# Studies on hydrolysis of chiral, achiral and racemic alcohol esters with *Pseudomonas cepacia* lipase: mechanism of stereospecificity of the enzyme

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**Steady-state kinetics of *Pseudomonas cepacia* lipase-catalysed hydrolysis of five analogous chiral and achiral substrates, *i.e.* (*R*)- and (*S*)-1-methyl-2-(4-phenoxyphenoxy)ethyl acetates (*R*)- and (*S*)-1a, (*R*)- and (*S*)-2-methyl-2-(4-phenoxyphenoxy)ethyl acetates (*R*)- and (*S*)-1b and 2-(4-phenoxyphenoxy)ethyl acetate 1c, were investigated in sufficiently emulsified reaction mixtures of water-insoluble substrates. The apparent Michaelis constant $K_m$ values were identical for all the esters, and no nonproductive binding was observed in these substrates. The apparent catalytic constants $k_{cat}$ were found to reflect the leaving abilities of the alcoholate ions for the fast-reacting enantiomers. These observations, based on the findings that acyl-enzyme intermediate formation was rate-determining in the overall reaction, strongly suggested that all the substrates are bound to the enzyme in the same manner whether or not the alcohol moiety has a medium-sized substituent $L_M$ at the stereocentre and that the breakdown of a tetrahedral intermediate is rate-determining in the acylation of the enzyme. Time courses were also studied for the hydrolysis of racemic 1-ethyl-2-(4-phenoxyphenoxy)ethyl acetate 1d together with 1a, 1b and 1c. The enzyme distinguished (*R*)-1d from its antipode perfectly and hydrolysed only the (*R*)-enantiomer. These results were interpreted to indicate that $L_M$ of the slow-reacting enantiomer is positioned close to the imidazole ring of the catalytic His and hinders $N^{\varepsilon 2}$ of the residue from forming a weak interaction with $O^1$ of the leaving alcohol and that the breakdown of the tetrahedral intermediate is thus inhibited.**

## Introduction

In recent years, lipases have attracted great attention as useful chiral catalysts for the preparation of enantiomerically pure compounds. This is partly because the enzymes catalyse hydrolysis and synthesis reactions of water-insoluble substances often encountered in practical synthesis.[1] *Pseudomonas cepacia* (previously classified as *P. fluorescens*) lipase (PCL) is one of the most frequently used enzymes for enantioselective resolutions to create chiral alcohols.[2,3] For secondary alcohols, the enzyme, together with a few other lipases, has been predicted to react with the enantiomer shown in Fig. 1 faster than the other enantiomer from an empirical rule based on the sizes of substituents at the carbon stereocentre.[2,4] The reliability of the proposed rule was demonstrated for the PCL-catalysed reactions of various kinds of secondary alcohols and their esters.[2,4a] The reactions are often highly enantioselective.[2,4a,5] Recently, some lipases including PCL were observed to hydrolyse primary alcohols esters with lower enantioselectives than analogous secondary alcohol esters.[6,7] Under these circumstances, an essential question has arisen: how such enantioselectivities of the lipases are caused. Active site models such as 'cubic-spaced model'[4b,7a,8] or 'two-site model'[9] were proposed to answer the question. Because of their simplicity, such models, especially the former, seemed to have been accepted favourably by organic chemists using enzymes synthetically. It is however most essential in the long run to explain the mechanism of the stereospecificity of the enzymes.

Kinetic measurements and inhibition studies have established that the pathway of lipase-catalysed hydrolysis reactions includes the formation and decomposition of an acyl-enzyme intermediate similar to that of serine proteases.[10] This can be described in terms of eqn. (1) as a minimal requirement, where

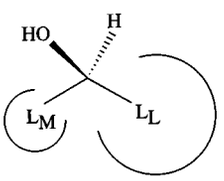



**Fig. 1** For secondary alcohols, the enantiomer shown reacts faster with lipases, including *Pseudomonas cepacia* lipase (PCL), than the other enantiomer

$$E + S \overset{K_S}{\rightleftharpoons} E \cdot S \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2 \quad (1)$$

E · S is the enzyme–substrate complex, ES′ is the acyl-enzyme, and $P_1$ and $P_2$ are the alcohol and acid portions of the ester substrate, respectively. X-Ray crystallographic studies confirmed that the active sites of lipases are formed by triads similar to those of serine proteases.[11] Acylation of lipases results from nucleophilic attack on the carbonyl carbon of the ester by the Ser residue of the catalytic triad and proceeds *via* the formation and breakdown of a tetrahedral intermediate,[12] as was observed for serine proteases.[13] This may be summarized by eqn. (2) where ET is the tetrahedral intermediate.

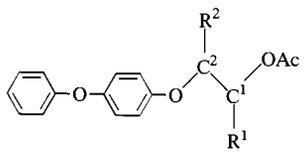
$$E + S \rightleftharpoons E \cdot S \rightleftharpoons ET \longrightarrow ES' + P_1 \xrightarrow{k_3} E + P_2 \quad (2)$$

In the course of studies on the PCL-catalysed hydrolysis of **1a** and **1b** for preparation of enantiomerically pure juvenile hormone analogues, we have found that the formation of the

**Table 1** Apparent kinetic constants of PCL-catalysed hydrolysis of chiral and achiral 2-(4-phenoxyphenoxy)ethyl acetates[a]

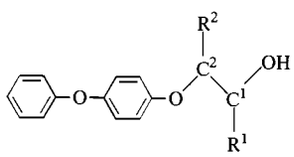
| Substrate | $K_m/10^{-3}$ mol dm$^{-3}$ | $k_{cat}/10^{-2}$ s$^{-1}$ | $(k_{cat}/K_m)$/ dm$^3$ mol$^{-1}$ s$^{-1}$ |
|---|---|---|---|
| (*R*)-**1a** | 8.6 ± 0.4 | 7.0 ± 0.3 | 4.9 ± 0.5 |
| (*S*)-**1a** | 11 ± 0.8 | 0.0027 ± 0.0002 | 0.0025 ± 0.0002 |
| (*R*)-**1b** | 4.9 ± 0.5 | 3.7 ± 0.3 | 7.5 ± 0.7 |
| (*S*)-**1b** | 6.8 ± 1.1 | 30 ± 5 | 43 ± 10 |
| **1c** | 5.2 ± 0.8 | 17 ± 8 | 33 ± 8 |

[a] pH 7.0 and 55.0 ± 0.5 °C; stirring rate 1000 rpm. Polyvinyl alcohol (0.2%, w/v) was added to each reaction mixture to stabilize the emulsion for kinetic measurements.




**1**

| | $R^1$ | $R^2$ |
|---|---|---|
| **1a** : | $CH_3$ | H |
| **1b** : | H | $CH_3$ |
| **1c** : | H | H |
| **1d** : | $C_2H_5$ | H |




**2**

| | $R^1$ | $R^2$ |
|---|---|---|
| **2a** : | $CH_3$ | H |
| **2b** : | H | $CH_3$ |
| **2c** : | H | H |
| **2d** : | $C_2H_5$ | H |

acyl-enzyme intermediate is rate-limiting in the overall reaction.[6] Accordingly, the apparent steady-state kinetic constants $K_m$ and $k_{cat}$ may be regarded as expressing the dissociation constant of the enzyme–substrate complex $K_s$ and the acylation rate constant $k_2$ [eqn. (1)] respectively, with the substrate being in great excess to enzyme. It may be useful to investigate the steady-state kinetics in these reactions since the enzyme stereospecificity may be reflected in both the binding situation of the substrate and the acylation step of the enzyme.

In this article, we report the kinetics and time courses of the PCL-catalysed hydrolysis reactions of analogous chiral, achiral and racemic 2-(4-phenoxyphenoxy)ethyl acetates in order to eludicate the mechanism of the stereospecificity of the enzyme.

## Results and discussion

### Estimation of kinetic constants on water-insoluble substrates

Table 1 shows the apparent kinetic constants pertaining to (*R*)- and (*S*)-1-methyl-2-(4-phenoxyphenoxy)ethyl [1-(4-phenoxyphenoxy)-2-propyl] acetates (*R*)- and (*S*)-**1a**, (*R*)- and (*S*)-2-methyl-2-(4-phenoxyphenoxy)ethyl [2-(4-phenoxyphenoxy)-1-propyl] acetates (*R*)- and (*S*)-**1b** and 2-(4-phenoxyphenoxy)-ethyl acetate **1c** evaluated at suitable substrate concentrations by use of the Eadie–Hofstee equation.

There were some specific issues concerning the estimation of kinetic constants in a biphasic system of water-insoluble substrate with lipases. It was suggested[14] that $K_m$ in the biphasic system should be expressed as the interfacial area per volume, because the kinetic constants vary depending upon the particle size of the emulsion. However, it was announced later[15] that the apparent $K_m$ expressed as moles per volume, measured under fixed conditions for emulsion formation, would be useful for kinetic analysis. We demonstrated previously[16] that an agitation at a stirring rate of more than a certain rpm value gave optimum activity in every assay by producing the finest substrate particles in the emulsion. The stirring rate of 1000 rpm was chosen by confirming that optimum initial reaction rate was achieved in the experimental runs. Polyvinyl alcohol (0.2%, w/v) was added to maintain a stable emulsion of the reaction mixture similarly to the system reported recently.[17] The surfactant appeared to be especially effective in the cases of relatively small amounts of the substrates being used. It is thus reasonable to express the kinetic constants $K_m$ and $k_{cat}$ obtained in this emulsion system as concentration (moles per volume).

Substrate concentrations used in the kinetic measurements were 3.5–100 mmol dm$^{-3}$, whereas those of the enzyme were *ca.* 5 μmol dm$^{-3}$ for (*S*)-**1a** and 10–70 nmol dm$^{-3}$ for the other substrates. Such great differences in concentrations between the substrate and the enzyme indicated that the substrate was in great excess with respect to the enzyme throughout the steady-state kinetic studies.

Important observations and indications as shown in Table 1 are as follows.

### Substrate binding situation

The comparison of $K_m$ (= $K_s$) with $k_{cat}/K_m$, algebraically equivalent to $k_2/K_s$, which is independent of nonproductive (unreactive) binding effects,[18] provides information as to the possibility of such nonproductive effects. There is substantially no nonproductive binding in this study since no characteristic behaviour was observed of such binding on the $K_m$ and $k_{cat}/K_m$ values. Thus, the observed enantioselectives [which are measured by the enantiomeric ratio $E = (k_{cat}/K_m)_{fast}/(k_{cat}/K_m)_{slow}$[19]] are not attributed to nonproductive binding. The $K_m$ value of (*S*)-**1a** may seem to be slightly larger than those of the other substrates. However, it is clear that the extremely low reactivity of (*S*)-**1a** does not result from such binding of the enantiomer.

The $K_m$ values of all five acetates are regarded as being almost identical whether or not the substrates have a medium-sized substituent $L_M$ at the stereocentre in the alcohol moiety, and regardless of the position and direction of the $L_M$. This may indicate that all substrates examined are bound to the enzyme in the same manner by the methyl group of the acid moiety, ester bond and the large substituent group $L_L$ including the ethyl backbone and that $L_M$ does not play an important role in the binding.

These results do not support the cubic-spaced model explaining that fast and slow reactions result respectively from good and poor fit of the enantiomers into the cubic-spaced binding sites.[4a,7a,8] The above findings are also incompatible with the two-site model in which $L_L$ and $L_M$ were tightly bound to the larger hydrophobic subsite $\rho_1$ and the smaller subsite $\rho_2$, respectively, and these bindings determine the position and the distance of the carbonyl carbon of the enantiomer to be attacked from the nucleophilic Ser in the catalytic site.[9]

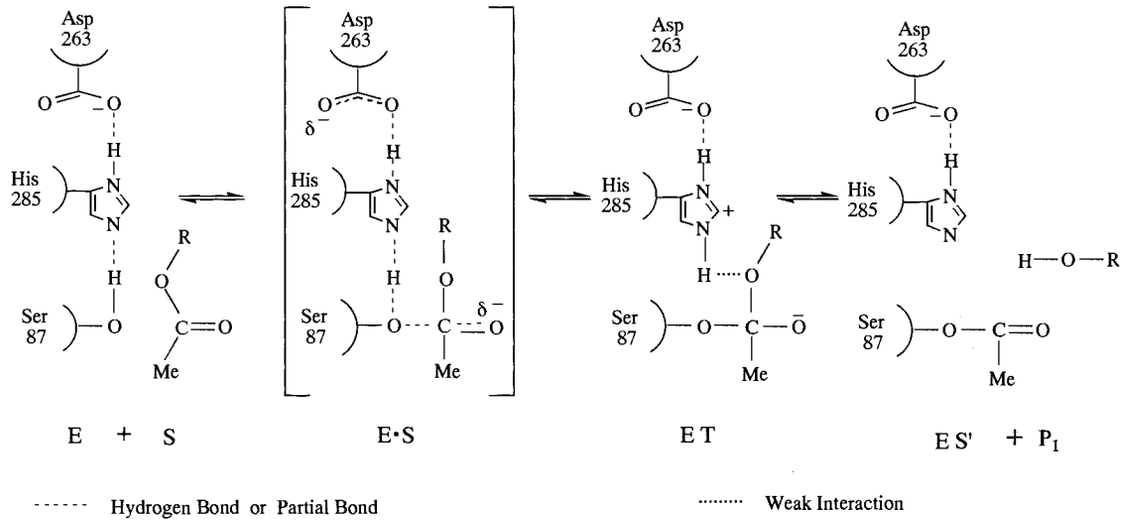
### Acylation rate constant

The different $k_{cat}$ values for at least four substrates, namely (*R*)- and (*S*)-**1a**, and (*R*)- and (*S*)-**1b**, confirm that the formation of the acyl-enzyme intermediate is rate-determining in the overall reaction since the rate of breakdown of the same acyl-enzyme intermediate should be identical.

It is obvious that the strict enantioselectivity in the hydrolysis of **1a** is due to the extremely small $k_{cat}$ value of the (*S*)-**1a**. A similar tendency was observed for the hydrolysis of a cyclic secondary alcohol ester with *Arthrobacter* sp. lipase in which the slow-reacting (*S*)-enantiomer was demonstrated to be a competitive inhibitor of its antipode.[9a,9b]

It is crucial to compare $k_{cat}$ with $k_{cat}/K_m$, which gives a true measure of an enzyme's overall preference for a particular substrate, when we discuss the $k_{cat}$ values of a number of substrates. The order of magnitude of the acylation rate constant for the fast-reacting enantiomers seems to be **1c** ≈ **1b** > **1a**, especially when the $k_{cat}$ values were compared with the $k_{cat}/K_m$, which reflect the rate-determining step more correctly than any other kinetic constants.

The observed reactivities may reflect primarily the leaving abilities of the alcoholate ions. The electron-releasing methyl group attached to $C^1$ will give the secondary alcohol **2a** a slightly lower leaving ability than the primary alcohol **2c** having no methyl group. On the other hand, the effect of the substituent at $C^2$ is screened to a considerable extent. Thus, the leaving




**Scheme 1**

ability of the primary alcohol **2b** can be nearly identical to that of **2c**.

**Rate-determining breakdown of ET (see Scheme 1)**

The above observations indicate that the breakdown of ET is rate-determining in the acyl-enzyme intermediate formation and hence in the overall reaction. If this is the case, the observed $k_{cat}/K_m$ values reflect most correctly the rate-determining breakdown of ET which is presumably the key step of the stereospecificity of the enzyme.

It is well accepted that the p$K_a$ value of the catalytic serine hydroxy nucleophile is significantly smaller (more acidic) than that of an ordinary aliphatic alcohol.[20] The p$K_a$ values of the alcohols **2a**, **2b** and **2c** can be roughly identical with that of ethyl alcohol (*ca.* 16). Such alcohols can be converted to good leaving groups by being protonated in nucleophilic substitutions on the carbonyl group.[21] Thus, in terms of the Hammond postulate, it is conceivable that the breakdown of ET is rate-determining in the acyl-enzyme intermediate formation. This is also in good accordance with previous findings in the acylation of the serine proteases by specific substrates.[13c] It was indicated that the breakdown of ET was the rate-determining step in the cases of peptide[13c] and ethyl ester[18a] substrates but that the formation of such an intermediate was rate-determining for labile *p*-nitrophenyl ester.[18a] Although the absence of ET was argued later in the acylation of chymotrypsin by specific methyl ester substrates,[22] this would not exclude the possibility that the formation and breakdown rates were of the same order of magnitude in these cases.

An assumption that the formation of ET is rate-determining is not consistent with the above observations at all. Since the mechanism of the acylation is considered to be the same in all five esters and there is no compelling reason to believe otherwise, the finding that all five substrates are bound to the enzyme in the same manner requires that the carbonyl carbons of the five esters are attacked identically by the catalytic serine nucleophile. It is very clear that the (*R*)- and (*S*)-configurations do not account for such a great difference in reactivities chemically.

**Perfect distinction between (*R*)- and (*S*)-1d**

Time courses are shown for the extent of hydrolysis of **1a**, **1b**, **1c** and 1-ethyl-2-(4-phenoxyphenoxy)ethyl [1-(4-phenoxyphenoxy)-2-butyl] acetate **1d** and for the enantiomeric excess (ee) of the liberated alcohol **2d** in Fig. 2.

The **2d** is obtained with 100% ee of the (*R*)-enantiomer regardless of the extent of hydrolysis. This indicates the enzyme distinguishes (*R*)-**1d** from its antipode perfectly. This also makes an interesting contrast with the case of **1a** in which the ee values of the liberated (*R*)-**2a** decreased with the hydrolysis

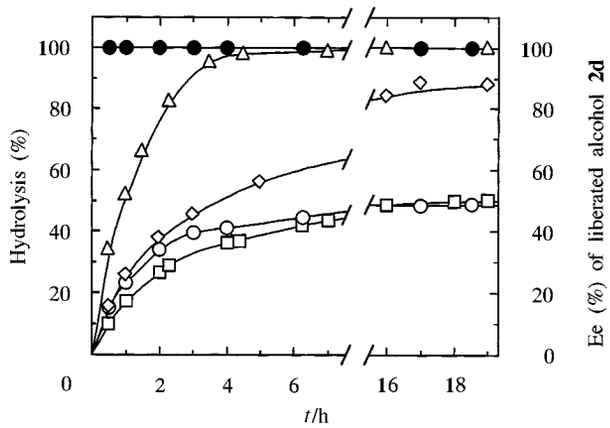



**Fig. 2** Time course of hydrolysis of **1a–d** with PCL. Reaction conditions: substrate, 4.37 mmol; PCL, 19.5 mg protein; phosphate buffer (0.2 mol dm$^{-3}$), making up the total volume of 50 cm$^3$ (pH 6.8 which was maintained constant with 1 mol dm$^{-3}$ NaOH solution); 40 °C; 1000 rpm. Hydrolysis degree (%) of **1a** (□), **1b** (◇), **1c** (△) and **1d** (○); and ee (%) of liberated (*R*)-**1d** (●).

extent very slightly (*e.g.* 99.7, 98.6 and 98.2% ee at 37.2, 46.3 and 50.6% hydrolysis, respectively). The different behaviour of **1d** from **1a** is entirely attributed to the substitution of $CH_3CH_2$ for $CH_3$ at $C^1$ of the alcohol moiety. It is remarkable that $L_M$, being less important in the binding, plays such a crucial role in the enantioselectivity.

The initial reaction rate of **1d** appears to be higher than that of **1a**. After reaching *ca.* 30% hydrolysis in 2 h, however, the reaction rate reduces drastically to be exceeded by that of **1a** and tends to 50% very slowly. It appears never to become greater than 50%, in contrast to the case of **1a**. The drastic decrease of the reaction rate may indicate that the enzyme was surrounded and bound tightly by large amounts of inhibitors in the final stage of the reaction.

The hydrolysis of **1c** proceeds smoothly until the substrate is consumed completely, as expected. The reactions of **1a** and **1b** are reproducible. The time dependent curves of the two substrates are identical to those previously reported[6] for all the differences of reaction scales.

**Hindrance to breakdown of ET by a medium-sized substituent**

The foregoing results and discussion indicate that $L_M$ of the slow-reacting enantiomer may be positioned close to the catalytic histidine side-chain in the PCL-catalysed hydrolysis of the series of 2-(4-phenoxyphenoxy)ethyl acetates. As a result, it hinders $N^{\varepsilon 2}$ of His 285 from forming a weak interaction with $O^1$ of the leaving alcohol in ET. Since a proton transfer from the

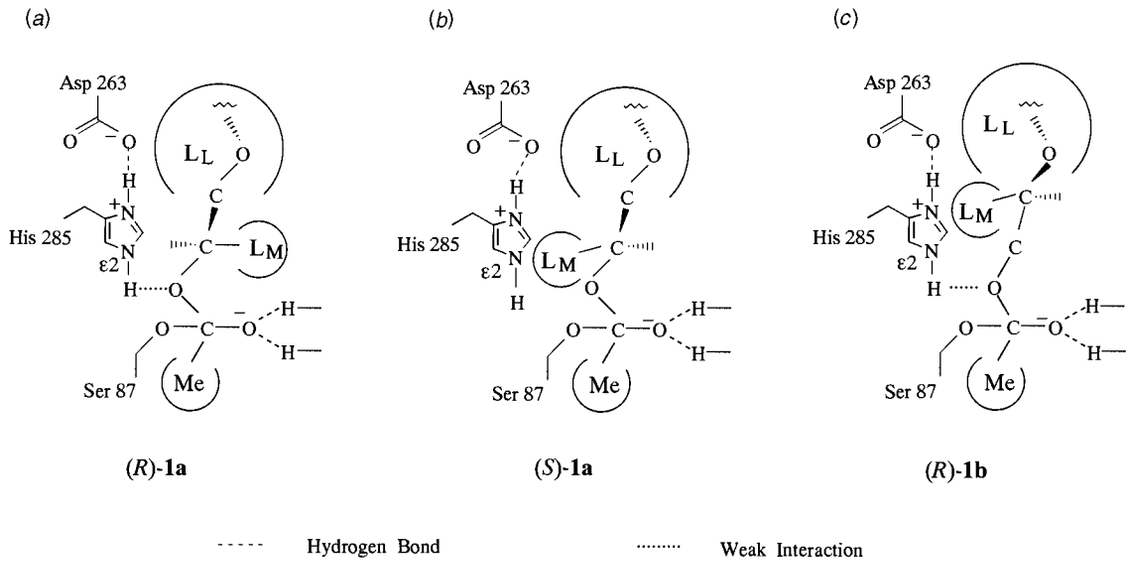



**Fig. 3** Schematic diagram of the proposed mechanism for the breakdown of ET. (*a*) For the fast-reacting enantiomer, a medium substituent ($L_M$) is positioned away from the imidazole ring of catalytic His 285, $N^{\varepsilon 2}$ of which forms a weak interaction with $O^1$ of the leaving alcohol. As a result, the breakdown of ET occurs through proton transfer to $O^1$. (*b*) $L_M$ of the slow-reacting enantiomer is positioned toward the His 285 side-chain and hinders the weak interaction with $O^1$. The breakdown of ET is retarded or inhibited consequently. It is understandable that a relatively large $L_M$ such as an ethyl group hinders completely once a sufficiently large $L_L$ is bound to the hydrophobic pocket of the enzyme tightly. (*c*) The hindrance may be smaller in the case of the primary alcohol than the corresponding secondary one since $L_M$ attaches to the farther $C^2$.

imidazole ring of the histidine to the leaving group leads to the breakdown of ET in a non-labile leaving group such as ethyl alcohol or amide, the lack of such an interaction and hence the protonation will cause slower or no breakdown of ET [Fig. 3(*b*)].

On the other hand, $L_M$ of the fast-reacting enantiomer may be positioned out of the imidazole ring of His 285 so that the breakdown of ET proceeds smoothly and may be faster than for the opposite enantiomer [Fig. 3(*a*)].

The proposed mechanism accounts very well for not only the results of (*R*)- and (*S*)-**1a** as above but also the lower enantioselectivity in the primary alcohol **1b** ($E = 5.8$) and the perfect distinction between (*R*)-**1d** and (*S*)-**1d** ($E = \infty$). In the case of (*R*)-**1b**, the methyl group close to His 285 is located farther from $O^1$ of the leaving group than that of (*S*)-**1a** so that the hindrance will be smaller than in the case of (*S*)-**1a** [Fig. 3(*c*)], whereas in the case of **1d**, the larger ethyl group at $C^1$ of the (*S*)-enantiomer may hinder $N^{\varepsilon 2}$ of His 285 from forming a weak interaction with the $O^1$ completely once the substrate is bound tightly to the enzyme by $L_L$, the ester bond and the methyl group of acetic acid.

### Combination with X-ray structural study

Recently, the X-ray structures of *Candida rugosa* lipase (CRL)–inhibitor complexes were studied for the hydrolysis of menthyl esters.[12a] Analysing the bond distances in the complexes, it was proposed[12b] that the isopropyl substituent (a part of $L_L$) of the slow-reacting enantiomer pointing toward the imidazole ring of the catalytic histidine made $O^1$ of menthol lie too far from the $N^{\varepsilon 2}$ to form a hydrogen bond and that this lack of a hydrogen bond brought about the slower reactivity of the enantiomer.

It is interesting that the two studies done with different methods reached the same point, that the lack of protonation causes the slower reaction of one enantiomer. This is remarkable because the two enzymes belong to different families of lipase and have different binding sites[3,5,23] and substrate specificites.[2,24]

This study shows that investigation of the enzyme reactions, including kinetics of chiral and achiral substrates, is useful in understanding the mechanism of action of the lipase although ET was not observed directly. Obviously, combining knowledge of the X-ray structures and of the reactions provides much stronger tools to elucidate the mechanism of the specificity of lipases. The measurement of the X-ray structures on the PCL–inhibitor complexes, for instance, may facilitate our understanding of the mechanism of the stereospecificity of the enzyme. This study may be a step in that direction.

## Experimental

$^1$H NMR spectra were obtained at 60 MHz on a Hitachi R-24B. Tetramethylsilane was used as internal standard; *J* in Hz. GLC and high performance liquid chromatography (HPLC) were recorded with a Shimadzu CR-1A and LC-6A, respectively. Melting points are uncorrected.

### Chemicals

Preparation procedures of 1-methyl-2-(4-phenoxyphenoxy)-ethanol **2a** and 2-methyl-2-(4-phenoxyphenoxy)ethanol **2b** were as described.[6]

2-(4-Phenoxyphenoxy)ethanol **2c** was prepared in a similar manner to **2b** with several modifications. To a solution of 4-phenoxyphenol (10.0 g, 54 mmol) and potassium carbonate (14.8 g, 107 mmol) in anhydrous *N*,*N*-dimethylformamide (50 $cm^3$), ethyl monochloroacetate (6.91 g, 56 mmol) was added dropwise with stirring for 10 min, the mixture then being stirred for 15 h at room temp. The reaction mixture was poured into cold water (200 $cm^3$) and extracted with diethyl ether (200 $cm^3$). The extracts were washed with water, dried over anhydrous magnesium sulfate and concentrated to give crude ethyl (4-phenoxyphenoxy)acetate (EPA, 12.4 g, 45 mmol) as a colourless liquid. To a suspension of lithium aluminium hydride (2.60 g, 68 mmol) in anhydrous tetrahydrofuran (THF, 50 $cm^3$) was added the crude EPA in anhydrous THF (20 $cm^3$) with stirring for 30 min at 0 °C, and then for 1 h at room temp. After cooling to 0 °C again, cold water (10 $cm^3$) and then cold dilute hydrochloric acid (50 $cm^3$) were added to the mixture slowly. After stirring for 30 min, the reaction mixture was poured into water (200 $cm^3$) and extracted with diethyl ether (200 $cm^3$, twice). The organic phase was washed with water, dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography to give **2c** (9.95 g, 43 mmol) as colourless crystals, mp 65.5 °C; $\delta_H(CDCl_3)$ 2.6–2.9 (1H, m, OH), 3.7–4.3 (4H, m, $CH_2CH_2$) and 6.6–7.5 (9H, m, Ar H).

1-Ethyl-2-(4-phenoxyphenoxy)ethanol **2d** was prepared in a manner similar to **2a** with the replacement of 1-chloropropan-2-ol by 1,2-epoxybutane. After washing, drying and concentrating the reaction mixture of sodium 4-phenoxyphenoxide and 1,2-epoxybutane, the residue was chromatographed on silica gel to give **2d** (1.65 g, 6.4 mmol); $\delta_H(CDCl_3)$ 1.0 (3H, t, *J* 6), 1.3–1.9 (2H, m, $CH_2Me$), 2.3 (1H, br s, OH), 3.5–4.2 (3H, m, 1-H and 2-H) and 6.5–7.5 (9H, m, Ar H).

Acetylation of **2a** and **2b** and purification of **1a** and **1b** have already been described.[6] The alcohols **2c** and **2d** were acetylated and purified in the same manner as **1a**. From the same GLC analysis as in the case of **1a** and **1b**, the chemical purities of the acetates **1c** and **1d** were estimated to be higher than 99 and 97%, respectively. All other chemicals used were of reagent grade.

(*R*)- and (*S*)-**1a** and (*R*)- and (*S*)-**1b** were prepared through the enzyme-catalysed hydrolysis reactions of **1a** and **1b**, respectively. For the preparation of (*R*)- and (*S*)-**1a**, PCL was used because of its strict enantioselectivity.[6] The vigorous stirring was halted at 50.5 to 51% hydrolysis to stop the reaction of the emulsified reaction mixture. The resulting mixture of (*R*)-**2a** and (*S*)-**1a** was extracted with toluene. The extract was separated into (*R*)-**2a** and (*S*)-**1a** through column chromatography on silica gel using 5:1 hexane–ethyl acetate as eluent. Effluent fractions containing (*R*)-**2a** or (*S*)-**1a** were concentrated independently of each other to give the compounds. The (*R*)-**2a** thus obtained was recrystallized from hexane to yield (*R*)-**2a** of a high ee value (>98% ee). The acetylation of (*R*)-**2a** was carried out in the same manner as for **2a** to give (*R*)-**1a** having a chemical purity of >99%. The concentrated (*S*)-**1a** was confirmed to have >99% chemical purity and >98% ee. For the preparation of (*R*)- and (*S*)-**1b**, on the other hand, lipase from *Chromobacterium viscosum* (CVL) was used because the enzyme showed the greatest enantioselectivity and the highest activity for **1b**.[6] The enzymatic reaction was stopped at 20–25% hydrolysis to obtain (*S*)-**2b** having ⩾85% ee. The (*S*)-**2b** was separated from the unhydrolysed acetate by silica gel column chromatography and recrystallized from hexane for improvement of the ee value. The (*S*)-**2b** thus obtained was acetylated to give (*S*)-**1b** of >99% chemical purity and >98% ee. To obtain (*R*)-**1b** having a sufficiently high ee value, the enzymatic reaction was stopped at 75–80% hydrolysis. The unhydrolysed acetate was separated from the liberated alcohol by silica gel column chromatography. However, the acetate thus obtained did not yet have ee as high as >98%. The enantiomeric ester was therefore hydrolysed by potassium methoxide and the (*R*)-**2b** produced was recrystallized from hexane. After improvement of the ee value, the alcohol was acetylated again to give (*R*)-**1b** having a chemical purity of >99 and >98% ee.

### Enzymes

PCL was provided from Amano Pharmaceutical Co., Aichi [Lot No: LPHO-5512, 1010 unit $mg^{-1}$ where one unit of lipase activity was defined as the amount of enzyme that liberated 1 μmol equiv. of fatty acid from Fatgen (20% sesame oil emulsion, Dainippon Pharmaceutical Co.) in 1 min at pH 7.0 and 37 °C]. The enzyme was described to be pure at least as a lipase.[24] The activity expressed on Fatgen was indeed more than ten times higher than that of the enzyme used previously.[25] The enzyme consists of a single chain protein and contains neither sugar nor lipid.[26] The protein concentration of the enzyme was determined by the method described previously.[25b] All of the protein was assumed to have catalytic activity. A molecular mass of 33 000[26] was used in this study. The value was in good agreement with that evaluated for *Pseudomonas glumae* lipase[23] in which the corresponding amino acids were identical to those in PCL, except for one amino acid residue. CVL was obtained from Asahi Chemical Co., Shizuoka (Lot No: LP-656-s) and used only for the preparation of both (*R*)- and (*S*)-**2b**. The two enzymes were used without further purification.

### Initial rate of hydrolysis

The kinetic studies were done by measuring the initial rates of hydrolysis of chiral and achiral substrates (*R*)- and (*S*)-**1a**, (*R*)- and (*S*)-**1b** and **1c**. The initial rates were measured using a pH-stat autotitration system (Radiometer Co., Copenhagen: Titrator, TTT80; Autoburette, ABU80 and pH meter, PHM82) as was described previously[27] with some modifications. The substrate solution consisted of a suitable amount (10–300 mg) of each substrate, 1 $cm^3$ of McIlvaine buffer solution (pH 7.0), 1 $cm^3$ of 2% polyvinyl alcohol and distilled water. The amount of water was adjusted to make the total volume of the solution 9 $cm^3$. After 10 min of incubation of the solution at 55 ± 0.5 °C, the enzymatic reaction was started by addition of PCL solution (1 $cm^3$) at a stirring rate of 1000 rpm to achieve a sufficiently emulsified reaction mixture.[16] The liberated acetic acid was titrated with 0.01 or 0.05 mol $dm^{-3}$ NaOH back to the initial pH. The reaction temperature was chosen to avoid partial solidification of **1c** at high concentrations. Thermal inactivation was not observed at that temperature.[6] Spontaneous hydrolysis was subtracted in every assay although it was not very significant. Amounts of PCL used for (*R*)-**1a**, (*R*)- and (*S*)-**1b** and **1c** were 3.54–22.9 μg of protein in each reaction run, whereas 1.55 mg of protein was used for (*S*)-**1a**, in inverse relation to the reactivities of the substrates.

The values of $K_m$, $k_{cat}$ and $k_{cat}/K_m$ were calculated by a computer program for the least-squares analysis of an Eadie–Hofstee plot. Error limits for these values reflect the standard deviation calculated from the least-squares analysis and do not reflect possible sources of systematic error such as pH and ionic strength dependent perturbations.

### Time course of hydrolysis

The following reaction mixture was prepared to examine the time courses of the PCL-catalysed hydrolysis of **1a–d**. The mixture contained 4.37 mmol of the water-insoluble substrate, 19.5 mg of PCL and 0.2 mol $dm^{-3}$ of phosphate buffer solution (pH 6.8) to make up the total volume of 50 $cm^3$. The enzymatic hydrolysis was started by emulsifying the mixture by stirring at 1000 rpm. The mixture was kept at 40 °C under $N_2$ atmosphere. The pH was kept constant during the reaction with 1 mol $dm^{-3}$ NaOH solution. 50 $mm^3$ of the emulsion were pipetted out at certain reaction times. The liberated alcohol and the remaining substrate were extracted with toluene and analysed.

### Analysis

The determination of the hydrolysis degree by GLC analysis and the enantiomeric analysis of the liberated alcohols using HLPC have already been described in detail.[6] Retention times were 12.4 and 13.6 min for (*R*)- and (*S*)-**2d**, respectively. The peaks of (*R*)- and (*S*)-**2d** were completely separated from each other as found in the cases of both **2a** and **2b**.

The establishment of the absolute configurations of (*S*)-**2a** as well as (*R*)-**2b** has been described previously.[6] The absolute configuration for (*S*)-**2d** [or (*R*)-**2d**] was not determined from a chiral starting material synthetically. The peaks of (*R*)- and (*S*)-**2d** were assigned tentatively from the order of the retention times of (*R*)- and (*S*)-**2a**, considering the similarity of the molecular structures. The assignment was in agreement with the predicted enantiopreference of the enzyme from the empirical rule (Fig. 1).

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