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Imine Synthesis by Engineered D-Amino Acid Oxidase from Porcine Kidney

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Cite This: *ACS Omega* 2025, 10, 2212−2221

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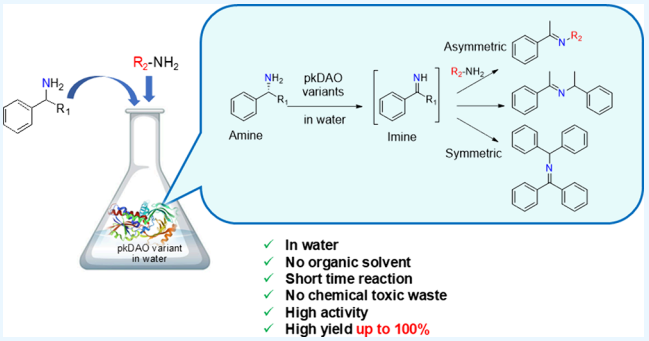
ABSTRACT: Various symmetric and asymmetric imines were synthesized using the novel amine oxidase, obtained as variants of D-amino acid oxidase (pkDAO) from porcine kidney (Y228L/R283G) and (I230A/R283G). Active primary imines produced as intermediates in the oxidation of methylbenzylamine (MBA) derivatives were trapped by aliphatic, aromatic amines and diamines as nucleophiles forming new imines. (*R*)-Fluoro-MBA was the best substrate for symmetric imine synthesis, providing almost stoichiometric conversion (100 mM) and achieving nearly 100% yield. Several (*R*)-MBA derivatives were used as substrates, and the corresponding symmetric and asymmetric imines were synthesized. The turnover number of *N*-benzylidenebenzylamine synthesis from benzylamine was calculated to be 1.61×10^5 (number of moles of reactant consumed per mole of catalyst/h), which is more than 10^3 higher than metal-, photo-, and organo-catalysts reported so far. The diastereomers of bis(1-phenylethyl)amine, the reduced products of (*R*)-MBA, were identified as a mixture of 84.9% (*R*,*R*)-bis(1-phenylethyl)amine and 15.1% (*R*,*S*)-bis(1-phenylethyl)amine to consider the reaction mechanism.



INTRODUCTION

Since the discovery of D-amino acid oxidase (EC 1.4.3.3) in mammalian tissues by Krebs,[1] it is believed that the products of the catalysis of D- and L-amino acid oxidases from amino acids are keto acid, ammonia, and hydrogen peroxide, recognizing that the real product is the corresponding imino acids, which are soon hydrolyzed in water.[1−3] Mechanisms of amine oxidases (AOxs) involving the prosthetic group FAD have been intensively studied.[4−8] Hafner and Wellner reported deracemization reaction using L-alanine and L-lysine, utilizing the reactive imino acids as intermediates of the reactions with pkDAO and L-amino acid oxidase from *Crotalus adamanteus* in the presence of sodium borohydride as a chemical reductant.[9,10] The utilization of the intermediate imino acid has been studied by reduction with chemical reductants or by imine reductases. The stoichiometric deracemization (enantiomerization) of racemic pipecolic acid was demonstrated by Soda et al., producing the L-enantiomer by deracemization by formation of stable cyclic imine Δ1-piperideine-2-carboxylate,[11,12] followed by enzymatic imine reduction to synthesize chiral L-pipecolinic acid as a drug intermediate.[13,14] Beard and Turner[15,16] and others[17] developed effective production methods for (*R*)- or (*S*)-amino acids, by L-amino acid oxidase and D-amino acid oxidase, respectively, using mild reducing reagents such as $NaBH_3CN$ or $NaBH_4$. They synthesized wide varieties of (*R*)-amines by deracemization using *S*-selective monoamine oxidase variants (MAO-N) from *Aspergillus niger*.[18−21] They also reported the deracemization of 4-chlorobenzhydrylamine (4-CBHA) with variant D11C of MAO-N using $NH_3−BH_3$ as a reductant.[22]

We explored the ability of D-amino acid oxidases to develop a new AOx and designed a deracemization reaction of MBA to form (*S*)-MBA.[23−25] By introducing two alteration sites (Y228L/R283G) in the active site of a D-amino acid oxidase from porcine kidney (pkDAO), based on the structure of the inhibitor benzoic acid (PDB: 1VE9), we successfully created an *R*-stereoselective AOx that does not exist in nature (PDB: 3WGT).[23] We demonstrated the deracemization of racemic amines to form (*S*)-amines using a variant of pkDAO in the presence of a reducing agent, such as $NaBH_4$ in the reaction system. Further expansion of the substrate specificity of pkDAO was achieved by the alteration (I230A/R283G) based on the crystal structures of pkDAO (Y228L/R283G) and the variant I230A/R283G (PDB: 5WWV), accommodating much bulkier (*S*)-4-CBHA.[26,27] pkDAO (I230A/R283G) was successfully used to synthesize (*R*)-4-CBHA via deracemization.[27] Development and use of *R*-6-hydroxy-D-

Received: October 7, 2024
Revised: December 28, 2024
Accepted: December 30, 2024
Published: January 10, 2025

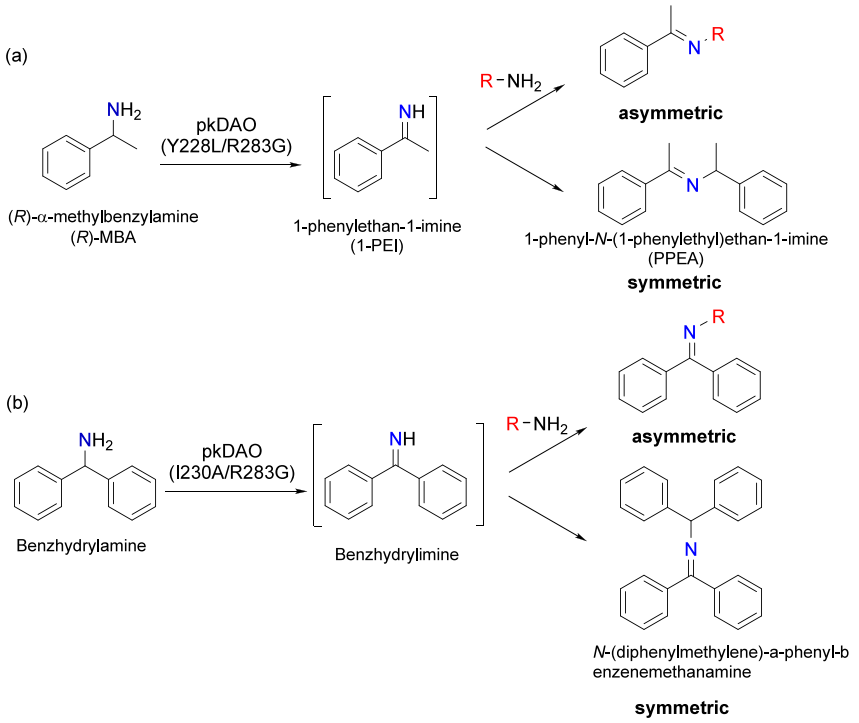
Table 1. Comparison of Typical Catalysts Developed for *N*-Benzylidenebenzylamine Synthesis from Two Molecules of Benzylamine

catalyst	benzylamine	catalyst details	solvent	supply of O_2	temperature (°C)	light	time (h)	yield (%)	reference
D-amino acid oxidase variant (Y228L/R283G)	1 mmol[a]	D-amino acid oxidase variant (2 units = 0.11 mg)	water (pH 9), 10 mL	atmospheric oxygen, dissolved in water	20	no	1	96	this work
D-amino acid oxidase variant (Y228L/R283G)	1 mmol	D-amino acid oxidase variant (2 units = 0.11 mg)	water (pH 9), 10 mL	atmospheric oxygen, dissolved in water	20	no	1	89	this work
$Au(OAc)_3$ + CeO_2	0.4 mmol	$Au(OAc)_3$ + CeO_2 (2.6 mg) on Al_2O_3 support (118.6 mg)	chlorobenzene, 2 mL	O_2	108	no	16	89	34
Cu(0)	1 mmol	red copper (10 mg), NH_4Br (1 mmol), 1,10-phenanthroline (1.5 mmol)	toluene, 2 mL	O_2	100	no	24	>99	53
$Fe(NO_3)_3$ and TEMPO	2 mmol	$Fe(NO_3)_3$ (5 μmol) and TEMPO (5 μmol)	toluene, 0.5 mL	atmospheric	80	no	24	97	42
monomodal copper aluminum mixed metal oxide (MMO)	3 mmol	100 mg	neat, 3 mL	atmospheric	100	no	24	>99	41
Cu/Chitosan beads *tert*-butyl hydroperoxide (TBHP)	0.5 mmol	10 mg and TBHP(5 mmol)	acetonitrile, 2 mL	atmospheric	80	no	0.5	98	43
Cu^I/topaquinone mimic	0.02 mol	0.04 mol	methanol, 10 mL	atmospheric	r.t.	no	10	96	51
few layer C_3N_4	0.5 mmol	15 mg	acetonitrile, 5 mL	atmospheric	25	light (λ> 420 nm)	1	99	48
Ru^{II}-polypyridyl metal−organic framework	0.1 mmol	5 mg	acetonitrile, 3 mL	O_2	r.t.	Light (λ> 440 nm)	3	>99	39
TiO_2, arizarin red dye (ARS) and TEMPO	0.3 mmol	10.7 mg ARS-TiO_2 (0.002 mmol of ARS)	acetonitrile, 5 mL	atmospheric	25	blue LED	2	96	35
metal-free two-dimensional porphyrin-based covalent organic framework	0.2 mmol	10 mg	acetonitrile, 2 mL, and chlorobenzene, 20 μL	atmospheric	25	LED (λ: 400−830 nm)	1.2	97	55
metal-free salicylic acid derivative on silica gel	3 mmol	4,6-dimethoxysalicylic acid (0.15 mmol)	toluene, 1.5 mL	atmospheric	90	no	6	95	54
metal-free nitrogen doped carbon nanosheets	1 mmol	15 mg	DMSO:H_2O (1:0.5), 0.5 mL	atmospheric	60	no	40	>99	56
ionic liquid (TEAOAc)	0.25 mmol	0.4 g	neat	O_2	50	no	1	97	50

[a](*R*)-MBA is the substrate with 1-phenyl-*N*-(1-phenylethylidene)ethanamine (PPEA) as the product.

https://doi.org/10.1021/acsomega.4c09160
ACS Omega 2025, 10, 2212−2221

Scheme 1. Synthesis of Symmetric and Asymmetric Imines Using D-Amino Acid Oxidase from Porcine Kidney (pkDAO) Variants (a) with pkDAO (Y228L/R283G) and (b) with pkDAO (I230A/R283G)



nicotine oxidase for the preparation of *S*-amines are also reported.[28]

Besides these reduction reactions on the active imine intermediate, we recently developed a new enzymatic method for α-alkyamino acid synthesis by discovering the oxidative cyanation reaction on the active imine, using the variant pkDAO (Y228L/R283G) in the presence of nitrilase with wide substrate specificity.[29] Furthermore, during the course of the studies on the oxidative cyanation reactions, we discovered that this variant catalyzes imine (1-phenyl-*N*-(1-phenylethylidene)ethanamine (PPEA)) synthesis from two molecules of methylbenzylamine ((*R*)-MBA).[30]

It is reported that around 40−45% of the small molecule pharmaceuticals and many other industrially important fine chemicals and agrochemicals contain chiral amine fragments.[31] Imines are one of the frequently used starting materials for the synthesis of chiral amines[32] and generally synthesized by chemical oxidation of primary or secondary amines, condensation of amines with aldehydes or ketones, and oxidative condensation of amines via aldehydes or ketones.[33] There are a number of reports on the oxidative coupling of benzylamine as a typical model reaction with several catalysts, which typically involve metal catalysts, such as Au,[34] Ti,[35] Bi, Mo,[36] W,[37] Os,[38] Ru,[39] Nb,[40] Cu,[41] Fe,[42] etc., often in the presence of light. Metal oxides are also used with several organic supports such as Cu/chitosan,[43] mesoporous Cs/MnOx,[44] etc.

To avoid contamination of metals used as the catalysts, metal-free organo-catalysts such as graphene,[46] acetylene-bridged triazine framework,[47] carbon nitrides,[45,48] and porous organic polymer[49] are used in the presence of light. The use of ionic liquid is unique as a catalyst for the same reaction.[50] However, the conditions to use these catalysts described above still require harsh conditions such as high temperatures and organic solvents. Therefore, the mimic of biocofactor Cu/topaquinone has been studied in the same reaction.[51] The efficiency of enzymes is much higher compared with these catalysts because enzyme reactions are done under ambient temperature in water, and scaling up of the production of biocatalysts is easy.[52] Therefore, biocatalytic imine synthesis can be developed as an alternative sustainable method that is simple, effective, and environmentally benign. As the authors know, there has been no report on the enzymatic coupling of benzylamine besides our studies.[30] Table 1 shows the comparison of typical catalysts developed so far to synthesize (1-phenyl-*N*-(1-phenylethylidene)ethanamine (PPEA)) from two molecules of methylbenzylamine.

The turnover number of the pkDAO variant is calculated as follows. The substrate benzylamine (0.5 mmol) was converted by dimerization to *N*-benzylidenebenzylamine in 89% yield. Knowing the molecular mass of one subunit of holo pkDAO, 39,972 Da, the turnover number (TOF) of the reaction is calculated as follows: 0.45×10^{-3} /1 (mol/h) converted by 0.11×10^{-3}/39,972 mol of the enzyme. TOF = 1.61×10^{5} (number of moles of reactant consumed per mole of catalyst/h). When the substrate is (*R*)-MBA, the TOF of the same reaction is similarly calculated to be 1.74×10^{5}. They are ca. 3 $\times 10^{3}$ higher than one of the most efficient catalysts,[43] reported so far. TOFs of benzylamine dimerization under the catalysis of $Au(OAc)_3$ + CeO_2, mesoporous nonomodal copper aluminum mixed metal oxide (MMO), and Cu/chitosan beads are reported to be 3.2,[34] 0.27,[41] and 57.7[43] h^{-1}, respectively. Scaling up of imine production in the gram scale is reported by Dong et al.[54]

In this study, we aimed to explore the synthesis of various imines by the nucleophilic addition of amines to the extremely active but rather stable aromatic imines formed in the reactions of pkDAO variants. Symmetric imines can be produced when MBA derivatives attack active primary imine intermediates. Heterocoupled imines were produced in gram scales when primary amines, including aliphatic/alkyl, aromatic, and

https://doi.org/10.1021/acsomega.4c09160
ACS Omega 2025, 10, 2212−2221

diamines, were used as nucleophiles. In the heterologous coupling of two amine compounds, one of the nucleophiles was not a substrate of pkDAO, in competition with the homologous coupling of the substrates. The reaction was performed in a simple manner in aqueous solution using a one-pot system (Scheme 1). We further distinguished and clarified the structures of diastereomers of bis(1-phenylethyl)amine (BPEA) from the imine synthesized by the addition of a reductant, which showed two compounds with the same mass spectrum (MS) in gas chromatography−mass spectrometry (GC-MS) analysis.

MATERIALS AND METHODS

General information, materials, the chemical synthesis of reference compounds, and analyses are provided in the Supporting Information. (*R*)- or (*S*)-MBA, (*R*)-ethylbenzylamine ((*R*)-EBA), acetophenone, (*R*)- and (*S*)-4-fluorobenzylamine (FMBA), (*R*)- and (*S*)-4-chloro-α-methylbenzylamine (Cl-MBA), (*R*)-(+)-α-4-dimethylbenzylamine ((*R*)-DMBA), and benzylamine (BA) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). (*R*,*R*)-, (*R*,*S*)-, and (*S*,*S*)-BPEA were chemically synthesized. Various amine donors, including ethylamine, *n*-butylamine, *n*-hexylamine, *n*-heptylamine, and cyclopropylamine, were purchased from Tokyo Chemical Industry Co., Ltd. All the other chemicals were of commercially available grade.

The secondary imine (*R*)-MBA and other derivatives including (1*E*)-1-(4-chlorophenyl)-*N*-[1-(4-chlorophenyl)-ethyl]ethan-1-imine (FFEI), (1*E*)-1-(4-methylphenyl)-*N*-[1-(4-methylphenyl)ethyl]ethan-1-imine, (*E*)-*N*-benzyl-1-phenylmethanimine, (1*E*)-1-phenyl-*N*-(1-phenylpropyl)propan-1-imine, and PPEA were synthesized by an oxidation reaction catalyzed by pkDAO as standard or reference compounds. The enzymatic reactions were performed with 50 mM each of primary BA derivatives, including (*R*)-MBA, (*R*)-EBA, (*R*)-FMBA, (*R*)-Cl-MBA, (*R*)-DMBA, and BA, pH 9.0 set by 2 N HCl, 2 units of pkDAO (Y22L/R283G) at 20 °C for 1 h in 10 mL reaction. Subsequently, the reaction mixture was stopped and extracted three times with ethyl acetate. The organic phase was dried over $MgSO_4$, filtrated, and evaporated. The compound was analyzed by ^{1}H NMR (400 MHz, $CDCl_3$).

Enzyme Production and Purification. *Escherichia coli* JM109 (DE3) harboring pUC-18/pkDAO (Y228L/R283G[23] or I230A/R283G[27]) was incubated at 37 °C for 12 h in a 5 mL LB medium containing 80 μg/mL ampicillin. The grown cells (5 mL) were transferred into 500 mL of the LB medium containing 80 μg/mL ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside as the final concentration. Subsequently, the 500 mL culture was incubated at 37 °C for 24 h for pkDAO gene expression. The *E. coli* cells were harvested by centrifugation (12,000*g*, 20 min) and washed with 10 mM potassium phosphate buffer (KPB, pH 8.0) containing 0.1% (v/v) 2-mercaptoethanol.

The cells were suspended in five volumes of the same buffer and disrupted by sonication for 20 min at 180 W using an Insonator 201 M (Kubota Co., Tokyo, Japan). After the cell debris was removed by centrifugation at 15,000*g* for 20 min at 4 °C, the supernatant was obtained from the cell-free extract (crude enzyme). The enzyme in the cell-free extract was then fractionated using an ammonium sulfate precipitant at 20−35% saturation, and the fraction was suspended in 10 mM KPB (pH 8.0) containing 0.1% (v/v) 2-mercaptoethanol and dialyzed against the same buffer. The dialyzed enzyme solution was applied to a DEAE-Toyopearl 650 M column (φ 6.0 × 13 cm). The absorbed enzyme was eluted using a linear gradient of 0−0.5 M NaCl. The enzyme solution was then saturated to 20% using ammonium sulfate and applied to a Butyl-Toyopearl column (φ 3.0 × 22.0 cm). Linear gradient elution was performed using 10 mM KPB buffer containing 20−0% ammonium sulfate saturation. Purity was confirmed by the presence of a single band in the SDS-PAGE analysis. Protein concentrations were measured using a Pierce BCA protein assay. The specific activity toward (*R*)-MBA was calculated to be 18.3 units/mg with (*R*)-MBA as a substrate.

Enzyme Assay. The oxidase activity was assayed at 30 °C by measuring quinonimine dye formation by determining the absorbance at 505 nm with a spectrophotometer. The reaction mixture contained the substrate, 100 mM KPB (pH 8.0), 2 mM phenol, 1.5 mM 4-aminoantipyrine, 2 units of horseradish peroxidase, and 10% (v/v) dimethylsulfoxide. One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of hydrogen peroxide per min with the (*R*)-MBA as a substrate.

Identification of Diastereomers of BPEA. To distinguish the diastereomers of BPEA, BPEA synthesis was designed using the following two reaction systems: (i) One-step enzymatic reaction with oxidation and reduction *in situ*; 150 mM (*R*)-MBA (pH 9.0) with 2 units of pkDAO (Y228L/R283G) and 100 mM $NaBH_4$ as reductant in 1 mL of the reaction mixture, which was incubated at 20 °C for 1 h. The reaction was stopped, extracted with *n*-hexane (0.5 mL), and analyzed by HPLC. (ii) Two-step reaction: After the enzymatic reaction, the imine product was extracted with *n*-hexane and then reduced with $NaBH_4$. The reaction was conducted with 150 mM (*R*)-MBA (pH 9.0) with 2 units of pkDAO (Y228L/R283G) without the addition of a reductant in 1 mL of the reaction mixture, which was incubated at 20 °C for 1 h. Subsequently, 0.5 mL of *n*-hexane was added to stop the enzymatic reaction and extract the synthesis product (PPEA). The chemical reaction was then performed by adding 100 mM $NaBH_4$ to 1 mL of methanol. The reaction was performed at 50 °C for 1 h. The synthetic product was extracted with *n*-hexane (0.5 mL), and the diastereomer was analyzed by HPLC.

Identification of the Synthetic Product from Enzymatic Reaction with GC-MS. The extracted products were analyzed by using GC-MS (Agilent Technologies). GC-MS spectra were obtained using HP-5975C Inert XL EI/CI MSD with a triple-axis Detector at 75 eV, coupled with a 7890A GC system, equipped with an HP-5 ms column (30 m × φ 0.25 mm; 0.25 μm in film thickness), operated in the spiteless mode at 40 °C for 2 min, then programmed to increase at 10 °C/min to 290 °C, and finally held at this temperature for 5 min. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. GC and GC-MS data were processed using an Agilent ChemStation (Hewlett-Packard Co., Chongqing, China) with reference to an MS database (Wiley ninth/NIST 2011 MS Library (Agilent Technologies, Santa Clara, CA, USA)).

Symmetric and Asymmetric Imine Synthesis with Various Amines. Various primary amines, such as alkyl amines; aromatic amines; alkyl diamines; alkyl amines including ethylamine, *n*-butylamine, *n*-hexylamine, and *n*-heptylamine; aromatic amines including cyclopropylamine; (*R*)-FMBA; and alkyl diamines, including ethyl diamine, hexyl diamine, and heptyl diamine, were estimated as amine donors in the enzymatic reaction of pkDAO and the trapping reaction

https://doi.org/10.1021/acsomega.4c09160
ACS Omega 2025, 10, 2212−2221

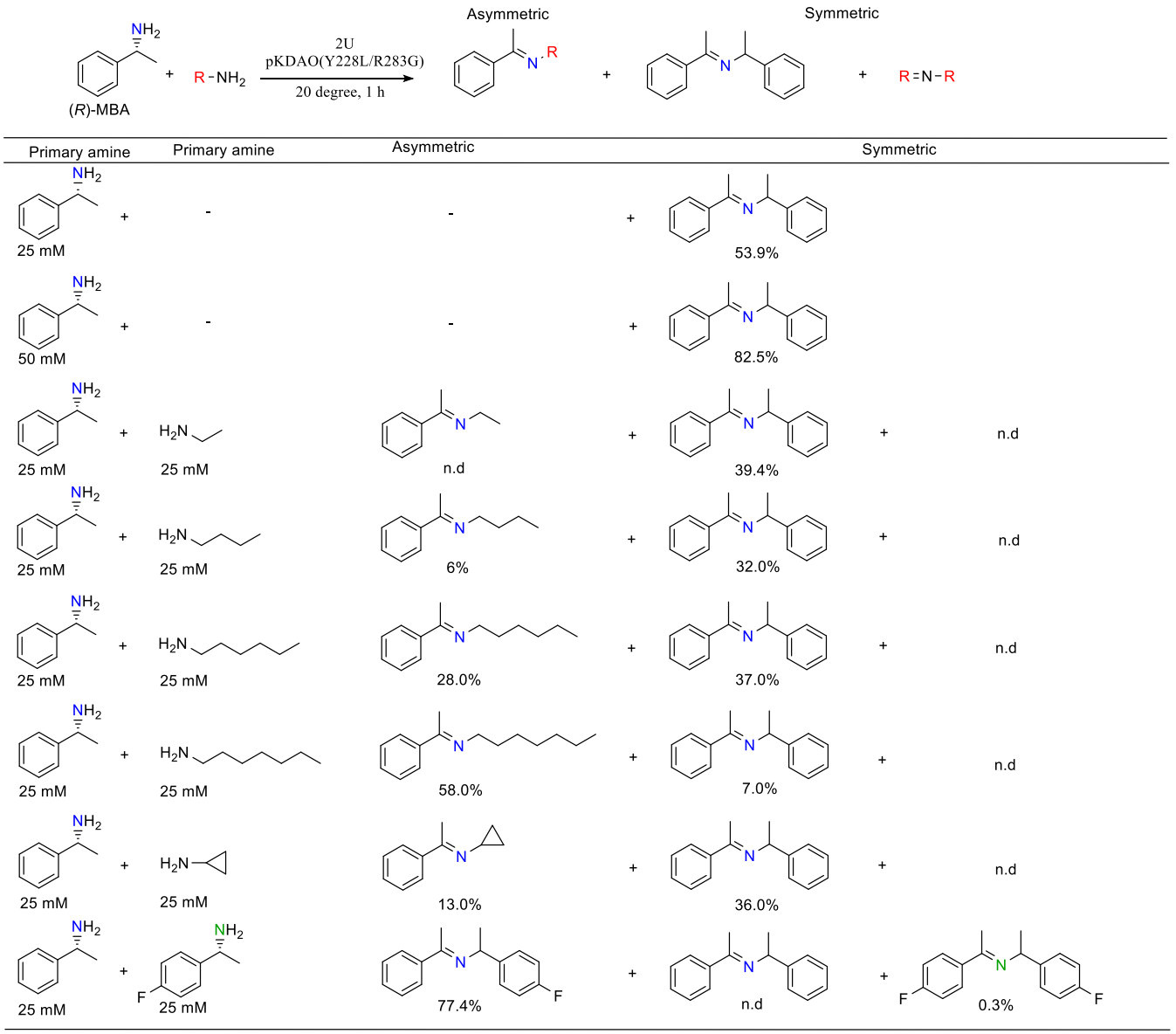


Figure 1. Synthesis of symmetric and asymmetric imines by the oxidation of (*R*)-MBA with variant pkDAO (Y228L/R283G).

in a one-pot reaction system. Amines were prepared in distilled water to obtain a final concentration of 1 M. The pH was adjusted to 9.0 with 2 N HCl. Imine synthesis was performed in a 1 mL reaction mixture of 25 mM (*R*)-MBA, 25 mM primary amines, and 2 units of purified pkDAO (Y228L/R283G). The synthesis reactions were performed in a shaking incubator (50 rpm) at 20 °C for 1 h. After the completion of the reaction, the reaction mixture was extracted using *n*-hexane (0.5 mL). The products were analyzed by GC-MS as described above.

Symmetric and Asymmetric Imine Synthesis with Various Diamines. The reaction of diamino acids with diaminohexane and benzhydrylamine was evaluated using pkDAO (I230A/R283G). The reaction was performed in two steps. In the first step, 25 mM diaminohexane and 25 mM benzhydrylamine were reacted with 4 units of pkDAO (I230A/R283G) in 1 mL of water, at pH 9.0 (2 N HCl) and 20 °C for 1 h. Subsequently, 100 μL of the sample was taken, and half a volume of *n*-hexane was added to stop the reaction and extracted. The samples extracted in the first step were analyzed by GC-MS. Simultaneously, the reaction was subjected to a second step by adding 20 mM benzhydrylamine (pH 9.0) and 4 units of pkDAO (I230A/R283G). The reaction mixture was incubated at 20 °C for 1 h. Subsequently, the reaction was stopped, and the product was extracted by adding *n*-hexane (0.5 mL). The product from the second step was analyzed by GC-MS.

Symmetric Imine Synthesis with Various MBA Derivatives. The reaction was conducted with 25 mM MBA derivatives, including (*R*)-MBA, (*S*)-MBA, (*R*)-EBA, (*R*)-FMBA, (*S*)-FMBA, (*R*)-Cl-MBA, (*S*)-Cl-MBA, (*R*)-DMBA, and BA using 2 units of pkDAO (Y228L/R283G) in water at a 1 mL working volume at pH 9.0. The synthesis reactions were performed at 20 °C for 1 h. After the completion of the reaction, the reaction mixture was extracted using *n*-hexane (0.5 mL). The products were analyzed by GC-MS as described above.

Gram-Scale Synthesis of Symmetric Imine with Methylbenzylamine Derivatives. The gram-scale reaction was conducted using 100 mM MBA derivatives, including (*R*)-

https://doi.org/10.1021/acsomega.4c09160
ACS Omega 2025, 10, 2212−2221

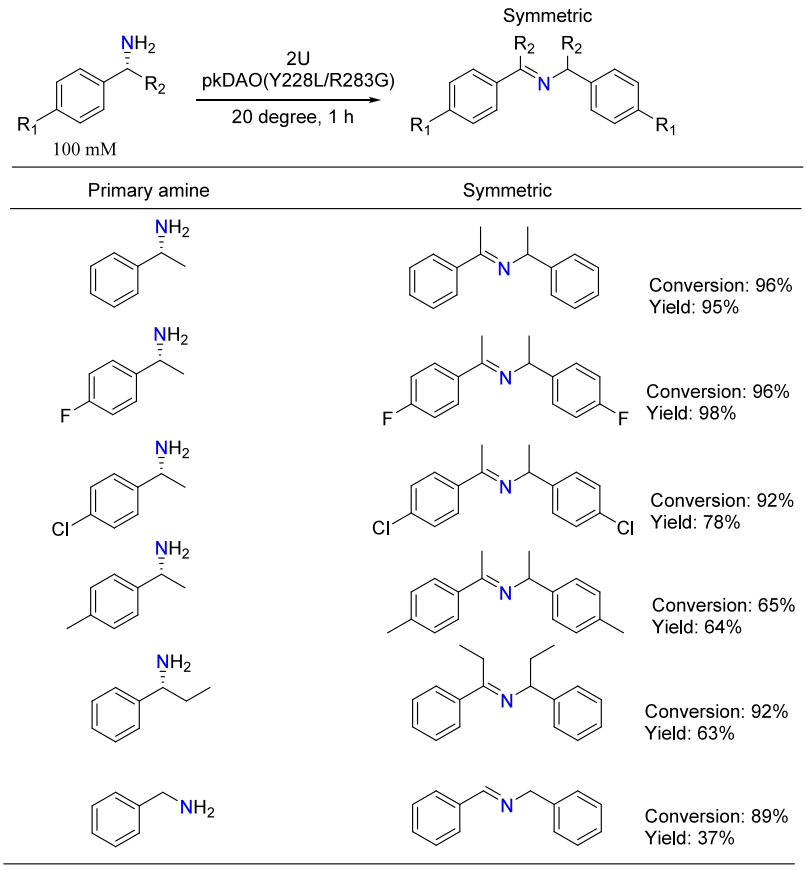


Figure 2. Synthesis of symmetric imines from (*R*)-MBA derivatives using pkDAO (Y228L/R283G).

MBA, (*R*)-EBA, (*R*)-FMBA, (*R*)-Cl-MBA, BA, and (*R*)-DMBA, with 2 units of pkDAO (Y228L/R283G). The reactions were performed in 10 mL of water at pH 9.0 and were incubated by shaking at 150 rpm and 20 °C for 1 h. The reaction was stopped, and the product was extracted three times with ethyl acetate. The organic layer was extracted and dried over anhydrous brine (saturated with NaCl in distilled water) and $MgSO_4$. The solvent was evaporated under a vacuum. The crude extract was dissolved in $CDCl_3$ and analyzed by ^{1}H NMR. The conversion was calculated from ^{1}H NMR of the starting material (primary amine) that converted into the product (secondary imine) in the reaction. Yield was calculated from the amount of products (secondary imine) obtained from the reaction relative to the theoretical maximum amount that could be produced based on the amount of starting material.

Optimization for the Symmetric Imine Synthesis of (*R*)-FMBA. The optimization for imine synthesis was studied using 5−250 mM (*R*)-FMBA, pH 9.0, which was set with 2 N HCl, and 0.5−5.0 units of pkDAO (Y228L/R283G) in a 1 mL reaction. The synthesis reactions were conducted at 20 °C for 10−250 min. The products were extracted and analyzed by GC-MS as described above.

RESULTS AND DISCUSSION

Oxidative Coupling of (*R*)-MBA Derivatives in the Gram Scale. We explored a wide range of substrates, including aliphatic/alkyl, aromatic, and diamines, to demonstrate the generality of the reaction for imines with variant pkDAO in a one-pot system (Figure 1). The reactivities of several primary amines in a mixture of 25 mM (*R*)-MBA and 25 mM of other primary amines (aliphatic/alkyl, aromatic, and diamines) as a nucleophilic amino donor in the aqueous reaction (1 mL) under the optimal oxidative condition (pH 9.0, 20 °C for 1 h)[30] were investigated using 2 units of pkDAO (Y228L/R283G).

We investigated how the nucleophilic addition of homo- and heterologously coupled reactions results in the syntheses of symmetric and asymmetric imines, respectively. The reactions were compared with those of enzymes inactivated by heat treatment or without nucleophilic amines. The reaction proceeded smoothly to furnish the expected homologously coupling products at high yields (82.5%) along with a symmetrical imine (PPEA) in almost all reactions generated from oxidative homologous couplings of the amine substrate ((*R*)-MBA).

Furthermore, as shown in Figure 1, the desired asymmetric imine produced by cross-coupling of (*R*)-FMBA was identified as (1*E*)-1-(4-fluorophenyl)-*N*-[1-(4-fluorophenyl)ethyl]ethan-1-imine (FFEI) with a 77.4% yield. Interestingly, the symmetric imine (PPEA) was not observed in the reaction of (*R*)-MBA with (*R*)-FMBA. These results showed that pkDAO (Y228L/R283G) exhibited high selectivity for oxidation reactions with MBA and its derivatives. In contrast, cyclopropylamine afforded the asymmetric imine in a lower yield (13%). The yields of the asymmetric imines synthesized from MBA and aliphatic/alkyl amines (which are not direct substrates of the pkDAO variants) followed the following order: *n*-heptylamine (58%) > *n*-hexylamine (28%) > *n*-butylamine (6%). Ethylamine was not among the substrates the aliphatic/alkyl amines used, and secondary imines were not detected. We observed the tendency that the yield of the

https://doi.org/10.1021/acsomega.4c09160
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coupling reaction of MBA was decreased with the amines having a shorter alkane chain, meaning that the reaction was not so much dependent on the nucleophilicity. Considering the very hydrophobic active site of the variant of D-amino acid oxidase (Y228L/R283G), surrounded by π electrons of F242, Y224, and the isoalloxazine ring of FAD (PDB: 3WGT),[23,26,27] the small methylamine and other alkylamines have lower accessibility to the active sites, reflecting the substrate specificity of the variant, which prefers aromatic amines. Furthermore, not only the active site but subsites would also be there in the parent enzyme pkDAO, preferring the original substrates in the order of D-Pro (100%), D-Phe (84%), D-Met (75%), D-Ala (40%), D-Ile (35%), D-Val (28%), D-Leu (21%), and Gly (0%).[57] The nonsubstrate *n*-alkylamines with longer chain lengths would have an affinity toward the active site, although they are not the substrate for the pkDAO variant.[24] The substrate, such as *n*-hexylamine, which has a closer structure with D-Met, reacted with the first product imine (1-PEI) as soon as it was formed and released to the medium at the surface of the enzyme. We monitored the evidence that there is no diastereomeric preference in the coupling reaction of the substrate, as suggested in Figure S2, which means that the product 1-PEI reacts with the nucleophile amine from both sides of the 1-PEI plane. The local concentration of *n*-hexylamine might be higher similar to the substrate MBA at the surface of the pkDAO. A detailed kinetic study must be done on the preference of coupling substrates.

There are only five reports[43,44,49,51,56] on the heterologous coupling of the amines among the 23 literature reports we cited,[34−56] and the varieties of the counterpart amines tested are not so wide. The amines attacking the imine are aniline derivatives,[43] aniline and benzylamine derivatives,[44] aniline derivatives, *n*-hexylamine, *n*-dodecylamine, phenyethylamine,[49] and several alkylamines such as *n*-hexylamine, cyclopropylmethylamine without description on the yield,[51] and diaminobenzene with cyclization to form benzimidazole.[55] The reaction mixture contained 25 mM (*R*)-MBA, 2 units of variant pkDAO (Y228L/R283G), and 25 mM primary amines at pH 9.0 adjusted by HCl, and it was incubated at 20 °C for 1 h in a 1 mL reaction mixture. The products were identified by gas chromatography−mass spectrometry (GC-MS). The result showed that the synthesis of the product FFEI was maximally optimized with 2 units/mL enzyme (Figure S1).

Two BPEA isomers with the same MS (225.25 *m*/*z*) were detected by GC-MS analysis of the reaction mixture of (*R*)-MBA after reduction with $NaBH_4$. These were identified by HPLC, and the retention times were compared with those of standard (*R*,*R*)-, (*R*,*S*)-, and (*S*,*S*)-BPEA. Two procedures for the reaction ((1) *in situ* reduction and (2) reduction of the imine after isolation) were designed to show the vicinity effect of reduction in the environment of the enzyme reaction to form the diastereomers. The exact ratios of (*R*,*S*)-BPEA and (*R*,*R*)-BPEA were 84.9 and 15.1%, respectively, in reaction (1), and the ratios were similar at 83.2 and 16.8%, respectively, in reaction (2) (Figure S2). Therefore, the uneven distribution would not have been caused by the enzyme stereoselectivity.

To illustrate the synthetic utility, gram-scale production of symmetric imines was performed, and the structures of the products were confirmed by ^{1}H NMR. As shown in Figure 2, symmetric imines were produced from 100 mM (*R*)-MBA, (*R*)-EBA, (*R*)-FMBA, (*R*)-Cl-MBA, BA, and (*R*)-DMBA with 2 units of pkDAO (Y228L/R283G). The reactions were performed in 10 mL of water at pH 9.0 with 2 N HCl and then incubated at 20 °C for 1 h. The reaction proceeded smoothly to furnish the expected self-coupling products in high yields, with lesser amounts of ketones or aldehydes as byproducts.

The large-scale homologous coupling of (*R*)-FMBA gave an excellent yield (98%) and conversion (96%) under the selected conditions (Figure 2). Imine synthesis from (*R*)-FMBA was optimized to maximize the production yield. The reaction was optimized with 5−200 mM (*R*)-FMBA, 0.25−5.0 units of the variant pkDAO, and 5−240 min of reaction time (Figure S3a−c). The product (approximately 50 mM FFEI) was maximally synthesized from 100 mM (*R*)-FMBA (pH 9.0) with 2 units of pkDAO (Y228L/R283G) at 20 °C for 60 min. Electron-withdrawing substitutes such as chloro- and fluoro- at the para-position of the MBA increased the yield of the dimerization reaction, while electron-donating substituent methyl decreased the yield, in accordance with our proposed reaction mechanism that the 1-PEI acts as the electron acceptor.[30] In the reactions reported so far, opposite results were obtained, i.e., electron-donating substitutes gave faster coupling velocities than the electron-withdrawing ones of benzylamine.,[35,40,53] or almost the same,[52] possibly because of mechanisms involving radicals with photocatalysts.

The product formation decreased with increasing (*R*)-FMBA concentrations >150 mM, probably because the variant pkDAO was inhibited by high concentrations of the substrate. The secondary imine product was degraded to acetophenones, ammonia, and primary amines when the incubation time was increased to >120 min. In our study, product degradation was observed when the incubation time was extended beyond 120 min. This prolonged exposure to reaction conditions has likely enhanced hydrolysis. Evidence of degradation included the detection of byproducts such as acetophenones, ammonia, and primary amines after 120 min of incubation. The degradation is unlikely to have been directly caused by hydrogen peroxide or ammonia generation, as we have already evaluated that there is no improvement by addition of catalase to the reaction mixture. Our results indicated that the same amount of secondary imine was formed in reactions both with and without catalase, suggesting that these factors did not significantly influence the reaction outcome. The reaction mixture contained 25 mM amines, 2 units of variant pkDAO (Y228L/R283G) at pH 9.0 adjusted by HCl, and incubated at 20 °C for 1 h in a 10 mL reaction mixture. The numbers indicate the yield of the product, as determined by ^{1}H NMR spectroscopy.

These results indicate that longer incubation times are not suitable for efficient imine production. Therefore, to achieve a high yield, the reaction time was fixed at 60 min. Furthermore, the imine from (*R*)-FMBA could be optimally synthesized in high yields (nearly 100%) using one unit of enzyme. When pkDAO (Y228L/R283G) was used in the reaction with (*R*)-MBA as the substrate and diaminohexane as the nucleophile, no secondary imine product was observed. In contrast, in the reaction of the variant pkDAO (I230A/R283G) with benzhydrylamine, a symmetric secondary imine (*N*-(diphenylmethylene)-α-phenylbenzenemethanamine) was produced in a 2.5 mM concentration from the 25 mM substrate (20% yield) (Figure S4). With diaminohexane, a small amount of secondary imine was detected in a two-step reaction (Figure S5). The reaction of FAD-dependent amino acid oxidase yields the corresponding keto acids, ammonia, and H_2O_2. However, the real products from amino acids are imino acids, which are

https://doi.org/10.1021/acsomega.4c09160
ACS Omega 2025, 10, 2212−2221

hydrolyzed nonenzymatically by medium water to yield keto acids.[1−3]

Water is counted as a substrate in this reaction. Hafner and Wellner showed that hydrazine and semicarbazid function as nucleophiles to form hydrazine and semicarbazone amino acids, respectively, in reactions of pkDAO and L-amino acid oxidase from snake venom.[10] We have previously shown that pkDAO and L-amino acid oxidase from *Crotalus atrox* catalyze the synthesis of 2-amino-2-cyano-3-phenylpropanoic acid from potassium cyanide and D- and L-phenylalanine, respectively.[23] Furthermore, we demonstrated the deracemization reaction of MBA by the action of variants of pkDAO (Y228L/R283G) and (I230A/R283G) to form optically active (*S*)-MBA in the presence of a reducing agent such as $NaBH_4$.[23] These results clearly indicated the presence of an active primary imine intermediate such as an imino (phenylethylanimine [1-PEI]) compound. Besides the reduction of imines, we extended this reaction to synthesize various α-cyanated amino acid derivatives with pkDAO (Y228L/R283G) in the presence of nitrilase AY487533, which acts on substrates with substitutions at the α-carbon of nitriles.[29] Several α-alkyl amino acids were successfully synthesized. During further studies on oxidative cyanation reactions, we discovered the formation of a trace amount of the byproduct PPEA, optimized the reaction conditions, and clarified the mechanism of imine synthesis.[30] Symmetric imine PPEA was produced from active primary imine 1-PEI as an intermediate (Scheme 1). The secondary imine (PPEA) was formed by homologous coupling with an unreacted primary amine (amino donor) via nonenzymatic nucleophilic addition. To understand the reaction mechanism, the origin of one nitrogen molecule in PPEA (secondary imine) was confirmed to be from ^{15}N-labeled *n*-hexylamine as the nucleophile (Scheme 1).

Imines are a group of compounds that are useful in several applications and are normally synthesized by using chemical methods under harmful conditions. In this study, we not only optimized the reaction to synthesize larger amounts of homologous-coupled imines but also explored heterologous-coupled asymmetric imine synthesis by the oxidation reaction of variants of pkDAO. The reactions were performed using several primary amines, including aliphatic, aromatic, and diamines. These results indicate that MBA derivatives are good substrates for symmetric imine synthesis with pkDAO (Y228L/R283G) in the oxidation reaction under aqueous conditions. Imine (FFEI) was synthesized with an approximately 100% yield from (*R*)-FMBA, without ketones or aldehydes detected in the reaction mixture. In contrast, this reaction does not occur in the presence of aliphatic or alkyl amines only. This result is supported by our previous reports[23,24,26−30] that several primary amines, including aromatic and aliphatic/alkyl, and cyclic amines such as ethylamine, butylamine, and hexylamine are not substrates for pkDAO variants.

Our results showed that pkDAO (Y228L/R283G) and (I230A/R283G) have a high ability to produce symmetric and asymmetric imines in an ecofriendly manner in a one-pot reaction. Since the α-cyanated phenylalanine derivative has been detected with unmodified pkDAO and knowing that natural amines can be the substrate of nucleophilic addition to the active imine species generated by the action of pkDAO, there is a possibility that some amino adducts of amino acids might be produced *in vivo* when exposed to amino acid oxidases.[10] We have shown to utilize the active imines formed in the amino acid oxidase or AOx reactions and developed enzyme variants that act on aromatic amines, with the product primary imines being rather stable in water, to allow the syntheses of considerable amounts of symmetric and asymmetric aromatic imines. This study indicates that many amino acid oxidases including the well-utilized D-amino acid oxidase and AOxs developed as analytical elements have wide possibilities for synthetic applications.[58−61]

CONCLUSIONS

In summary, we discovered abilities of variants of pkDAO (Y228L/R283G) and (I230A/R283G) to synthesize considerable amounts of various symmetric and asymmetric imines based on our mechanistic and structural studies. We showed for the first time that a wide range of nucleophiles can be substrates of pkDAO, suggesting new possibilities for FAD-dependent AOxs, including amino acid oxidases.[1−3,40−43] Some of the imines were synthesized on a gram scale, providing an environmentally friendly strategy for the preparation of imines and secondary amines. The turnover numbers (TOF) of *N*-benzylidenebenzylamine and PPEA synthesis were calculated to be 1.61×10^5 and 1.74×10^5/h, respectively, which are more than 10^3 higher than previously known metal-, photo-, and organo-catalysts reported so far.[34−51,53−56] Thus, we demonstrated for the first time the merits of the imine synthesis by enzyme catalysis by pkDAO variants, showing an extremely high TOF number compared with the conventional catalysts, in water used as a medium, at low room temperature. It is easy to scale up by mass production of the enzyme by culture of the *E. coli* transformant.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c09160.

Materials and equipment, enzyme production and purification, enzyme assay, synthetic procedures for symmetric and asymmetric imines, GC-MS chromatograms and MS spectra of imines, and ^1H NMR spectra of imines (PDF)

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https://doi.org/10.1021/acsomega.4c09160
ACS Omega 2025, 10, 2212−2221

Complete contact information is available at:
https://pubs.acs.org/10.1021/acsomega.4c09160

Author Contributions

W.K. and Y.A. designed the experiments, analyzed the results, and wrote the manuscript. W.K. and S.S. performed the experiments. G.I. helped with the identification of the chemicals.

Notes

The authors declare no competing financial interest.
P00371 · OXDA_PIG

■ ACKNOWLEDGMENTS

This work was supported by grant-in-aid for Scientific Research (S) and (A) from the Japan Society for the Promotion of Science (grant nos. 17H06169 and 22H00361, respectively) and the grant from the Toyama Pharmaceutical Valley Development Consortium to Y.A.

■ ABBREVIATIONS

pkDAO, D-amino acid oxidase from porcine kidney; AOx, amine oxidase; BPEA, bis(1-phenylethyl)amine; PPEA, (1-phenyl-*N*-(1-phenylethylidene)ethanamine; FFEI, (1*E*)-1-(4-chlorophenyl)-*N*-[1-(4-chlorophenyl)ethyl]ethan-1-imine; MBA, α-methylbenzylamine; CBHA, chlorobenzhydrylamine; EBA, ethylbenzylamine; FMBA, fluorobenzylamine; Cl-MBA, chloromethylbenzylamine; DMBA, dimethylbenzylamine; BA, benzylamine

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https://doi.org/10.1021/acsomega.4c09160
ACS Omega 2025, 10, 2212−2221