM), *N*-methylpiperidine-3-carboxylate methyl ester (MP3CM), and *N*-methylpiperidine-4-carboxylate methyl ester (MP4CM). CarEst3 could also catalyze the hydrolysis of piperidine carboxylate esters and displayed the highest specific activity toward MP2CM (167 mU·mg^{-1}), followed by MP3CM (142 mU·mg^{-1}) and finally MP4CM (127 mU·mg^{-1}) (Figures 1 and S7). CarEst3 demonstrated an ee_p of 64.2% toward MP2CM, while it almost lost the enantioselectivity toward MP3CM when the ester group was in the *meta*-position (Figure 1), indicating the importance of the positions of the substituents in enantioselective recognition.

3-Cyclohexene-1-carboxylate esters contain a nearly symmetric hexatomic ring, which is difficult for enzymes to discriminate. Recombinant CarEst3 is an efficient biocatalyst for the synthesis of chiral 3-cyclohexene-1-carboxylate derivatives and, to the best of our knowledge, provides the highest recorded substrate load and enantioselectivity to date. To elucidate the mechanism underlying the high enantioselectivity for CHCM substrates, the homologous modeling structure of CarEst3 was constructed, and ligands, either (*R*)- or (*S*)-CHCM, were docked into its active center. Multiple molecular dynamic simulations (100 ns ×5) were performed. The ΔG values toward (*R*)- and (*S*)-CHCM were -20.5 ± 1.2 and -17.6 ± 1.0 kcal·mol^{-1}, respectively (Table 1). CarEst3 displayed a lower ΔG value and a higher binding affinity toward (*R*)-CHCM, which is consistent with its low K_M value. Average snapshots of (*R*)- and (*S*)-CHCM were retrieved from molecular dynamic simulation trajectories. (*R*)-CHCM was well-accommodated into the active center by a $\pi-\pi$ interaction between the phenyl ring of F78 and the double bond of the substrate, a hydrophobic interaction between L249 and the cyclohexene of the substrate, and multiple interactions between H139, D200, H325, and the CHCM methyl group (Figure 2A). With respect to (*S*)-CHCM, the ester chain rotated to the other side to form a reactive conformation due to steric hindrance (Figure 2B). This conformational switch likely resulted in the breakage of the $\pi-\pi$ interaction between the substrate and F78, although the methyl group of CHCM could also be stabilized by L249 and H325. All these changes contribute to the lower binding affinity of (*S*)-CHCM to CarEst3 compared to that of (*R*)-CHCM. It should be noted that CarEst3 is the most efficient biocatalyst identified so far for discriminating nearly symmetric (*R*)- and (*S*)-CHCM. Rational engineering is in progress to further increase its enantioselectivity.

Commercial enzymes, including PLE, HLE, and porcine pancreatic lipase (PPL), have been reported with relatively high enantioselectivities. However, they are not reliably reproducible because they consist of mixtures of enzymes; for example, PLE consists of six isoenzymes. Furthermore, the space-time yields of the commercial enzymes are lower than 20

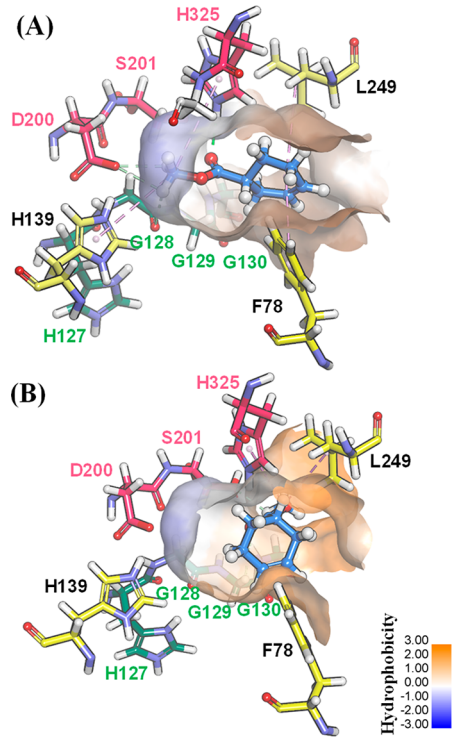

Figure 2. Interaction of (A) (*R*)-CHCM and and (B) (*S*)-CHCM with residues in the substrate binding pocket of CarEst3; red stick, catalytic residues; green stick, oxyanion hole; yellow stick, residues with interactions; and blue stick, CHCM.

g·L^{-1}·d^{-1} (Table S3). The variant Mu3 of BioH was previously constructed to catalyze the kinetic resolution of *rac*-CHCM.[17,18] However, the substrate loads are relatively low (<10 g·L^{-1}), and the S/C ratio of 2 g·g^{-1} and the low space-time yield are not acceptable for industrial applications. Among biocatalysts capable of the enantioselective hydrolysis of *rac*-CHCM to (*S*)-CHCM, CarEst3 identified in this study exhibits the highest substrate tolerance (700 g·L^{-1}), S/C value (3500 g·g^{-1}), and space-time yield (538 g·L^{-1}·d^{-1}), demonstrating its great potential in industrial manufacturing. Taken together, the novel bacterial carboxylesterase CarEst3 identified by genome mining enriches the currently available biocatalytic toolbox for the synthesis of chiral carboxylic acids.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.1c00714.

General experimental procedures and spectral data (PDF)

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Notes

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