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Enantioselective oxidation of amphetamine by copper-containing quinoprotein amine oxidases from *Escherichia coli* and *Klebsiella oxytoca*

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Abstract

The enantioselective properties of copper-containing quinoprotein amine oxidase (EC 1.4.3.6) from *Escherichia coli* K12 and *Klebsiella oxytoca* in the kinetic resolution of (*R*,*S*)-1-phenyl-2-aminopropane, amphetamine, have been determined. Determination of the enantiomeric ratio, $E = (k_{cat}/K_M)^R/(k_{cat}/K_M)^S$, the ratio of specificity constants for the enantiomeric substrates, can be accomplished in several ways. For practical reasons, we calculated *E* using non-linear regression analysis of initial rate data obtained at a fixed overall concentration of amphetamine mixtures of chiral composition ranging from 0 to 50% (*R*)-(−)-amphetamine [Jongejan et al., Recl. Trav. Chim. Pays-Bas 110 (1990) 247]. It is found that both enzymes catalyze the enantioselective oxidation of amphetamine with *E*-values of sufficient magnitude ($E \approx 15$) which may open the possibility for future application of amine oxidase-catalyzed kinetic resolutions of racemic amphetamine. The preference for the (*R*)-enantiomer of amphetamine is in agreement with the pro-*S* specificity that has been observed for the conversion of 2-phenylethylamine. Rationalization of this observation, based on the structure of the *E. coli* amine oxidase, is discussed.

Keywords: Amphetamine; *(R,S)*-1-phenyl-2-aminopropane; Kinetic resolution; Enantiomeric ratio; Copper-containing quinoprotein amine oxidase; *Escherichia coli*; *Klebsiella oxytoca*

1. Introduction

Even though the ultimate [2] "Jekyll & Hyde" case presented by (*R*)-thalidomide's sedative and (*S*)-thalidomide's teratogenic activity may be the exception rather than the rule, it is generally acknowledged that the enantiomers of chiral bioactive compounds may have widely different activities [3]. Current emphasis on the importance of chirality in the production of pharmaceuticals, agrochemicals and food additives is further exemplified by rapid developments in the area of "chirotechnology" [4–8].

Of the drugs that are active in the hormonal and central nervous system, the enantiomers of 1-phenyl-2-aminopropane, amphetamine and its phenyl-sub-

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stituted derivatives, are known to have distinct pharmacological properties [9]. In particular, (*S*)-(+)-amphetamine, dexedrine, has greater activity than the (*R*)-(−)-enantiomer as a locomotor stimulant, as a hyperthermic agent and in its ability to elicit stereotypic behavior. It is anticipated that the effects of amphetamine are related to the metabolism of catecholamines in the central neurons containing norepinephrine and dopamine. Factors of importance are the release of the endogenous amines from the nerve terminal, the inhibition of neuronal uptake of amines and the inhibition of oxidative deamination of the amines by monoamine oxidase. A number of studies have indicated that the inhibition of neuronal uptake of dopamine and norepinephrin by amphetamine is enantioselective. Enantioselectivity has been attributed to the presence of two types of neurons distributed in different regions of the brain. The potency of (*R*)-(−)-amphetamine over the (*S*)-(+)-enantiomer in inhibiting uptake and release of biogenic amines and monoamine oxidase activity in rat brain has been reported [10–12].

Chemical synthesis of (*R,S*)-amphetamine by reduction of the oxime of benzylmethylketone has been patented [13]. A medium-yield one-pot synthesis by reacting phenylacetone and ammonia in the presence of aluminum grit has been described [14].

(*S*)-(+)-amphetamine has been prepared by resolution of the racemic mixture with D-tartaric acid followed by acid treatment [15]. The use of enantioselective hydrolases in the resolution of racemic amines by acylation–deacylation strategies is well documented. High enantioselectivities have been measured in the conversion of natural and non-natural amino acids, good selectivities have been found for other chiral amines [16–19]. To our knowledge kinetic resolutions of amphetamine by this approach have not been reported. ω-Transaminase screened from soil microorganisms has been used for the enzymatic kinetic resolution of α-methylbenzylamine [20–21].

Aromatic primary amines including tyramine, dopamine and phenylethylamine, are widely distributed in nature. Microorganisms such as *Escheichia coli* and *Klebsiella oxytoca* are able to use these aromatic amines as a carbon and/or nitrogen source. In most cases, their catabolism is initiated by oxidative deamination. Amine oxidases that catalyze dioxygen-linked oxidations ($RCH_2NH_2 + O_2 + H_2O \rightarrow RCH{=}O + NH_3 + H_2O_2$) occur in prokaryotes and eukaryotes including fungi, plants and mammals, as constitutive as well as inducible enzymes [22]. Two classes appear to be involved: flavin-containing monoamine oxidases (EC 1.4.3.4) and copper-containing quinoprotein amine oxidases (EC 1.4.3.6). The copper-containing amine oxidases form a diverse group with few characteristics that are specific for all enzymes so classified. For some time, it was believed that pyrroloquinoline quinone (PQQ) functioned as the organic cofactor [23]. It has now been unequivocally established that tyrosine-derived topaquinone is the organic cofactor [24]. The structures of the copper quinoprotein amine oxidases from *E. coli* [25,26] and pea seedling [27] have recently been determined by X-ray crystallography.

Copper quinoprotein amine oxidases display broad substrate specificity, exhibiting stereochemical heterogeneity in the conversion of pro-chiral amines [28]. Amine oxidase from pea seedling has been shown to catalyze the abstraction of the pro-*S* hydrogen from C(1) of benzylamine, tyramine and dopamine [29–33]. Porcine plasma amine oxidase, on the other hand, is pro-*R* specific for tyramine and dopamine [32]. Bovine plasma amine oxidase is pro-*S* specific for benzylamine, *p*-hydroxybenzylamine and 3-methylbutylamine [31,34–35] but is non-stereo specific for tyramine and dopamine [32,36]. The different stereochemistry has been rationalized by assuming that the latter substrates have two binding modes at the active site of plasma amine oxidase. The enzyme processes the pro-chiral substrate by mirror-image-binding modes in which the substrate is bound in such a way that the cofactor can abstract the protons from the same side of the substrate, regardless of the chirality of the bonds involved in cleavage during the catalytic reactions [37].

Considering the interesting stereochemistry of copper quinoprotein amine oxidases in the conversion of pro-chiral substrates, we decided to investigate their enantioselectivity with respect to chiral amines. We chose amphetamine as a model compound and the amine oxidases from *E. coli* and the closely related enzyme isolated from *K. oxytoca* [38–39] as enzymes for which abstraction of the pro-*S* hydrogen has been rationalized on the basis of structural arguments [26].

2. Materials and methods

2.1. Chemicals

2-Phenylethylamine, tyramine, tryptamine, dopamine, histamine and *n*-butylamine were obtained from commercial suppliers (Merck, Fluka, Aldrich). (*S*)-(+)-amphetamine, and (*R*,*S*)-amphetamine of > 99.5% chemical and enantiomeric purity were kind gifts of Dr. Huizer, Forensic Laboratory Rijswijk, NL. All chemicals used in this study were of the highest quality commercially available. Solutions were prepared in demineralized water.

2.2. Enzymes

Amine oxidases from *E. coli* K12 (LMD 93.68 and 93.69) and *K. oxytoca* (LMD 72.65; ATCC 8724) were purified as described [39,40]. Protein concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient of 1.7×10^6 cm^2/mo1.

2.3. Kinetic measurements

Amine oxidase activity was determined polarographically using a Clark type oxygen electrode (Biological Oxygen Monitor). The electrode was calibrated prior to use in air-saturated 0.1 M sodium/potassium phosphate buffer, pH = 7.0 and oxygen-free solution containing sodium dithionite, respectively. The assay mixture (1.5 ml) consisted of air-saturated 0.1 M sodium/potassium phosphate buffer, pH = 7.0 and the enzyme, thermostatted at 30°C. The reaction was started by the addition of substrate. The initial rate of oxygen consumption was monitored.

Rate measurements were performed for varying concentrations of the substrate at constant enzyme concentrations. Fitting of the experimental data of each substrate was achieved by applying non-linear regression analysis to the plots of reaction rates versus substrate concentrations. The enantioselectivity of the amine oxidases in the oxidation of amphetamine was determined from the initial rate data obtained with different ratios of the enantiomers at a fixed total concentration of 2.5 and 25 mM, respectively, using the non-linear regression software SIMFIT [1].

3. Results and discussion

The stereospecificity of the C_α-proton abstraction of pro-chiral aromatic amines catalyzed by various copper-quinoprotein amine oxidases has been determined by NMR spectroscopy using deuterium-labeling strategies [28,32,33,37]. Although qualitatively reliable specificities can be deduced, it must be realized that the accuracy of the NMR signal quantitation may well be off by a few percent. As a result of the highly skewed confidence intervals for *E*-value determinations [41], specificities that are judged absolute by NMR spectroscopy may actually correspond to $E \geq 15$. Determination of the enantiomeric ratio of *E. coli* and *K. oxytoca* amine oxidases by measuring the ratio of the specificity constants for (*S*)- and (*R*)-amphetamine would appear to be straightforward. However, limited availability of amphetamines due to restrictive regulations prohibited us from obtaining both enantiomers in enantiopure form. Instead, we obtained chemically pure racemic amphetamine and pure (*S*)-(+)-enantiomer by courtesy of the Dutch Forensic Laboratory, Rijswijk, NL. Considering certain limitations in the amounts of purified enzyme, interference of products formed during the reaction (aldehyde and hydrogen peroxide) and the need for proper analytical procedures, determination of the *E*-value by monitoring the enantiomeric excess value of residual substrate ee_s versus the extent of conversion ξ in a batch kinetic resolution of racemic substrate, according to Chen and coworkers [42–43], was not feasible. Analysis of initial rate data obtained at fixed overall concentration of amphetamine of chiral composition ranging from 0 to 50% of (*R*)-(−)-enantiomer [1] on the other hand, appeared to be a promising alternative. In order for this method to be applicable, certain conditions must be met. Interference of substrate inhibition phenomena has been recognized as a factor of importance [1,44]. The kinetic features of amine oxidases purified from *E. coli* and *K. oxytoca* were tested for several amines. Both enzymes showed high activity with 2-phenethylamine, tyramine,

Table 1
Apparent kinetic parameters of amine oxidase from *K. oxytoca*[a]

Substrate	K_M (μM)	V_{max}^b (μmol/min)	K_I (mM)
Phenylethylamine	3	92	0.75
Tyramine	4	79	1.3
Tryptamine	4	73	1.4
Dopamine	29	115	2.6
Histamine	330	56	30
Butylamine	270	86	7.9
(+/−)-Amphetamine	830	36	17

[a] Amine oxidase from *E. coli* shows kinetic constants within 5% of those displayed for the enzyme from *K. oxytoca*.
[b] Assay mixtures contained 1 μg electrophoretically pure enzyme.

tryptamine and dopamine as substrates, moderate activities were observed for histamine, *n*-butylamine and (*R*,*S*)-amphetamine (Table 1). Other amines such as benzylamine, methylamine, cadaverine, putrescine were not oxidized.

High levels of substrate inhibition are observed for 2-phenethylamine, tryptamine, tyramine and dopamine. Histamine, *n*-butylamine and (*R*,*S*)-amphetamine, on the other hand, show moderate inhibition. Examples are shown in Fig. 1.

Apparent K_M and K_I values calculated on the basis of minimal competitive inhibition kinetics are collected in Table 1. The enzymes have very low K_M values for 2-phenethylamine, tyramine and tryptamine. These values are much lower than those reported in the literature for other amine oxidases such as methylamine oxidase from *Arthrobacter* P1 [45], benzylamine oxidase from *Candida boidinii* [46], monoamine oxidase from *Klebsiella aerogenes* [47] and benzylamine oxidase from *Saccharomyces cerevisiae* [48]. Similar K_M values for benzylamine oxidation have been reported, however; for the benzylamine/putrescine oxidase from *Pichia pastoris* in the oxidation of putrescine and cadaverine and for benzylamine oxidase from *Candida boidinii* [46]. Substrate inhibition patterns similar to those observed for the *K. oxytoca* and *E. coli* amine oxidases have been reported for the oxidases of human placental DAO and porcine PAO [49]. This behavior has been attributed to the presence of a non-catalytic inhibitory substrate binding site [50]. Other explanations have been proposed (see Mondovi [49] for a review).

As mentioned above, substrate inhibition poses a problem with respect to the *E*-value determination

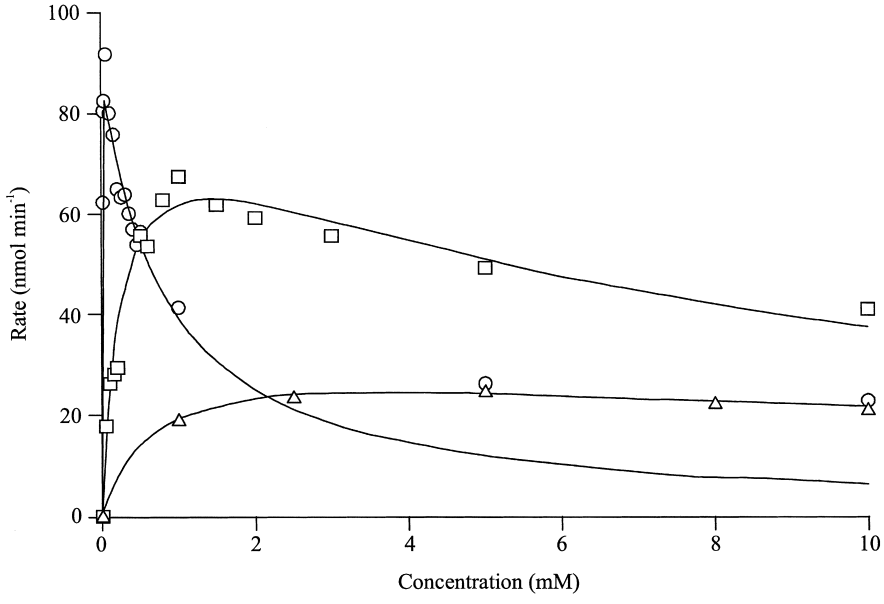


Fig. 1. Initial reaction rates as a function of substrate concentration for amine oxidase from *K. oxytoca* in the conversion of phenethylamine (○), butylamine (□) and (*R*,*S*)-amphetamine (△). Simulated curves were generated by fitting the data to the expression $r = V_{max}[S]/(K_M + [S] + [S]^2/K_I)$. Similar values (within 5%) are observed for the amine oxidase from *E. coli*.

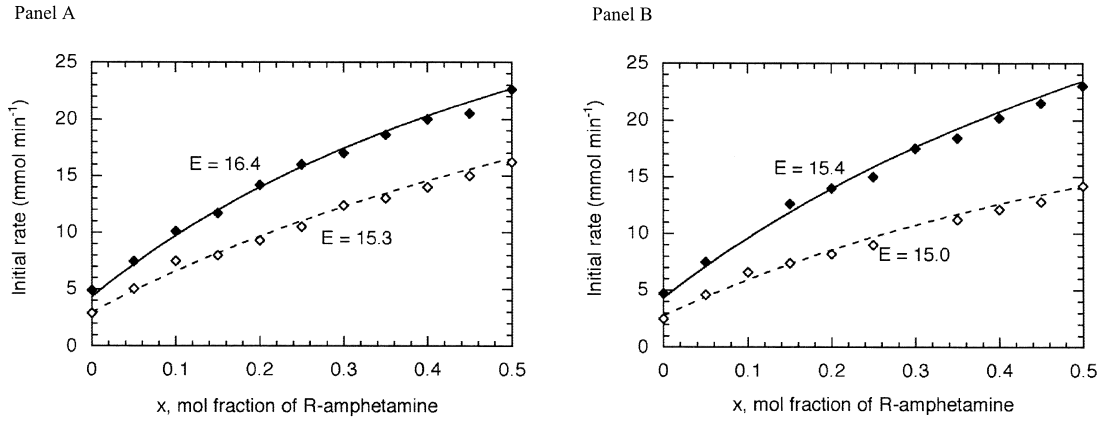


Fig. 2. *E*-values determined for amine oxidase from *K. oxytoca* (Panel A) and *E. coli* (Panel B) in the kinetic resolution of amphetamine using different total concentrations of substrate mixtures. Total concentration of amphetamine: 2.5 mM — solid line (fit), (♦) (data); 25 mM — broken line (fit), (◇) (data). Accuracy of *E*-values is ±3 (standard deviation).

by initial rates according to Jongejan and coworkers [1,41]. In order to derive the *E*-value as the ratio of specificity constants [42,51]:

$$E = \frac{(k_{cat}/K_M)^R}{(k_{cat}/K_M)^S} \quad (1)$$

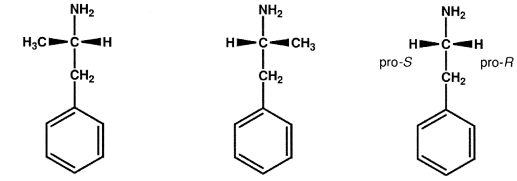
reduction of the kinetic equations for the bi-bi ping-pong reaction catalyzed by amine oxidases, into the form appropriate for the evaluation of initial rate data from enantiomer mixtures [41]:

$$E = \frac{r_R(r_x - r_S)}{r_S(r_R - r_x)} \times \frac{1-x}{x} \quad (2)$$

is required. In this equation r_R and r_S represent the initial rates of the fast- and slow-reacting enantiomers, respectively, and r_x the initial rate of the mixture with mole fraction x of the (*R*)-enantiomer. *E* is the enantiomeric ratio as defined Eq. (1). In deriving Eq. (2), use is made of the linear dependence of both the numerator and denominator of the rate equations on the mole fraction x. In principle, this linearity will be biased by substrate inhibition kinetics involving squared terms in the denominator [1]. However, it can be shown that for those cases where the departure from linearity interferes with the validity of Eq. (2), numerically different *E*-values should be obtained for mixtures with net concentrations of order K_I and of order $0.1 \times K_I$, respectively. From the data presented in Fig. 2, showing *E*-values of equal magnitude for a substrate concentration of 2.5 (well below the apparent K_I of 17 mM) and 25 mM (slightly above the apparent K_I),respectively, it can be concluded that $E \cong 15$is a reliable value for the enantioselectivity of these amine oxidases in the oxidation of amphetamine.

In addition, it is found that the copper quinoprotein amine oxidases from *E. coli* and *K. oxytoca* possess a distinct preference for the oxidation of the (*R*)-enantiomer of amphetamine. This finding is in complete agreement with the pro-*S*-specificity reported for abstraction of the C_α-hydrogen of prochiral substrates, e.g. 2-phenethylamine, by these enzymes. Relevant configurations are compared in Fig. 3.

In order to gain insight into the factors of importance for the enantioselectivity of *E. coli* and *K. oxytoca* in the oxidation of amphetamine, molecular modeling of the enzyme active site has been performed. Preliminary results have been obtained using the crystal structure of the complex between the amine oxidase from *E. coli* (ECAO) and the cova-



S-(+)-Amphetamine (Dexedrine) *R*-(-)-Amphetamine Phenylethylamine

Fig. 3. Configurations of (*S*)-(+)- and (*R*)-(−)-amphetamine compared with pro-chiral 2-phenethylamine.

lently-bound inhibitor, 2-hydrazinopyridine (HP pdb entry RISPUSF) [26] as a template. Hydrogens were added using standard procedures implemented in Insightll/Discover (MSI). In order to visualize possible covalent binding modes of (*R*)- and (*S*)-amphetamine, the 2-hydrazinopyridine moiety was replaced with either one of the amphetamine enantiomers. Relaxation of the resulting structures by molecular dynamics (50 K) while keeping all atoms other than those of the phenylisopropyl group constrained to their original positions was not feasible. Instead, structures had to be positioned manually in order to relieve major clashes. In agreement with the observations published for the molecular model in which 2-phenethylamine was bound to the cofactor in the enzyme active site [26] the (*R*)-amphetamine adduct could be accommodated with the benzyl group sticking out from the D4 domain into the interfacial region between the D4 and D3 domains. Interactions involved mainly the phenolic side chain of Tyr 381, an interaction that might actually contribute to lowering of the transition state energy by hydrophobic interactions (not considered in the CVFF force field used). The adduct of the (*S*)-enantiomer, however, experienced substantial interactions with residues located around the active site entrance. In particular, the C_α-methyl group of the substrate is too close to Tyr 381, while the phenyl group interferes with Gly 464 and Val 463. For both enantiomers, structural waters present in the published structure need not be removed in order to accommodate the substrates. The relevant part of the active site residues is depicted in Fig. 4. Comparison of the crystal coordinates measured for the free and HP-inhibited enzyme suggests that major conformational changes accompany substrate entrance and binding. For this reason, more realistic relaxation and minimization strategies were not considered.

The conformational freedom of the amine oxidase cofactor has been investigated in more detail recently. Murray and coworkers [52] showed that the aspartate (Asp 383) functioning as the active site base in the proton abstraction from the adduct of the quinone form of the TPQ cofactor and the substrate has a distinct role in maintaining the cofactor in a productive orientation. Similar observations have been reported for the corresponding aspartate (Asp 319) in the TPQ-containing copper amine oxidase

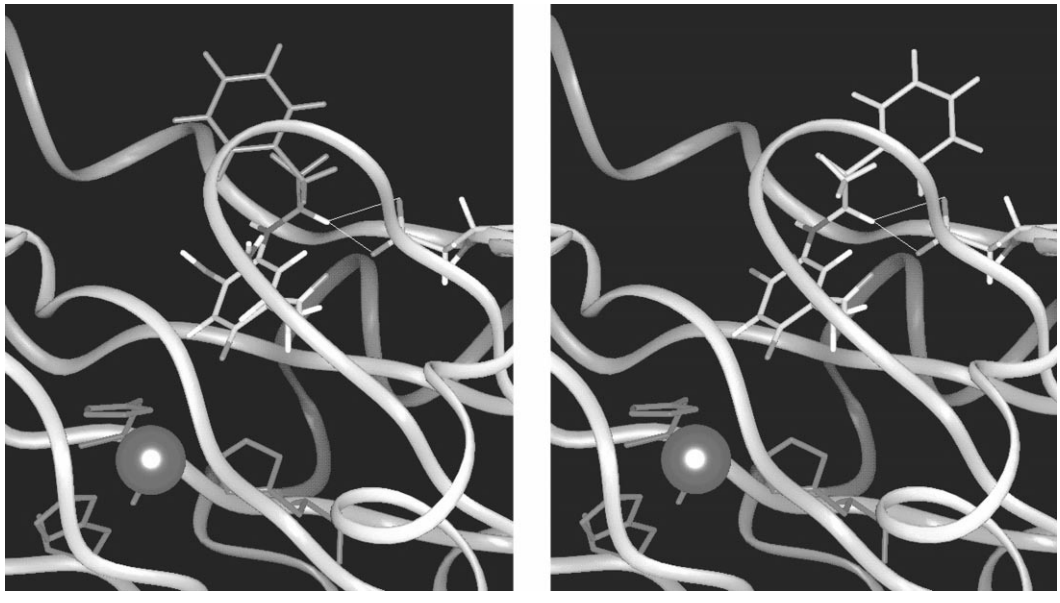


Fig. 4. Molecular model of *E. coli* amine oxidase active site region containing (*S*)-amphetamine (Panel A) and (*R*)-amphetamine (Panel B) covalently linked to the TPQ cofactor. Copper (CPK) and histine ligands (stick representation) in lower left corner, distances between Asp 383 (protruding from the right) and amphetamines (phenyl groups pointing North) bound to the TPQ cofactor (central position) are drawn as white lines. Pictures generated in InsightII (MSI).

from *Hansenula polymorpha* [53]. Whether the observed mobility extends to the residues lining the active site remains to be established.

4. Concluding remarks

The specificity of copper-containing amine oxidases for the removal of the enantiotopic pro-*S*, viz. pro-*R* hydrogen from the C_α-position of pro-chiral amines has been given considerable attention in the past. We show that the pro-*S* specificity observed for the amine oxidases from *E. coli* [28] and *K. oxytoca* (G. Alton and M.M. Palcic, pers. commun.) in the oxidation of benzylamine also applies to the enantioselective oxidation of amphetamine. It appears that molecular modeling provides a rational for this behavior. Since quantification of the enantioselectivity is straightforward when suitable methods are available, determination of the enantioselectivity of copper-containing amine oxidases for other chiral amines may contribute to the further understanding of these enzymes. From a practical point of view, kinetic resolution of racemic amphetamines using copper-containing amine oxidases may offer an interesting route to the enantiopure compounds.

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