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Deracemization of 2-Methyl-1,2,3,4-Tetrahydroquinoline Using Mutant Cyclohexylamine Oxidase Obtained by Iterative Saturation Mutagenesis

Guangyue Li,[†,∥] Jie Ren,[†,∥] Peiyuan Yao,[†] Yitao Duan,[†] Hailing Zhang,[†] Qiaqing Wu,[†] Jinhui Feng,[†] Peter C. K. Lau,*[,‡,§,▽] and Dunming Zhu*[,†]

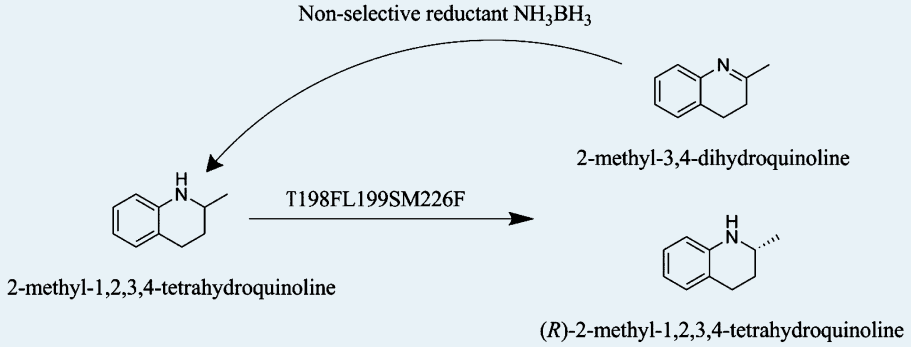
[†]National Engineering Laboratory for Industrial Enzymes and Tianjin Engineering Center for Biocatalytic Technology, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, 32 Xi Qi Dao, Tianjin Airport Economic Area, Tianjin 300308, People's Republic of China

[‡]National Research Council Canada, 6100 Royalmount Avenue, Montreal, Quebec, H4P 2R2, Canada

[§]Departments of Chemistry and Microbiology & Immunology, McGill University, Montreal, Quebec, H3A 2B4, Canada

[▽]FQRNT Centre in Green Chemistry and Catalysis, Montreal, Quebec, Canada

Supporting Information



ABSTRACT: The current toolkit of biocatalysts for the production of enantiomerically pure chiral amines is largely restricted to amine transaminases, ammonia lyases, or the genetic variants of monoamine oxidase N(MAO-N) from *Aspergillus niger*. Flavin-dependent amine oxidases have the apparent advantage of using molecular oxygen as a stoichiometric oxidant and their reactions are irreversible. To expand the toolkit and increase the substrate spectrum of a bacterial and flavin-dependent cyclohexylamine oxidase (CHAO) to enable deracemization of secondary amines, saturation mutagenesis of 11 amino acid residues located around the cyclohexanone substrate within a distance of 5 Å, followed by iterative saturation mutagenesis of four beneficial mutants, were performed. Screening with 2-methyl-1,2,3,4-tetrahydroquinoline as the substrate generated two improved CHAO variants, T198FL199S and T198FL199SM226F, that exhibited up to 406 times higher catalytic efficiency than the wild-type CHAO. Besides, high stereoselectivity for 2-methyl-1,2,3,4-tetrahydroquinoline and other 2-substituted-1,2,3,4-tetrahydroquinolines was demonstrated. In particular, deracemization of 2-methyl-1,2,3,4-tetrahydroquinoline by *Escherichia coli* whole cells expressing CHAO mutant T198FL199SM226F led to the production of (*R*)-2-methyl-1,2,3,4-tetrahydroquinoline with high yield (76%) and enantiomeric excess (ee, 98%). Tetrahydroquinolines are important building blocks of natural and synthetic products useful in the pharmaceutical and agrochemical industries.

KEYWORDS: *biocatalysis, amine oxidase, iterative saturation mutagenesis, chiral amines, deracemization*

1. INTRODUCTION

Optically active 1,2,3,4-tetrahydroquinoline (THQ) derivatives are an important class of building blocks for the asymmetric synthesis of pharmaceuticals, agrochemicals, and natural products.[1−8] They are also employed as the chiral ligand for transition-metal catalysts in asymmetric organic synthesis.[9] Traditional methods for the preparation of optically active THQ derivatives involve asymmetric hydrogenation of quinoline derivatives with metal catalysts.[1,10−15] However, the use of hazardous materials such as metals raises safety concerns and increases production costs, because of the stringent regulatory restriction on the level of metal residues allowed in pharmaceutical products and the requirement of extra processing steps to remove them.[16]

The use of biocatalysts or enzymes as environmentally benign molecules to replace traditional chemical methods has

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Table 1. Kinetic Parameters of Wild Type Cyclohexylamine Oxidase (wt CHAO) and Its Variants

enzyme	substrate[a]	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
CHAO	**1**	0.56 ± 0.07	249.25 ± 14.45	445.09
	2	1.06 ± 0.14	0.18 ± 0.01	0.17
T198F	**1**	0.99 ± 0.12	84.1 ± 5.15	84.94
	2	0.69 ± 0.08	9.14 ± 0.33	13.24
L199T	**1**	0.91 ± 0.13	45.75 ± 5.75	50.27
	2	1.12 ± 0.13	13.63 ± 0.68	12.17
M226F	**1**	0.66 ± 0.06	92.95 ± 5.21	140.83
	2	0.62 ± 0.08	3.65 ± 0.16	5.89
Y459T	**1**	0.79 ± 0.11	9.84 ± 0.96	12.45
	2	0.29 ± 0.03	15.98 ± 0.46	55.11
T198FL199S	**1**	1.03 ± 0.13	17.42 ± 1.81	16.91
	2	0.36 ± 0.03	14.77 ± 0.45	41.03
T198FL199SM226F	**1**	0.99 ± 0.15	11.12 ± 1.63	11.23
	2	0.27 ± 0.04	18.63 ± 0.84	69.00

[a]Substrates **1** and **2** are (*S*)-1-phenylethanamine and 2-methyl-THQ, respectively.

been well-documented.[17−22] Besides, enzymes are generally known to impart exquisite chemoselectivities, regioselectivities, and stereoselectivities in the reactions they catalyze. To the best of our knowledge, a biocatalytic method for synthesis of optically active THQ derivatives has not been reported. Herein, we describe the genetic improvement of a previously cloned FAD-dependent cyclohexylamine oxidase (CHAO) derived from *Brevibacterium oxidans* IH-35A, and application of the CHAO variants in the deracemization of secondary amines, of which the wild type (wt) CHAO had virtually no activity but exhibited high substrate specificity toward alicyclic amines and *sec*-alkylamines.[23,24] In strain IH-35A, CHAO carries out the initial oxidative step of the degradation of the industrial chemical cyclohexylamine to cyclohexanone.[25,26] We combined rational structure-guided engineering of the 50 kDa monomeric CHAO protein with iterative saturation mutagenesis, coupled with functional screening, to arrive at beneficial mutants that are capable of deracemization of 2-methyl-THQ at a preparative scale.

2. RESULTS AND DISCUSSION

Engineering of CHAO for 2-Methyl-THQ. The monomeric structure of CHAO has a *p*-hydroxybenzoate hydrolase (PHBH) fold topology belonging to the flavin monooxygenase family of enzymes.[27] One of the structural features is that CHAO has a buried hydrophobic active site. Based on the crystal structure of CHAO-FAD-cyclohexanone (PDB ID: 4I59),[27] 11 amino acid residues (F88, T198, L199, M226, Q233, Y321, F351, L353, F368, P422, and Y459), located within a distance of 5 Å of the product cyclohexanone were selected for site-saturation mutagenesis. The resulting focused libraries were screened using 2-methyl-THQ as a substrate. To achieve at least a 95% probability of mutant coverage, more than 300 clones were screened in each case. As a result, screening of ∼4500 clones from the 11 libraries led to the identification of 55 clones with higher activity toward 2-methyl-THQ, compared to the native enzyme. Of these, four clones that exhibited >20 times specific activity toward 2-methyl-THQ, compared to the wt CHAO (not shown), were chosen and identified by DNA sequencing to have the respective mutation: T198F, L199T, M226F, and Y459T.

In order to identify the best combination mutant toward 2-methyl-THQ, iterative saturation mutagenesis (ISM)[28] was then performed on T198F, L199T, M226F, and Y459T. Consequently, 12 saturation mutagenesis libraries labeled in the following as T198F+L199*, T198F+M226*, T198F+Y459*, L199T+T198*, L199T+M226*, L199T+Y459*, M226F +T198*, M226F+L199*, M226F+Y459*, Y459T+T198*, Y459T+L199* and Y459T+M226*, [where the asterisk (*) represents NNK 32 codons degeneracy (*N* = A/C/G/T and *K* = G/T)][28] were created and screened. A positive mutant T198FL199S was selected and identified from the second round of saturation mutagenesis and screening, following which two additional saturation mutagenesis libraries (T198FL199S +M226* and T198FL199S+Y459*) were created. Screening of these libraries led to the identification of positive mutant T198FL199SM226F. The last round of saturation mutagenesis (T198FL199SM226F+Y459*) and selection did not yield any positive mutant. Hence, from four rounds of saturation mutagenesis and screening, the following four single substitution mutants (T198F, L199T, M226F, Y459T), a double mutant (T198FL199S), and a triple mutant (T198FL199SM226F) were used for further study.

Kinetic parameter. All mutants and wt CHAO were expressed in *E. coli* BL21 (DE3) at 25 °C as soluble proteins with similar expression level (see Figure S1a in the Supporting Information). The proteins were purified to near homogeneity in one chromatographic step using a DEAE FF crude column. The purified proteins gave the expected 50 kDa band in SDS−PAGE (see Figure S1b in the Supporting Information). In Table 1, the kinetic data of wt CHAO and the six mutants toward (*S*)-1-phenylethanamine and 2-methyl-THQ are compared. In all cases, the catalytic efficiency (k_{cat}/K_m) of the mutant proteins toward (*S*)-1-phenylethanamine substrate dropped significantly largely due to the higher Michaelis−Menten constant (K_m) and the corresponding lower k_{cat} values

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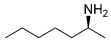
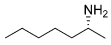
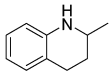
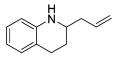
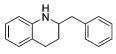
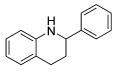
Table 2. Substrate Specificity of wt CHAO and Its Variants

Substrate	Structure	Relative activity (100%)[c]						
		CHAO	T198F	L199T	M226F	Y459T	T198FL199S	T198FL199SM226F
(*S*)-1-phenylethanamine		3705.8	1228.7	759.3	1243.8	119.0	182.5	117.4
(*R*)-1-phenylethanamine		Trace[a]	Trace	Trace	Trace	Trace	Trace	Trace
(*S*)-heptan-2-amine		2330.1	706.1	557.3	1009.2	ND[b]	ND	ND
(*R*)-heptan-2-amine		Trace	Trace	Trace	Trace	ND	ND	ND
(*S*)-1,2,3,4-tetrahydronaphthalen-1-amine		5316.3	2244.7	6520.9	6347.2	520.9	671.3	666.8
(*R*)-1,2,3,4-tetrahydronaphthalen-1-amine		Trace	Trace	Trace	Trace	Trace	Trace	Trace
2-methyl-THQ		Trace	100	100.7	29.1	145.0	129.9	258.4
2-allyl-THQ		Trace	18.8	30.1	Trace	41.9	28.7	21.1
2-benzyl-THQ		Trace	12.1	11.5	Trace	32.6	25.4	15.6
2-phenyl-THQ		ND	Trace	Trace	ND	17.1	ND	ND

[a]Trace = trace activity (<0.01 U/mg). [b]ND = no detectable activity. [c]100 = the specific activity (0.1 U/mg) of T198F for 2-methyl-THQ was defined as 100%.

compared to the wt CHAO. In contrast, with respect to 2-methyl-THQ substrate, all mutant enzymes displayed 78−406 times higher catalytic efficiency (k_{cat}/K_m) than the wt CHAO. In this case, all the mutants displayed improved k_{cat}, and in the case of the triple mutant up to 103 times higher, than the wt CHAO. Except for L199T, the K_m value of all mutants decreased in the range from 35% to 75% for 2-methyl-THQ.

With respect to 2-methyl-THQ and single amino acid substitution mutants, Y459T showed a significantly higher catalytic efficiency (55.11 min^{-1} mM^{-1}) than T198F, L199T, and M226F. However, no cooperative effect was detected for the combination of Y459T with other amino acid residues in the subsequent saturation mutagenesis and screening (not shown). In contrast, although M226F provided the lowest improvement in catalytic efficiency, its combination with two other point mutations produced the most active variant: T198FL199SM226F. A plausible explanation for this may come from the crystal structure of CHAO revealing that the aromatic side chains of F88, Y215, Y321, F368, and Y459 form a hydrophobic cavity for substrate binding, while the amino acid residues T198, L199, M226, and F351 are located on the other side of the substrate binding site, separating the substrate binding site from a second cavity which is closer to the protein surface.[27] As such, we observed that substitution of the amino

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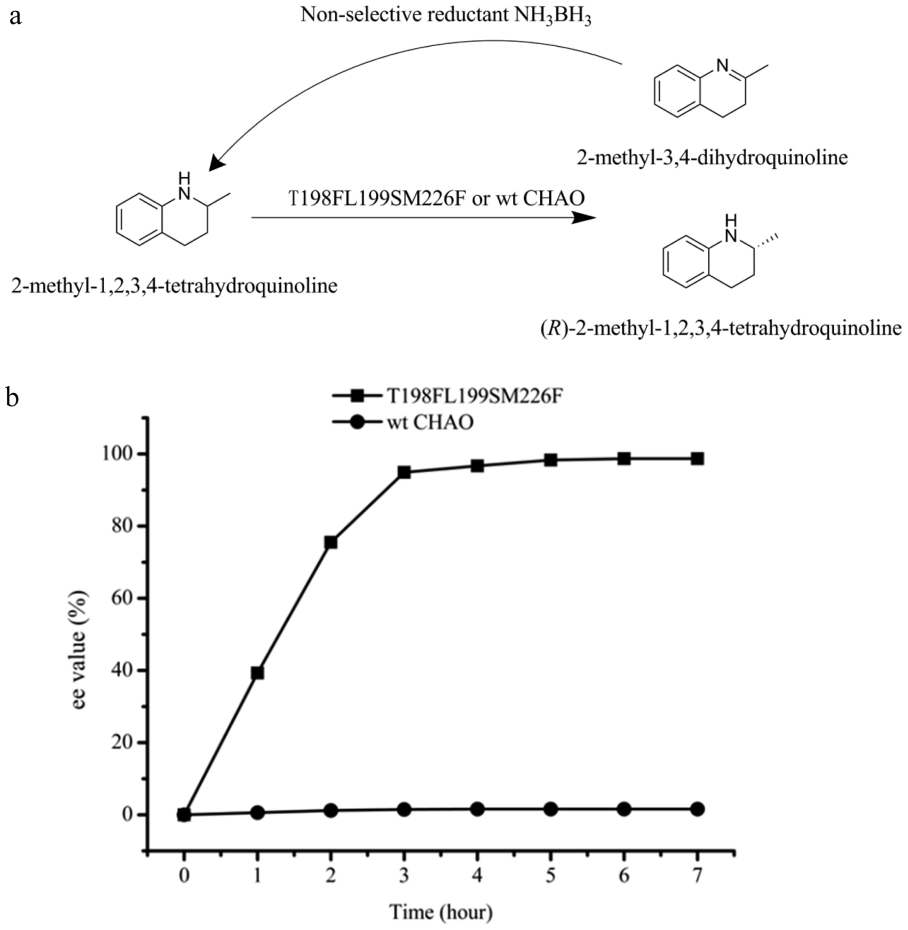

Figure 1. (a) Scheme and (b) time course of the deracemization of 2-methyl-THQ by employing a cyclic sequence of enantioselective oxidation with CHAO or its mutant T198FL199SM226F and nonselective reduction with $NH_3{\cdot}BH_3$. *E. coli* BL21 (DE3) cells expressing mutant T198FL199SM226F or wt CHAO were used.

acid residues T198, L199, and M226 resulted in cooperative effect on the enzyme activity. On the other hand, Y459 appears "isolated" from the residues T198, L199, and M226, and, hence, no synergistic effect was observed in this case.

Substrate Profiling. Because enzyme substrate profiling provides useful information for our understanding of an enzyme and its application, a series of amine substrates with diverse structural features were examined with the purified evolved enzymes and the wt CHAO (see Table 2). Consistent with previous studies where a similar set of substrates was used,[23,24] the wt CHAO prefers (*S*)-enantiomer to the (*R*)-enantiomer of primary amines, and exhibited only trace activity toward secondary amines. Compared to the wt enzyme, the mutants had much higher activity toward secondary amines, e.g., 2-methyl-THQ and other 2-substituted THQs, but lower activity toward primary amines. The reactivity toward 2-substituted THQs is of the following order: methyl > allyl > benzyl > phenyl substituents. This ordering suggests that the steric factor significantly affects the enzyme activity. Among the mutants, T198FL199SM226F rendered the highest activity toward 2-methyl-THQ, but Y459T was most active toward other 2-substituted THQs.

When individual *R*- and *S*-enantiomers of chiral primary amines were examined, both wt CHAO and mutant CHAOs were found to have much higher activity for the (*S*)-enantiomers than the corresponding (*R*)-enantiomers, indicating the high enantioselectivity of the enzymes. The same was true for the mutant enzymes toward secondary amines when kinetic resolutions of 2-methyl-THQ, 2-allyl-THQ, and 2-benzyl-THQ were carried out with the following respective set of mutant enzymes: Y459T, T198FL199S, and T198FL199SM226F; Y459T and T198FL199S; and Y459T. In all cases, one enantiomer was oxidized to the corresponding imine completely (see Figure S2 in the Supporting Information).

Biocatalytic Synthesis of (*R*)-2-Methyl-THQ in High Yield and Enantiomeric Excess. *E. coli* whole cells of the triple mutant T198FL199SM226F were prepared and in combination with the nonselective chemical reducing agent ($NH_3{\cdot}BH_3$) deracemization of 2-methyl-THQ was achieved compared to the null effect of wt CHAO (Figure 1). The time course study (Figure 1b) showed that the reaction with the triple mutant was fast and linear in the first three hours, resulting in up to 95% enantiometric excess (ee) value. The deracemization process plateaued in the next 3 h, with a modest increase in ee to 98%, but no apparent improvement afterward. As a result, (*R*)-2-methyl-THQ was isolated after 6 h of reaction, and the yield and ee value were found to be 76% and 98%, respectively. The nonreactivity of wt CHAO in the deracemization of 2-methyl-THQ was consistent with the result reported by Leisch et al.[23] This is the first report that optically active (*R*)-2-methyl-THQ is synthesized with high isolated yield and ee value by an amine oxidase.

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3. CONCLUSION

Iterative saturation mutagenesis of CHAO led to the successful isolation of a triple CHAO mutant (T198FL199SM226F) that efficiently catalyzed the deracemization of 2-methyl-THQ to give (*R*)-2-methyl-THQ with high isolated yield and enantiometric excess (ee) value. This expands the biocatalytic repertoire of wild type CHAO (wt CHAO), whose original substrate spectrum does not include secondary or tertiary amines. Five other CHAO beneficial mutants were also isolated, although exhibiting varying success in accommodating secondary amines. The *R*-enantiomer produced by T198FL199SM226F is expected to provide valuable access to a variety of THQ derivatives of pharmaceutical or clinical importance, whereby the THQ nucleus or quinoline, per se, has been described as a "privileged" scaffold or substructure in many biologically active natural products and therapeutic agents used in cancer drug development.[29−33]

EXPERIMENTAL SECTION

CHAO Mutant Library Construction. Eleven (11) amino acid residues located around cyclohexanone and within a distance of 5 Å in the crystal structure of CHAO−FAD−cyclohexanone (PDB ID: 4I59) were selected as mutation targets. Subsequently, oligonucleotide primers were designed with degenerate codon NNK for the desired saturation mutagenesis (see Table S1 in the Supporting Information). Each mutant library was constructed using the PCR-based Quick Change method.[34] The resulting mutant plasmids were each transformed to *E. coli* BL21 (DE3) and the transformants were plated on HiBond-C Extra membranes placed on LB agar plates containing 100 μg/mL ampicillin and 0.5 mM IPTG, respectively. The plates were incubated at 37 °C for 24 h. The resulting first round of mutant libraries were screened with 2-methyl-THQ as the substrate, as described below. From four positive mutants, a second round of saturation mutagenesis was performed at the three other respective amino acid residues. This second-round saturation mutagenesis and screening produced a double mutant that later led to a triple mutant with improved enzyme activity. Further saturation mutagenesis of the triple mutant and the screening did not result in any positive mutant.

Screening of the Mutant Libraries with 2-Methyl-THQ. The plate assay method described by the Turner group was followed.[35,36] Specifically, the Hi-BondC Extra membranes containing the clones were pulled from the LB agar plates and kept at −20 °C for 24 h to lyse the cells. The membranes were incubated at room temperature for 12 h with an assay mixture containing 1 tablet of diaminobenzidine (DAB), 1 mL of potassium phosphate buffer (1 M, pH 7.0), 20 μL of 2-methyl-THQ (100 mM) solution, 30 μL of horseradish peroxidase (5 mg/mL), 10 mL of 2% agarose, and water (up to 20 mL). Positive clones were selected and inoculated on LB agar plates (100 μg/mL ampicillin) every 2 h. The selected positive clones were subjected to activity measurement (see below) and the mutations were identified by DNA sequencing (BGI Company, Beijing, PRC) and amino acid sequence verification.

Expression and Purification of CHAO and Its Variants. CHAO and its variants were expressed in *E. coli* BL21 (DE3) cells, as previously described.[24] The recombinant proteins were purified by ion interaction chromatography on an AKTA Purifier 10 system with DEAE FF crude column (GE, USA), as reported in our previous study.[24] The protein samples were collected and stored at −20 °C. Protein concentration was measured using the Bradford method.[37]

Activity Assay. Enzyme activity was determined by a modified procedure as previously described.[22,38] The enzyme specific activities were assayed using a SPECTRAMAX M2e system (MD, USA) at 30 °C. Initial rates of the reaction were measured via the absorbance of a dye (ε = 29.4 mmol L^{-1} cm^{-1}) at 510 nm, which was produced by the action of horseradish peroxidase with the liberated hydrogen peroxide from the oxidation of the amine by CHAO or variants, 4-aminoantipyrine, and 2,4,6-tribromo-3-hydroxybenzoic acid. The assay mixture (total volume of 0.2 mL) contained 174 μL of phosphate buffer (50 mM, pH 6.5), 2 μL of a 2,4,6-tribromo-3-hydroxybenzoic acid stock solution (20 mg/mL in DMSO), 2 μL of 4-aminoantipyrine stock solution (15 mg/mL in H_2O), 2 μL of an amine stock solution (1 M in DMSO), and 2 μL of a horseradish peroxidase stock solution (5 mg/mL). The reaction was started by the addition of 20 μg of enzyme in 20 μL of phosphate buffer (50 mM, pH 6.5). [Note that one enzyme unit (U) was defined as the amount of enzyme that produced 1 μmol of hydrogen peroxide per minute.] The activity assays were performed in triplicate with the appropriate control experiments.

Determination of Kinetic Parameters. The kinetic parameters were obtained by measuring the initial velocities of the enzymatic reaction and curve-fitting according to the Michaelis−Menten equation using GraphPad Prism 5 software (GraphPad Software, Inc.). The activity assay was performed in a mixture containing a varying concentration of (*S*)-1-phenylethanamine (0.1−2 mM) or 2-methyl-THQ (0.1−5 mM). All experiments were conducted in triplicate.

Kinetic Resolution of Racemic Secondary Amines with *E. coli* Whole Cells. The kinetic resolution of racemic secondary amines (see Table S2 in the Supporting Information) was carried out as follows: the cell pellet from 4 mL ferments (the ferments were prepared as described above, the cell density of ferments was detected and shown in Table S2 in the Supporting Information) was resuspended in 2 mL of phosphate buffer (50 mM, pH 6.5), and the substrate (15 mM) was added and mixed. The mixture was shaken at 200 rpm and 30 °C on an orbital shaker for 20 h. The pH of the reaction mixture was carefully adjusted to 10 with 5 M NaOH solution and extracted with dichloromethane (DCM). The organic layers were dried over anhydrous sodium sulfate and filtered to give a sample for analysis. The product was identified by comparison with authentic samples in an Agilent Model 1200 liquid chromatography system with a Chiracel OJ-H column (4.6 mm × 250 mm, Daicel Chiral Technologies Co., Ltd.). A mixture of hexane and isopropyl alcohol (with 0.5% ethanolamine) (9:1) was used as the eluent at a flow rate of 0.8 mL/min and the column temperature was controlled at 30 °C.

Deracemization of 2-Methyl-THQ using Recombinant Cells of Mutant T198FL199SM226F. Deracemization of 2-methyl-THQ was carried out as follows: the cell pellet of mutant T198FL199SM226F or wt CHAO from 25 mL of ferments (the ferments were prepared as described above, the cell density of ferments was OD_{600} = 4.32 for T198FL199SM226F and OD_{600} = 4.51 for wt CHAO) was resuspended in 25 mL of phosphate buffer (50 mM, pH 6.5). 2-Methyl-THQ (50 mg) and borane−ammonia complex (30 mg) were added and mixed. The mixture was shaken at 200 rpm and 30 °C on an orbital shaker and the reaction was monitored every hour. When deracemization was finished, the pH of the

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reaction mixture was carefully adjusted to a value of pH 2 with 1 M HCl solution. The suspension was centrifuged (10 000*g*, 15 min) and the supernatant was basified to pH 10 with 5 M NaOH solution. The aqueous layer was extracted three times with 25 mL of DCM. The organic layers were combined and concentrated (25 mL), then extracted 3 times with 25 mL of HCl solution (1 M). The suspension was centrifuged (10 000*g*, 15 min) and the supernatant was basified to pH 10 with 5 M NaOH solution again. The aqueous layer was extracted three times again with 25 mL of DCM. The combined volume of organic layer was dried over anhydrous sodium sulfate and filtered. Removal of the solvent gave a product with 76% yield (38 mg), which was identified as (*R*)-2-methyl-THQ by comparison of the retention time on a HPLC with that of the authentic sample. An ee value of 98% was obtained as determined by chiral HPLC analysis as described above. The retention times for (*S*)- and (*R*)-2-methyl-THQ were 11.29 and 12.32 min, respectively. The absolute configuration for (*R*)-2-methyl-THQ was determined by comparison of its optical rotation with the literature. $[\alpha]_D^{20}$+86.3(*c* = 1.1, chloroform); lit $[\alpha]_D^{20}$+80.8(*c* = 1.0, chloroform).[39]

■ ASSOCIATED CONTENT

Supporting Information

Preparation of three 2-substituted THQ compounds, the primers used for site-saturation mutagenesis, preparation of whole cells for kinetic resolution of 2-substituted THQs, and the HPLC spectra of the reaction mixtures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*Fax: +86 22 84861996. E-mail: zhu_dm@tib.cas.cn (D. Zhu). *Fax: 514 3987052. E-mail: Peter.Lau@mcgill.ca (P. C. K. Lau).

Author Contributions

‖These authors contributed equally.

Notes

The authors declare no competing financial interest.

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