Predictive Rules for the Enantioselectivity of Hydrolases towards Alcohols and Amines

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To Mops, Pops, Phillip and Marc

thank you for always being there for me

&

For my "medium boy", Julien

with love big like the sky

Mirror

When you look
into a mirror
it is not
yourself you see
but a kind
of apish error
posed in fearful
symmetry.

John Updike

Abstract

To help organic chemists use enzymes as synthetic reagents, guidelines are needed to help them choose an appropriate enzyme.

We proposed reliable empirical substrate rules that predict the stereochemical outcome of reactions catalyzed by hydrolases. These rules, developed through a combination of substrate screening and literature surveys, are based on the difference in size of the substituents at the stereocenter of substrates. One proposed rule predicts the enantiopreference of cholesterol esterase, lipase from *Pseudomonas cepacia*, and lipase from *Candida rugosa* towards secondary alcohols and their esters.

A similar rule is proposed to predict the enantiopreference of subtilisins towards isosteric primary amines of the type NH_2CHRR'. The enantiopreference of lipases towards primary amines and of subtilisins toward secondary alcohols is reviewed. Lipases and subtilisins have opposing enantiopreferences towards both secondary alcohols and primary amines. We also observed that the regioselectivity of subtilisin is opposite to that of lipases. We offer a rationalization to explain these opposing selectivities, based on known crystal structures of lipases and subtilisin.

A similar rule is also found to predict the enantiopreference of lipase from *Pseudomonas cepacia* towards primary alcohols; this rule excludes substrates having an oxygen atom directly attached to the stereocenter. The favored enantiomer of primary alcohols is opposite to the favored enantiomer of secondary alcohols. Experiments conducted on substrates with two stereocenters and molecular modeling studies suggest that both classes of alcohols bind in the same regions of the active site.

We proposed a method to enhance enantioselectivity of lipases and esterases towards secondary alcohols. This technique, based on increasing the difference in size of the substituents at the stereocenter, was successfully applied

to the preparation of two useful chiral synthons: (*S*)-(-)-4-acetoxy-2-cyclohexen-1-one and esters of (*R*)-lactic acid.

Using a similar approach, we designed a synthetic scheme for the preparation of both enantiomers of a useful C_2-symmetric synthon. The key step is an acylation reaction catalyzed by lipase from *Candida rugosa*. (1*R*,4*R*)- and (1*S*,2*S*)-bicyclo[2.2.1]heptan-2,5-diones were obtained with high optical purity.

Résumé

Les chimistes organiciens ont besoin de nombreuses données pour les aider à trouver l'enzyme qu'ils recherchent.

Nous avons proposé et validé des règles empiriques qui permettent de prédire, à partir du substrat, la stéréochimie du produit obtenu lors de réactions catalysées par les hydrolases. Ces règles, mises au point grâce à nos résultats et à ceux de la littérature, sont basées sur la différence de taille des substituants portés par le carbone asymétrique du substrat. Une des règles que nous proposons permet de déterminer l'énantiosélectivité de l'estérase du cholestérol et de lipases issues de *Pseudomonas cepacia* et *Candida rugosa,* vis-à-vis d'alcools secondaires et de leurs esters correspondants.

Selon le même principe, nous pouvons prédire l'énantiospécificité des subtilisines pour des amines isostériques primaires du type H_2NCHRR'. Nous avons révisé les connaissances concernant l'énantiosélectivité des lipases vis-à-vis des amines primaires et des subtilisines vis-à-vis des alcools secondaires. Les lipases et les subtilisines font preuve d'énantiosélectivité opposée envers à la fois les alcools secondaires et les amines primaires. Nous avons également observé que leur régiosélectivité est différente. La connaissance de leurs structures cristallines respectives nous a permis d'expliquer ces différences de sélectivités.

Une règle similaire nous a également permis de prédire l' énantiosélectivité d'une lipase issue de *Pseudomonas cepacia* vis-à-vis des alcools primaires. Cette règle n'est pas valable pour des substrats ayant un atome d'oxygéne lié directement au carbone asymétrique. Dans le cas des alcools primaires, la configuration absolue de l'énantiomère majoritairement obtenu est opposée à celle observée pour les alcools secondaires. Des expériences réalisées sur des substrats ayant 2 centres asymétriques et des études de modélisation moléculaire suggèrent que toutes les classes d'alcools se fixent dans la même région du site actif de l'enzyme.

Nous avons proposé une méthode permettant d'augmenter l'énantiosélectivité des lipases et des estérases pour les alcools secondaires. Cette technique, basée sur l'augmentation de la différence de taille des substituants portés par le carbone asymétrique, a été appliquée avec succès à la préparation de 2 synthons chiraux recherchés : le (*S*)-4-acétoxy-2-cyclohex-1-one et les esters de l'acide (*R*)-lactique.

En utilisant une approche similaire, nous avons proposé un schèma synthétique pour la préparation des 2 énantiomères d'un synthon important, de symétrie C_2. L'étape clef est une réaction d'acylation catalysée par une lipase issue de *Candida rugosa*.. Les composés (1*R*, 4*R*) et (1*S*, 2*S*)-bicyclo [2.2.1]heptan-2,5-dione ont été obtenus avec une pureté optique elevée.

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Acknowledgements

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I wish to thank my many collegues, past and present, both in Otto Maass, Room 346 and at the URA 400, for their friendship and for helping me in so many ways. In particular, I would like to mention Didier Buisson, Gaetan Caron, Louis Cuccia, Lana Janes, Joanne Krupa, Isabelle Lacroix, Cecile Moussa, Christine Ng, and Alessio Serreqi. I also owe many thanks to those students in Prof. G. Just's and Prof J. Chin's with whom I had many helpful discussions. In addition, I would like to thank Christine Guérard for her enormous help in translating the abstract into french.

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Last but not least I would like to thank two people who have been very special to me for many years. My dearest friend T.H., if it wasn't for those initials, this thesis may never have come to be, and Yves, who has always supported me in more ways than one.

Contributions of Authors

Chapters 2, 3, 4, and 5 have been published and the typeset versions appear as appendices to each chapter.

The work in Chapter 2 was done under the supervision of my research director and with the collaboration of two undergraduate students. A portion of this work was initially started during my undergraduate honours research project. My contribution to the research described in Chapter 2 consisted of the following. The synthesis of *cis*-1,4-diacetoxy-2-cyclohexene,(±)-1α,4α-diacetoxy-2β,3α-dibromocyclo-hexane, (*S*)-(-)-4-acetoxy-2-cyclohexexen-1-one, and all of their derivatives. I performed all the enantioselective hydrolysis studies on these cyclic substrates. I did the large scale asymmetric synthesis of optically-pure (*S*)-(-)-4-acetoxy-2-cyclohexen-1-one and the determination of its enantiomeric purity and absolute configuration. I interpreted all the experimental and structure determination data obtained for these cyclic compounds. In addition, I also helped with the literature survey required for the development of the rule. Aviva T. Rappaport and Louis A. Cuccia performed all the experimental work associated with the lactate ester derivatives. Aviva T. Rappaport also obtained the enantioselectivity data for cholesterol esterase shown in Table 1.

I conducted the research described in Chapters 3 and 4, under the guidance my research director Prof. R. J. Kazlauskas. Chapter 5 is an account of the research I did under the supervision of Prof. R. Azerad at the Université René Descartes, Paris, France.

Although the writing of the published articles was a combined effort between myself and Prof. Kazlauskas or Prof. Azerad, I did the bulk of the writing of the papers for which I am first author.

Note to reader

This doctoral thesis is presented as a manuscript-based thesis and is formatted according to the McGill University guidelines for thesis preparation.

The area of bioorganic chemistry covered in the introduction of this thesis developed very quickly during the time that the research described in this thesis was on going. Therefore, for the sake of continuity and clarity, some of my work has been included as part of the literature review in the introduction.

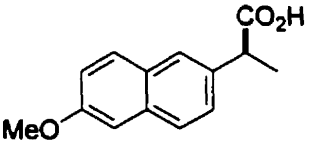
Chapter 1

General Introduction

1.1 The importance of hydrolases in the chiral world

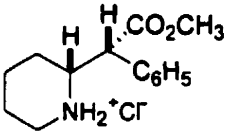
The synthesis of optically pure compounds has been one of the most challenging tasks of chemists for many decades. Enantiomers are molecules that are non-superimposable mirror images. A mixture that contains equal amounts of each enantiomer is called a racemate or racemic mixture. Enantiomers have the same physical properties, other than their ability to rotate plane polarized light in different directions, and their chemical properties are identical except when they interact with a chiral environment. For this reason, their separation is difficult and often involves long and complicated processes. However, the importance of obtaining optically pure compounds has been demonstrated repeatedly, especially in the pharmaceutical and agrochemical industries.[1]

For example, in the pharmaceutical industry, it is necessary to assess the bioactivity of each enantiomer of a chiral compound separately; one enantiomer may have the desired biological activity while the other may be inactive or have a different activity. This minimizes the possibility of undesirable or dangerous side effects that may be caused by one of the enantiomers but not the other. If one of the enantiomers is inactive, chemical pollution of the body may result from the ingestion of more drug than is necessary. For example, the *S* enantiomer of naproxen, a non-steroidal anti-inflammatory drug, is 28 times more effective than the *R* enantiomer and 3 times more effective than the racemate.[2]



(*S*)-(+)-naproxen

In 1997, at least 50% of the top selling pharmaceutical drugs were single-enantiomers,[3] and the number, for synthetically-derived drugs, is expected to reach 75% by the year 2000.[4] In addition to the development of new optically active drugs, many drugs that have already been approved and marketed in their racemic form are being redeveloped for marketing as single isomers. For example, Ritalin, a drug used for children with attention deficit disorder, has been marketed as the racemic *threo*-diastereoisomer of methylphenidate. It is now being developed as the enantiomerically-pure, (2*R*,3*R*), *threo*-isomer, in order to minimize the side effects of insomnia and appetite suppression.[3]



(2*R*,3*R*)-methylphenidate

One factor that must be considered is the severe environmental chemical pollution can result from the production of enantiomerically-pure material. This, in itself, can become a health concern. Therefore, in endeavoring to find cost-effective methods of manufacturing optically-pure compounds, researchers must consider any potential harmful effects to the environment.

Great advances have been made in the domain of biocatalysis over the past decade and, as a result, chemists have become more aware of the usefulness of

enzymes in the preparation of optically-pure compounds.[5,6,7] Enzyme catalysis has proven to be a good solution to the problem of efficiency and environmental acceptability. As a result, enzymes have found many applications in academic research and in industry, including the food, surfactant, agricultural, and pharmaceutical industries.

Enzymes are globular proteins having an active center that is capable of catalysis, Figure 1.1. One of the most important features of enzymes is that they have the intrinsic ability of chiral recognition. That is, they are capable of stereoselectively catalyzing chemical reactions, producing optically active products.

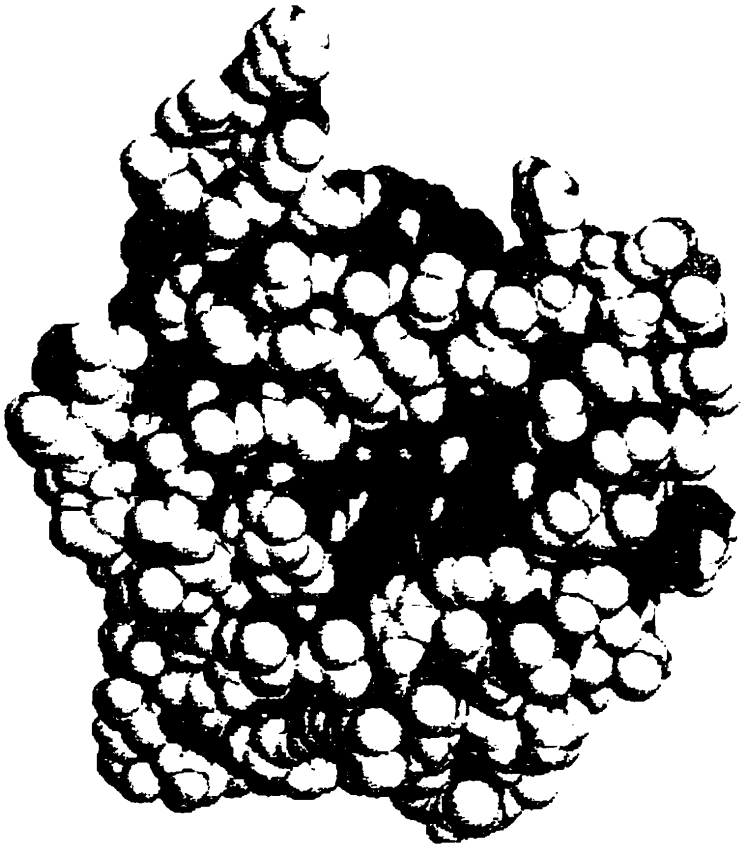


Figure 1.1. The crystal structure of lipase from *Pseudomonas cepacia* shows the globular shape of enzymes. The catalytic triad residues are situated at the bottom of the substrate binding site. Serine is shown in orange, histidine in blue, and the speck of red corresponds to the aspartic acid residue. This figure was created using RasMol v2.6 and coordinates from the PDB data files (accession code 4lip).[8]

Hydrolases are a class of enzymes that catalyze the cleavage or formation of esters, amides, lactones, lactams, epoxides, nitriles, anhydrides, and glycosides. There are over 600 hydrolases identified, of which at least 125 are commercially available. They are one of the most widely used types of enzymes due to the fact that they do not require the use of expensive and sensitive cofactors. Hydrolases are also highly stable and can be used under mild and unusual conditions, consequently, they are easy to manipulate. They often show a high tolerance for varying substrate structure and yet maintain high enantioselectivity. Finally, many hydrolases are inexpensive and readily available. Lipases, esterases, and proteases all belong to the family of hydrolases. Lipases, whose natural substrates, lipids, possess a chiral alcohol group, are the most commonly used. Two of their main advantages are that they are highly stable in organic solvents and there are a large number of them available.

The work of Sih, *et al*[9] illustrates the usefulness of hydrolases in synthetic organic chemistry. They prepared an important intermediate in the synthesis of taxol, a potent antitumor drug, with high optical purity using a simple chemoenzymatic process. The key step in this process was the lipase-catalyzed hydrolysis of the acetyl ester of 3-hydroxy-4-phenyl-β-lactam, Figure 1.2.

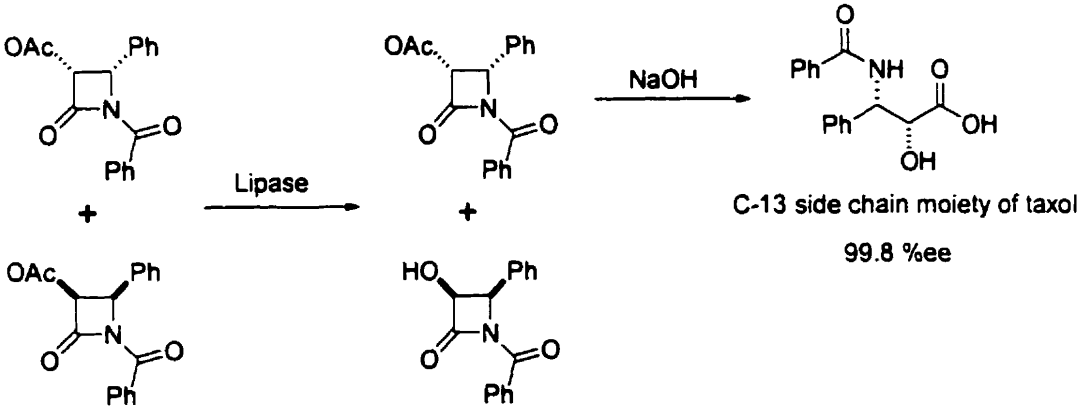


Figure 1.2. Chemoenzymatic preparation of an important intermediate in the synthesis of taxol.

Another example is given in Figure 1.3. The lipase-catalyzed resolution of a useful chiral synthon, 4-*endo*-hydroxy-2-oxabicyclo[3.3.0]oct-7-en-3-one, afforded both enantiomers with high optical purity. This resolution allowed for the efficient synthesis of two important chiral compounds: (-)-carbovir, an anti-HIV agent, was synthesized from the (-)-hydroxylactone and (+)-brefeldin A, an anti-fungal agent, was obtained from the other enantiomer.[10]

In addition the use of enzyme catalysis has often considerably cut the cost of producing enantiomerically-pure intermediates. For example, Baust-Timpson reduced the production costs for both isomers of α-phenethyl alcohol by 75%, using an enzyme-catalyzed resolution method.[3]

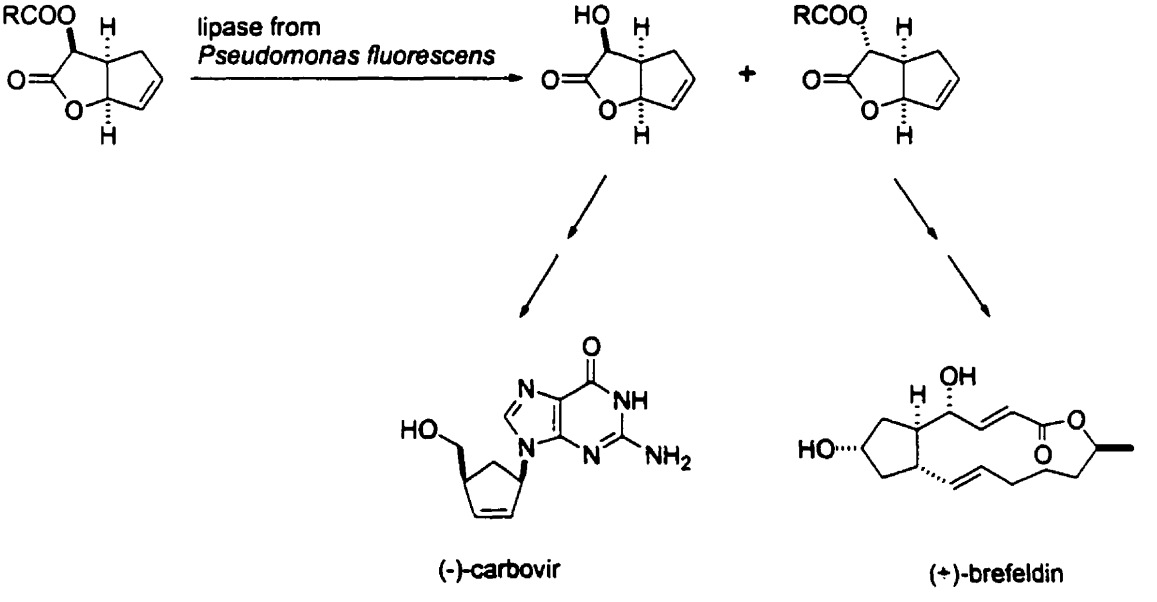


Figure 1.3. Two useful single-enantiomer chiral drugs can be obtained via the lipase-catalyzed resolution of a hydroxylactone.

The use of enzymes as tools in organic synthesis is becoming more popular, however, despite their many advantages, many chemists are still somewhat reluctant to use them. This is in part due to the fact that the great number of enzymes available is overwhelming, making the task of choosing an appropriate enzyme seem, at first, rather daunting. In order to make enzymes more appealing to chemists, predictive models and rules have been developed to facilitate the selection of an enzyme for a given substrate and transformation. These models and rules are based on the study of hydrolase substrates and non-substrates as well as the binding sites, the specific areas on or within enzymes to which a substrate is bound as it undergoes catalytic reaction.

This thesis describes the development of rules used to predict the stereochemical outcome of transformations of alcohols and amines by various hydrolases. A method to enhance the selectivity of hydrolases was developed, based on one of these rules. The value of these rules is demonstrated by their application in the preparation of several useful chiral synthons.

1.2 Enantioselective reactions

The types of selectivities that enzymes exhibit are chemoselectivity, regio- and diastereoselectivity, and enantioselectivity. Enantioselectivity[11] is the type of selectivity most studied in this thesis, although the other selectivities are also examined to a lesser extent.

The chiral environment of the active site allows for hydrolases to conduct enantioselective reactions. There are two major categories of enantioselective reactions catalyzed by hydrolases: kinetic resolutions and asymmetric syntheses.* A kinetic resolution is the selective transformation of only one enantiomer of a racemic substrate (Figure 1.4a). An asymmetric synthesis involves the transformation of a meso or prochiral substrate into a chiral product (Figure 1.4b,c).

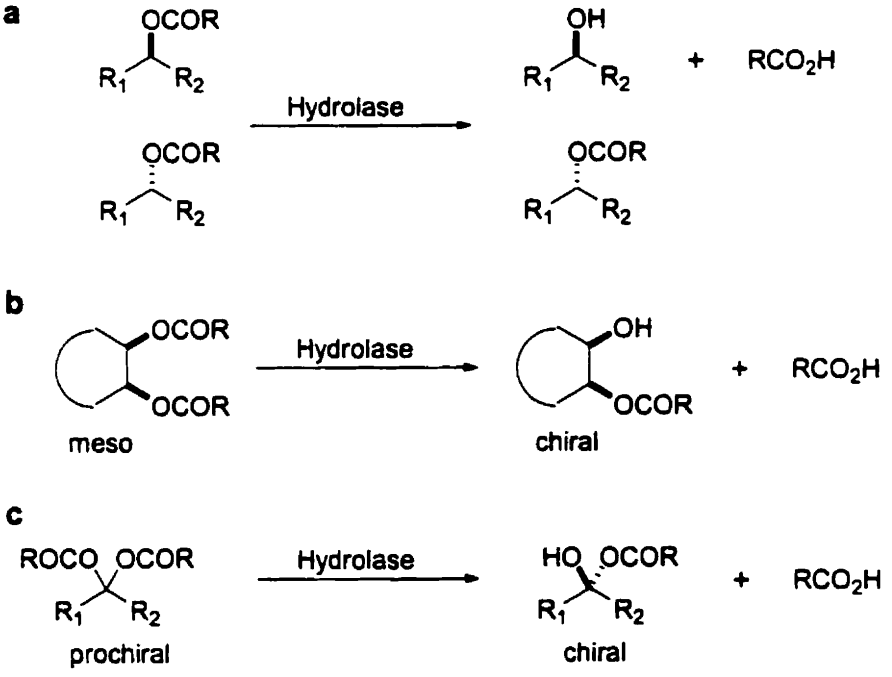


Figure 1.4. (**a**) Hydrolytic kinetic resolution (R_1 different from R_2). (**b**) Hydrolytic asymmetric synthesis starting from a meso molecule. (**c**) Hydrolytic asymmetric synthesis starting from a prochiral molecule.

* Although most of the discussions throughout this thesis refer to the kinetic resolution of chiral substrates the same reasonings, proposals, etc. can be applied to asymmetric syntheses.

If a hydrolase's enantioselection process was always perfect, only one optically pure product would ever be obtained, however, hydrolases make errors. The ability of a hydrolase to discriminate between one enantiomer of a given substrate and the other, in a kinetic resolution, can be measured quantitatively. This quantity is defined as E, the enantiomeric ratio. It is a measure of the enantioselectivity of the enzyme. For example, an E of 1 means that the hydrolase transforms each enantiomer in a 1:1 ratio, giving a racemic product; the enzyme does not have a preference for either enantiomer. An E of 50, on the other hand, means that the hydrolase reacts with the enantiomers in a 50:1 ratio, yielding a product with high optical purity. In the case of asymmetric syntheses, the competition is between enantiotopic groups of a given substrate rather than between enantiomers.

Kinetic resolutions

Since the substrate in a kinetic resolution is racemic, one could theoretically obtain the optically pure product with a maximum yield of 50% and, in addition, the other enantiomer could be isolated as the residual substrate, also in a 50% yield (see Figure 1.4a). However, since hydrolases make mistakes in enantiodiscrimination, the enantioselectivity varies for each hydrolase and for each substrate, and consequently differs for each reaction.

The enantiomeric ratio can be expressed by mathematical formulas derived from steady state Michaelis-Menton kinetics for irreversible reactions.[12] In a kinetic resolution, two enantiomeric substrates, A and B, compete for the active site of the enzyme, E, as follows:

$$E + A \underset{}{\overset{(K_M)_A}{\rightleftharpoons}} EA \overset{(k_{cat})_A}{\rightleftharpoons} E + P$$

$$E + B \underset{}{\overset{(K_M)_B}{\rightleftharpoons}} EB \overset{(k_{cat})_B}{\rightleftharpoons} E + Q$$

The enzyme turnover number, k_{cat}, denotes the maximum number of substrate molecules that one active site transforms into product per unit time. The Michaelis constant, K_M, represents the substrate concentration at half the maximum reaction rate.

The initial rates of formation of the enantiomeric products P and Q are defined as

$$v_A = \frac{d[A]}{dt} = k_{cat}[EA] \quad \text{and} \quad v_B = \frac{d[B]}{dt} = k_{cat}[EB] \tag{1.1}$$

When k_{cat} is slow then K_M is a binding constant:

$$(K_M)_A = \frac{[E][A]}{[EA]} \quad \text{and} \quad (K_M)_B = \frac{[E][B]}{[EB]} \tag{1.2}$$

Then, by substitution,

$$v_A = \left(\frac{k_{cat}}{K_M}\right)_A [E][A] \quad \text{and} \quad v_B = \left(\frac{k_{cat}}{K_M}\right)_B [E][B] \tag{1.3}$$

The ratio of the initial rates defines the enantiomeric ratio

$$E = \frac{v_A}{v_B} = \frac{d[A]/dt}{d[B]/dt} = \frac{\left(k_{cat}/K_M\right)_A [A]}{\left(k_{cat}/K_M\right)_B [B]} \tag{1.4}$$

If a substrate is racemic, [A] and [B] are initially present in equal amounts, therefore they cancel out. Integration yields

$$E = \frac{\ln\left(\frac{[A]}{[A_0]}\right)}{\ln\left(\frac{[B]}{[B_0]}\right)} = \frac{\left(k_{cat}/K_M\right)_A}{\left(k_{cat}/K_M\right)_B} \qquad (1.5)$$

where $[A_0]$ and $[B_0]$ are the initial amounts of each enantiomer, and [A] and [B] are the amounts of unreacted substrate. 'A' is the faster-reacting enantiomer. k_{cat}/K_M is an apparent second order rate constant that includes a kinetic constant associated with substrate binding. It relates the reaction rate to the concentration of unbound enzyme. This second order rate constant is known as the specificity constant and it is used to examine the efficiency and selectivity of an enzyme for a given substrate. Therefore, as shown above in equation 1.5, the enantiomeric ratio or enantioselectivity, E, is the ratio of the specificity constants for each enantiomer. A high E results from sufficiently different specificity constants for each enantiomer in a given reaction.

During a kinetic resolution, the optical purity of both the product and the substrate vary as the reaction proceeds. Sih *et al.*[13] derived formulas that relate enantiomeric excess to the extent of conversion. The substrate concentration values, A, A_0, B, and B_0, in equation 1.5, were substituted by the extent of conversion, c, and the enantiomeric excess of either the product, ee_p, or of the unreacted substrate, ee_s. These values are more easily measured experimentally than k_{cat} and K_M. Since,

$$c = 1 - \frac{A + B}{A_0 + B_0} \qquad (1.6)$$

and

$$ee_s = \frac{A + B}{A - B} \quad \text{and} \quad ee_p = \frac{P + Q}{P - Q} \qquad (1.7)$$

then, by substitution of equations (1.6) and (1.7) into equation (1.5),

$$E = \frac{\ln[(1-c)(1-ee_s)]}{\ln[(1-c)(1+ee_s)]} \qquad (1.8)$$

and

$$E = \frac{\ln[1-c(1+ee_p)]}{\ln[1-c(1-ee_p)]} \qquad (1.9)$$

Equations 1.8 and 1.9 can be combined to give equation 1.10. The latter equation does not require the percent conversion of the reaction. Experimentally, enantiomeric excesses can be measured more accurately than the extent of the reaction, so equation 1.10 is often more accurate.

$$E = \frac{\ln\left[\dfrac{1-ee_s}{1+(ee_s\ ee_p)}\right]}{\ln\left[\dfrac{1+ee_s}{1+(ee_s\ ee_p)}\right]} \qquad (1.10)$$

Due to the logarithmic nature of these equations, high values of E (i.e. E>50) are much more difficult to measure accurately than lower E values.

It is convenient to graphically illustrate the dependence of the ee_s and ee_p on the %conversion, Figure 1.5. These graphs show that, for a given E, the optical purity of the product decreases as the reaction proceeds, whereas the optical purity

of the remaining substrate increases. Thus, using these graphs, a chemist can decide at what point a given reaction should be stopped in order to obtain optimal enantiomeric purity of both the product and the remaining starting material. For example, a chemist could stop a hydrolytic reaction at less than 50% conversion to isolate the product when it is at its highest optical purity. Hydrolysis of the remaining substrate can then be continued to over 50% conversion, in order to reach its optimum optical purity. Thus both isomers are obtained with high enantiomeric excess. It must be noted, however, that in the case of a reaction for which the E is low, only the substrate can be obtained with high enantiomeric excess, albeit with low yield. Obviously, the higher the E, the better.

On account of the dependence of optical purity on the extent of reaction, it is necessary to compare the enantiomeric ratios of two kinetic resolution reactions as opposed to their enantiomeric excesses. Except in one particular case, see Section 1.7, E is constant for a given reaction.

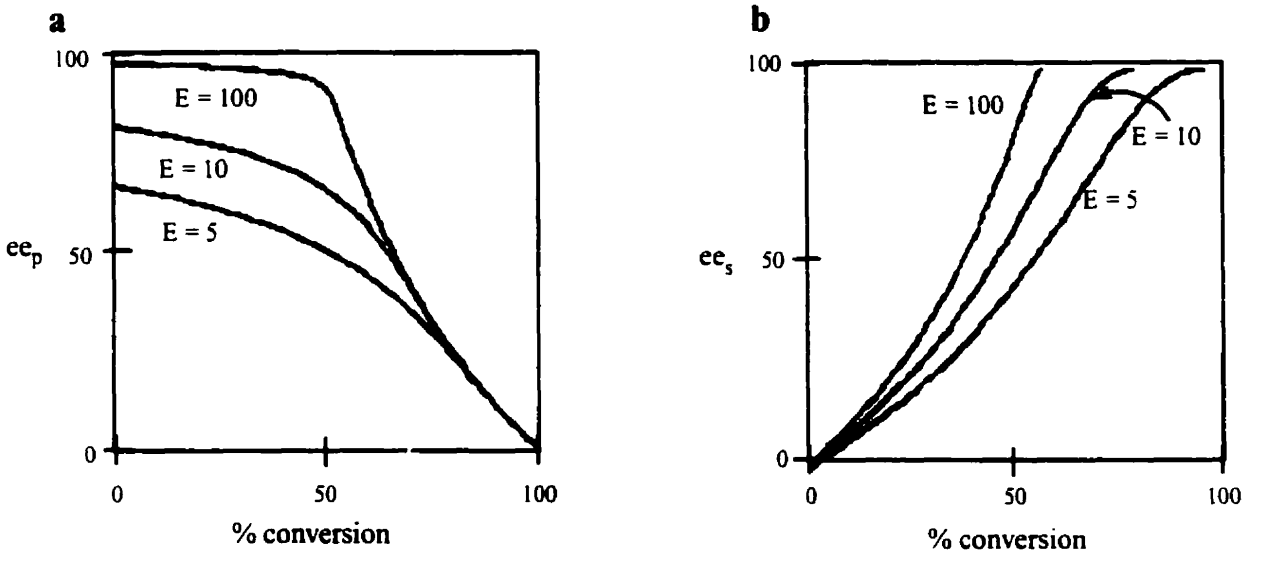


Figure 1.5. Graphical representations of the dependence of optical purity on the extent of conversion. (**a**) enantiomeric excess of the product as a function of percent conversion. (**b**) enantiomeric excess of the remaining substrate as a function of percent conversion.

The above equations and graphs apply to irreversible reactions. An enzyme-catalyzed hydrolysis reaction is considered to be irreversible because, as dictated by the law of mass action, the great excess of water shifts the thermodynamic equilibrium of the reaction to the right. In addition, ionization of the leaving carboxylic acid in water, at pH 7, also drives the reaction, see Figure 1.4.

However, for a many reasons, it is often more desirable to conduct the reverse reaction, i.e. a kinetic resolution involving the acylation of an alcohol in an organic solvent[14], Figure 1.6. Among the advantages of non-aqueous enzyme catalysis is improved enantioselectivity, increased solubility and stability of substrates, and simple recovery of the enzyme by filtration. Unfortunately, the low concentrations of acyl donor available in these esterification or acyl-transfer reactions shift the thermodynamic equilibrium such that the reactions become reversible, Figure 1.7.

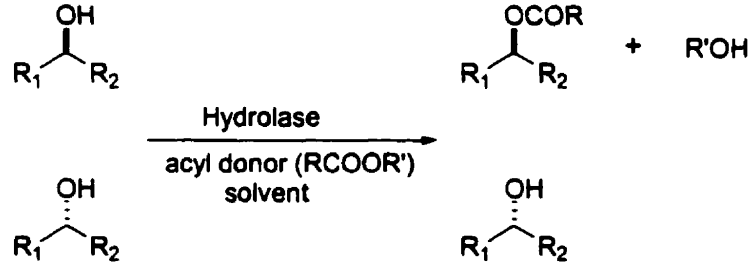


Figure 1.6. Hydrolase-catalyzed esterification (R'=H) and acyl-transfer (R'=alkyl, aryl, etc.).

Kinetic analyses of a reversible system are more complicated and as a result the calculation of E requires the inclusion of the equilibrium constant[15]. When such reactions are reversible, the optical purity of both the product and the residual substrate is lowered as the reaction proceeds. In order to avoid these undesirable complications, irreversible conditions must be forced upon an enzyme-catalyzed acylation reaction. There are two methods of accomplishing this: 1) use an excess

of the acyl donor and 2) use a special acyl donor that ensures irreversible conditions. Figure 1.8 shows an example of the latter method. Under these quasi-irreversible conditions, the same equations for the calculation of E can be used as for the irreversible hydrolysis reactions. It must be kept in mind, however, that in practice irreversible conditions are only mimicked under approximately 40% conversion.[16]

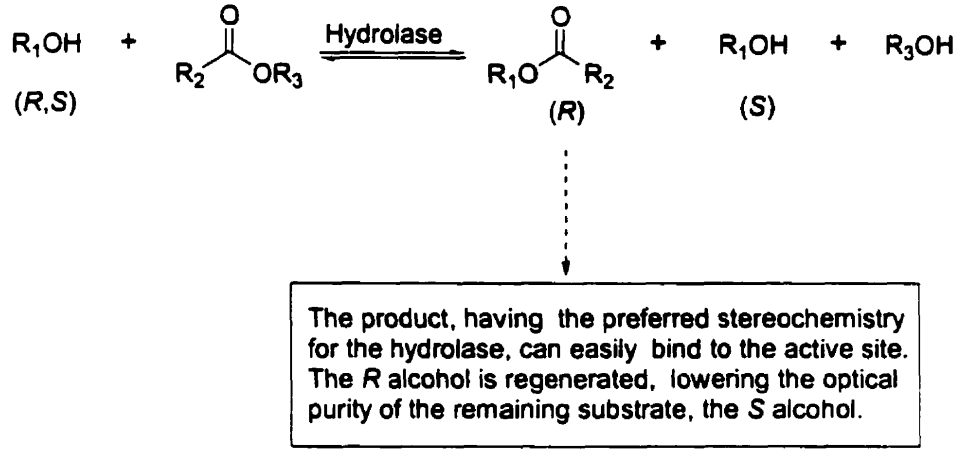


Figure 1.7. The reversibility of hydrolase-catalyzed acyl-transfer reactions leads to a decrease in the optical purity of the remaining substrate. The *R* and *S* configurations are assigned arbitrarily.

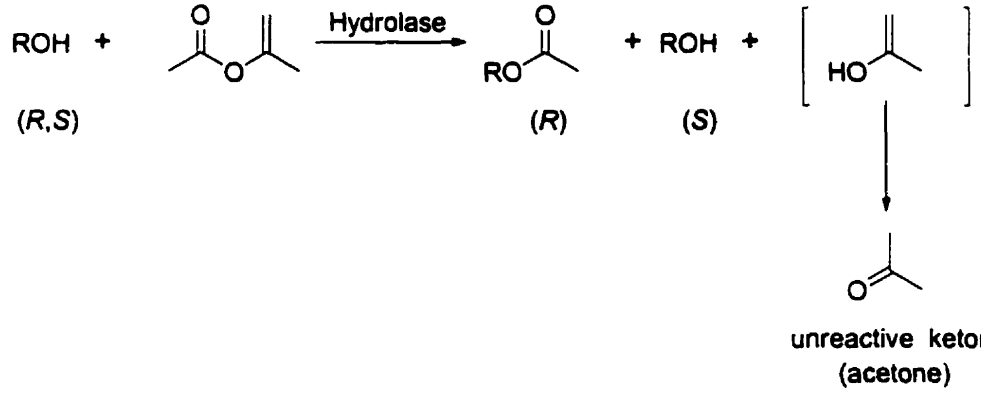


Figure 1.8. The use of *i*-propenyl acetate as acyl donor results in the formation of an unstable enol which tautomerizes to an unreactive ketone, thus making the reaction irreversible. The *R* and *S* configurations are assigned arbitrarily.

Lipase-catalyzed acylations of amines have also been conducted, Figure 1.9. In this case, reversibility is not a problem, because under these conditions lipases do not hydrolyze amide bonds, due to their greater stability. However, care must still be taken in choosing the acylating agent, to avoid chemical acylation.

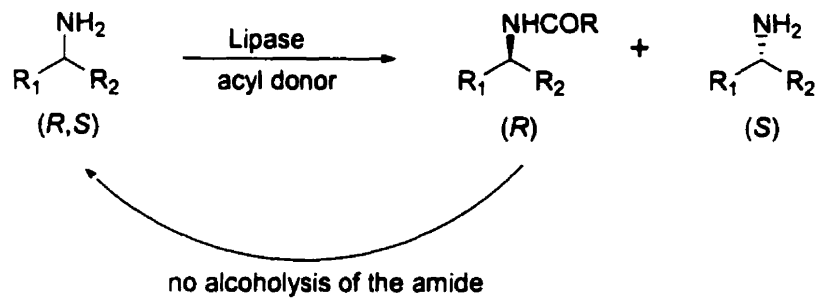


Figure 1.9. Acylations of amines catalyzed by lipases are considered to be irreversible reactions.

One important aspect of kinetic resolutions is that optical purity can be enhanced by a process known as recycling.[13] This involves the chemical re-esterification or hydrolysis, in the case of an acyl-transfer, of the product isolated from a first hydrolase-catalyzed reaction. This regenerated non-racemic substrate is re-submitted to hydrolysis by the hydrolase, resulting in a product with increased optical purity. The disadvantage is that the chemical yield is lowered during this process. This is just one example of the many ways hydrolase-catalyzed kinetic resolutions can be manipulated to enhance optical purity.[5,6]

Asymmetric syntheses

An asymmetric synthesis is the transformation of a meso or prochiral starting material (Figure 1.4b,c). The preference that the enantiomer shows for one enantiotopic group over the other determines the selectivity of the reaction. The enantiomeric ratio is given by equation 1.11.[17]

$$E = \frac{1 + ee}{1 - ee} \qquad (1.11)$$

Since the substrate fits into the active site in an optimal position before transformation of the reactive group occurs, theoretically one should be able to obtain quantitative conversion to a single chiral product, if the reaction is continued until all the substrate is consumed. However, in reality a second reaction may occur, Figure 1.10.

In the first step, the enantiomeric excess of the product is independent of the extent of conversion, as there is no competition between enantiomers. The optical purity is solely dependent on the ability of the enzyme to discriminate between the enantiotopic groups. If the reaction proceeds to the second step, a kinetic resolution occurs with the product, lowering the yield. If this secondary reaction does occur, equation 1.11 no longer holds. Nevertheless, since the secondary reaction often occurs with the minor enantiomer, the optical purity of the desired product is often increased, Figure 1.10. One disadvantage of asymmetric syntheses, as opposed to kinetic resolutions, is that only one enantiomer is accessible. However, the other enantiomer can be obtained if the reverse reaction is conducted, although the reverse reaction may not give the same results in terms of activity and selectivity.

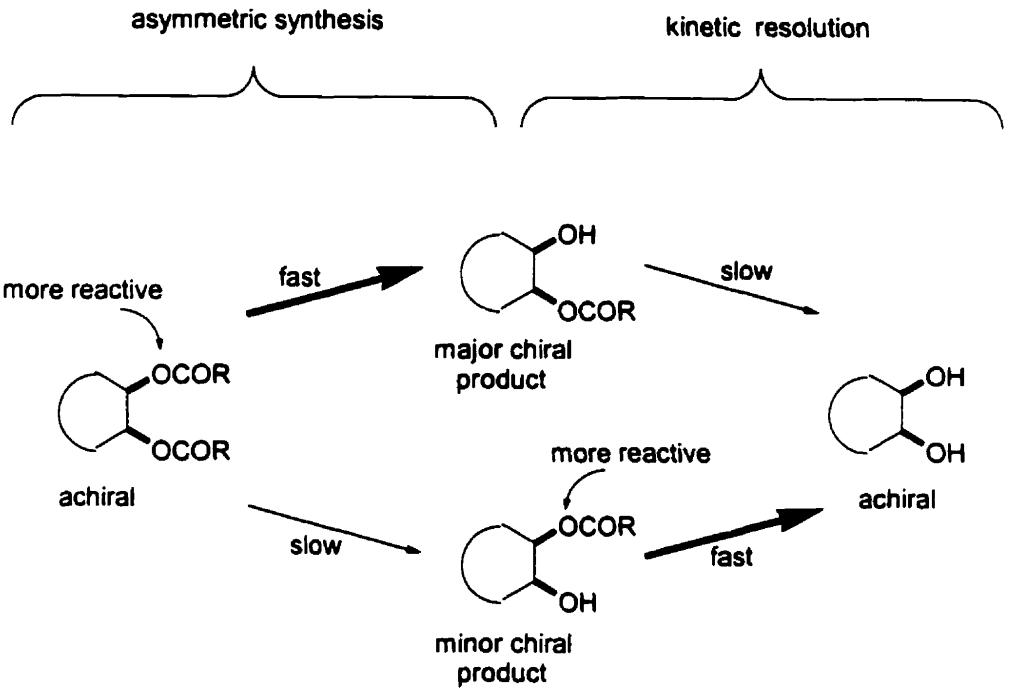


Figure 1.10. Asymmetric synthesis starting from a meso compound. The minor product from the first step of a hydrolase-catalyzed hydrolysis of a meso diester reacts more quickly in the second step. The total amount of the minor enantiomer is decreased, resulting in an increase in the optical purity of the major mono-ester product.

Selectivity at two stereocenters

The selectivity of a lipase towards a substrate with two stereocenters is examined in Chapter 3. The appendix of Chapter 3 describes the calculations employed in the quantitative determination of both the diastereoselectivity and the selectivity at each stereocenter for such substrates.

1.3 Structure and reaction mechanism

The hydrolases of primary focus in this thesis are lipase from *Candida rugosa* (CRL),[18a] lipase from *Pseudomonas cepacia* (PCL),[18b] pancreatic cholesterol esterase (CE), and subtilisin. Subtilisin is a serine protease, whereas lipases and esterases are serine esterases. Serine proteases and serine esterases belong to the family of serine hydrolases.

Structure of lipases and esterases

The X-ray structures of many serine esterases, including the three mentioned above, have now been solved, however none of these three-dimensional structures were known when the research described in this thesis was begun. As X-ray structures have become available, it has become apparent that although the amino acid sequence of all lipases and esterases is very different, they all have an astonishing similarity in their three-dimensional structure[19]. All lipases and esterases that have been studied have a series of β-strands and α-helices that are folded in a similar manner, see Figure 1.11 for an example.[20] This fold is called the α/β hydrolase fold.[21] It is comprised of a central β-sheet, formed by a series of eight predominantly parallel β-strands, that is flanked on either side by α-helices. The β-sheet has the usual left-handed twist found in proteins. Each enzyme has unique extrusions from this core structure. Figure 1.12 shows the basic pattern of the α/β hydrolase fold.

The catalytic machinery located within the active site of hydrolases is made up of the "catalytic triad" residues and the "oxyanion-stabilizing" residues. The catalytic triad residues, serine, histidine, and aspartic acid or glutamic acid, are in the same order in the amino acid sequence of all esterases and lipases. Consequently, they have the same orientation in the three-dimensional structure of

each enzyme because of the common α/β fold. The catalytic triad is situated at the C-terminal edge of the β-sheet. The nucleophilic serine is always situated at the elbow of a sharp turn of the loop between strand 5 and helix C, the acid, asp or glu, is in a loop following strand 7, and histidine is the first amino acid after a reverse turn following strand 8. In a few lipases, the catalytic acid does occur elsewhere (e.g. human pancreatic lipase). The "oxyanion hole", containing the oxyanion stabilizing residues (residues that stabilize a transition-state intermediate formed during catalysis) is also situated in approximately the same place in all the serine esterases: in a turn between strand 3 and helix A. The geometry of the catalytic triad is maintained by an extensive hydrogen bonding network.

This specific arrangement of the catalytic machinery and the α/β folds provides a chiral environment within the active site and, therefore, the selective embedding of a substrate. Due to the common structural framework of serine esterases, the binding sites have conserved elements. For instance, studies suggest that the secondary alcohol binding site is made up of two principle regions: a large hydrophobic pocket and a medium-sized pocket that is more polar in character. At least partially as a result of this, lipases exhibit in a common enantiopreference towards secondary alcohols. This will be discussed in detail in the following sections.

However, the uniqueness of each enzymes' amino acid sequence leads to differences in the details of their refined three-dimensional structure and differences in their individual electronic character. It is these differences that render each enzyme unique in its ability to bind various substrates and differentiate between isomers of a particular substrate. The detailed structure of each individual lipase and esterase and the method by which they distinguish between enantiomers is discussed in the following sections.

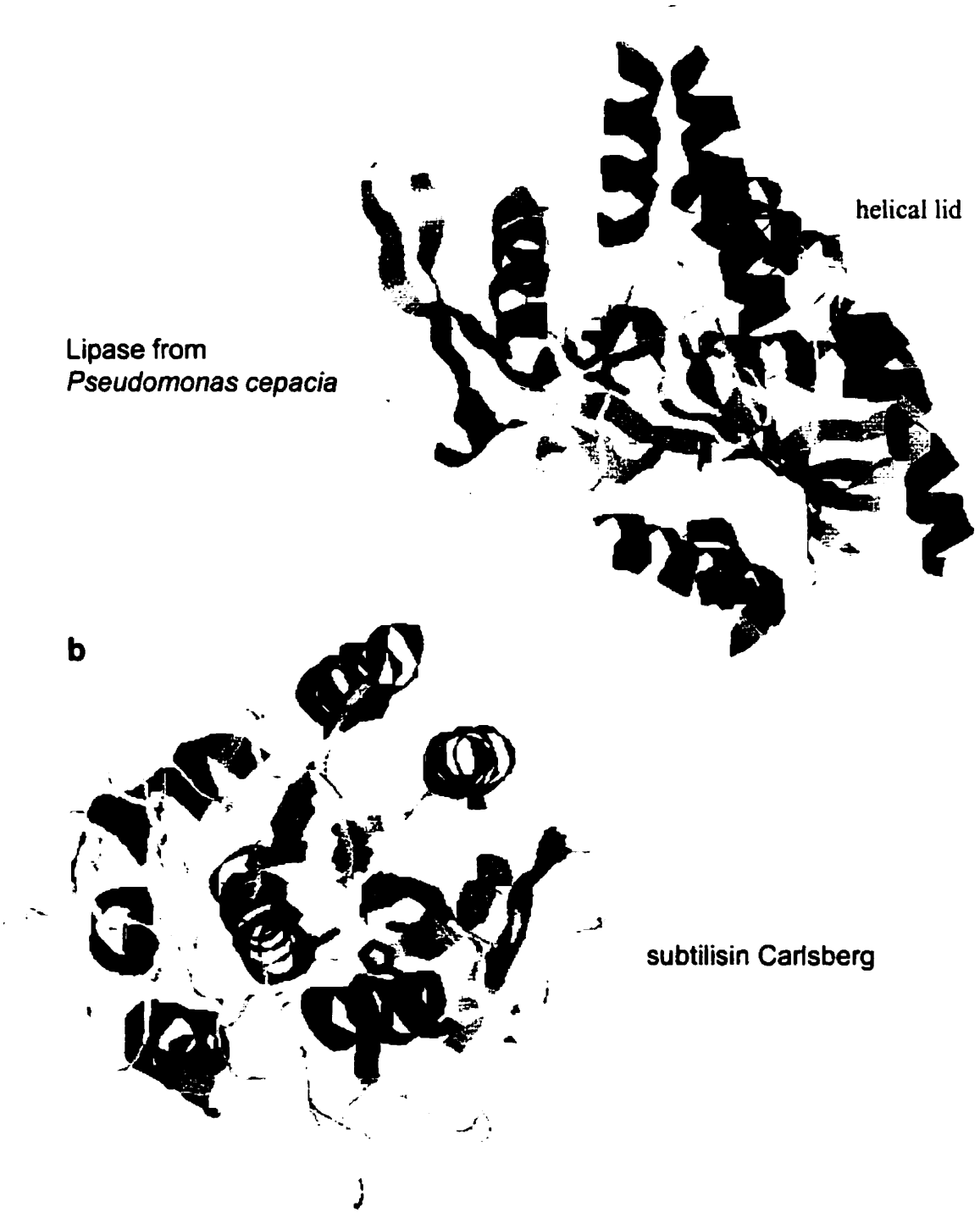


Figure 1.11. Ribbon diagrams of the three-dimensional structures of lipase from *Pseudomonas cepacia* **(a)** and subtilisin Carlsberg **(b)** showing the typical α/β hydrolase and α/β subtilase folds, respectively. The catalytic triad residues are coloured as follows: Ser, orange; His, blue; Asp, red. If the page is turned sideways to the left, the diagrams have the same orientation as the schematic drawings in Figure 1.12. This figure was created using RasMol v2.6 and coordinates from the PDB data files (accession codes for PCL and subtilisin Carlsberg are 4lip and 2sbc, respectively).[8]

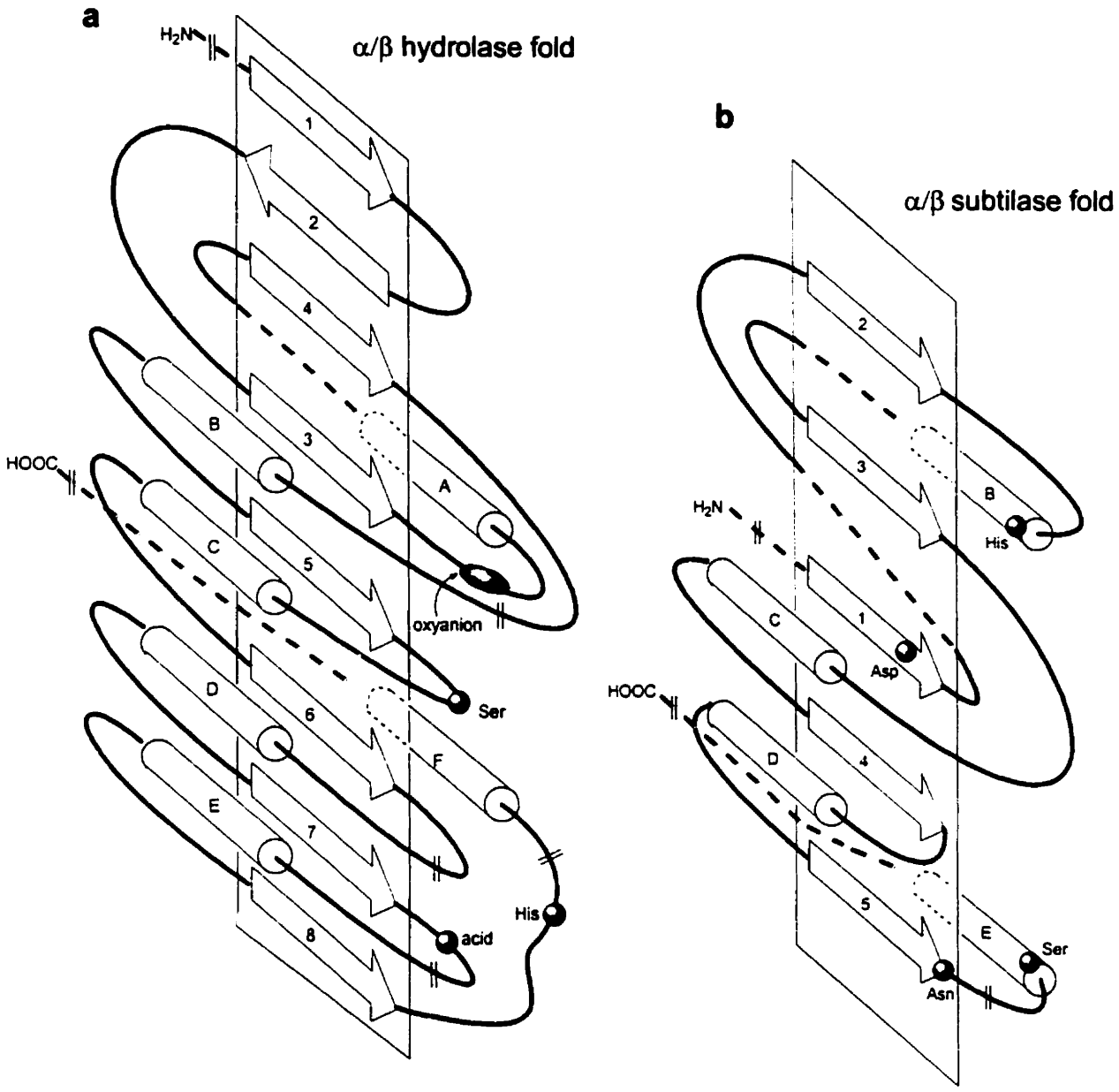


Figure 1.12. Schematic drawing of the α/β hydrolase **(a)** and α/β subtilase **(b)** folds showing the relative position of the catalytic site residues and the oxyanion hole residues. The double slashes correspond to places were there may be extrusions that are unique to each hydrolase. For example, a comparison with Figure 1.11 shows that PCL has additional α-helices and β-strands and subtilisin Carlsberg has additional α-helices.

Whereas esterases exhibit normal Michaelis-Menton kinetics, acting upon water-soluble substrates, the catalytic activity of most lipases sharply increases when in contact with lipid-soluble substrates at a lipid/water interface.[22] This "interfacial activation" is probably the result of a change in the conformation of

the lipase upon lipid contact. Three-dimensional crystal structures show that the active sites of most lipases are completely buried under an α-helical "lid" situated on the surface of the enzymes, see Figure 1.11a.[23] When these lipases are in the aqueous phase, this lid is closed, limiting access to the active site. When the lipases come into contact with the lipid phase, the lid opens, making the catalytic machinery and binding site available to the lipid-soluble substrate. In some lipases, the oxyanion hole is formed when this lid is opened. This lid does not seem to play a role in determining the enantioselectivity of lipases.[24]

Structure of subtilisin

The amino acid sequence and X-ray structure of subtilisin, a serine protease possessing esterase activity, has also been determined.[25] The core secondary structures of subtilisin are folded in what is known as the α/β subtilase fold,[26] that is, it is not an α/β hydrolase, Figure 1.11b. This fold consists of a β-sheet comprised of five parallel β-strands flanked by four α-helices, two α-helices on either side of the plane formed by the β-sheet, Figure 1.12b. An interesting feature of subtilisin is that one of the α-β-α motifs in this region is left-handed rather than the usual right-handed. This is necessary to place the catalytic histidine within the active site. Histidine is on helix B and must be on the same side of the β-sheet as the other two catalytic residues, serine and aspartic acid. Unlike the α/β hydrolases, the catalytic residues are not located on the loops joining the α-helices and β-strands but rather within the secondary structures themselves. Histidine and serine are located on the first turn of helix B and helix E, respectively. Aspartic acid is found within strand 1 and asparagine which forms part of the oxyanion hole, along with the main chain nitrogen atom of the catalytic serine, is on the very tip of the carboxy end of strand 5.

Figure 1.13. The active sites of subtilisins and lipases are approximate mirror images: the catalytic serine is on the opposite side of the plane formed by the imidazole ring of the catalytic histidine. **A)** The catalytic triad of subtilisin BPN'. **B)** The catalytic triad of lipase from *Pseudomonas cepacia*. The catalytic triad is viewed from the substrate binding site, in both cases. This figure was created using RasMol version 2.6 and coordinates from the PDB data files (accession codes for subtilisin BPN' and PCL are 2ST1 and 2lip respectively.[8]

Although the catalytic triad residues are the same as those found in serine esterases, the serine nucleophile lies on the opposite side of the plane formed by the imidazole ring of the histidine residue. Therefore, the three-dimensional orientations of the catalytic machinery (including the oxyanion hole) in serine esterases and serine proteases are approximately the mirror images[21], Figure 1.13.

Catalytic mechanism

The mechanism for serine hydrolases has been well established.[12,27,28] The mechanism (Figure 1.14) involves the nucleophilic attack of the serine hydroxyl group on the carbonyl carbon of an ester (or amide) of the non-covalently bound substrate, resulting in the formation of a tetrahedral intermediate (T_d1). Lipases attack on the *Re* face of esters and *Si* face of amides (the priority of the groups changes).[29] Subtilisin attacks on the *Si* face of esters and *Re* face of amides.

Through hydrogen bonding, the His residue functions to reduce the pK_a of the serine hydroxyl moiety, making it more available for attack. As the tetrahedral intermediate is being formed, the serine hydroxyl proton is transferred to the His residue. However, it is thought that the proton remains hydrogen bonded to both the serine oxygen and the substrate oxygen atom of the tetrahedral intermediate, serving to facilitate bond cleavage and leaving group departure.[29,30] The negatively charged Asp(Glu) residue stabilizes the positively charged imidazole ring. Several residues known as the oxyanion stabilizing residues are also present within the active site. These residues serve to stabilize the oxyanion of the tetrahedral intermediate by forming two hydrogen bonds with the negatively charged carbonyl oxygen. Breakdown of the tetrahedral intermediate (T_d1) releases the alcohol (or amine) leaving group, with His donating its proton to the leaving group, and results in the formation of the acyl-enzyme intermediate.

The attack of a nucleophile on the acyl-enzyme results in the formation of a second tetrahedral intermediate (T_d2) and in the subsequent release of the acyl group. If the nucleophile is water as in the case of a hydrolysis reaction, an acid is released. During the formation of the second tetrahedral, His is again protonated and when the T_d2 breaks down, the His proton is transferred back to Ser, regenerating the free enzyme.

Kinetic studies have indicated that it is the breakdown of the first tetrahedral intermediate that is the rate determining step in the overall reaction of hydrolyses.[31] In section 1.5, we will discuss how it is this step that determines the enantioselectivity of the hydrolase towards alcohols.

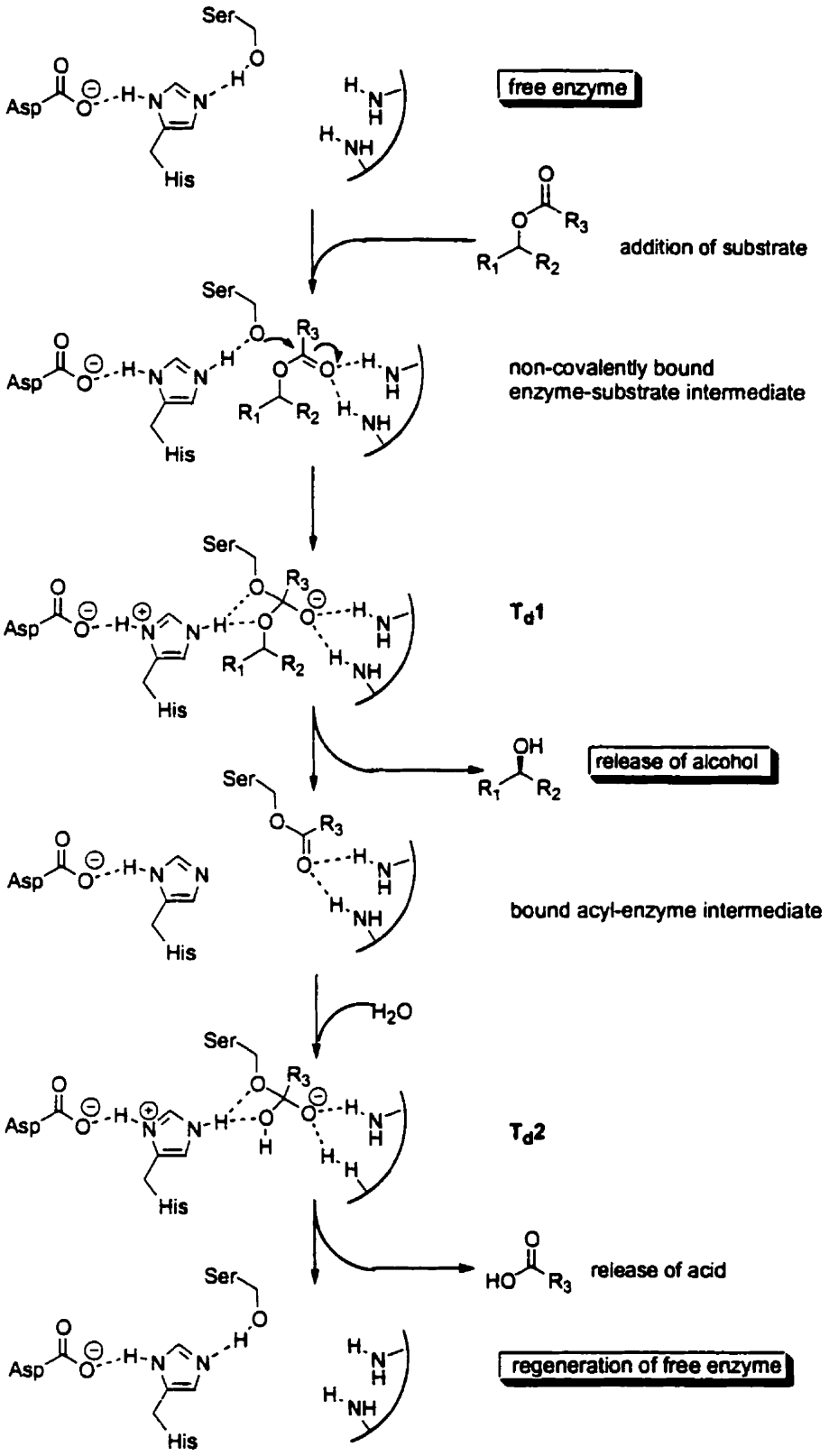


Figure 1.14. Catalytic mechanism for serine hydrolases. In some hydrolases Asp is replaced by Glu and the oxyanion stabilizing residues may differ.

The kinetics and mechanism of the reverse reaction, acylation of an alcohol or amine, have also been examined.[16,32,33] In this case, the acyl-enzyme is formed by the attack of catalytic serine residue on the acylating agent. In the second step, the chiral alcohol (or amine) nucleophile attacks the carbonyl carbon of the acylated serine, forming the second tetrahedral intermediate. The breakdown of this tetrahedral intermediate, also thought to be rate determining, results in the release of the ester (or amide) product.

1.4 Principal structural elements of selected hydrolases

The following is a description of five synthetically useful hydrolases that are focussed on in this thesis. These hydrolases have been used in the kinetic resolutions or asymmetric syntheses involving one or all of the following substrates: secondary alcohols, primary alcohols, and primary amines

The following descriptions show the similarity between the binding sites of hydrolases. A close-up picture of the substrate binding sites referred to below is shown in the following section (section 1.5).

Lipase from *Candida Rugosa*

X-ray crystal structures for lipase from *Candida rugosa*,[18a] a fungal lipase, have been obtained for both the open-lid[34] and closed-lid[35] forms as well as with bound inhibitors.[29,36] CRL consists of a 538 amino acid sequence and weighs approximately 63 kDa.[37]

The X-ray crystal structure of CRL covalently bound to a transition state analogue, mimicking the cleavage of a secondary alcohol ester, provided an insight into the important structural and electronic elements of the lipase's active site. The active site can be simply described as a hydrophobic cavity in the lipase's surface. The crevice is oval in shape, measuring approximately 7 Å x 12 Å. Within this cavity is a large "pocket", open to the solvent, lined with hydrophobic side chains and a medium-sized pocket lined with both polar and hydrophobic groups. The medium pocket faces the floor of the crevice where the catalytic triad, Ser209-His449-Glu341, is situated. The "oxyanion hole" consists of the Gly123 Gly 124, Ala210 residues. The acyl chain of the ester binds partially in a tunnel projecting towards the center of the protein. This tunnel, unusual among hydrolases, allows the binding of a chain containing up to 18

carbon atoms. There is a well-defined lid that covers the active site when the lipase is in an inactive conformation.

Pseudomonas lipases

Many different lipases have been isolated from the *Pseudomonas* family of bacterial microorganisms: lipases from *Pseudomanas cepacia*[18b] (PCL, lipase P, P-30, PS, LPL-80, LPL-200S, SAM-II), *Pseudomonas fluorescens*[38] (PFL, lipase YS, AK, AKG), and *Pseudomonas glumae* (*Chromobacterium viscosum*[39]), *Pseudomonas sp.* (lipase AK-10). Of these lipases, PCL is the one most focussed upon in this thesis.

Although the X-ray structure of *Pseudomonas glumae* was reported several years ago[40], it is only very recently that an X-ray structure has been obtained for PCL[41]. PCL, which consists of a 320 amino acid sequence[42] and weighs about 33 kDa, was found to measure approximately 30 Å x 40 Å x 50 Å. When the helical lid is open, a large hydrophobic surface, surrounding a deep active site cleft, is exposed. The active site serine is located in the center of the cleft. The opening of the cleft is oval-shaped, measuring 10 Å x 25 Å across, whereas the cresent-shaped bottom of the cleft measures about 4 Å across. The cleft is approximately 15 Å deep. The catalytic triad is consists of Ser87, His286, and Asp264 residues and the oxyanion hole is formed by the Gln88 and Leu17 residues.

Three binding sites were observed in the crystal structure of PCL complexed with a glycerol derivative transition-state analogue.[30] The acyl-chain binding cleft is a well-defined hydrophobic groove measuring approximately 8 Å x 10 Å. There is a large binding pocket that has a small hydrophilic region at the bottom of the cleft and a larger hydrophobic region towards the surface of the enzyme. There is also a smaller pocket that is less hydrophobic.

Lipase B from *Candida antarctica*

The three-dimensional crystal structure of Lipase B from *Candida antarctica* (CAL-B) was determined by Uppenberg *et al.* both with and without a covalently bound inhibitor within the active site.[43,44] CAL-B is a fungal enzyme that consists of a 318 amino acid sequence and weighs 33 kDa. Its overall dimensions are approximately 30Å x 40Å x 50Å.

The catalytic triad, consisting of Ser105, His224, and Asp187, is situated at the bottom of a deep, narrow substrate binding site. The oxyanion stabilizing residues, Gln106 and Thr40, are thought to form three stabilizing hydrogen bonds to the tetrahedral intermediate. Two hydrophobic side chains protrude slightly into the binding side separating it into two 'channels': one for the alcohol side of the substrate and the other for the acyl portion. There is a medium pocket towards the floor of the alcohol side of the binding pocket as well as three hydrophobic regions towards the surface of the enzyme.

Although there is a short α-helix near the entrance to the binding site, there is no indication that it acts as a lid by blocking the site when no substrate is present. CAL-B does not display the interfacial activation that is typical for many lipases.

Cholesterol esterase

Cholesterol esterase, also known as bile-salt activated lipase, is secreted by the pancreas of vertebrates as well as from the milk of various mammals.

Cholesterol esterase, unlike lipases, which require a water-organic interface, requires the presence of bile-salts for activation. This bile-salt dependence places cholesterol esterase between lipases and other esterases. It can act both upon water-soluble substrates and hydrophobic substrates.

The crystal structure of bovine pancreatic cholesterol esterase, one of the most commonly used and studied esterases, has recently been solved by two independent research groups. Wang *et al.*[45] solved the structure of the native dimer and its complex with a bile salt, while Chen *et al.*[46] solved the structure for a mutant monomer . Since the mutation (removal of N-linked glycosylation sites) occurred far from the active site, the properties of the mutant strain were the same as for the native esterase.

Bovine pancreatic cholesterol esterase is similar to CRL. It is made up of 579 amino acid residues and weighs approximately 63 kDa. The protein core has a typical lipase α/β hydrolase fold consisting of 11 β-strands flanked by 15 α-helices. The catalytic triad, consisting of Asp320, His435, and Ser194, is located near the center of the molecule. Ala195, Gly107, and Ala108 are thought to be the oxyanion stabilizing residues. The active site is located in a large, deep hydrophobic pocket with a surface area of 504 $Å^2$. As with CRL there seems to be a tunnel-shaped pocket for acyl-chain binding.

The environment around the active site is different from lipases. Cholesterol esterase lacks the amphipathic, helical lid found in lipases that blocks the entrance to the active site binding pocket in the inactive lipase conformation. Instead, in the position that corresponds to the lid of lipases, is a short truncated loop made up of two anti-parallel β-strands. This loop (residues 64-80) does not block the entrance to the active site and is too rigid to change conformation. It is therefore unlikely to play any role in the activation of CE. Instead, two different modes for the activation of CE were proposed.

Chen *et al.* examined an inactive conformation of CE. They found that the last six C-terminal amino acids of the enzyme were located within the active site pocket, physically hindering substrate binding and diverting the putative oxyanion binding site from the catalytic serine. They suggest that displacement of the C-

terminal plug is mediated either by bile-salt binding at either of two bile-salt binding sites present or simply by substrate binding. Displacement of the C-terminus exposes the hydrophobic binding site and permits the oxyanion hole to attain the position required for catalysis. The protein is then in its active conformation.

Wang *et al.* examined a conformation of CE that had been activated by bile salts. They were unable to interpret any electron density for the last 32 C-terminal residues. They suggested that this was due to the conformational flexibility of the C-terminal region. Instead, Wang et al. found that the active site was partially blocked by a short non-helical loop (residues 116-124) whose conformation seems to be dependent on bile-salt binding. They described the presence of two bile-salt binding sites, one proximal to the active site and the other distal. The loop blocks the entrance to the active site when bile-salt is absent. It appears that when bile-salt binds in the site closest to the active site, the loop moves out of the way, allowing sufficient space for the substrate to enter the binding pocket. The position of the oxyanion hole does not alter and remains correctly formed for catalysis in both conformations. Wang *et al.* concluded that both the distal bile-salt binding site and the C-terminal region are unrelated to the catalytic process.

Although these proposals are vastly different, they are both similar to lipase activation in that a small portion of the protein must move to allow substrate binding. In one case it is a short loop that blocks the active site and in the other it is the C-terminal region.

Subtilisin

Subtilisins are serine proteases that are produced by various species of bacilli. They are commonly used in laundry detergents to aid in stain removal. Subtilisins have been extensively studied because of their commercial value.

Subtilisin BPN' from *Bacillus amyloliquefaciens* and subtilisin Carlsberg from *Bacillus licheniformis* are probably the most frequently used. Subtilisin Carlsberg has one less residue (274 amino acids) than subtilisin BPN' (275 amino acids) and differs at 84 residues.[25] The information given below is valid for both of these subtilisins.

Subtilisin consists of a single polypeptide chain that, as discussed in the previous section, is folded into an α/β structure that is quite different from the α/β hydrolase fold of lipases and esterases. It is an essentially spherical molecule that measures approximately 42 Å in diameter and weighs about 27 kDa. The catalytic triad, situated at the bottom of a large, open, shallow groove in the protein's surface, is comprised of Ser221, His64, and Asp32 residues. They are situated outside of the carboxy ends of the central β-sheet. The amide moeity of Asn155 and the main-chain nitrogen atom of the catalytic serine form the oxyanion hole.

The S_1 site is analogous to the acyl binding site of lipases and esterases and the S_1' site is analogous to the site in which the alcohol substituents bind.[47] The S_1 site is more neutral than hydrophobic and shows a preference for large aromatic moieties.[48]

1.5 How do hydrolases discriminate between enantiomers?

The detailed study of substrate and active site characteristics has led to an insight into the molecular basis by which hydrolases distinguish between enantiomers of a substrate. One of the main objectives of this work is to give researchers clues as to how enantioselectivity can be improved.

Many of these studies have been conducted with primary and secondary alcohols and their analogues. Chiral primary and secondary alcohols, unsymmetrical by nature, often have two substituents that are substantially different in size and electronic character. Lipases, esterases, and subtilisin have binding pockets that also differ in size, shape and electronic character, as shown in Figure 1.15. Researchers have attempted to discover whether hydrolases differentiate between enantiomers by 'recognizing' the difference in size of the substituents, difference in their electronic characteristics, or whether it is some other structural feature of the substrate or lipase that determines enantioselectivity. The examination of hydrolase crystal structures with bound transition-state analogues as well as molecular modeling studies have been extremely useful for gaining information about the shape, size, and electronic character of the catalytic machinery and binding site.*

Secondary alcohols

Importantly, as described in Chapter 2, all lipases and esterase studied thus far, have a common enantiopreference towards secondary alcohols, although they exhibit differing grades of enantioselectivity. It is suggested that this common

* It must be noted that all of the following studies were carried out after the work described in Chapters 2. When the empirical substrate rule was proposed, no X-ray structures were available for the lipases considered here. Furthermore, the research described in Chapter 3 was carried out before an X-ray structure was available for PCL.

enantiopreference is due to the common characteristics of the alcohol substrate binding sites: the presence of a large hydrophobic pocket and a smaller more hydrophilic pocket, figure 1.15. It is these pockets that are referred to in the following discussion.

But why do these enzymes prefer one enantiomer to the other? Several research groups have proposed molecular level explanations for the enantioselectivity of lipases toward secondary alcohols. All of the researchers referred to below agree that the preferred enantiomer of secondary alcohols bind with the larger substituent in the large hydrophobic pocket and the medium substituent in the smaller more hydrophilic pocket, of the active site. They do not agree on where the substituents of the less favored enantiomer bind. Their proposals and rationalizations for how and why lipases make 'mistakes', by reacting with the disfavored enantiomer, differ. The first four proposals (Cygler *et al.*, Nishizawa, *et al.*, Ema *et al.*, and Nakamura, *et al.*) agree that enantiodiscrimination stems from differences in the reactivity of the two enantiomers in the transition state. They also agree that the large substituent of both enantiomers must bind in the large hydrophobic pocket and point out towards the surface of the enzyme. The following two proposals (Uppenberg *et al.*, Rottici *et al.*, Orrenius *et al.*, and Zuegg, *et al.*) agree that enantiodiscrimination stems from the preferential binding mode of one enantiomer of the substrate.* One enantiomer is disfavored because it must orient its substituents in such a way as to cause unfavorable interactions within the binding pockets. That is, the large and medium substituents switch places.

* This does not imply that enantiodiscrimination is necessarily based on differences in the binding constants.

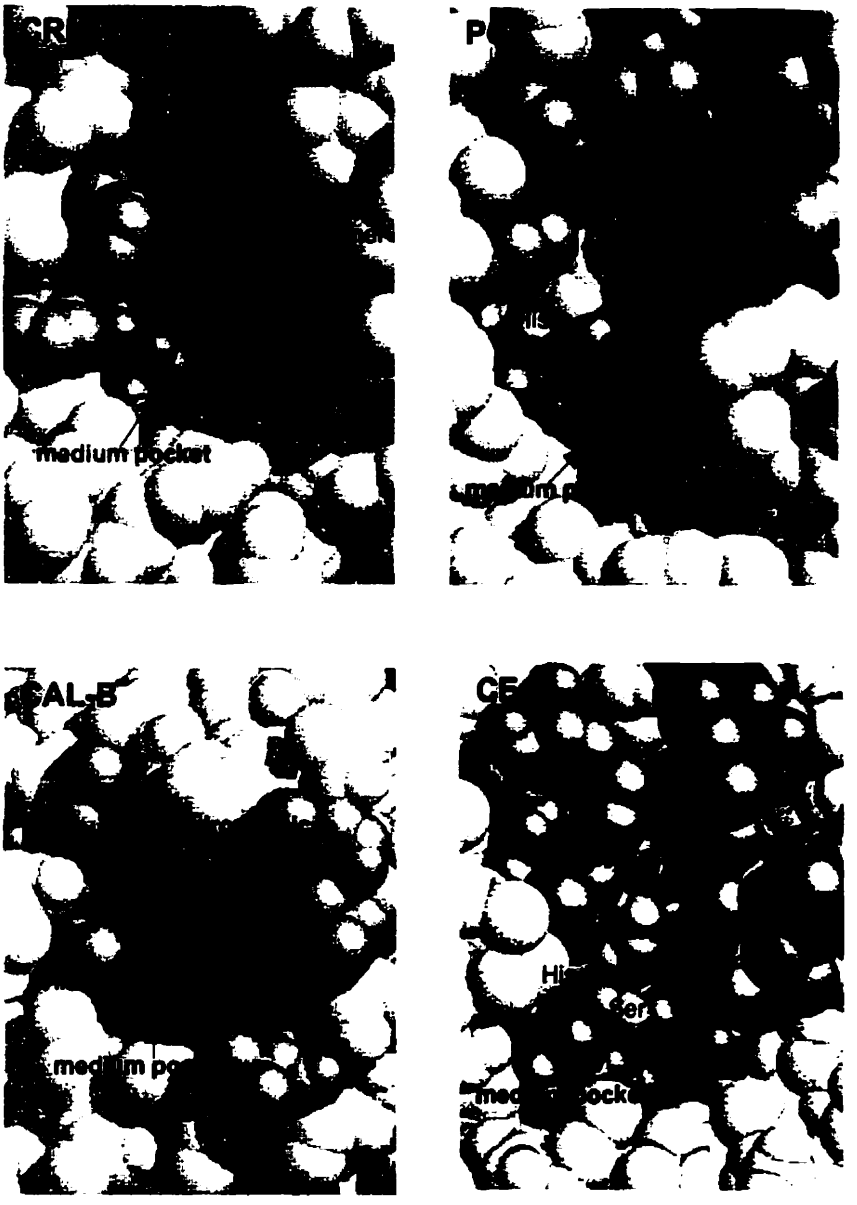


Figure 1.15. A close-up view of the substrate binding sites of CRL, PCL, CAL-B, CE. The view is straight in, towards the active site. Our interpretation of the three-dimensional crystal structures shows that these enzymes all have a medium-sized binding pocket (red) that is situated close to the bottom of the active site and a large pocket (green) that extends further out towards the surface of the enzyme. The medium pocket is lined with both hydrophilic and hydrophobic amino acid residues. These residues always include the one before the catalytic serine, the one after the catalytic histidine, and one next to the oxyanion-stabilizing residues. The large pocket is lined predominantly with hydrophobic residues. CRL has a deep, spacious tube-like binding site with a narrow tunnel for the acyl moiety. The binding site of PCL is relatively wide towards the surface but becomes narrow and crescent-shaped near the catalytic triad. CAL-B has a very deep and narrow binding site, with the medium pocket being barely accessible to the solvent. CE has the most solvent-accesible binding site. It is shallow and wide with a deep narrow gorge for the acyl moiety. These pictures were created using RasMol v2.6 and coordinates from the following PDB files: 1lpm (CRL), 4lip (PCL), 1lbs (CAL-B), 1aql (CE).[8]

Cygler *et al.*[29] obtained data on the structural and electronic characteristics of lipase from *Candida rugosa* by studying the crystal structure of the lipase with a phosphonate derivative of menthol covalently bound to the active site. This derivative mimics the first tetrahedral intermediate formed during hydrolysis of secondary alcohol esters. Individual crystal structures, for each enantiomer of the menthol derivative bound to the active site, were solved. From this, they were able to propose a method by which the lipase distinguishes between the two enantiomers. In the case of the favored enantiomer, the larger side of the menthyl ring, containing an isopropyl moiety, binds in the large hydrophobic pocket of CRL and the smaller side binds in the smaller less hydrophobic pocket, Figure 1.16. A hydrogen bond is formed between the Nε2 atom of the catalytic histidine and both the alcoholic oxygen of the substrate and the nucleophilic oxygen of the serine residue, Figure 1.17. This hydrogen bond network stabilizes the tetrahedral intermediate and facilitates deprotonation of the serine and protonation of the alcohol leaving group. The position of the disfavored enantiomer in the active site is such that although its large substituent still binds within the hydrophobic pocket, the isopropyl moiety is directed towards the catalytic histidine. The orientation of the histidine imidazole ring is distorted, permitting only the hydrogen bond to the serine residue to form, Figure 1.17. The stability of the tetrahedral intermediate is decreased and protonation of the leaving alcohol is hampered, delaying release of the alcohol product or resulting in the reformation of the starting material. Thus one enantiomer is preferentially hydrolyzed.

Figure 1.16. Active site of CRL with the covalently-bound, fast-reacting enantiomer of the phosphonate inhibitor. The isopropyl moiety binds in the large pocket and the methyl group binds in the medium pocket. This figure was created using RasMol v2.6 and PDB file 1lpm.[3]

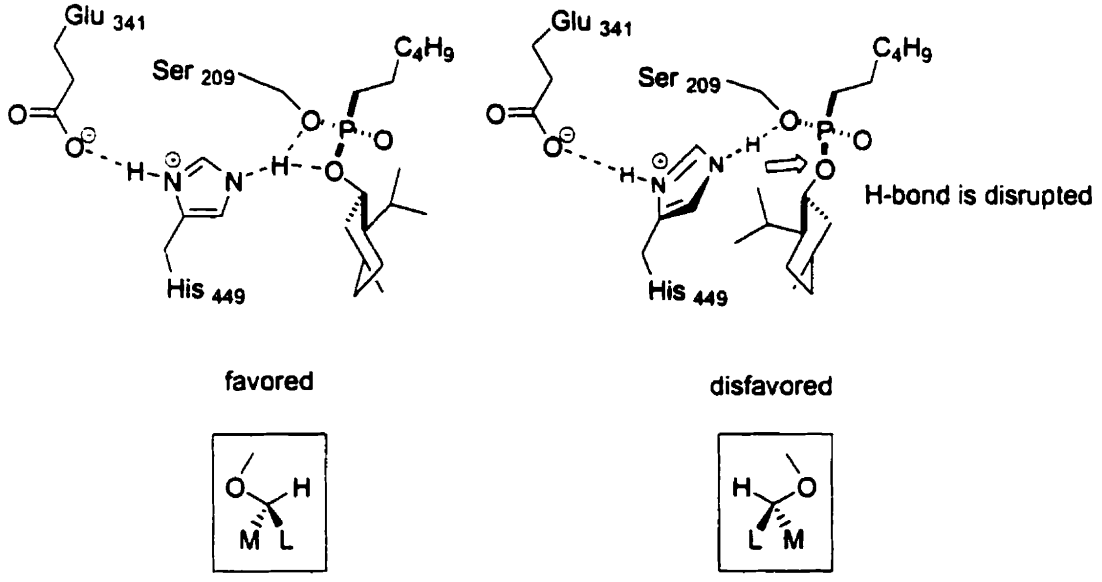


Figure 1.17. Schematic representation of both enantiomers of the menthol inhibitor bound to CRL. The alcohol oxygens of the two enantiomers point in different directions. The slightly different orientation of the large group within the hydrophobic pocket, disrupts the catalytically essential hydrogen bond in the case of the disfavored enantiomer.

Nishizawa *et al.* came to a similar conclusion from kinetic studies of PCL-catalyzed hydrolyses of secondary alcohol esters.[31] They found that the apparent K_M values were identical for several different substrates but that the k_{cat} values were substantially different. It was suggested that enantiodifferentiation is a result of one enantiomer reacting faster than the other, and that the two enantiomers bind to the same extent. Nishizawa *et al.* proposed that the large substituent of both enantiomers binds tightly to the same site and but that the smaller substituent of the slow reacting enantiomer orients itself in such a way as to hinder hydrogen bond formation between His and the alcoholic oxygen.

Nakamura *et al.*[49] and Ema *et al.*[32] carried out kinetic studies on the reverse reaction, acylation of secondary alcohols, catalyzed by lipases from *Pseudomonas cepacia* and *Rhizomucor miehei*, respectively. Both groups proposed that the enantioselectivity of lipases towards secondary alcohols originates from a differences in V_{max} rather than K_M. However their mechanistic interpretations differ, as discussed below.

Using a combination of kinetic studies and energy calculations, Ema *et al.* proposed that enantiomer differentiation is dependent solely on the relative stability of the transition states of each enantiomer, while ignoring any influence that the size, shape, and electronic characteristics of the substrate may have. The transition states considered correspond to the breakdown of the second tetrahedral intermediate, resulting in the release of the acylated alcohol. In the transition state, the bond between the alcoholic oxygen and the chiral carbon of the substrate have a *gauche* conformation with respect to the bond connecting the nucleophilic serine to the carbonyl carbon of the ester. According to the stereoelectronic theory[50], this is necessary for the efficient cleavage of the ester C-O bond. When the fast-reacting enantiomer adopts this conformation, the large substituent is directed towards the solvent and the proton attached to the stereocenter is *syn* with

the carbonyl oxygen, Figure 1.18. When the slow enantiomer adopts a *gauche* conformation, the large substituent must be situated in the same way to avoid disturbance of the 'triangular wall' formed by the catalytic Ser and His residues and an adjacent His residue. The medium substituent is then forced into a position that causes repulsive non-bonding interactions with the carbonyl oxygen. Rotation of various dihedral angles to reduce steric strain, leads to a destabilization of the transition state and a disturbance of the catalytic histidine and the hydrogen-bonding network. The transition state of the disfavored enantiomer is therefore at a higher energy level. One disadvantage of this theory is that it does not take into account the size and shape of the binding site; only a few residues in the active site are considered. If chiral recognition at the binding step could be completely ignored then the degree of stereoselectivity of all lipases should be the same; this is obviously not the case.

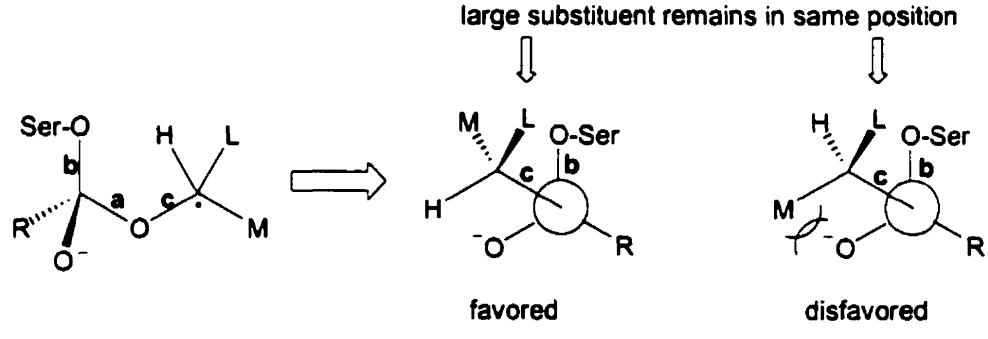


Figure 1.18. Simplified schematic representation of the proposal given by Ema *et al.* The view along bond **a** for the favored and disfavored enantiomers is shown. Bonds **b** and **c** are *gauche* in both cases and the large substituent points outward. For the disfavored enantiomer, repulsive interactions occur between the carbonyl oxygen and the medium substituent. Note that this is not a transition state model of the enzyme-substrate complex, but rather a simple view of the relative positions of pertinent groups.

Nakamura *et al.*[49] suggested that large aromatic substituents are 'anchored' in a large pocket close to the catalytic site. That is, the large pocket of PCL is lined with phenyl rings and has a special affinity for an aromatic substituent. In the case

of the favored enantiomer, the medium substituent and the hydrogen atom attached to the stereocenter are bound in their respective pockets, and the hydroxyl group is positioned correctly for acylation. It is suggested that an aromatic large substituent of the disfavored enantiomer is also subjected to the 'anchoring effect'.[51] That leaves the option of either the hydroxyl group binding easily in the medium pocket, in an unreactive position, or being situated at the catalytic position, Figure 1.19. In the latter position, however, the medium substituent is forced into the sterically constricted pocket meant for the hydrogen atom. The lower enantioselectivity that is observed for substrates having a flexible alkyl chain as the large substituent is accounted for by the fact that they are not subjected to the 'anchor effect'. The lack of an aromatic group increases the freedom of movement of the large substituent within its pocket. The unfavorable steric interactions of the medium group within the small pocket can be therefore be alleviated, allowing the disfavored enantiomer to become a better substrate.

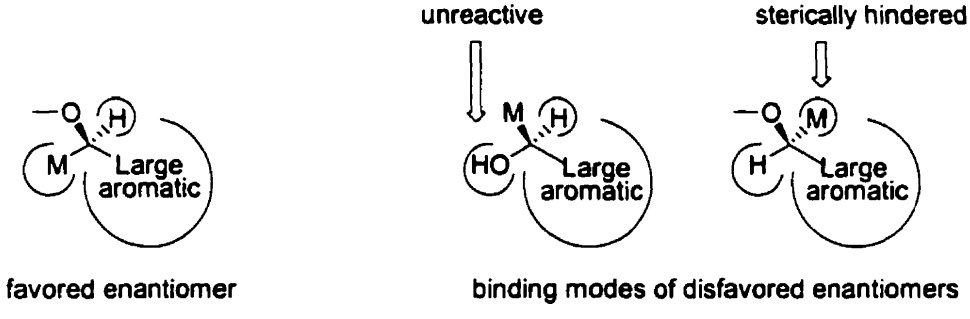


Figure 1.19. Nakamura *et al.* proposed that the large aromatic group of both the favored and disfavored enantiomers is anchored in the large pocket. Two binding modes of the disfavored enantiomer are possible, but only one is catalytically viable.

This dependence of enantioselectivity on non-steric interactions within the large pocket of PCL has also been observed from substrate screening studies. For example, Honig *et al.*[52] found that in the case of a heterocycle such as 3-

hydroxytetrahydrofuran acetate, which, other than for the presence of the ring oxygen is symmetrical, the preferred product has an R configuration. This observation led them to suggest that the heteroatom must interact electrostatically within the large pocket. Carrea *et al.* suggested that the degree of enantioselectivity can be dependent on $\pi-\pi$ interactions between a phenyl group on the substrate and an amino acid moiety in the binding site.[53] Rotticci *et al.*[54] found that the addition of a halogen atom to the large side of aliphatic secondary alcohols greatly increased the enantioselectivity of *Candida antarctica* lipase B (CAL-B), whereas addition of the halogen to the smaller side resulted in a decrease.

The following two proposals focus on the notion that enantiodiscrimination is based on the preference for a given orientation of the substrate substituents within the binding site.

Uppenberg *et al.*[44] carried out crystallographic studies with an achiral covalently-bound inhibitor and molecular modeling studies with chiral substrates, using CAL-B. The tetrahedral intermediates of two different secondary alcohol substrates were embedded into the active site. The presence of the hydrogen bond between the catalytic His and the alcoholic oxygen was observed for the favored enantiomers of both substrates. Interestingly, however, it was also present for the disfavored enantiomer of one of the substrates. This led them to the assumption that there must also be other factors governing enantioselectivity.

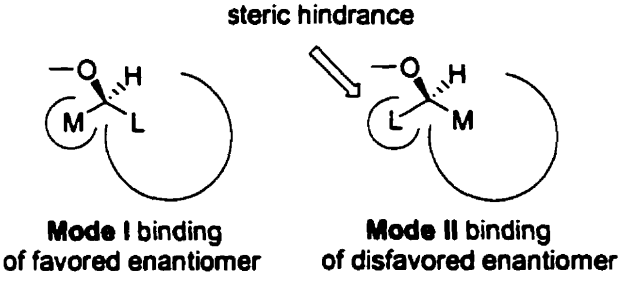


Figure 1.20. Binding modes I and II for secondary alcohols. Mode II has unfavorable steric interactions.

This study, as well as later experiments combining substrate screening with molecular modeling that were carried out by some of the same researchers (Rotticci *et al.* [55] and Orrenius *et al.*[56]), indicated that size restrictions of the binding pockets are important. It was suggested that there are two modes of binding for the substrates, Mode I and Mode II, Figure 1.20. Favored enantiomers bind as in Mode I, with the medium substituent in the medium sized pocket. The disfavored enantiomer must bind as in Mode II to be catalytically productive. In this latter mode, the large substituent is placed in the sterically restricted medium pocket. When the large substituent is not very big, it can fit more easily into the medium pocket, lowering the preference for Mode I binding. However, as the large group is increased in size, accommodation within the medium pocket becomes more difficult; the enantioselectivity is enhanced because Mode II binding is less likely. They also suggested that unfavorable electrostatic interactions can occur in the medium pocket if a halogenated substituent is present, as discussed above. The presence of a halogen atom on the large substituent, probably increases the enantioselectivity by making Mode II binding less likely because of both unfavorable types of interactions within the medium pocket.

Zuegg *et al.*[24] analyzed the low energy conformations of both enantiomers of secondary alcohols within the active site of both PCL and CRL. They proposed that enantiomer differentiation is based on the size of the substituents. Secondary alcohols exhibit what they call H-alignment, Figure 1.21a. The hydrogen atom at the stereocenter of both enantiomers binds in the same site. The large substituent of one enantiomer is then forced to bind in the medium pocket, causing unfavorable steric interactions. The two possible orientations for the substrate are the same as proposed by Rotticci *et al.* and Orrenius *et al.*, above.

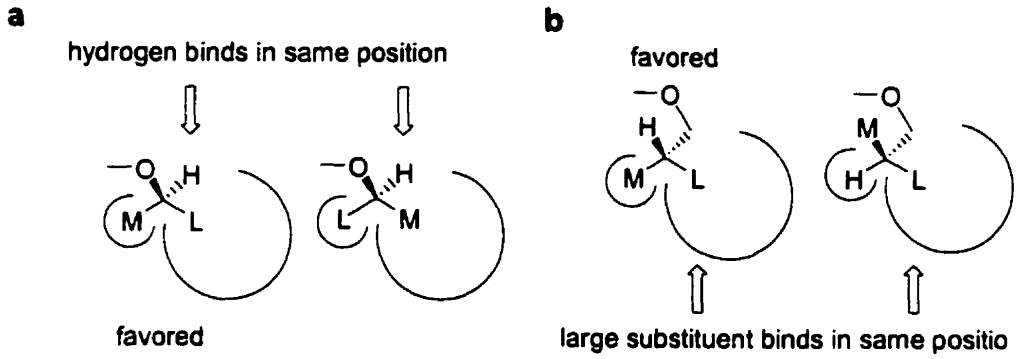


Figure 1.21. Secondary alcohols, **(a)**, exhibit H-alignment, whereas primary alcohols, **(b)**, exhibit L-alignment. The large and medium substituents of both classes of alcohols bind in the same respective sites.

Primary alcohols

As described in Chapter 3, PCL prefers the opposite enantiomer of primary alcohols. We give a proposal for this opposite enantiopreference based on studies carried out with substrates having two stereocenters. Three independent groups of researchers have performed crystallographic and molecular modeling analyses using PCL. Their proposals for the molecular basis by which PCL differentiates between enantiomers of primary alcohols and rationalizations for its opposite preference for primary and secondary alcohols are discussed below.

Zuegg *et al.*[24] proposed that the favored enantiomer of secondary alcohols and primary alcohols bind in approximately the same manner: the larger substituent at the stereocenter in a large hydrophobic pocket and the medium substituent in a smaller more hydrophilic pocket, Figure 1.21. They suggest that the preferred primary alcohol is opposite to the preferred secondary alcohol because of the extra carbon between the stereocenter and the alcohol hydroxyl group. This is in agreement with the research described in Chapter 3. Primary alcohols exhibit L-alignment. That is, the large substituents bind in the same

pocket for both enantiomers. For the disfavored enantiomer, this forces the hydrogen atom into the hydrophilic pocket and the medium substituent out of the pocket towards the solvent. In this proposal, these unfavorable binding modes are the basis for enantiodifferentiation.

In the previously discussed studies conducted by Nishizawa *et al.*,[31] it was also suggested that both substituents of primary and secondary alcohols bind in the same regions of the active site of PCL. They proposed that the enantioselectivities for primary alcohols are lower than for secondary alcohols because the medium substituent is further away from the stereocenter and therefore does not disrupt hydrogen bonding to the catalytic histidine to the same extent. This results in less of a difference between the transition state reactivities of the two enantiomers.

Tuomi and Kazlauskas[57] proposed that primary and secondary alcohols bind in different regions of PCL, as a result of molecular modeling of transition state analogues. The secondary alcohols bind as described above, but the large substituents of primary alcohols bind in an "alternate" hydrophobic pocket that is fairly far removed from the hydrophobic pocket that binds large substituents of secondary alcohols, Figure 1.22. In addition to molecular modeling, kinetic studies were performed. They studied two different primary alcohol substrates, one with an oxygen attached to the stereocenter and one without. This was done in an attempt to discover why, as will be discussed later, an existing rule can predict the enantiopreference of PCL towards primary alcohols without an oxygen at the stereocenter, but it cannot be applied to those with an oxygen. In the case of the substrate lacking an oxygen at the stereocenter, the favored enantiomer has a lower K_M than the disfavored one, but the k_{cat} values are approximately the same. Molecular modeling shows that the favored enantiomer has better hydrophobic contacts: the medium substituent of the disfavored enantiomer is situated outside the medium pocket, Figure 1.23.

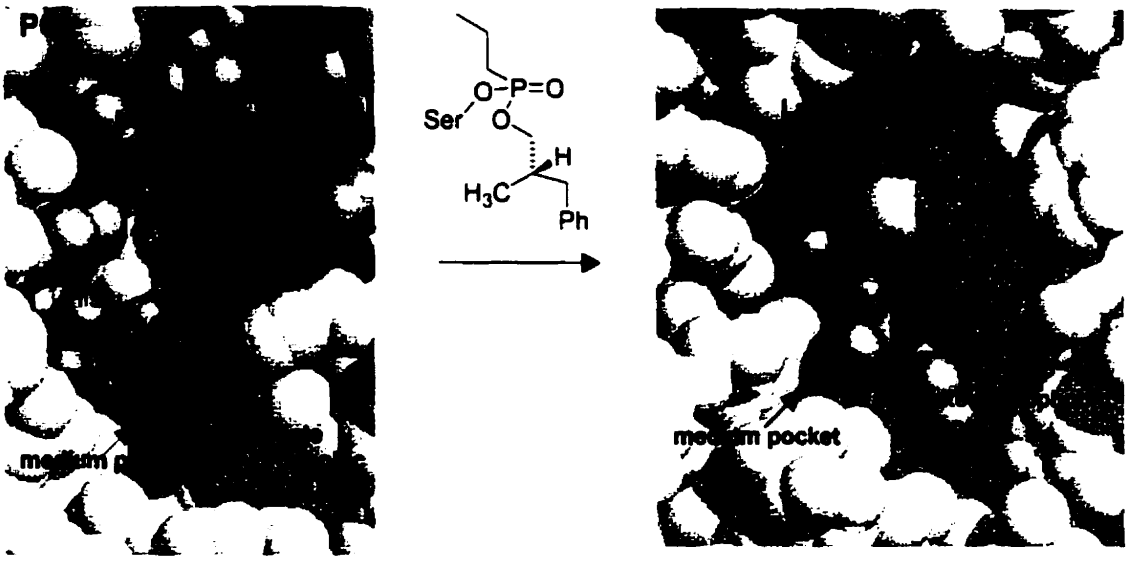


Figure 1.22. The active site of PCL without (left) and with (right) an embedded primary alcohol phosphonate transition state analogue. According to Tuomi and Kazlauskas the large substituent of primary alcohols, in this case a phenyl group, binds in an alternate hydrophobic pocket. In the right hand picture, the colours have been omitted for clarity. The viewing angle of the two pictures is slightly different.

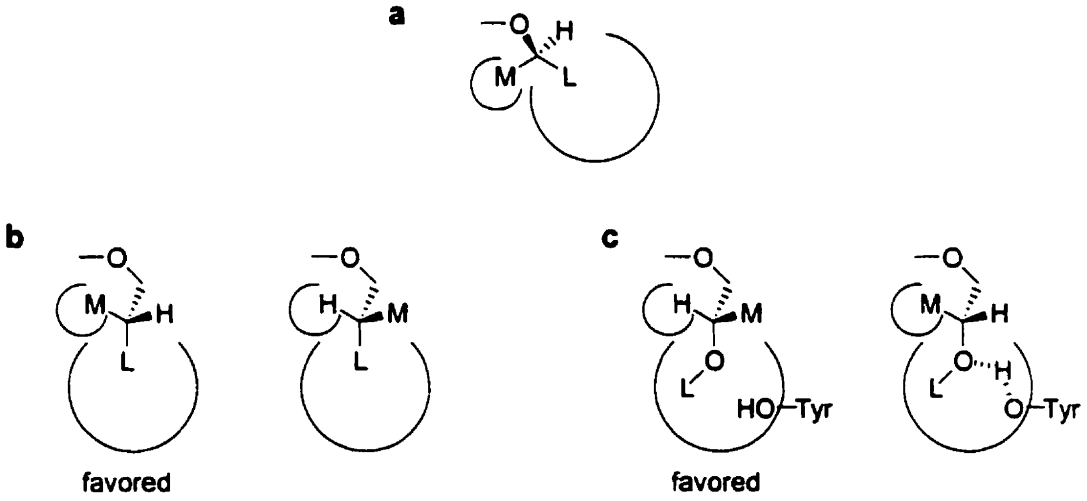


Figure 1.23. **(a)** The preferred orientation of a secondary alcohol substrate. **(b)** Proposed orientations for each enantiomer of a primary alcohol substrate with an oxygen at the stereocenter. **(c)** Proposed orientations for each enantiomer of a primary alcohol lacking an oxygen at the stereocenter: the disfavored enantiomer forms a hydrogen bond to tyrosine. The large substituent of primary alcohols binds in an alternate hydrophobic pocket that does not correspond to the hydrophobic pocket for the large substituents of secondary alcohols. The medium substituents bind in the same pocket for both classes of alcohols.

PCL prefers the opposite enantiomer of the substrate having an oxygen at the stereocenter.[58] The kinetic studies showed that both k_{cat} and K_M are greater for the preferred enantiomer. Molecular modeling showed that the disfavored enantiomer has better binding: both substituents are in their respective pockets and, in addition, there is a hydrogen bond between the phenoxy oxygen of the substrate and the phenol hydroxyl moiety of a tyrosine residue in the 'alternate' hydrophobic pocket, Figure 1.23. Although the hydrophobic contacts of the favored enantiomer are poorer, the difference in k_{cat} is much greater than the difference in K_M, as a result the favored enantiomer reacts faster. It is suggested that the lower k_{cat} of the disfavored enantiomer is due to the hydrogen bond to the tyrosine residue; it is possible that the reverse reaction is promoted due to the tighter binding of the substrate. In all of the above binding modes, the catalytically essential hydrogen bonds were maintained. In this proposal, both k_{cat} and K_M are important in determining enantiopreference.

Lang *et al.*[30] identified three pockets within the substrate binding site of PCL by examining its crystal structure with a covalently-bound triacylglycerol analogue. The three binding sites were defined as follows: HA, the acyl binding pocket; HH, a large hydrophobic pocket with a small hydrophilic region; and HB, a smaller slightly hydrophobic pocket.* The crystal structure was obtained with only the faster-reacting, R, enantiomer of the inhibitor. Molecular modeling studies were done to determine how the slower-reacting, S, enantiomer would bind in comparison. It was observed that the *sn*-2 moiety of the fast-reacting enantiomer binds in the HH pocket and forms a hydrogen bond between the carbonyl oxygen and the O_γ of a Thr residue. However, the *sn*-3 moiety of the slow enantiomer is bound in this pocket. Since the environment near the stereocenter of the two substituents is different, unfavorable steric interactions

* It appears that the HH pocket corresponds to the alternate hydrophobic pocket of Tuomi and Kazlauskas.

occur for the *S* enantiomer, instead of the stabilizing hydrogen bond. It is suggested the presence of these unfavorable interactions might account for the preferential reaction of the *R* enantiomer. The interactions of the HB pocket with the inhibitor are fewer and weaker and therefore of less consequence. In this proposal, the two substituents switch places and one binding mode is preferred. It is rationalized that the size and nature of the portion of the substituents that is close to the stereocenter is of great importance in the discrimination of enantiomers.

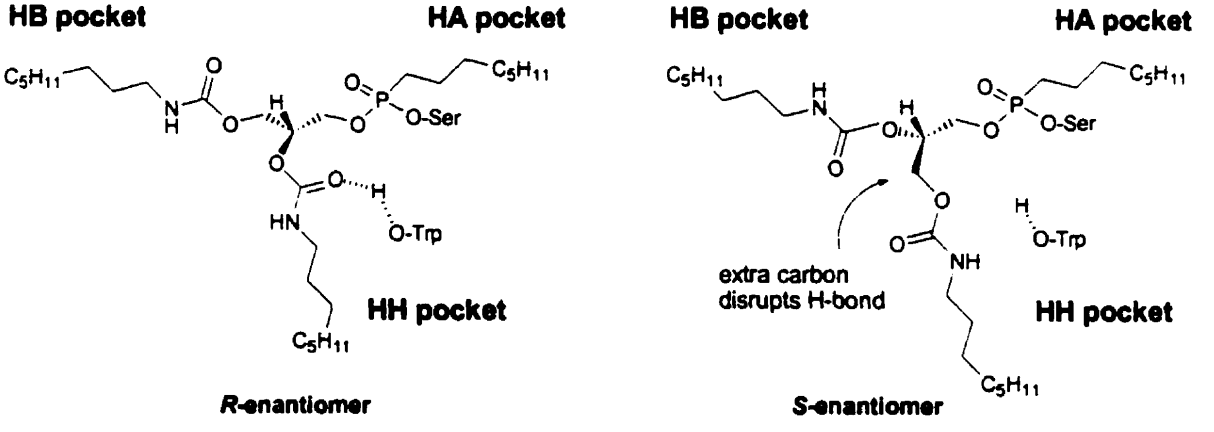


Figure 1.24. A stabilizing hydrogen bond is formed when the *sn*-2 moiety of the preferred, *R*, enantiomer binds in the HH pocket. In the case of the *S* enantiomer, it is the *sn*-3 moiety that binds in the HH pocket: its structure does not allow for hydrogen bonding to the Trp residue.

To summarize the studies done with primary alcohols, Tuomi and Kazlauskas disagree with Zuegg *et al.* and Nishizawa *et al.* on how primary alcohols bind as compared to secondary alcohols, however they all agree that the large substituent remains in same position for both enantiomers of a primary alcohol. Zuegg *et al.* and Lang *et al.* suggest that differences in binding are of primary importance in enantiodiscrimination. Nishizawa *et al.* suggest that it is

differences in reactivities of the transition state. However, Tuomi and Kazlauskas propose that both factors influence enantiopreference.

Subtilisin

As will be discussed in the following section, subtilisin favors the opposite enantiomer of secondary alcohol substrates as compared to lipases and esterases. In Chapter 4, we propose that this is due to the fact that the catalytic histidine is on the opposite side of the binding pockets, as shown in Figure 1.25.

Figure 1.25. A comparison of this figure with Figure 1.15 shows how the catalytic histidine of subtilisin is on the opposite side of the binding pockets, in comparison to its position in lipases and esterases. This figure was created with RasMol v2.6 and PDB file 1sbc.[8]

Although fewer studies have been conducted with subtilisin, to determine how it distinguishes between enantiomers, it seems as though rationales similar to those for lipases can be applied. Colombo *et al.*[59] modeled two secondary alcohol substrates within the active site of subtilisin Carlsberg. The same hydrogen bond network between the catalytic His residue, the alcohol oxygen of the substrate, and the nucleophilic Ser oxygen was found to be present for the favored enantiomer but not for the disfavored one. In addition, a favorable stacking arrangement was

observed between the imidazole ring of the catalytic His and the phenyl ring of the fast-reacting enantiomer.

Crystal structures of subtilisin with boronic acid based transition-state analogues bound to the active site, have been studied.[48] However since these inhibitors mimic amino acids, it is the S_1 subsite, corresponding to the acyl binding site of lipases and esterases, that was probed. This study is beyond the scope of this thesis.

Although many different proposals have been made, there is still not any one clear answer to the question of how enantiodifferentiation occurs and what determines the level of enantioselectivity. It would seem as though there is a fine balance between the many structural and electronic characteristics of both the substrates and the hydrolases. In addition, it is possible that there is a different explanation for different substrates and different enzymes. In spite of this, a reliable prediction of the stereochemical outcome of many hydrolase-catalyzed reactions is possible.

1.6 Predicting enzyme selectivity

In recent years chemists have attempted to make enzymes more accessible and alluring for the production of optically pure materials by developing rules that predict the enantioselective outcome of enzyme-catalyzed reactions. As there are a great number of hydrolases available, reliable rules and models greatly simplify the screening process involved in choosing the most appropriate hydrolase for a given transformation.

Despite the vast amount of information that has recently become available a a result of crystal structure analysis, in practice it is still difficult to use this data to predict the enantiopreference of a hydrolase. Therefore, although most of the following rules were proposed before X-ray structures became available, they are still very valuable to the organic chemist. So much so, that rules and models have even been developed after crystal structures have been solved.

One of the first rules available was developed by Prelog to predict the enantioselectivity of yeast-catalyzed reductions of ketones[60]. This rule is based solely on the size of the substituents at the stereocenter and is reliable enough to use for the determination of absolute configurations[61]. After the first few rules for hydrolases were proposed about ten years ago, a plethora of rules and models have been developed as a result of extensive screening studies and molecular modeling studies.

The primary objective of the work described in this thesis was to develop general models for predicting the stereochemical outcome of hydrolase-catalyzed reactions; specifically, the hydrolysis of esters and esterification of primary and secondary alcohols. The application of these rules led us to propose a method to increase enantioselectivity as well as allowing us to prepare several optically-pure useful chiral synthons.

There are two basic categories of predictive rules or models: substrate rules and box-type models. Substrate rules illustrate which substituent characteristics are preferred by hydrolases. Some are detailed and apply only to a single class of substrates with a particular enzyme, and therefore have a limited degree of usefulness. Others are very general and apply to a vast variety of substrates and several hydrolases. The box-type models, either two- or three-dimensional, attempt to map the active site. These models tend to give more detail about the shape, size, hydrophobicity, and electronic character of the hydrolase binding site. However, they are often harder to apply to a given substrate and are more specific to one particular hydrolase. The following review of proposed rules and models is divided into five categories: substrate rules and active site models for secondary alcohols, substrate rules and active site models for primary alcohols, and substrate rules for primary amines.*

Secondary alcohols: Predictive substrate rules

Among the first substrate rules to be proposed were detailed and specific to one enzyme and one class of substrate. Xie *et al.* proposed two rules that predict the favored enantiomer in PCL-catalyzed hydrolyses of cyclic and bicyclic systems.[62] The first is a three-site model specifically for cyclic or bicyclic diols[63] and the second is a stereomodel using a Newman projection of cyclic or bicyclic systems[64] (Figure 1.26).

Oberhauser *et al.* proposed a detailed substrate model for CRL-catalyzed resolutions of bicyclic secondary alcohols. [65] This model, which is restricted to bicyclo[2.2.1]heptanols and bicyclo[2.2.2]octanols, is shown in Figure 1.24. This model has recently been refined by Faber *et al.*, using comparative molecular field analyses, to include to quantitative estimations of the enantioselectivity of CRL towards this group of substrates.[66]

*The reader may wish to consult Figures 1.15 and 1.25 while reading this section, to compare the rules and models with the substrate binding sites of the hydrolases.

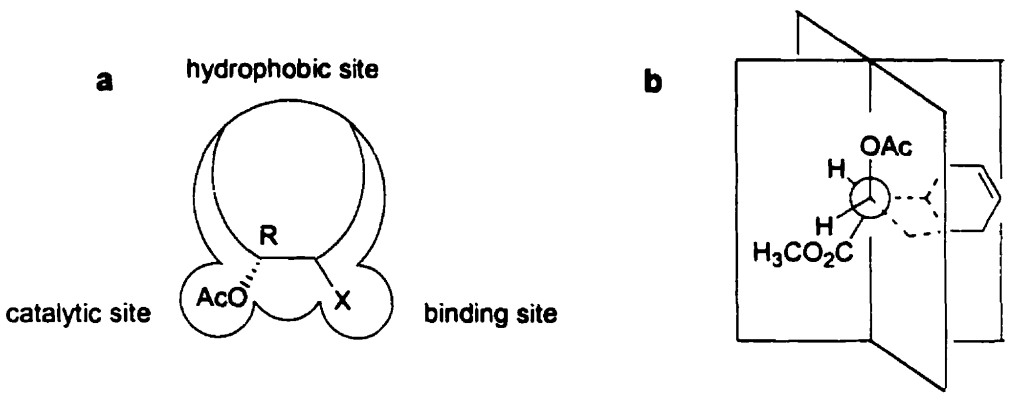


Figure 1.26. **a)** Three-site model for PCL. The hydrophobic site is for cyclic ring systems and the binding site is for an alcoxycarbonyl or acetate group. **b)** Stereomodel for PCL. Binding occurs in the right-hand volume when the Newman projection of the substrate is drawn with the acetate upwards.

The refined mathematical substrate model gives a representation as to which areas of the substrate are sterically favored, as well as which areas are favored for a negative charge and which for a positive charge. For example, the top of the bridge atom is an area that is favored for a negative charge but sterically disfavored. When all the structural and electronic requirements of the substrate are met, a good enantioselectivity is predicted with the preferred product being the enantiomer shown in Figure 1.27.

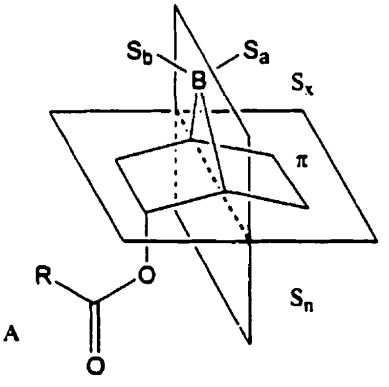
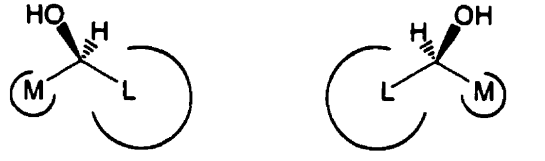


Figure 1.27. Model for bicyclic substrates of CRL. The sites and substituents are defined as follows: **A**: reaction site (must be *endo*). **B**: bridge (may contain hetero atoms). S_a and S_s: *anti* and *syn* substituents (may be an ester, ether, or acetal group). S_x: *exo*-substituent (may be large). S_n: *endo*-substituent (must be very small). π: π-site (π-electron in this site enhance enantioselectivity). The mathematical model gives more detailed space and electrostatic restrictions.

The development of a more simple rule that predicts the stereochemical outcome for hydrolase-catalyzed reactions with secondary alcohols and their esters is detailed in Chapter 2. This is a very general qualitative rule (Rule A, Figure 1.28) which is based on the difference in size of the substituents at the stereocenter of the substrate. It is not an attempt to map the active site but rather simply a rule to predict which substrate of a racemic pair (or which alcohol moiety of a meso or prochiral diol) will react preferentially.



Rule A: Lipases **Rule B**: Subtilisin

Figure 1.28. Simple predictive substrate rules for secondary alcohols.

The great advantage of this rule is its generality and simplicity to apply. One must simply draw the substrate in question such that the alcohol (or ester) group is pointing out of the page towards the reader as shown in Figure 1.28. A line is then drawn down the middle of the molecule and the enantiomer with the large group on the right side of the line is the one that will react preferentially. A similar was first proposed by Ziffer *et al.*[67] to predict the enantiopreference of lipase from *Rhizomucor nigricans* in the hydrolysis of a variety of cyclic and aromatic secondary alcohol esters. Through extensive literature surveys and substrate screenings we found that this rule also predicts the enantiopreference of three other hydrolases, namely cholesterol esterase (CE), lipase from *Candida rugosa* (CRL), and lipase from *Pseudomonas cepacia* (PCL), see Chapter 2. In addition, the same or similar rule has been shown to hold true for at least 8 other hydrolases.[68,69] In the case of CRL, the rule can only be applied to cyclic

substrates. This rule has been found to be extremely reliable; at least 93% accurate. The validity of this rule was confirmed by the crystallographic analysis of CRL which, as described in the previous sections, shows the presence of a large and a medium pockets in the active site. Thus, the active site resembles the rule.

Although this rule is very reliable, a certain amount of discretion must be used when applying the rules. For instance, a long unbranched alkyl chain is acceptable in the "M" position probably because the chain can fold and point out towards the solvent, therefore only occupying a small volume of space within the binding site.

Additional substrate mappings carried out with several lipases resulted in more details about the spatial constraints required for a substrate. Exl, *et al.*[70] concluded that CRL accepts larger flat substrates (to a limit of 9.2 Å) than PCL (to a limit of 7.1 Å) but generally with lower enantioselectivities. From a detailed substrate analysis with PCL, Theil *et al.*[71] determined that the good substrates have a long and flat or unbranched substituent, whereas poor substrates are sterically crowded close to the stereocenter. In addition, enantioselectivity was found to be highly dependent on the substitution pattern of an aryl ring (Figure 1.29). Kim and Cho[72] found that lipoprotein lipase from *Pseudomonas aeruginosa* prefers substrates whose smaller group is linear and up to three carbons long and whose larger substituent is nonlinear and preferably contains a hydrophobic ring.

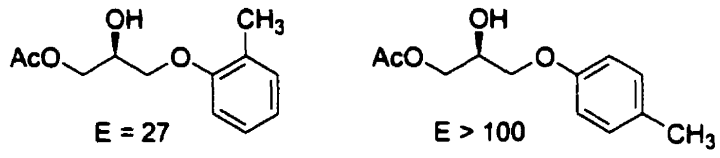


Figure 1.29. The enantioselectivity of PCL differs for *ortho* and *para* substituted substrates.

In Chapter 4 we review how the protease, subtilisin[73], prefers the enantiomer opposite to the one favored by lipases.[74] Therefore, a predictive rule opposite to Rule A is applicable for subtilisins (Rule B, Figure 1.28). This rule was found to be 85% accurate in predicting the stereochemical outcome of reactions with secondary alcohols and their esters.

Secondary alcohols: Active site models.

The next generation of models that were developed to predict hydrolase enantiopreference are the two-dimensional box type. These active-site models are also based on the size of groups at the stereocenter of secondary alcohol substrates. They are not as simple to use as the rules described above,. however they include more detailed size restrictions. Each model is also specific to one given hydrolase.

At about the same time that the large/medium rule described in Chapter 2 (rule A) was published, a two-dimensional box-type model was developed for lipase AK (model C, Figure 1.30) from *Pseudomonas fluorescens*.[75] The first step in using this model is to fit the hydroxyl functionality of the substrate into the catalytic site (the OH pointing out from the page). The next step is to determine which enantiomer best fits into the active site model. If any part of a molecule goes beyond the boundaries of the model, it is not a substrate. The enantiomer with the best fit is predicted to be the major product. This model was based primarily on reactions with unsaturated alcohols. This model was also used to explain experimental observations for PCL and *Chromobacterium viscosum* (*Pseudomonas glumae*)[76]

A similar model was proposed to predict the enantiopreference of lipase YS[77] from *Pseudomonas fluorescens* (model D, Figure 1.30). It is used in exactly the same way as the one proposed for lipase AK. This model was developed by studying a large variety of substrates: acyclic, cyclic, and bicyclic secondary and primary alcohols. The top perspective of the model shows that there is a large

pocket on the right and a smaller pocket on the left. It was proposed that the right-hand pocket is more polar because the lipase showed high enantioselectivity for substrates having a phenyl moiety with small polar substituents.

The same type of model was also proposed for Lipase QL[78] from *Alcaligenes sp.* (model E, Figure 1.30).To use this model one must place the hydroxyl group into the catalytic site; the stereocenter hydrogen into the H_S site (small hydrophobic site); and the two substituents into the H_L site (large hydrophobic site). H_L has a large pocket (H_{Lr}) on the right and a small pocket (H_{Ll}) on the left.

H_{Lr} is more polar in character. Both H_{Lr} and H_{Ll} are wider than the pockets of lipase AK and lipase YS. Again, the enantiomer with the best fit is predicted to react preferentially.

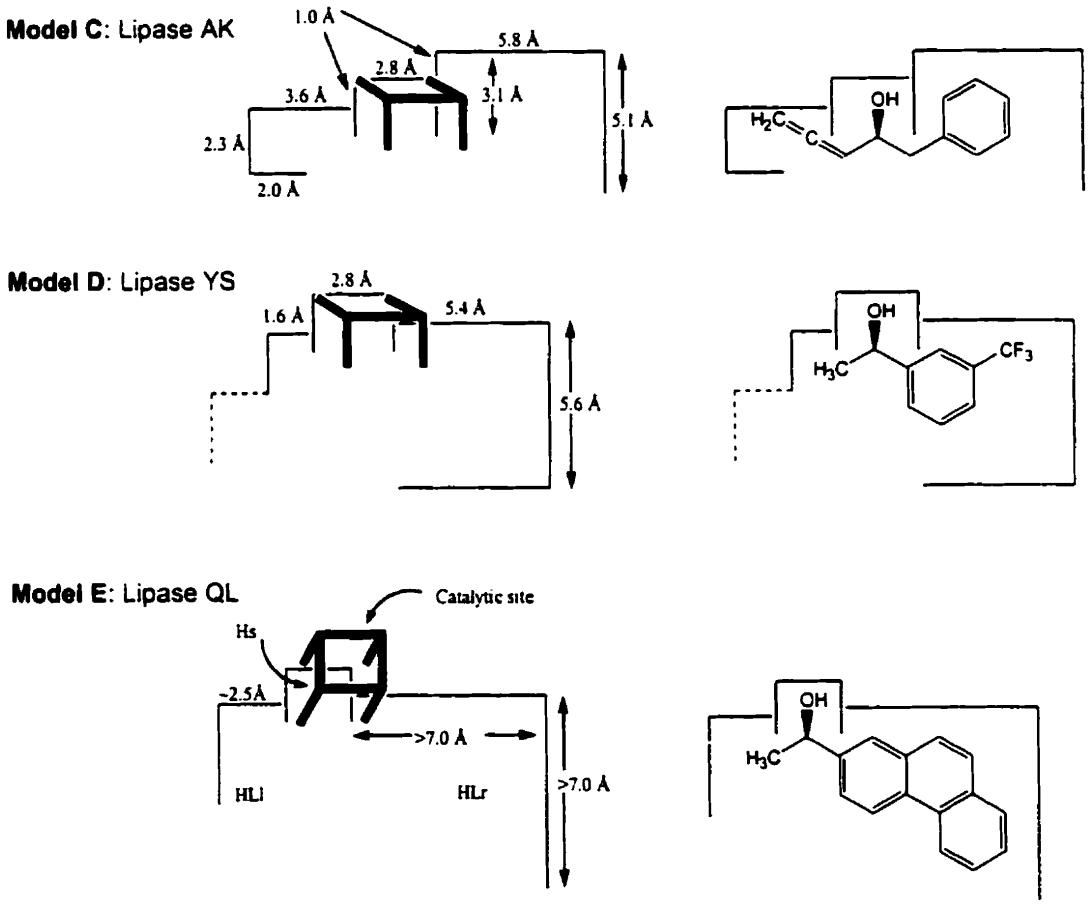


Figure 1.30. Lipase active site models for secondary alcohols.

Very recently, through a combination of substrate mapping and molecular modeling, two three-dimensional active site models were independently proposed for PCL. Both models were proposed prior to knowledge of its crystal structure.

Lemke *et al.* investigated the enantioselectivity of PCL towards 3-(aryloxy)propan-2-ols.[79] This research was a continuation of the previously described work carried out by Theil *et al.*[71] A comparison of the experimentally obtained enantiomeric ratios with a superposition of the low-energy conformers of both substrates and non-substrates led them to the propose Model F (Figure 1.31). According to this model, the two hydrophobic pockets are very different in shape. One has a spherical shape and the other is a long tube with a limited diameter. The former pocket accommodates bulky groups such as phenyl, phenoxymethyl, and substituted aryl derivatives, wherease the latter binding site accepts only stretched substituents such as acetoxymethyl and (n- hexadecanoyloxy)methyl. Therefore, it was suggested that it is not only size that determines enantioselectivity but also shape.

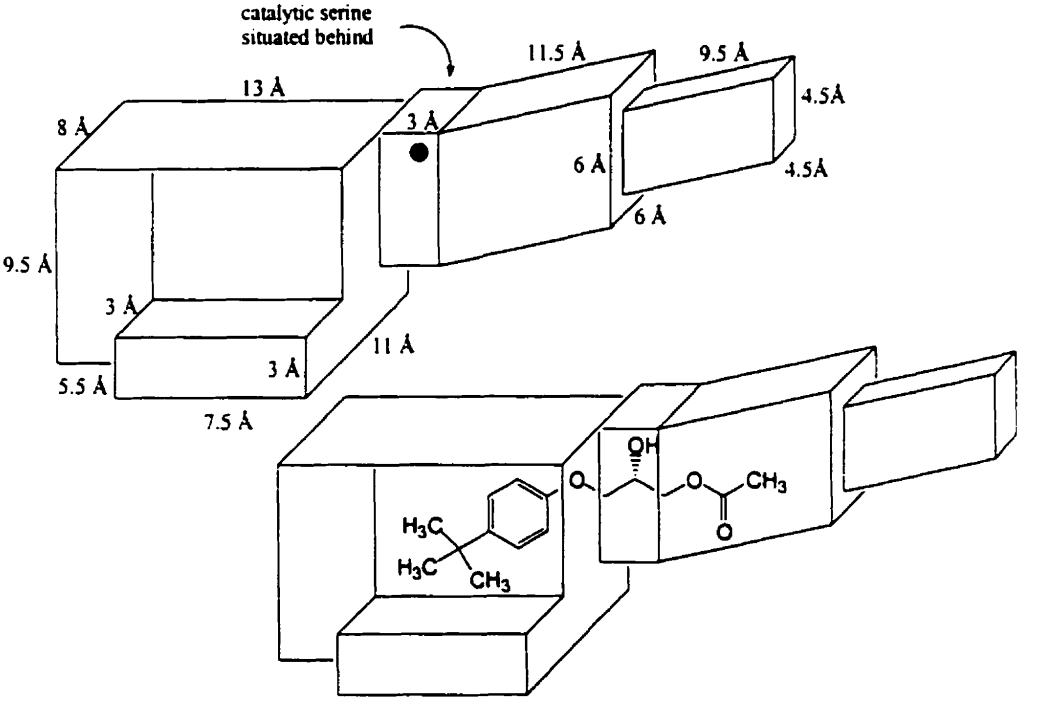


Figure 1.31. Model F: active site model for PCL.

Grabuleda[80], et al. carried out similar computational studies on a wide variety of secondary alcohol substrates. They also came to the conclusion that there are two different shaped binding pockets: a large hydrophobic pocket (H_L) and a smaller tunnel-shaped less hydrophobic pocket (H_S), Model G, Figure 1.32. The hydroxyl group binds in the H_H pocket, pointing outwards toward the front of the pocket. Both models are essentially the same, however the dimensions of Model F are greater overall.

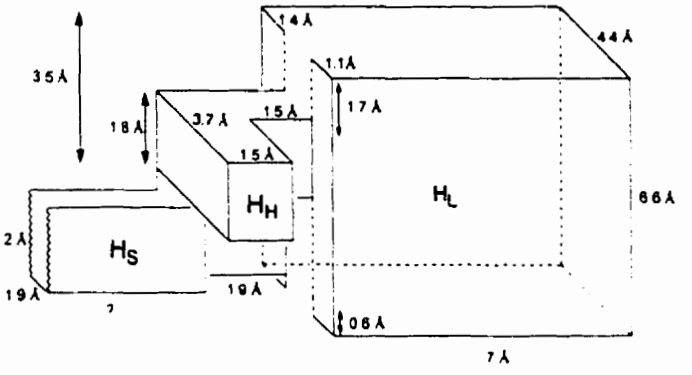


Figure 1.32. Model G: Active site model for PCL

Jones *et al*[81] developed a detailed box-type model of the active site of porcine liver esterase (PLE). Although this model was based on reactions with esters of racemic acids (carboxylic esters), it has been successfully applied to PLE-catalyzed hydrolyses of racemic diacetates of bicyclic compounds (2° alcohols).[82] The boundaries of the original model have since been slightly modified[83] and it is the final model which is shown in figure 1.33 (model H).

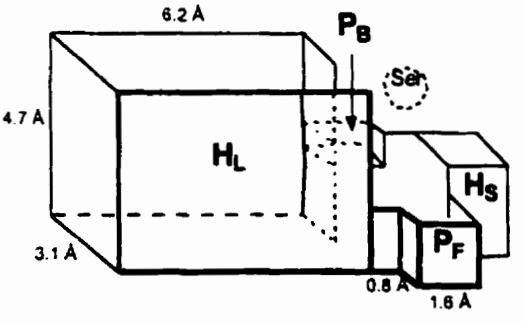


Figure 1.33. Model H: active site model for PLE.

As can be seen in Figure 1.34, all the above box-type models have one side that is larger than the other and therefore they resemble the empirical substrate rule, Rule A, in this manner.

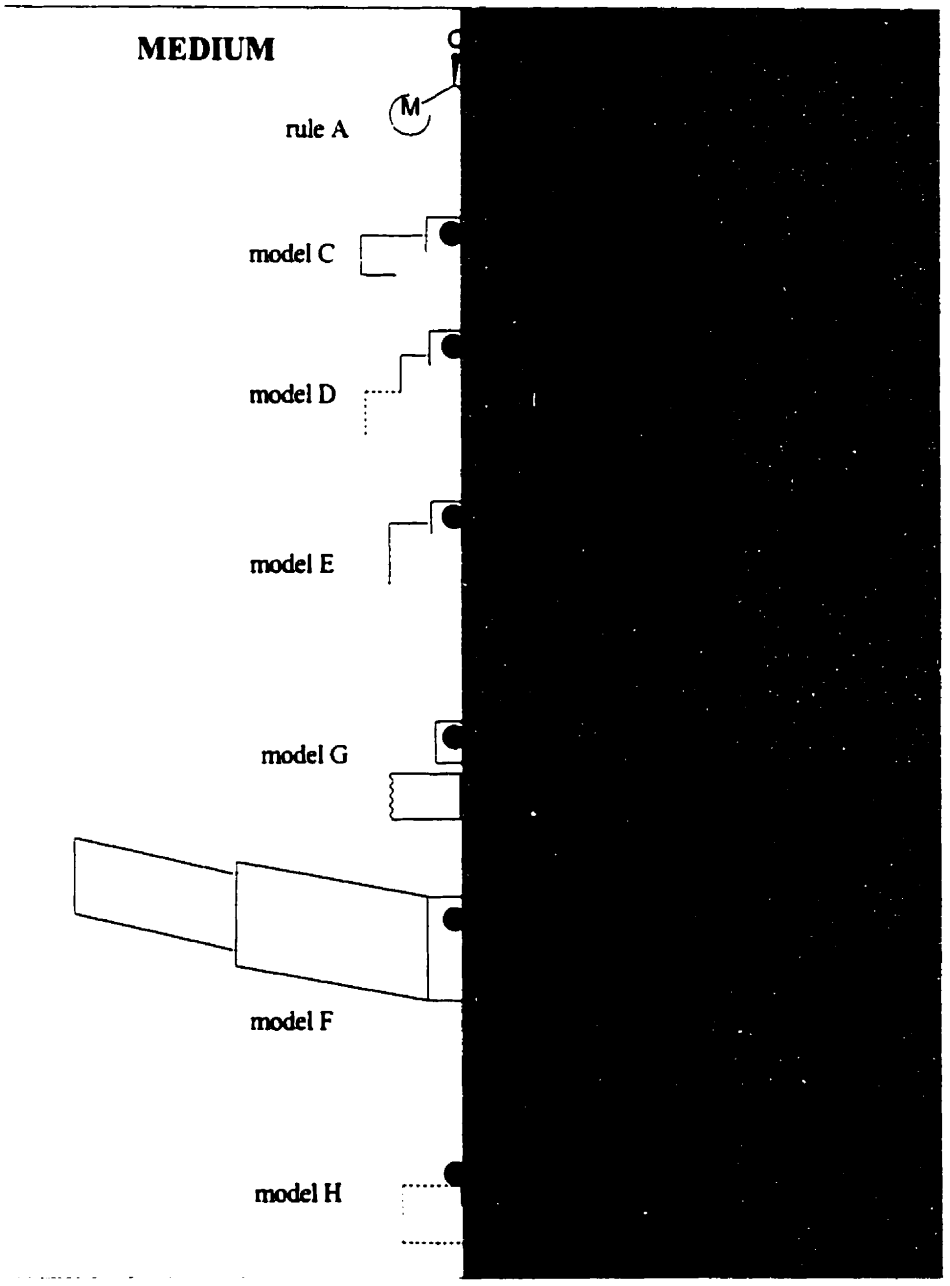


Figure 1.34. A comparison of the box-type models for secondary alcohols shows that their overall shape is similar to substrate rule A. The models have been simplified and redrawn such that the alcohol binding site, represented by the black circle, is pointing upwards and out of the page.

Primary alcohols: Predictive substrate rules

There are fewer examples of hydrolases-catalyzed transformations involving primary alcohols because enantioselectivities tend to be lower than for secondary alcohols. For this reason, fewer rules and models have been developed and they are generally less reliable.

Chapter 3 describes how the empirical rule for secondary alcohols (Rule A, Figure 1.28) was extended to β-branched primary alcohols. This rule (rule I, Figure 1.35) is opposite to the secondary alcohol rule; that is, the CH_2OH points into the page, away from the reader when the large substituent is on the right and the medium substituent is on the left. It applies solely to PCL and only reliably predicts which enantiomer of a substrate will react preferentially when the substrate does not have an oxygen attached directly to the stereocenter. In such cases, the rule is 89% accurate.

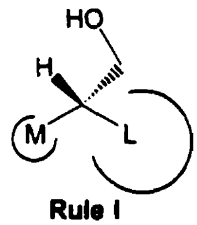


Figure 1.35. Simple rule to predict the enantiopreference of PCL towards primary alcohols.

Jones *et al.*[84] have recently shown that subtilisin shows an enantiopreference opposite to lipases for β-branched primary alcohols. This rule, Rule J, Figure 1.36, is also opposite to the predictive rule for subtilisin towards secondary alcohols, Rule B, Figure 1.28. They also found that the enantiopreference was reversed, once again, for subtilisin-catalyzed reactions with γ-branched primary alcohols, Rule K.*

* This rule was based on only one example with low selectivity.

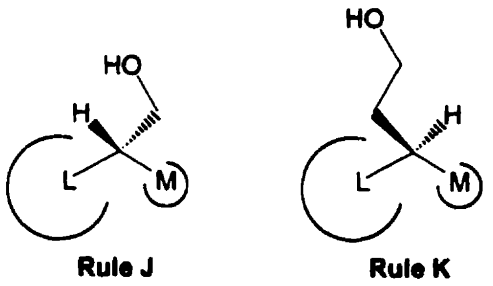


Figure 1.36. Rules to predict the enantiopreference of subtilisin *Bacillus lentus* towards β-branched primary alcohols (Rule J) and γ-branched primary alcohols (Rule K).

Several rules have been proposed to predict the stereochemical outcome of reactions catalyzed by PPL. However, due to many discrepancies, none of the rules are reliable enough to be useful to the organic chemist. In fact, rule L and rule M, Figure 1.37, are essentially mirror images. This contradiction of rules has often been referred to as the 'PPL dilemma'.

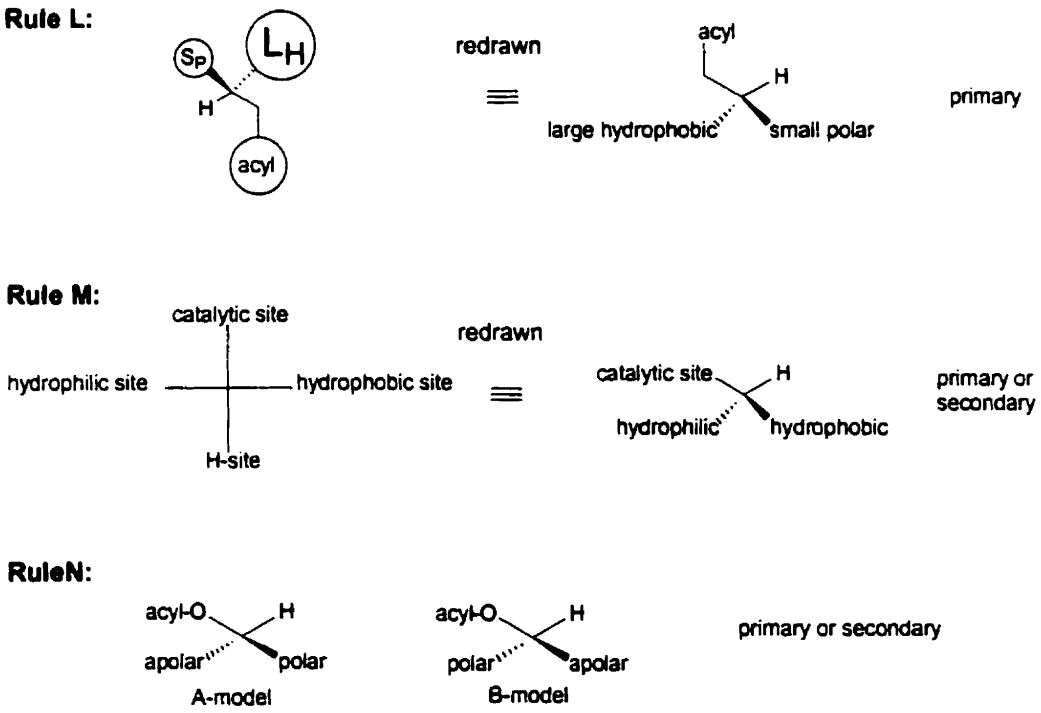


Figure 1.37. Rules to predict the enantiopreference of PPL towards primary and secondary alcohols.

The rule proposed by Hultin and Jones[85] is shown in Figure 1.37, Rule L. The acyl site corresponds to the catalytic site. The L_H pocket accepts sterically demanding substituents that are essentially hydrophobic in character. The S_P pocket is much smaller, accepting polar substituents no bigger than an acetoxymethyl group. The S_P site will also accept small non-polar substituents such as a methyl group.

The rule proposed by Ehrler and Seebach[86] does not include any size factor. This rule, Rule M, is simply applied by comparing the relative hydrophobicities of the substituents at the stereocenter. The rule is drawn as a Fisher projection with the catalytic site for esters of primary or secondary alcohols at the top and the stereocenter hydrogen at the bottom. The preferred enantiomer then has a hydrophobic group on the right and a hydrophilic group on the left.

When the rules are redrawn as shown in Figure 1.37, one can easily see that they are basically enantiomeric. Wimmer[87] attempted to clarify the problem by comparing the successfulness of A-model and B-model of rule N with both Rule L and Rule M, in predicting the preferred enantiomer. Wimmer specified that these rules are to be applied by comparing the polarity of the portion of the substituents that is in the immediate vicinity of the stereocenter. He also stated that the relative size of substituents is of lesser importance than the polarity. Wimmer deduced that although the A-model was more reliable, neither was completely successful.

Although Rules M and N are for secondary alcohols as well as primary alcohols, it has been suggested that pure PPL has no activity towards secondary alcohol esters and that it is contaminants within crude PPL that are responsible for hydrolysis.[85,88]

Primary alcohols: Active site models.

The box-type model that was proposed for reactions with secondary alcohols catalyzed by lipase YS (model D, Figure 1.30) was also found to apply to reactions with primary alcohols. It must be noted that none of the primary alcohols screened contained an oxygen at the stereocenter. Figure 1.35 shows how primary alcohols are placed within this model. By comparing Figures 1.30 and 1.38, it can be seen that this model also predicts that the opposite enantiomer is preferred for primary alcohols.

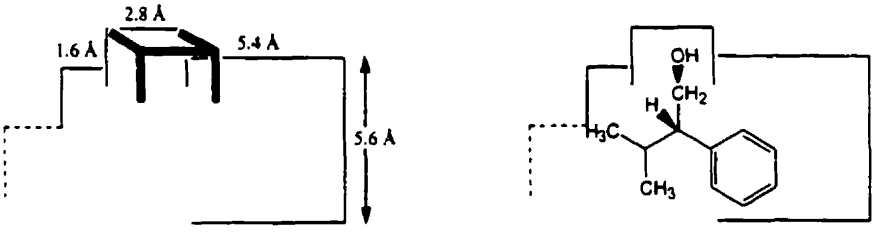


Figure 1.38. Model D for lipase YS applied to primary alcohols.

A two-dimensional box-type active site model has been proposed to predict the enantioselectivity of lipase AKG from *Pseudomonas sp.*[89]. This model is very specific to a certain group of substrates, namely, α,α-disubstituted 1,2-diols, a group of primary alcohols having a tertiary OH at the stereocenter. This model is shown in figure 1.39, model O. The R (reaction) pocket is the catalytic site for the primary hydroxyl group (this hydroxyl must point into the page, away from the reader). The tertiary hydroxyl group points out of the page, towards the reader. The F (flat) pocket accepts only flat, nearly planar, side chains (e.g. allyl group) in an extended conformation. Phenyl substituents can be positioned in the S (small) pocket. Substituents positioned in the F pocket must not be aliphatic, in order to obtain a good fit. Phenyl substituents must not be substituted in the para position, because they become too large for the S pocket.

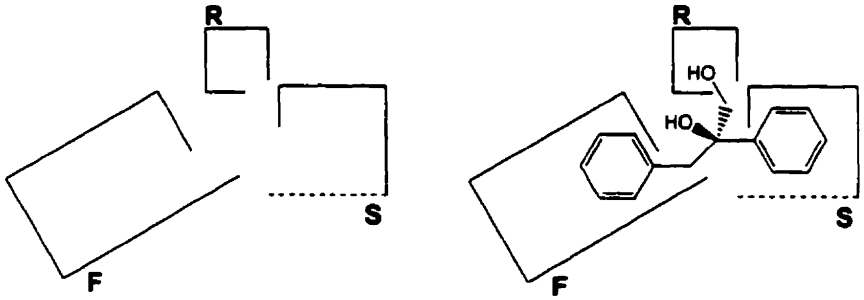


Figure 1.39. Model O: for predicting the enantiopreference of lipase AKG towards α,α-disubstituted 1,2-diols

A box-type active site model has also been proposed for lipase from *Rhizopus delemar*[90]. This model, Model P, Figure 1.40, is specifically for derivatives of *meso*-bis(acetoxymethyl)cyclopentane (meso diacetates of primary alcohols). Both the top view and the side view of the model are shown. The nucleophilic serine residue is positioned at the "S". The acetyl function of a substrate must be positioned here when the substrate is fitted into the box, for hydrolysis to occur.

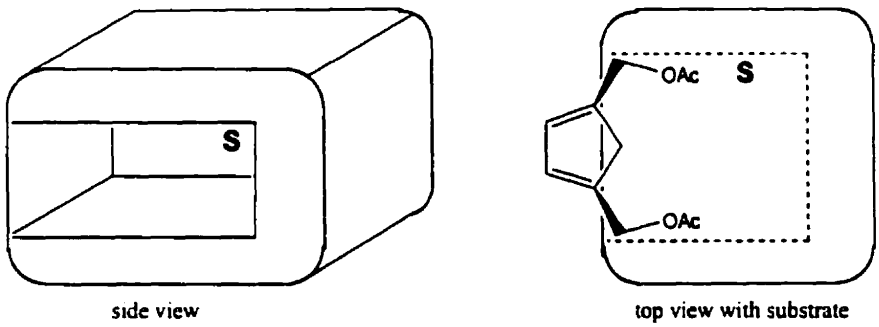


Figure 1.40. Model P: for predicting the enantiopreference of lipase from *Rhizopus delemar* towards meso diacetates of primary alcohols.

Guanti *et al.* proposed a box-type active site model for PPL catalyzed reactions of primary alcohols, Model Q, Figure 1.41.[91] It was developed primarily for 2-substituted 1,3-diacetoxypropanes. According to this model, the active site is comprised of four pockets. Site A is the catalytic site and site C is a hydrophilic site that accepts the non-reacting acetoxy group or some other polar group. Site B and D are hydrophobic pockets. Site B is more structurally and sterically selective than site D. Apolar chains prefer site B if they fit, especially if they have some form of unsaturation. It is a crude model with no indication as to the dimensions of the pockets, therefore it is difficult to apply. Guanti *et al.* state that the validity of this model must be confirmed by additional research.

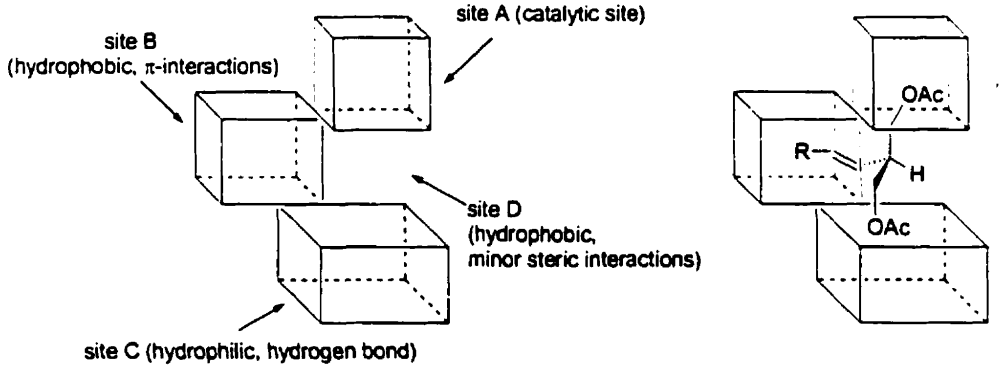


Figure 1.41. Model Q: Box-type model for PPL. The alkenyl substituent of 1,3 diacetoxypropanes binds in site B when it has a *trans* configuration because of favorable π-interactions, as shown. The *cis*-alkenyl substituents must bind in site D because the position of the R group is such that unfavorable steric interactions occur in site B. Hence the opposite enantiomer is preferred.

Primary amines: predictive substrate rules.

The general rule for secondary alcohols (rule A) has been shown, see Chapter 4, to account for the enantiopreference of Lipase B from *Candida antartica* (CAL-B)[92], *Pseudomonas aaeruginosa* (PAL), and *Pseudomonas cepacia* (PCL) towards primary amines of the type NH_2CHRR'. Subtilisin shows the same opposite enantiopreference for primary amines as it showed for

secondary alcohols. Therefore rules R and S are useful for predicting the stereochemical outcome of reactions with primary amines, Figure 1.42.

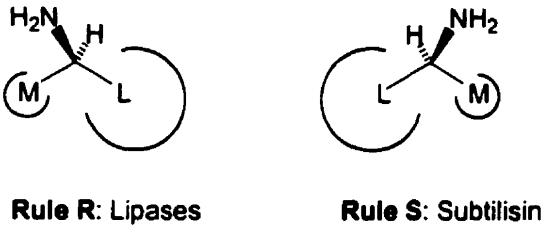


Figure 1.42. Rules to predict the enantiopreference of lipases and subtilisin towards primary amines.

As discussed in Section 1.5, researchers have used molecular modeling of substrates within active sites in an attempt to rationalize the enantiopreference and degree of enantioselectivity of hydrolases. However, as this area of research is still in its infancy, this method is difficult to use. Although the above rules and models are a simplistic view of hydrolase selectivity, in practice they provide a relatively easy means of predicting hydrolase enantiopreference.

1.7 Increasing hydrolase enantioselectivity

A number of methods have been found to alter the reaction rates, specificity, and selectivity of enzymes. In particular, an entire area of research is devoted to improving the enantioselectivity of hydrolases through the modification of reaction conditions, alteration of the substrate, and mutation of the hydrolase itself. Significant improvements have been observed in all three cases, but no general method has been established. Unless otherwise noted, the following discussion is limited to studies involving hydrolyses and acylations of primary and secondary alcohols.

Modification of reaction conditions

Modifications of reaction conditions such as solvent, and temperature have led to surprisingly good improvements in hydrolase selectivity. Although there are some rules and rationalizations, the variation in enantioselectivity remains, for the most part, unpredictable.

Solvent

The influence of solvent on hydrolase-catalyzed transformations has been extensively investigated over the past decade.[93] There is no doubt that the nature of solvents influences enantioselectivity, however our understanding of the enzyme-substrate-solvent interactions involved is still rather limited. One distinct advantage of conducting reactions in organic solvent is that the enantioselectivity can often be increased, decreased, and even reversed by simply changing the solvent.[5,93c] The one drawback of this 'medium engineering' is that hydrolase activity is often greatly reduced in organic solvents as compared to reactions carried out in water.[94]

An example of the need to optimize the solvent for a given reaction is given in Chapter 5. The enantioselectivity of a lipase-catalyzed esterification of a bicyclic alcohol increased >100 fold upon changing the solvent from chloroform to toluene.

Researchers have attempted to correlate the physicochemical characteristics of solvents with the selectivity of hydrolases.[93c] In one study, Fitzpatrick and Klibanov found a correlation between the enantioselectivity of subtilisin Carlsberg towards secondary alcohols and both the dielectric constant and dipole moment of the solvent. [95] No correlation with hydrophobicity (log P) was observed, nor was any correlation found when subtilisin was replaced by porcine pancreatic lipase. In this study, a 20-fold increase in enantioselectivity was observed upon changing the solvent from acetonitrile to dioxane, in the transesterification of 1-phenyl ethanol with vinyl butyrate. The effect of solvent on the selectivity of subtilisin Carlsberg, a protease from *Aspergillus oryzae*, and a lipase from *Pseudomonas sp.* towards prochiral primary alcohols was examined in a later study.[96] A correlation between prochiral selectivity and solvent hydrophobicity was observed. In this case, the influence of solvent dipole moment was ruled out by conducting reactions in two isomeric solvents having different polarities but the same hydrophobicity; similar prochiral selectivities were obtained. It was suggested substrates having a large hydrophobic substituent bind more tightly within the hydrolase's hydrophobic pocket in the presence of hydrophilic solvents, leading to a more stereoselective reaction. That is, changes in selectivity are based on changes in the solvation of the substrate. They also found that a hydrophobic additive in the reaction medium competed for binding in the hydrophobic pocket, resulting in decreased selectivity.

An inversion of enantioselectivity was observed for lipase from *Pseudomonas cepacia*[97] upon variation of the solvent, but no correlation was found between the enantioselectivity and the hydrophobicity of the solvent.

Carrea and co-workers, as well as several other groups, have found a complete lack of correlation between the enantioselectivity of several lipases, towards primary alcohols and secondary alcohols, and the physicochemical characteristics of solvents.[93c]

Enantioselectivity varied greatly with respect to solvent in the resolution of amines catalyzed by lipase *Pseudomonas cepacia* and a lipase from *Candida antarctica*.[98] However, there was no relationship between the degree of enantioselectivity and the log P or dielectric constant of the solvent.

Recently, Ke *et al.*[99] suggested that prochiral selectivity can be correlated with the relative solvation energies of the pro-*R* and pro-*S* binding modes of the substrate in the transition state. They found that prochiral selectivity in various organic solvents can be quantitatively predicted using equation 1.12. This equation was derived by theoretical means and experimentally confirmed.

$$\log [(k_{cat}/K_M)_{pro\text{-}S} / (k_{cat}/K_M)_{pro\text{-}R}] = \log (\gamma'_{pro\text{-}S}/\gamma'_{pro\text{-}R}) + \text{const} \quad [1.12]$$

$\gamma'_{pro\text{-}S}$ and $\gamma'_{pro\text{-}R}$ are defined as the activity coefficients of the desolvated fragment of the substrate in the pro-*S* and pro-*R* enzyme bound transition states, respectively. These values are calculated by first conducting modeling studies to determine which part of the bound molecule is not accessible to the solvent. The activity coefficient of a model molecule that resembles the unsolvated portion of the substrate is then determined. Very good correlation was found for cross-linked crystals of chymotrypsin and subtilisin, but not for their lyophilized or precipitated forms. It was proposed that this is due to the fact that while the conformation of

the enzymes in their crystallized forms does not change as the solvent is varied, experiments indicated that reversible conformational changes do occur for the other forms. Later experiments[100] showed that the solvent dependency of the prochiral selectivity is dominated by the $(k_{cat}/K_M)_{pro\text{-}R}$ term (for their substrates, where R is the favored product) and that both k_{cat} and K_M are equally involved. It was also found that the activity of the enzyme was not decreased in the solvents for which selectivity was increased. These experiments were only carried out on one type of substrate, prochiral primary diols having a benzyl or substituted benzyl group at the stereocenter.

Colombo *et al.*[59] found that this method was unsuccessful when applied to their substrates. They studied the enantioselectivity of both cross-linked crystalline and lyophilized subtilisin towards two very different chiral secondary alcohols. They found no correlation between the enantiomeric ratio and the ratio of the activity coefficients for the unsolvated portions of the substrate.

In addition to Ke *et al.*'s proposal that the selectivity of hydrolases in organic solvents is dependent solely on the energetics of substrate solvation, two other hypotheses have been put forth. It has been suggested that selectivity could be affected by solvent molecules bound within the active site.[101,102,103] These solvent molecules could disturb the normal binding mode of the substrate through steric and electrostatic interactions, thus influencing the selectivity. Another suggestion is that variation in solvent leads to changes in the conformation of the enzyme.[104,105] This, in turn, would lead to changes in enzyme-substrate interactions that could affect selectivity.

Significant increases in enantioselectivity have also been observed upon the addition of an organic co-solvent to hydrolytic reactions. Generally, it seems that esterases and proteases favor the addition of a water-miscible hydrophilic solvent, whereas lipases favor the addition of a water-immiscible hydrophobic solvent.[6]

However, Hansen *et al.*[106] found that the enantioselectivity of a hydrolysis, catalyzed by lipase B from *Candida antarctica*, could be raised ten-fold by the addition of approximately 20% *tert*-butanol or acetone. In this case the lipase's performance is improved by the addition of a hydrophilic water-miscible co-solvent. Many more examples of the use of co-solvents exist.[107]

Secundo *et al.* observed an increase in enantioselectivity as a function of substrate conversion for the PCL-catalyzed transesterification of sulcatol with vinyl acetate, in chlorinated solvents. This interesting phenomenon did not occur in non-chlorinated solvents, nor did it occur for other substrates studied. This is just another example that emphasizes the complex nature of enzyme-substrate-solvent interactions.

Temperature

Researchers have also improved enantioselectivity by either increasing or decreasing the temperature of a given reaction.[5,6,108]

The difference between the free energies of activation of one enantiomer and another, directly related to the enantiomeric ratio, is temperature dependent. Therefore, a rationalization of temperature effects has been proposed using theoretically derived equations based on the thermodynamics of enzyme-catalyzed reactions.[108] Using the mathematical model,

$$\ln E = - \Delta\Delta G^{\ddagger} / (RT) = \Delta\Delta S^{\ddagger} / R - \Delta\Delta H^{\ddagger} / (RT)$$

the enantioselectivity could be optimized once the $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ are known. The temperature at which there is no enantioselectivity (E = 1) is defined as $T_{rac} = \Delta\Delta H^{\ddagger} / \Delta\Delta S^{\ddagger}$. Theoretically, at temperatures below T_{rac}, the $\Delta\Delta H^{\ddagger}$ term dominates and enantioselectivity should increase with decreasing temperature

whereas at temperatures above T_{rac}, the $\Delta\Delta S^{\ddagger}$ dominates and enantioselectivity should increase with increasing temperature. In addition, if $\Delta\Delta H^{\ddagger}$ is the major contributing factor of $\Delta\Delta G^{\ddagger}$ for the desired reaction, then high selectivies will be obtained at the lowest temperatures. On the other hand if $\Delta\Delta S^{\ddagger}$ is the larger term, then the best selectivity will be obtained at the highest temperatures. Obviously, the temperature extremes are limited by the stability of the enzyme and substrate.

The validity of this mathematical model was confirmed by Sakai *et al.*[109], who studied the effects of temperature on the lipase-catalyzed transesterifications of a series of primary and secondary alcohols: the highest enantiomeric ratios were observed at temperatures as low as -40°C.

The method by which an enzyme is prepared was found to influence the effect of temperature on enantioselectivity. Both an increase and a decrease in enantioselectivity was observed with an increase in temperature, for subtilisin catalyzed reactions.[110] This was dependent upon the method used for the preparation of the enzyme.

Other reaction parameters

It is thought that pH should influence the enantioselectivity of enzyme-catalyzed hydrolyses.[6,7,111] The ionization state of an enzyme can easily be changed by varying the pH of its environment. The conformation of the enzyme would be altered and, as a result, substrate enantioselectivity would theoretically be modified. Surprisingly, however, very little research has been done in this domain and no useful conclusions have been made.

The water activity, related to the amount of water that is bound to an enzyme in a given reaction medium, has also been observed to influence both activity and enantioselectivity.[5,6,93c]

Substrate modification

As discussed previously, the ability of hydrolases to distinguish between enantiomers is very dependent on the structural elements of the substrate, such as steric bulk and electronic characteristics. Therefore, variation in substrate structure can significantly influence enantioselectivity. The empirical rule proposed in Chapter 2 for secondary alcohols (Rule A) was successfully used to redesign substrates. The enantioselectivity of hydrolases, towards poorly resolved substrates, can be enhanced by increasing the difference in size between the two substituents at the stereocenter. The steric bulk of the 'large' substituent can be increased by the introduction of a group that can easily be substituted or removed. Two such examples are described in Chapter 2. A number of other researchers have used this method succesfully.[5] For example, both Rotticci *et al.*[112] and Adam *et al.*[113] increased the enantiomeric ratio of lipase-catalyzed esterifications of secondary alcohols, by adding steric bulk using a trimethylsilyl protecting group. In addition Rotticci *et al.*, gradually increased the size of the large substituent and, other than for one exception, observed an enhancement in enantioselectivity.

The acyl portion of a substrate can also be altered to improve enantioselectivity. It has been shown that an increase in the acyl chain length in hydrolytic reactions involving the resolution of alcohols can increase enantioselectivity. However, one study showed that for CRL, there is a minimum in selectivity when an acyl carbon chain length of 6 is used.[114] Variation of the acyl donor in transesterification reactions in organic solvent can greatly influence the degree of enantioselectivity. This was observed in the research described in Chapter 5. Another method that has been used to enhance enantioselectivity is the "bichiral method".[115] This involves the resolution of a racemic alcohol protected by a chiral acyl group[116].

Enzyme modification

A lot of research is being done in the modification of enzymes by various techniques to increase the enantioselective potential. These include chemical modification[57,117], site-directed mutagenesis[118], and directed evolution. [119] However a detailed review of these methods goes beyond the scope of this thesis.

References and Notes

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Chapter 2

When the work for this thesis was started, organic chemists were just beginning to realize the synthetic utility of hydrolases for the generation of chiral molecules. Very little research had as yet been done in this field and there was a great need for guidelines to help chemists select an appropriate lipase for a given transformation. In particular, simple means of predicting the stereochemical outcome of hydrolase-catalyzed transformations were imperative.

This chapter presents the elaboration of a simple, reliable rule that predicts the enantiopreference of three serine esterases in transformations involving secondary alcohols and their esters.

First, the rule was proposed on the basis of data accumulated from an exhaustive literature survey of reactions catalyzed by Cholesterol esterase (CE), lipase from *Pseudomonas cepacia* (PCL), and lipase from *Candida rugosa* (CRL). The validity of the proposed was then confirmed by additional experiments.

A strategy for improving the enantioselectivity for a given secondary alcohol substrate was developed and proven to be successful through the study of enantioselective reactions involving two sets of secondary alcohol substrates and their derivatives.

This paper has been highly quoted and describes some of the earliest work in the field of predictive enzyme rules.

Note: The text of this article has been reformatted in accordance with the Thesis Preparation Guidelines. A photocopy of the published version is included in the appendices.

A Rule To Predict Which Enantiomer of a Secondary Alcohol Reacts Faster in Reactions Catalyzed by Cholesterol Esterase, Lipase from *Pseudomonas cepacia*, and Lipase from *Candida rugosa*.[1]

Romas J. Kazlauskas,* Alexandra N. E. Weissfloch, Aviva T. Rappaport, and Louis A. Cuccia

Abstract: The enantioselectivity of the title enzymes for more than 130 esters of secondary alcohols is correlated by a rule based on the sizes of the substituents at the stereocenter. This rule predicts which enantiomer of a racemic secondary alcohol reacts faster for 14 of 15 substrates of cholesterol esterase (CE), 63 of 64 substrates of lipase from *Pseudomonas cepacia* (PCL), and 51 of 55 cyclic substrates of lipase from *Candida rugosa* (CRL). The enantioselectivity of CRL for acyclic secondary alcohols is not reliably predicted by this rule. This rule implies that the most efficiently resolved substrates are those having substituents which differ significantly in size. This hypothesis was used to design syntheses of two chiral synthons: esters of (*R*)-lactic acid and (*S*)-(-)-4-acetoxy-2-cyclohexen-1-one, **70**. As predicted, the acetate group of the methyl ester of lactyl acetate was hydrolyzed by PCL with low enantioselectivity because the two substituents, CH_3 and $C(O)OCH_3$, are similar in size. To improve the enantioselectivity, the methyl ester was replaced by a *t*-butyl ester. The acetate group of the *t*-butyl ester of lactyl acetate was hydrolyzed with high enantioselectivity (E > 50). Enantiomerically-pure (*R*)-(+)-*t*-butyl lactate (>98% ee, 6.4 g) was prepared by kinetic resolution. For the second example, low enantioselectivity (E < 3) was observed in the hydrolysis of *cis*-1,4-diacetoxycyclohex-2-ene, a meso substrate where the two substituents CH_2CH_2 and CH=CH, are similar in size. To improve enantioselectivity, the size of the CH=CH substituent was increased by addition of Br_2. The new substrate was hydrolyzed with high enantioselectivity (E > 65) using either CE or CRL. Enantiomerically-pure **70** (98% ee) was obtained after removal of the bromines with zinc and oxidation with CrO_3/pyridine.

Introduction

Microorganism- and enzyme-catalyzed syntheses and resolutions are among the best methods for the preparation of enantiomerically-pure compounds.[2] Enantioselective hydrolyses and transesterifications are especially useful because they are efficient, can be carried out on a large scale, and apply to a wide range of substrates. Unfortunately, there is little X-ray structural information available for the esterases and lipases that are used for these preparations;[3] thus, the appropriate enzyme is usually chosen by screening.

In order to streamline screening, substrate models have been developed using substrate selectivity data. Some models attempt to define the shape and hydrophobic character of the active site and are used to predict which new substrates will be transformed by the enzyme and whether the reaction will be enantioselective. In most cases, this type of model is only accurate for substrates that are similar to those already tested. For example, a model for PLE[4] predicts its selectivity with α,β-substituted carboxylic acids,[5] and a model for CRL predicts its selectivity for bicyclo[2.2.1]heptanols and bicyclo[2.2.2]octanols.[6] In some cases, more general models which define the sizes of hydrophobic pockets near the active site have been developed. These can be used to predict reactivity for a wider range of substrates.[7]

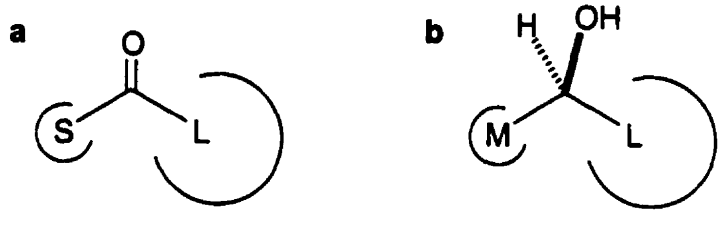

Figure 1. (**a**) Prelog's rule predicts that the yeast *Culvaria lunata* preferentially adds H_2 to the front side of ketones having the shape indicated. (**b**) An extension of Prelog's rule to hydrolases. For esters of secondary alcohols, the enantiomer shown reacts faster with CE, PCL and CRL than the other enantiomer.

The simplest models for enzyme selectivity, more accurately referred to as rules, predict only which enantiomer reacts faster usually based on either the size or hydrophobicity of the substituents at the stereocenter. The earliest example of a such a rule is Prelog's rule which predicts the enantioselectivity of the reduction of ketones by the yeast *Culvaria lunata* based on the size of the two substituents at the carbonyl, Figure 1.[8] The advantage of this rule is that it applies to a wide range of substrates, but the disadvantage is that there are exceptions to this rule. Nevertheless, this rule is sufficiently reliable to be used for the determination of absolute configurations[9] and has been used to redesign substrates in order to improve the enantioselectivity.[10]

A rule similar to Prelog's rule has been proposed for a hydrolase. For 47 esters of racemic secondary alcohols, a rule based on the sizes of the substituents at the stereocenter predicts which enantiomer is hydrolyzed faster in cultures of the yeast *Rhizopus nigrigans.*[11] This rule has been used to determine the absolute configuration of secondary alcohols.[12] However, this microorganism has not been used for large-scale synthesis.

Similar rules have been proposed for two commercially-available lipases: *Mucor meihei* [13] (MML) and *Arthrobacter.*[14] It is not yet clear how useful these rules will be because they are based on only six substrates for the *Mucor* enzyme and only two substrates for the *Artthrobacter* enzyme. A "two-site model" that has been recently proposed for cyclic substrates of PCL also appears similar to the rule discussed herein.[15]

This paper proposes a rule based on the sizes of the substituents for three hydrolases, Figure 1. This rule is supported by 14 out of 15 substrates for CE, 63 out of 64 substrates for PCL, and 51 out of 55 cyclic substrates for CRL and has an accuracy of ≥93%. This rule is the first attempt to predict the enantioselectivity of CE. For PCL, this rule is similar to the "two-site model" for cyclic substrates,[14]

but the evidence presented in this paper shows that this rule accounts for the enantioselectivity of acyclic secondary alcohols as well. For CRL, this rule is the first general rule and complements the model for bicyclic substrates.[6]

Besides correlating a large amount of experimental data, this rule also suggests a strategy for improving the efficiency of resolutions catalyzed by these enzymes: secondary alcohols having substituents which differ significantly in size should be more efficiently resolved than secondary alcohols having substituents which are similar in size. This hypothesis was used to design the preparations of enantiomerically-pure *t*-butyl lactate and (*S*)-(-)-4-acetoxy-2-cyclohexen-1-one.

Lactate esters are used as chiral starting materials and chiral auxiliaries. For example, lactate esters were used in the preparation of (*S*)-2-arylpropionic acids, a class of non-steroidal anti-inflammatory drugs,[16] α-N-hydroxy-amino acids,[17] chiral enolates,[18] and chiral auxiliaries for an enantioselective Diels-Alder reaction.[19] Polylactides containing interpenetrating networks of poly-(*R*)-lactide and poly-(*S*)-lactide are stronger than those containing racemic chains.[20]

Although enantiomerically-pure (*S*)-(+)-lactic acid is readily available, the unnatural enantiomer, (*R*)-(-), remains expensive. Enantiomerically-pure (*R*)-lactic acid can be prepared by D-lactate dehydrogenase-catalyzed reduction of pyruvate,[21] fermentation, or microbial destruction of the *S* enantiomer.[22] The resolution of lactate esters using PCL described in this paper yields material of high enantiomeric purity and is inexpensive and simple to carry out. An enzyme-catalyzed resolution of lactate esters has been reported previously using an enzyme from a similar microorganism.[23]

The second example is an enantioselective synthesis of (*S*)-(-)-4-acetoxy-2-cyclohexen-1-one, a new compound that should also be useful for synthesis. The analogous (4*S*)-*t*-butyldimethylsiloxy derivative has been prepared in six steps from quinic acid[24] and has been used for the synthesis of the cholesterol lowering

drugs ML 236A and compactin.[25] The procedure described below for the acetate derivative is simpler. Since the analogous cyclopentenone - 4-acetoxy-2-cyclopenten-1-one - has been widely used in synthesis,[26] the six-membered analog should also find many uses.

Results

A Survey of Enantioselectivity for Esters of Secondary Alcohols. Pancreatic CE catalyzed the hydrolysis of the acetates of the secondary alcohols listed in Table 1. The measured values of enantiomeric excess and % conversion were used to calculate the enantioselectivity, E, which indicates the degree to which the enzyme prefers one enantiomer over the other.[27] Several examples from the literature are also included in Table 1. Chart 1 indicates the structure of the fast-reacting enantiomer. The chart and tables are arranged so that the larger group is always on the right side as predicted by the rule in Figure 1b. CE showed no enantioselectivity toward **40**, but for the other fourteen examples the rule correctly predicts which enantiomer reacts faster. The overall accuracy of the rule for CE is 14 of 15 substrates or 93%; the single exception, the *t*-butyl ester of lactyl acetate, will be introduced and discussed below.

Enantioselectivity data for PCL was gathered from the literature, Table 2. This list includes all secondary alcohols prepared using lipase P from Amano Pharmaceutical. Reactions using other lipases from *Pseudomonas* (e.g. AK, K-10 or SAM-II) are not included. Patent literature is also not included. The reaction conditions used for the examples listed in Table 2 include both hydrolyses in aqueous solution as well as transesterifications and esterifications in organic solvents. The structures of the substrates in Table 2 include acyclic secondary alcohols and cyclic secondary alcohols in rings ranging from four- to seven-membered. For 63 of the 64 substrates, the rule in Figure 1 correctly predicts the fast-reacting enantiomer. The single exception, indicated by '(ent)' in the enantioselectivity column, was one of five substrates which showed low enantioselectivity, $E \leq 3$. Thus, for PCL the rule predicts which enantiomer reacts faster with 98% accuracy.

Chart 1. Structures for Tables 1-3

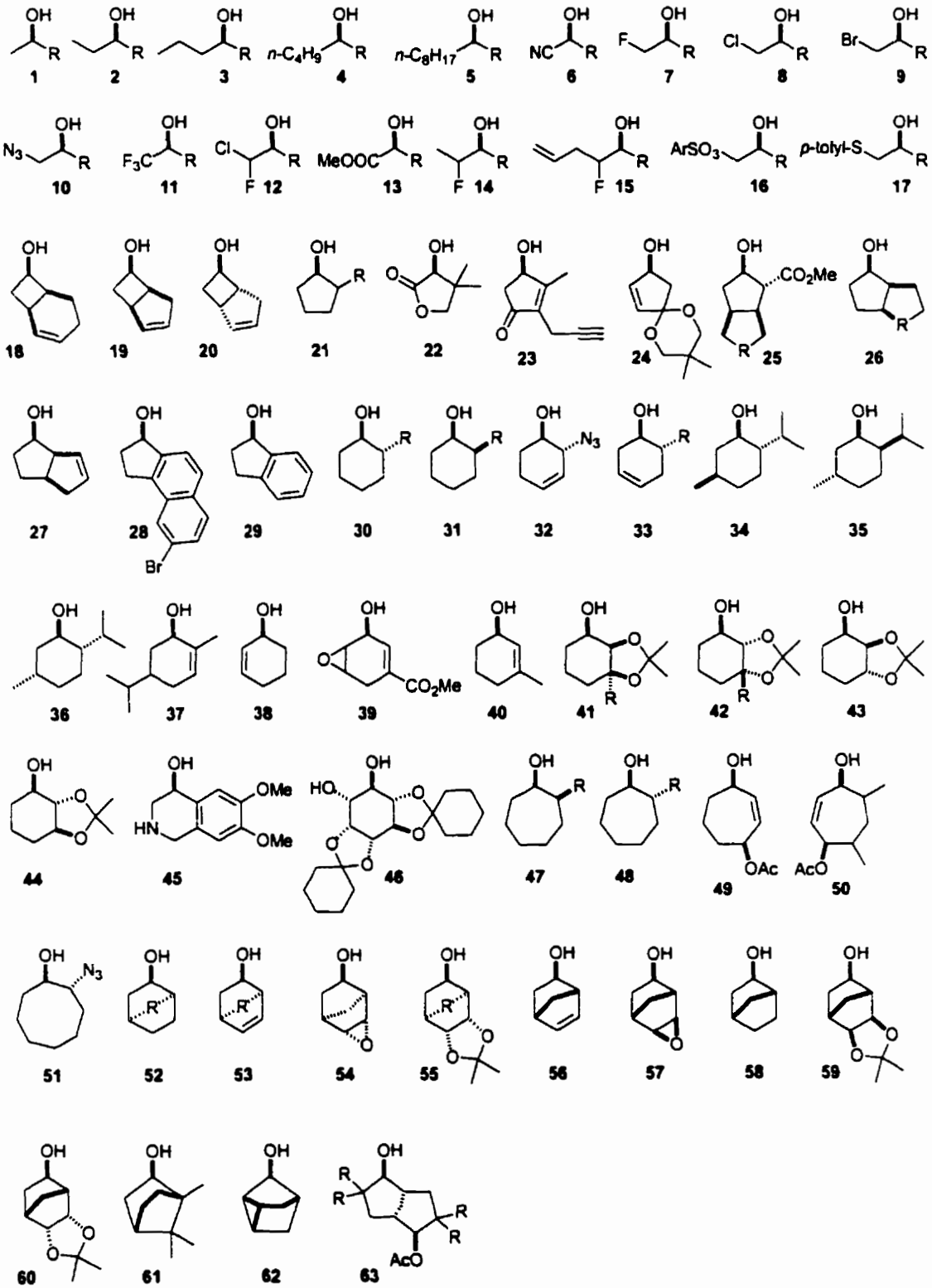

Table 1. Enantiospecificity of Bovine Pancreatic Cholesterol Esterase for Acetates of Secondary Alcohols.

Struc	R	Initial Rate[a] (units/mg)	Conversion (%)	Enantiomeric Excess (%)[b]	E Enantiospecificity
1	Ph	0.31	43	69	8.8 [c]
1	n-C_6H_{13}	0.36	46	35	2.6 [d]
1	$(CH_3)_2CH=C(CH_3)_2$	0.50	46	45	4.3 [e]
2	Ph	0.12	34	6.2	4.6 [f]
28		0.02	-	-	2.9 [g]
29		0.56	29	65	6.3 [d]
30	CH_3	0.32	50	94	>100 [h, i]
30	OAc	0.20	44	56	10 [j]
30	OH	0.59	48	59	6.8 [j]
31	CH_3	0.59	53	46	4.4 [h, k]
34		0.21	44	96	>100 [d]
39	*cis*	0.04	-	-	14 [l]
39	*trans*	0.07	-	-	37 [l]
40		0.60	50	0	1
46		-	-	-	37 [m]

a. For hydrolysis of the corresponding acetate ester unless otherwise noted. Unit = μmol of ester hydrolyzed/min. *b.* The enantiomeric purity of the product alcohol was determined by derivatizing with isopropyl isocyanate and separating the enantiomers by capillary GC using an XE-60-(*S*)-valine-(*S*)-α-phenylethylamide column (Chromopak). *c.* The product alcohol showed $[\alpha]_D$ (obsd) = +40.5° (c 1.2, hexane) indicating the *R* configuration: lit. (*S*)-(-), Klyne, W.; Buckingham, J. *Atlas of Stereochemistry*, Chapman and Hall: London 1974, p. 26. *d.* Absolute configuration was determined by comparison to an authentic sample. *e.* The product alcohol showed $[\alpha]_D$ (obsd) = -5.3° (c 2.2, hexane) indicating the *R* configuration: lit. *S*-(+), $[\alpha]_D$ = +16.1° (neat): Jacques, J.; Gros, C.; Bourcier, S. *Absolute Configurations of 6000 Selected Compounds with One Asymmetric Carbon Atom* Stuttgart: G. Thieme, **1977**; Levene, P. A.; Haller, H. L. *J. Biol. Chem.* **1929**, *83*, 177-183. *f.* The product alcohol showed a positive rotation (hexane) indicating the *R* configuration: lit. (*S*), $[\alpha]_D$ = -39° (neat): MacLeod, R.; Welch, F. J.; Mosher, H. S. *J. Am. Chem. Soc.* **1960**, *82*, 876-880. *g.* Kazlauskas, R. J. *J. Am. Chem. Soc.* **1989**, *111*, 4953-4959. *h.* The enantiomeric purity was determined by formation of the Mosher's ester and integration of the ^{1}H-NMR signals for the ring CH_3 groups; Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543-2549. *i.* The product alcohol showed $[\alpha]_D$ (obsd) = -8.7° (c 1.2, CH_2Cl_2) indicating the *R* configuration: lit. *R*-(-), Beard, C.; Djerassi, C.; Elliott, T.; Tao, R. C. C. *J Am. Chem. Soc.* **1962**, *84*, 874-875. *j.* Hydrolysis was caried out in aqueous solution saturated with sodium chloride. The %ee refers to unreacted starting material and was determined by HPLC of the benzoate derivative on a Chiralpak OT (Daicel, New York); Caron, G.; Kazlauskas, R. J., unpublished results. *k.* The product alcohol showed $[\alpha]_D$ (obsd) = -34° (c 1.6, hexane) indicating the *R* configuration: lit. *R*-(-), Beard, C.; Djerassi, C.; Elliott, T.; Tao, R. C. C. *J. Am. Chem. Soc.* **1962**, *84*, 874-875. *l.* As the butyrate ester at 0-5 °C: Pawlak, J. L.; Berchtold, G. A. *J Org Chem.* **1987**, *52*, 1765-1771. *m.* Liu, Y.-C.; Chen, C.-S. *Tetrahedron Lett.* **1989**, *30*, 1617-1620.

Table 2. Enantiospecificity of Lipase from *Pseudomonas Cepacia* (Amano P) for Esters of Secondary Alcohols.

struc	R	E	ref	struc	R	E	ref
1	Ph, CH_2Ph	>50	*a*	**12**	Ph, CH_2Ph, CH_2CH_2Ph	23 - >50	*k*
1	*n*-C_6H_{13}, *n*-$C_{10}H_{21}$	~12	*a*	**13**	$CH(CH_3)$-1-(2,6-dithiane)	>50	*l*
1	CH_2CN	29	*b*	**13**	*syn*-CH(Ar)(SAr')	>50	*m*
1	CH_2COOEt	~8	*c*	**21**	*cis*-COOEt, Oac *trans*-COOEt, OAc	>50	*n*
1	1-(2,6-dithiane), 1-(2,5-dithiolane), CH_2-1-(2,6-dithiane), CH_2-1-(2,5-dithiolane)	>50	*d*	**21**	*trans*-N_3	>50	*o*
1	CH_2-1-(2,6-dioxane)	2	*d*	**23**		>50	*h*
2	Ph	>50	*a*	**24**		>50	*p*
2	$CH(N_3)C_2H_5$ (*threo, erythro*)	>50	*e*	**25**	C=O, $C=CH_2$, HC=CH	>50	*n*
3	$CH(N_3)CH_2CH_2CH_3$ (*erythro*)	>50	*e*	**25**	1-(2,5-dioxolane)	3	*n*
4	CH_2N_3	2	*e*	**26**	C=O, $C=CH_2$, 1-(2,5-dioxolane)	>50	*q*
4	CH_2OSO_2Ar	25	*f*	**27**		>50	*r*
5	*syn*-$CH(Cl)CH_2COOEt$	50	*g*	**30**	COOEt, OAc, CN, C_6H_5	>50	*n, o, s*
6	*p*-C_6H_4OPh	>50	*h*	**30**	OH	17	*t*
6	Ph, CH_2CH_2Ph, CH=CHPh	18 - >50	*b*	**31**	OAc	8	*n*
8	Ph, 3,4-$(MeO)_2C_6H_3$	>50	*i*	**31**	COOEt	>50	*n*
8	CH_2OSO_2Ar	24	*f*	**33**	OAc	>50	*u*
9	2-naphthyl, 4-BrC_6H_4 4-$MeOC_6H_4$	>50	*i*	**45**		17	*v*
9	1-(3-bromo-5-isoxazolyl)	20	*j*	**47**	OAc	1	*n*
10	Ph	>50	*e*	**47**	CO_2Me	>50	*n*
10	*n*-C_6H_{13}	2 (ent)	*e*	**48**	CO_2Me, OAc	>50	*n*

a. Bianchi, D.; Cesti, P.; Battistel, E. *J. Org. Chem.* **1988**, *53*, 5531-5534; esterification with propionic anhydride in benzene; Nishio, T.; Kamimura, M.; Murata, M.; Terao, Y.; Achiwa, K. *J. Biochem. Tokyo* **1989**, *105*, 5110-5112; esterification with vinyl acetate, neat solution. *b.* Itoh, T.; Tagaki, Y. *Chem. Lett.* **1989**, 1505-1506; hydrolysis of β-methylthio- or β-phenylthioacetyl ester. *c.* Sugai, T.; Ohta, H. *Agric. Biol. Chem.* **1989**, *53*, 2009-2010; esterification with vinyl butanoate, 65 °C. *d.* Bianchi, D.; Cesti, P.; Golini, P. *Tetrahedron* **1989**, *45*, 869-876. *e.* Foelsche, E.; Hickel, A.; Hönig, H.; Seufer-Wasserthal, P. *J. Org. Chem.* **1990**, *55*, 1749-1753; hydrolysis of butyrates. *f.* Chen, C.-S.; Liu, Y.-C. *Tetrahedron Lett.* **1989**, 30, 7165-7168; transesterification of the butyrate in hexane. *g.* Tsuboi, S.; Sakamoto, J.; Sakai, T.; Utaka, M. *Chem. Lett.* **1989**, 1427-1428. *h.* Hirohara, H.; Mitsuda, S.; Ando, E.; Komaki, R. in *Biocatalysis in Organic Synthesis*, Tramper, J.; van der Plas, H. C.; Linko, P., Eds. Elsevier: Amsterdam, **1985**, 119-134. *i.* Hiritake, J.; Inagaki, M.; Nishioka, T.; Oda, J. *J. Org. Chem.* 1988, *53*, 6130-6133; esterification with *i*-propenyl acetate in *i*-propyl ether. *j.* Di Aminica, M.; De Micheli, C.; Carrea, G.; Spezia, S. *J. Org. Chem.* **1989**, *54*, 2646-2650; esterification with trifluoroethyl octanoate in 9:1 hexane/benzene. *k.* Yamazaki, T.; Ichikawa, S.; Kitazume, T. *J. Chem., Soc. Chem. Commun.* **1989**, 253-255. *l.* Suemune, H.; Mizuhara, Y.; Akita, H.; Oishi, T.; Sakai, K. *Chem. Pharm. Bull.* **1987**, *35*, 3112-3118. *m.* Akita, H.; Enoki, Y.; Yamada, H.; Oishi, T. *Chem. Pharm. Bull.* **1989**, *37*, 2876-2878; hydrolysis in water-saturated benzene, enzyme was immobilized on Celite. *n.* Xie, Z.-F.; Suemune, H.; Sakai, K. *J. Chem. Soc., Chem. Commun.* **1987**, 838-839; Xie, Z.-F.; Suemune, H.; Nakamura, I.; Sakai,

K. *Chem. Pharm. Bull.* **1987**, *35*, 4454-4459; Xie, Z.-F.; Nakamura, I.; Suemune, H.: Sakai, K. *J. Chem. Soc., Chem. Commun.* **1988**, 966-967. *o.* Hönig, H.; Seufer-Wasserthal, P.; Fülöp, F. *J. Chem. Soc., Perkin Trans. 1* **1989**, 2341-2345; hydrolysis of butyrate ester. *p.* Washausen, P.; Grebe, H.; Kieslich, K.; Winterfeldt, E. *Tetrahedron Lett.* **1989**, *30*, 3777-3778. *q.* Xie, Z-F.; Suemune, H.; Sakai, K. *Tetrhedron:Asymmetry* **1990**, *1*, 395-402. *r.* Klempier, N.; Faber, K.; Griengl, H. *Synthesis*, **1989**, 933-934. *s.* Schwartz, A.; Madan, P.; Whitesell, J. K.; Lawrence, R. M. *Org. Synth.* **1990**, *69*, 1-9. *t.* Caron, G.; Kazlauskas, R. J., unpublished results. *u.* Suemune, H.; Hizuka, M.; Kamashita, T.; Sakai, K. *Chem. Pharm. Bull.* **1989**, *37*, 1379-1381. *v.* Hoshino, O.; Itoh, K.; Umezawa, B.; Akita, H.; Oishi, T. *Tetrahedron Lett.* **1989**, *29*, 567-568.

Enantioselectivity data for CRL was also gathered from the literature, Table 3. The *Candida* list includes results using enzyme either from Sigma Chemical Co. or from Meito Sangyo (Lipase MY or OF-360); however examples where the absolute configuration of the product is uncertain were not included,[28] nor were examples from patents. The reaction conditions again include both hydrolyses in aqueous solution as well as transesterifications and esterifications in organic solvents.

For acyclic substrates of CRL the rule in Figure 1 does *not* reliably predict which enantiomer reacts faster. Only for about half - 14 of the 31 acyclic substrates - is the fast-reacting enantiomer predicted correctly; this proportion is close to that expected for random guesses. A large fraction of the acyclic substrates (12 of 31) showed low enantioselectivity, $E \leq 3$. Thus, the rule is not useful for acyclic substrates of CRL. Generalizations about this rule given below do *not* include acyclic substrates of CRL, but do include acyclic substrates of CE and PCL.

For cyclic substrates of CRL the rule reliably predicts which enantiomer reacts faster. These substrates include secondary alcohols in four- to eight-membered rings as well as secondary alcohols in bicyclic substrates. The rule predicts correctly for 51 of the 55 substrates where CRL was enantioselective, an accuracy of 93%. Three exceptions, indicated by '(ent)' as before, were among the thirteen cyclic substrates which showed low enantioselectivity, $E \leq 3$; one exception, **22**, showed moderate enantioselectivity, $E = 8$.[29]

Table 3. Enantiospecificity of Lipase from *Candida Rugosa* for Acetates of Secondary Alcohols.

struc	R	E	ref	struc	R	E	ref
1	C_2H_5	34	*a*	**33**	N_3	>50	*o*
1	*n*-C_6H_{13}	1.6	*b*	**34**		~10 - >50	*q, s*
1	2-furyl	5	*c*	**35**		>50	*q*
1	$CH_2(OCH_3)_2$	2.5	*d*	**36**		27	*q*
2	$CH(N_3)C_2H_5$ (*threo, erythro*)	12	*e*	**37**	*trans*	1	*t*
3	*erythro*-$CH(N_3)CH_2CH_2CH_3$	18	*e*	**37**	*cis*	25	*t*
4	CH_2N_3	2 (ent)	*e*	**38**		1.2	*t*
6	Ph	8 (ent)	*f*	**39**	*cis, trans*	6	*u*
6	*p*-C_6H_4OPh	12 (ent)	*g*	**40**		3.4	*d*
7	CH_2S-*p*-tolyl	1.3 (ent)	*h*	**41**	H, CH_3	>50	*v*
8	Ph	2	*f*	**42**	H	2	*v*
10	Ph	13	*e*	**42**	CH_3	26	*v*
10	*n*-C_6H_{13}	2	*e*	**43**		>50	*v*
10	*t*-C_4H_9	>50 (ent)	*e*	**44**		>50	*v*
11	CH=CHPh (*E, Z*)	>50 (ent)	*i*	**45**		20	*w*
11	$CH=CH(CH_2)_5CH_3$ (*E, Z*)	~16 (ent)	*i*	**48**	N_3	30	*o*
12	Ph	6	*j*	**49**		2.5	*x*
12	CH_2Ph, CH_2CH_2Ph	~3 (ent)	*j*	**50**	*cis, trans*	>50	*x*
13	Ph	>50 (ent)	*f*	**51**		>50	*o*
13	*anti*-$CH(CH_3)$-3-indolyl	3 - 40	*k*	**52**	CH_2	11	*y*
14	CH_2S-*p*-tolyl (*syn, anti*)	1.3 (ent)	*h*	**52**	CHCOOR', $CHOCH_2Ph$	3-10	*y*
15	CH_2S-*p*-tolyl (*syn, anti*)	1.2-2.1 (ent)	*h*	**53**	CH_2	35	*y*
16	Ph	>50 (ent)	*l*	**53**	$CHCO_2Me$, $C(OMe)_2$	~10	*y*
17	$CFPhCH_3$ (*syn, anti*)	6.5	*h*	**53**	O	>50	*y*
18		17	*m*	**54**		>50	*y*
19		>40	*n*	**55**	CH_2	22	*y*
20		1.2 (ent)	*n*	**55**	O	>50	*y*
21	*trans*-N_3	1.4	*o*	**56**		1	*y*
22		8 (ent)	*f*	**57**		1.4 (ent)	*y*
27		>50	*p*	**58**		1.5	*y*
30	CH_3, C_2H_5, *i*-C_3H_7, *t*-C_4H_9	>50	*q*	**59**		4	*y*
30	CN, NO_2, N_3	40 - >50	*o*	**60**		1.8	*y*
30	OAc, OH	2, 1 (ent)	*r*	**61**		>10	*z*
31	CH_3, *i*-C_3H_7	24	*q*	**62**		10	*aa*
32		20	*o*	**63**	H, CH_3	15	*bb*

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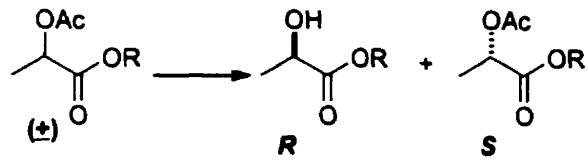
Thus, the rule predicts which enantiomer of a secondary alcohol reacts faster for ≥93% of both the cyclic and acyclic substrates of CE and PCL and the cyclic substrates of CRL. None of the substrates that reacts contrary to the rule shows enantioselectivity greater than eight and therefore none of the exceptions are synthetically useful.

This generalization implies that a substrate having substituents which differ significantly in size should be resolved with higher enantioselectivity than a substrate where the two substituents are similar in size. The data in Tables 1-3 suggests that this generalization is valid; however, it is difficult to test this

hypothesis quantitatively. Nevertheless, we used this hypothesis to redesign substrates that could be efficiently resolved by these enzymes.

Scheme I. Kinetic Resolution of Esters of Lactyl Acetate



Kinetic resolution of lactate esters. To resolve lactate esters we increased the size of the ester group until we found a substrate that was hydrolyzed with high enantioselectivity, Scheme I, Table 4. Lactyl acetate, R = H, was not a substrate for the three enzymes tested: activity <0.002 units/mg. The methyl ester of lactyl acetate, R = CH_3, was hydrolyzed with the *R* enantiomer reacting faster, but with very low enantioselectivity: the remaining starting material showed <32% ee at ~45% conversion. Accurate determination of the enantioselectivity was complicated by competing hydrolysis of the methyl ester which accounted for 55-80% of the disappearance of the starting material. The *t*-butyl ester of lactyl acetate, R = *t*-butyl, still showed low enantioselectivity with CE and CRL, but showed excellent enantioselectivity with PCL, E > 50. The lack of any increase in enantioselectivity for CRL is not surprising because the rule is not reliable for acyclic substrates of CRL. The unexpected result was that the enantioselectivity of CE was opposite of that predicted by the rule. The reason for this reversal is not known; this is the first substrate of CE that does not fit the rule in Figure 1. Even with this exception, the accuracy of the rule remains high: 14/15 substrates, 93%. The increase in enantioselectivity with PCL is consistent with the rule; indeed another lipase from *Pseudomonas* sp. (lipase K-10 from Amano) has been

reported to show excellent enantioselectivity when R = *t*-butyl.[23] Thus, increasing the size of the R group in Scheme I resulted in a substrate which was more efficiently resolved.

Table 4. Enantiospecificity of the Hydrolysis of Esters of Lactyl Acetate

	methyl ester				tert-butyl ester				
enzyme	rate[a]	%C[b]	%ee[c]	(*R*/*S*)[d]	rate[a]	%C[b]	%ee[c]	(*R*/*S*)[d]	E[e]
CE	0.18	42	8	*R*	0.06	32	21	*S*	3
CRL	0.30	49	32	*R*	0.01	40	26	*R*	4.4
PCL	0.01	40	24	*R*	0.35	50	89	*R*	51
PCL[f]					0.35	47	>98	*R*	>200

a. Initial activity of the enzyme in units/mg solid. Unit = μmol of ester hydrolyzed/min. *b*. The conversion refers to the amount of starting material consumed. This value was determined by ^{1}H-NMR after extraction of the reaction mixture with ethyl ether. *c*. Enantiomeric excess of the recovered starting material was determined by ^{1}H-NMR in the presence of Eu(hfc)$_3$. *d*. The absolute configuration of the preferred enantiomer was determined from the rotation of the recovered starting material; (*R*)-(-), Ito, S.; Kasai, M.; Ziffer, H.; Silverton, J. V. *Can. J. Chem.* **1987**, *65*, 574-582. *e*. Enantiospecificity, see ref. 27 for details. *f*. Hydrolysis of esters of lactyl butyrate. *g*. Enantiomeric excess of the product.

A preparative-scale resolution of *t*-butyl lactate was carried out using the butyrate instead of the acetate to simplify separation of the product alcohol and unreacted butyrate by fractional distillation. This change from acetate to butyrate also resulted in a further increase in enantioselectivity of PCL to >200. Hydrolysis of racemic *t*-butyl ester of lactyl butyrate (50 g) yielded the unnatural enantiomer, (*R*)-(+)-*t*-butyl lactate (6.4 g), with >98% ee after distillation.

Enantioselective synthesis of (*S*)-(-)-4-acetoxy-2-cyclohexen-1-one, 70. The five-membered 4-acetoxy-2-cyclopenten-1-one is a useful chiral starting material that can be prepared by an enzyme-catalyzed hydrolysis.[30] Acetylcholinesterase selectively hydrolyzes the *R* acetate in *cis*-1,4-diacetoxy-2-cyclopentene and the resulting olefinic alcohol is oxidized to the enone. An attempt to prepare the

corresponding six-membered compound by an analogous enzyme-catalyzed synthesis was not successful because the acetate was removed with only low to moderate selectivity (0-72% ee, Scheme II, Table 5).

Scheme II. Enzyme-Catalyzed Hydrolysis of **64** Showed Poor Enantioselectivity

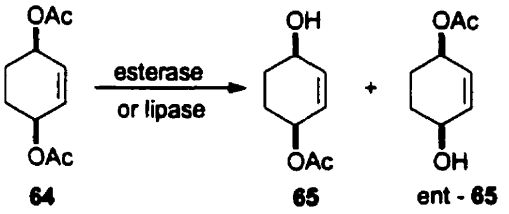


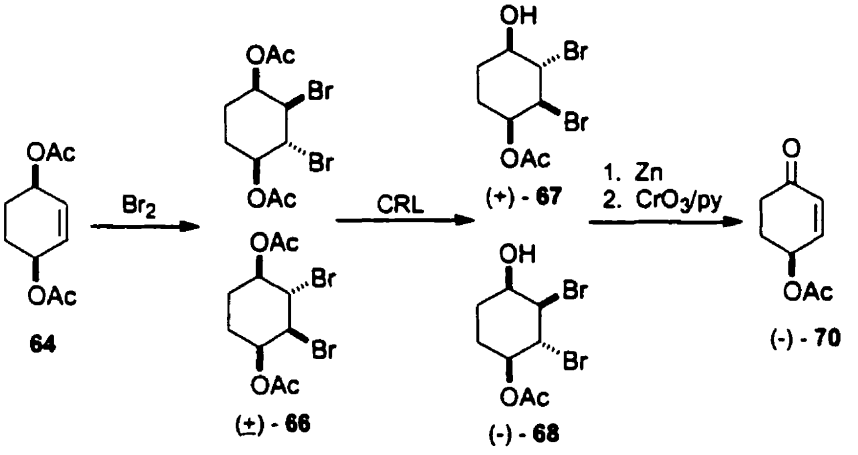
Table 5. Enantioselective Hydrolysis of *Cis*-1,4-diacetoxycyclohexane Derivatives.

enzyme [a]	substrate	rate [b]	product	%ee [c]
CE	**64**	0.6	**65**	32
CRL	**64**	4.1 [d]	ent-**65**	41
PCL	**64**	0.03	ent-**65**	72
PLE	**64**	18.2 [d]	**65**	62
ACE	**64**	18	**65** + ent-**65**	0
MML	**64**	0.0004	ent-**65**	14
CE	(±)-**66**	3.9	(+)-**67**, (-)-**68**	>97, >97
CRL	(±)-**66**	0.4 [d]	(+)-**67**, (-)-**68**	>97, >97
PLE	(±)-**66**	4.2 [d]	(+)-**67**, (-)-**68**	>97, >97
PCL	(±)-**66**	0.002	(+)-**67**, **68** + diol	14 [e]
ACE	(±)-**66**	0.57	(+)-**67**, (-)-**68** + diol [f]	85 [e], >95 [g]
MML	(±)-**66**	0.00005	(+)-**67**, **68** + diol [h]	87 [e]

a. ACE = acetylcholinesterase from electric eel, MML = lipase from *Mucor meihei*. *b*. Initial activity of the enzyme in units/mg solid. Unit = μmol of ester hydrolyzed/min. *c*. Determined by ^{1}H-NMR in the presence of Eu(hfc)$_3$. For the hydrolysis of (±)-**66**, the enantiomeric purity was determined for both **67** and **68** after separation by flash chromatography. The ^{1}H-NMR signals for the acetyl methyl group of the two enantiomers of **67** or of **68** are separated by 0.4 ppm in the presence of approximately one and a half equivalents of Eu(hfc)$_3$. With this excellent separation even 1.5 mol% of the other enantiomer can be detected as shown by a deliberate addition of racemate to an enantiomerically pure sample. *d*. In units/mg protein. *e*. For **67**. *f*. The ratio of isolated **68**: **67**: diol was approximately 1: 4: 8 after 0.9 equivalents of base had been consumed. *g*. For **68**. *h*. After 0.7 equivalents of base had been consumed, the major product was diol. Only traces of **68** were observed.

The rule suggests a possible reason for this difficulty. In the cyclopentene case, the substituents at the stereocenter - CH_2CHOAc and CH=CH - differ in size and can be distinguished by the enzyme; however, in the cyclohexene case, the substituents - CH=CH and CH_2CH_2 - are too similar in size to be distinguished by the enzyme. To increase the selectivity, bromine was added across the double bond of **64** to increase the difference in size of the substituents, Scheme III.

Scheme III. Preparation of **70** via the Dibromide Derivative



The highest yields for the addition of bromine to **64** were obtained using reaction conditions that favor free radical intermediates (CS_2, -78 °C, hv). Addition of bromine under conditions which favor ionic intermediates (polar solvents, dark) resulted in a mixture of products which may have resulted from intramolecular attack of an acetate on the bromonium ion intermediate. The addition of bromine to **64** yielded the *trans*-dibromide, **66**, identified from coupling constant of 9.3 Hz for the ^{1}H-NMR signals for the hydrogens at CHBr (δ 4.36, 4.27) indicating an axial-axial arrangement.[31] For the *cis*-dibromide a coupling constant of 2-3 Hz would be expected. This *trans* addition of bromine converted **64**, a meso compound, into a pair of enantiomers, **(±)-66**. The rule predicts

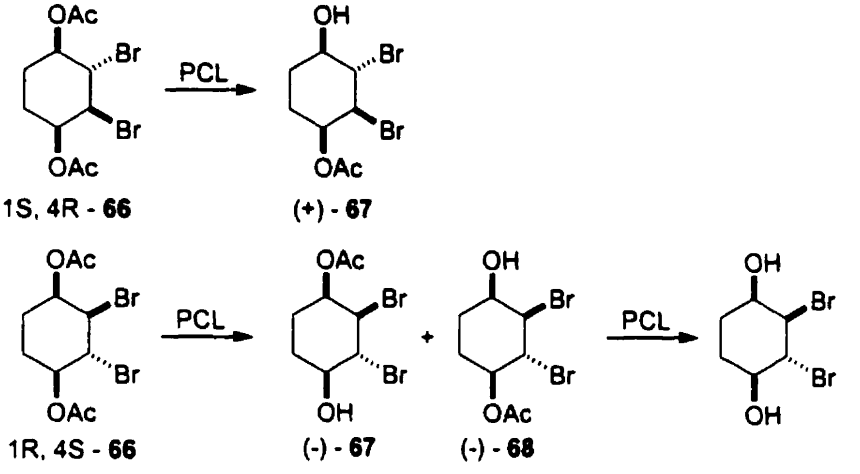
selective hydrolysis of the *R* acetate in both enantiomers yielding the two enantiomerically-pure diastereomers: **67** and **68**. Note that the two *R* acetates that are predicted to be hydrolyzed are diastereotopic: one *R* acetate is *cis* to an adjacent bromine whereas the other *R* acetate is *trans*.

With CE, CRL, or PLE as catalysts, the hydrolysis of (±)-**66** slowed and stopped after half of the acetates had been hydrolyzed. Analysis of the reaction mixture by TLC showed no remaining starting material, equal amounts of **67** and **68**, and traces of diol which resulted from hydrolysis of both acetates. The two products were enantiomerically pure as shown by ^{1}H-NMR in the presence of Eu(hfc)$_3$, Table 5. The opposite sign of rotation of (+)-**67** and (-)-**68** is presumably caused by the differing orientation of the adjacent bromine. Thus, for these three enzymes the hydrolysis of (±)-**66** proceeded as predicted by the rule.

When PCL was used as the catalyst, the reaction did not slow appreciably after half of the acetates had been hydrolyzed. The products isolated from a reaction stopped after half of the acetates had been hydrolyzed were unreacted starting material, (+)-**67** having low enantiomeric excess, a small amount of **68**, and a small amount of diol. Several other enzymes were also screened as possible catalysts, Table 5. MML and ACE[4] showed results similar to PCL. Formation of these products can be accounted for by the different reactions of the two enantiomers of **66** as shown in Scheme IV. For (1*S*, 4*R*)-**66**, removal of the *R* acetate is predicted by the rule. This acetate is also the more chemically reactive one because it is oriented *trans* to the adjacent bromine. Hydrolysis occurs as predicted and yields (+)-**67**. For the other enantiomer, (1*R*, 4*S*)-**66**, removal of the *R* acetate is also predicted by the rule; however, this acetate is the less chemically reactive one because it is oriented *cis* to the adjacent bromine. Hydrolysis of both acetates is observed: hydrolysis of the *R* acetate yields (-)-**68**, hydrolysis of the *S* acetate yields (-)-**67**. This last product accounts for the low enantiomeric excess of the

isolated **67**. Hydrolysis of both acetates yields the diol. Thus, the rule only partly accounts for the PCL-catalyzed hydrolysis of (±)-**66** due to the differences in chemical reactivity of the acetates caused by the differing orientation of the adjacent bromine. The rule in Figure 1 is too simple to include such effects.

Scheme IV. Hydrolysis of Racemic **66** Catalyzed by PCL



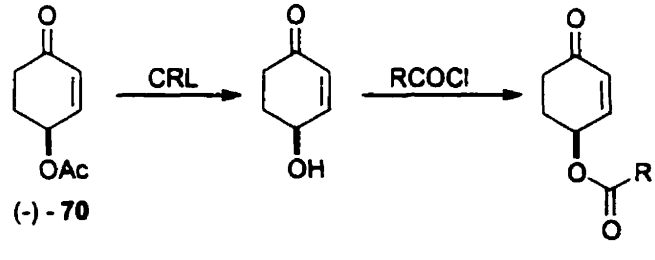
Of the three enzymes which showed excellent selectivity, CRL was chosen for the preparative-scale reaction because it is the least expensive on a unit basis. Hydrolysis of 26 g of (±)-**66** catalyzed by CRL yielded 20 g of enantiomerically-pure (+)-**67** and (-)-**68** in 82% yield as co-crystalline diastereomers. To complete the synthesis of **70**, the bromines were removed using zinc dust (82-90% yield) and the resulting olefinic alcohol was oxidized to the enone, **70**, with chromium trioxide/pyridine (84% yield). Other methods of oxidation either gave lower yields (MnO_2, 62%) or gave side products (Swern, PCC/NaOAc, DMSO/Ac_2O, DMSO/Ac_2O/py/CF_3COOH).

The enantiomeric purity of **70** was 98% as shown by ^1H and ^{19}F-NMR of the Mosher's ester derivative. This derivative was prepared by removal of the acetyl group either by CRL-catalyzed or base-promoted hydrolysis followed by reaction

with the acid chloride as shown in Scheme V. As a control, a racemic sample of **70** was also hydrolyzed using CRL and derivatized with the Mosher's acid chloride.[32] For the racemic sample, the diastereomers could be distinguished by ^{1}H-NMR (two well-separated multiplets for the proton at the carbinol carbon centered at δ 6.85) or by ^{19}F-NMR (two multiplets centered at δ -71.95). The ester derived from enantiomerically-pure **70** showed ~1% of the minor diastereomer by either method corresponding to 98% ee. Deliberate addition of material derived from racemic **70** confirmed that the small peaks were due to the other diastereomer.

Determination of the absolute configuration of 70. The absolute configuration of **70** was established to be (*S*)-(-) using the exiton chirality method.[33] The acetyl group of **70** was replaced with a benzoyl group as shown in Scheme V. The acetyl group was removed by an enzyme-catalyzed hydrolysis[31] and the product alcohol was treated with benzoyl chloride. The circular dichroism spectrum of the resulting benzoate showed a split Cotton effect, negative at 227 nm ($\Delta\varepsilon$ = -5.7) and positive at 192 nm ($\Delta\varepsilon$ = +4.0). This splitting indicates a left-handed screw sense between the benzoate and the enone chromophores, i.e. *S*. This assignment of absolute configuration is consistent with the expected enantioselectivity of the three enzymes and with previous assignments for a cyclopentenone[34] and a substituted cyclohexenone.[35]

Scheme V. Preparation of Derivative of **70**



Discussion

The major advantage of this rule is its simplicity. It is straightforward to use and correlates a large amount of experimental data because it applies to a wide range of substrates. It further suggests a strategy for improving the efficiency of resolutions: to increase the difference in size of the substituents at the stereocenter. Two tests of this strategy were successful because an efficiently resolved substrate/enzyme combination was found for both examples. The strategy of adding a large group to one side of a molecule resulted in a substrate that was efficiently resolved by a least one of the enzymes. Thus, screening a substrate with a large group as one of the substituents at the stereocenter appears to be more reliable than screening a substrate having substituents of similar size at the stereocenter.

The major disadvantage of this rule is that it does not account for subtleties in the selectivities of these enzymes. For example, it does not rationalize why only PCL and not the other two enzymes showed increased enantioselectivity for the *t*-butyl ester of lactyl acetate as compared to the methyl ester. The rule also does not predict the effect of additional stereocenters, for example, the *cis* vs. *trans* oriented bromine at C_2 of **66**. The sensitivity of PCL to this orientation rendered this enzyme unsuitable for the preparation of **70**, whereas the other two enzymes which were not sensitive to this orientation were suitable.

In spite of this disadvantage, a general rule such as that proposed in Figure 1 may be the most reasonable way to describe the active site of these enzymes. Structural data suggests that large conformational changes are required before the substrate can bind to the active site in pancreatic lipase and lipase from *Mucor meihei*.[3] Due to this flexibility it may never be possible to define an exact size and shape for the substrate binding region of lipases because this region may change

for each substrate. Consistent with this notion are reports that the enantioselectivity and conformation of CRL change upon treatment with bile salt and organic solvent[36] or with dextromethorphan.[37]

A second reason to use a general rule is that these enzymes may not be homogeneous. Sequencing of the gene for CRL showed several non-identical DNA sequences which code for this enzyme,[38] thus it is likely that the commercial enzymes are a mixture of isozymes. The enantioselectivities of isozymes of PLE were similar, but not identical;[39] a similar situation may hold for the isozymes of CRL. This heterogeneity may frustrate attempts to precisely define the size and shape of the active site, thus a general rule may be the most accurate way of describing the commercial catalyst.

A third reason for using rules and models is that even when the X-ray crystal structure of an enzyme is known, models are often used to predict enantioselectivity because they are simpler to use. For example, a high resolution X-ray crystal structure has been determined for alcohol dehydrogenase from horse liver, yet a cubic space model is usually used to predict its enantioselectivity.[40] Further, it remains difficult to predict which binding interactions are most important in an enzyme-substrate complex, thus it may remain difficult to predict enantioselectivity for an untested substrate even when the X-ray crystal structure is known. For example, the origin of the high selectivity for transfer of the *pro*-4*S* hydrogen of NADH catalyzed by lactate dehydrogenase ($>10^8$:1) is difficult to explain from the known crystal structure.[41] These rules and models may be used, along with X-ray crystal structures and molecular modelling, to determine which interactions are most important in determining the the enantioselectivity of these enzymes.

Experimental Section

General. Cholesterol esterase (bovine, 0.2 units/mg solid with 0.1 M cholesterol acetate in ethyl ether as substrate) was purchased from Genzyme Corp., Boston, MA. Lipase from *Candida rugosa* (L-1754, 0.2 units/mg solid using olive oil), porcine liver esterase (E-3126, 240 units/mg protein using ethyl butyrate) and acetyl cholinesterase (electric eel, C-3389, 28 units/mg solid with acetyl choline) were purchased from Sigma Chemical Co., St. Louis, MO. Lipase from *Pseudomonas cepacia* (lipase P30, 0.06 units/mg solid using olive oil) and lipase from *Mucor meihei* (lipase MAP-10, 0.05 units/mg solid using olive oil) were purchased from Amano International Enzyme Co., Troy, VI. Activated MnO_2 (Aldrich) was heated at 120°C for 24 h before use. Zinc dust (60 g) was activated by stirring for 1 min with 2% aq HCl. The dust was collected by vacuum filtration and washed with 120 mL of the following: 1 x 2% HCl, 2 x 95% ethanol, 1 x anhydrous ethyl ether. Elemental analyses were done by Guelph Laboratories, ON.

Enzyme-catalyzed hydrolyses. A rapidly-stirred suspension of substrate (1 mmol) in phosphate buffer (10 mM, 10 mL) containing enzyme (0.2-50 mg) was maintained at pH 7.0 by automatic titration with NaOH (0.1 N) using a Radiometer RTS 822 pHstat. Crystalline substrates were first dissolved in ethyl ether (10 mL). Sodium taurocholate (30 mg) was added to hydrolyses where CE was used as the catalyst. The rate of consumption of sodium hydroxide over the first 5% of the reaction was used to calculate the initial rates listed in Tables 1, 4, and 5. The reaction was stopped after the consumption of base indicated 20-50% conversion and the mixture was extracted four times with ethyl acetate. The combined extracts were washed with satd aq sodium bicarbonate, water, and brine, dried over magnesium sulfate and concentrated. The starting ester and alcohol were separated by flash chromatography and the enantiomeric purity was determined.

Determination of Enantiomeric Purity by Gas Chromatography. Alcohol (4 μL), isopropyl isocyanate (300 μL), and methylene chloride (300 μL) were heated at 100°C for 1.5 h in a tightly sealed glass vial. The completeness of the reaction was checked by TLC. Solvent and excess reagent were evaporated in a stream of nitrogen, the residue was diluted to 1 mL with dichloromethane and analyzed by gas chromatography using an XE-60-(S)-valine-(S)-α-phenylethylamide capillary column.

Determination of Enantiomeric Purity by ^{1}H-NMR. A 5 mg sample of the ester was dissolved in 0.5 mL of $CDCl_3$ in an NMR tube and the ^{1}H-NMR spectrum was obtained using a Varian XL-200 NMR spectrometer. Solid [3-heptafluoropropylhy-droxymethylene)-(+)-camphorato]europium(III), $Eu(hfc)_3$, was added in four portions and the spectra were obtained. A total of more than 1.3 equivalents of shift reagent was added to each sample.

Acetyl esters. Two to three equivalents of acetyl chloride were added dropwise to a stirred solution of alcohol in pyridine. Solid alcohols were dissolved in a mixture of ethyl ether and pyridine. Acetylation was complete after 10 min to 2 h as shown by TLC. The reaction mixture was washed twice with 10% sodium bicarbonate and once with water. If only one layer formed upon washing, the product was extracted into ether. The organic layer was dried with magnesium sulfate and concentrated by rotary evaporation. The esters were purified either by distillation or by flash chromatography.

(±)-Lactyl acetate was prepared using a literature procedure,[42] but substituting toluene for benzene. A mixture of racemic lactic acid (120 mL of 85% purity, 1.1 mol), glacial acetic acid (640 mL, 11.2 mol), toluene (80 mL), and concd sulfuric acid (0.40 mL) was refluxed with the continuous removal of distillate with a Dean Stark trap until a ^{1}H-NMR spectra of the distillate showed that no more water was present. Approximately 1 L of solution was removed during 40 h; acetic acid

(~600 mL) and toluene (~100 mL) were periodically added to the reaction to replace what was removed. The reaction mixture was neutralized with sodium acetate (1.6 g) and distilled under vacuum yielding 86 g (59%): bp 35-37 °C (0.2 torr) [lit.[43] bp 127 °C (11 torr)] ; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 10.6 (s, 1), 5.10 (q, 1, J = 7.1 Hz), 2.14 (s, 3), 1.53 (d, 3, J = 7.1 Hz).

(±)-Methyl ester of lactyl acetate. A suspension of potassium methoxide (3.8 g, 54 mmol) in dry ethyl ether (100 mL) containing (±)-2-acetoxypropionyl chloride[44] (7.3 g, 50 mmol) was stirred for 72 h. The reaction mixture was washed twice with satd sodium bicarbonate solution, the organic layer was collected, and the aqueous phase was extracted with ether (2 x 200 mL). The combined organic layers were dried with anhydrous magnesium sulfate, concentrated by rotary evaporation, and distilled giving 1.6 g (22%): bp 60-64 °C (~4 torr) [lit.[45] bp 64 °C (9.8 torr)]; ^{1}H-NMR (neat, 60 MHz) δ 4.8 (q, 1, J = 7 Hz), 3.5 (s, 3), 1.8 (s, 3), 1.2 (d, 3, J = 7 Hz).

(±)-*t*-Butyl ester of lactyl acetate. A two phase mixture of (±)-lactyl acetate (66 g; 0.5 mol), liquified isobutylene (120 mL, 1.5 mol), ethyl ether (85 mL), and concd sulfuric acid (4 mL) in a 500 mL pressure bottle was sealed with a rubber stopper wired securely like the cork of a champagne bottle and stirred for 7 h at room temperature until a single phase formed. The bottle was chilled in an ice-salt water bath or dry ice-acetone bath, opened and the contents were slowly added to a satd phosphate buffer (300 mL, pH 7). The pH of the buffer was maintained between 7 and 8 throughout the addition with a concd sodium hydroxide solution. The resulting solution was extracted with ethyl ether (3 x 500 mL). The combined ether extracts were dried over anhydrous potassium carbonate and filtered into a round bottomed flask that had been washed with a sodium hydroxide solution and rinsed with water to ensure the removal of trace acid. The ether and excess isobutylene were evaporated under vacuum and the resulting clear, slightly yellow

oil was distilled giving 81 g (86%): bp 95-100 °C (~1 torr); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 4.64 (q, 1, J = 7.1 Hz), 1.84 (s, 3), 1.21 (s, 9), 1.18 (d, 3, J = 7.1 Hz); ^{13}C-NMR ($CDCl_3$, 75.4 MHz) δ 169.4, 169.3 (**C**=O), 81.0 (**C**$(CH_3)_3$), 68.5 (**C**H), 27.3 (C(**C**$H_3)_3$), 20.0 (**C**H_3C=O), 16.3 (**C**H_3CH).

(±)-*t*-Butyl ester of lactyl butyrate. Butyryl chloride (244 mL, 2.36 mol) was added dropwise to stirred lactic acid (125 g of 85% purity, 1.18 mol). After addition was complete, the mixture was stirred overnight at room temperature. A ^{1}H-NMR spectrum of the reaction mixture indicated no remaining lactic acid. Excess butyryl chloride and butyric acid were removed by vacuum distillation leaving crude lactyl butyrate, 156 g (82%). A portion of this material (125 g, 0.78 mol) was treated with isobutylene (187 mL, 2.34 mol) as described above for the acetate derivative. Distillation yielded 51 g (36%): bp 94 °C (~2 torr); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 4.95 (q, 1, J = 7 Hz), 2.35 (t, 2, J = 7 Hz), 1.68 (m, 2), 1.45 (s + d, 9 + 3), 0.95 (t, 3, J = 7 Hz); ^{13}C-NMR ($CDCl_3$, 75.4 MHz) δ 173.6, 170.7 (**C**=O), 82.2 (**C**$(CH_3)_3$), 69.2 (**C**H), 36.1 (OC(O)**C**H_2), 28.1 (C(**C**$H_3)_3$), 18.5 (OC(O)CH_2**C**H_2), 17.1 (**C**H_3CH), 13.8 (CH_2CH_2**C**H_3).

***(R)*-(+)-*t*-Butyl lactate.** A suspension of racemic *t*-butyl ester of lactyl butyrate (50 g, 0.23 mol) in phosphate buffer (400 mL, 0.1 M, pH 7.0) containing PCL (1.0 g) was stirred at room temperature. The pH was maintained between 6.9 and 7.1 by automatic addition of NaOH (0.5 M). After 22 h, 215 mL of base had been added indicating 47% conversion. The suspension was saturated with sodium chloride and extracted with ethyl ether (4 x 750 mL). The combined extracts were dried over magnesium sulfate and concentrated by rotary evaporation to yield an oil, 42 g. Distillation yielded (*R*)-(+)-*t*-butyl lactate, 6.4 g (38% of theoretical yield): bp 51-54 °C (4 torr), [lit.[46] bp 46-47 °C (9 torr)]; oil which solidifies, mp 35-37.5 °C; $[\alpha]_D$ = +7.98 (*c* 1.7 CH_2Cl_2), [lit.[45] $[\alpha]^{20}_D$ = +9.48 (neat, l = 1)]; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 4.62 (q, 1, J = 7 Hz), 2.9 (s, br), 1.49 (s, 9), 1.38 (d, 3, J

= 7 Hz); ^{13}C-NMR ($CDCl_3$, 75.4 MHz) δ 175.8 (**C**=O), 82.6 (**C**(CH_3)$_3$), 67.2 (**C**H), 28.2 (C(**C**H_3)$_3$), 20.7 (**C**H_3CH); >98% ee by ^{1}H-NMR with Eu(hfc)$_3$ on the acetyl derivative. The limit of detection was determined by deliberate addition of racemic *t*-butyl ester of lactyl acetate to the NMR tube.

***cis*-1,4-Diacetoxy-2-cyclohexene, 64**, was prepared using Bäckvall's method[47] with the following changes. The acetic acid solution was heated to dissolve the palladium diacetate, then cooled prior to the addition of the other reagents. After the reaction completed, the solution was filtered through Whatman #41 paper on a Büchner funnel prior to extraction with pentane to minimize the formation of an emulsion.

(±)-1α, 4α-Diacetoxy-2β, 3α-dibromocyclohexane, 66. A solution of **64** (5.0 g, 25 mmol) in CS_2 (120 mL) was cooled to -78 °C in an acetone/dry ice bath and irradiated with a Phillips 150 watt reflector flood lamp placed 35 cm from the reaction mixture. A solution of Br_2 in CS_2 (3.9 M, 8.8 mL, 34 mmol) was added to the stirred reaction mixture in one portion. After 50 min, TLC showed the presence of a small amount of starting material, however longer reaction time did not result in its disappearance. The reaction mixture was diluted with cold chloroform (-20 °C, 700 mL) and subsequently washed with satd aq Na_2SO_3 (2 x 70 mL), water (70 mL), and brine (2 x 80 mL). The reaction mixture must remain cold until after the first washing with Na_2SO_3. The organic phase was dried over magnesium sulfate and concentrated by rotary evaporation. Recrystallization (ether/hexanes) yielded white crystals, 7.35 g (81%): mp 71.5-72 °C; R_f = 0.3 (4:1 hexanes/ethyl acetate); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 5.32 (m, 1, H4); 5.01 (ddd, 1, H1); 4.36 (dd, 1, $J_{1,2}$ = 9.0 Hz, $J_{2,3}$ = 9.3 Hz, H2); 4.27 (dd, 1, $J_{3,4}$ = 2.7 Hz H3); 2.15 (s, 3, C**H**$_3$); 2.11 (s, 3, C**H**$_3$); 2.20-2.00 and 1.70-1.85 (2m, 4, H5, H5', H6, H6'). ^{13}C-NMR ($CDCl_3$, 75.4 MHz) δ 169.7, 169.6 (**C**=O); 73.9, 70.7 (**C**HOAc); 54.1 (**C**HBr); 26.1, 25.3 (**C**H_2); 21.0, 20.9 (**C**H_3). IR (Nujol mull) 1751, 1731,

1257, 1231, 1024 cm^{-1}. MS (CI, NH_3) m/z 374 ($M+NH_4^+$, 46%); exact mass 373.96018 ($C_{10}H_{18}Br_2NO_4$ requires 373.96026, 2 ppm error). Anal. Calcd. for $C_{10}H_{14}Br_2O_4$: C, 33.55; H, 3.94. Found: C, 33.14; H, 3.84.

(1*S*)-(+)-1α- acetoxy-2α, 3β-dibromo-4α-hydroxycyclohexane, (+)-67, and (1*S*)-(-)-1α-acetoxy-2β, 3α-dibromo-4α-hydroxycyclohexane, (-)-68. Lipase from *Candida rugosa* (15 g) was added to a stirred mixture of dibromodiacetate, (±)-**66** (26 g, 73 mmol), aq phosphate buffer (260 mL, 0.1 M, pH 7.00), and ethyl ether (10 mL). Aliquots of a 0.5 M NaOH solution were added as required to maintain the pH of the mixture between 6.95 and 7.05. After three days a total of 1 equivalent (73 mmol) of base had been added. The mixture was extracted with ethyl acetate (4 x 750 mL) and the combined extracts were washed with satd aq sodium bicarbonate (600 mL), and brine (600 mL). The organic phase was dried over magnesium sulfate and concentrated by rotary evaporation. Recrystallization of the crude residue from 115 mL of CH_2Cl_2/hexanes (35: 65 v/v) yielded co-crystalline diastereomers **67** and **68**, 18.7 g (82%): mp 97-104 °C. A sample of the diastereomers was separated by flash chromatography (4:1 hexanes/ethyl acetate). **(+)-67**: mp 125.5 °C; R_f = 0.34 (3:2 hexanes/ethyl acetate); >97% ee by ^{1}H-NMR with $Eu(hfc)_3$. $[\alpha]_D$ = +141° (c 1.6, CH_2Cl_2); ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 5.31 (m, 1, H1); 4.29 (dd, 1, $J_{2,3}$ = 10.8 Hz, $J_{3,4}$ = 8.4 Hz, H3); 4.19 (dd, 1, $J_{1,2}$ = 2.6 Hz, H2); 3.79 (m, 1, H4); 2.66 (br, 1, OH); 2.15 (s, 3, OAc); 1.97-2.17 (m, 2, H5, H6); 1.65-1.88 (m, 2, H5' and H6'). ^{13}C-NMR ($CDCl_3$, 75.4 MHz) δ 169.6 (**C**=O); 74.4 (**C**OAc); 71.6 (**C**OH); 63.0 (**C**HBrCHOH); 54.6 (**C**HBrCHOAc); 27.0, 26.6 (**C**H_2); 20.9 (**C**H_3). IR (Nujol mull) 3409 (br), 1728, 1260, 1072 cm^{-1} Anal. Calcd. for $C_8H_{12}Br_2O_3$: C, 30.41; H, 3.83. Found: C, 30.03; H, 3.54. **(-)-68**: mp 122 °C; R_f = 0.48 (3:2 hexanes/ethyl acetate); >97% ee by ^{1}H-NMR with $Eu(hfc)_3$. $[\alpha]_D$ = -125° (c 1.6, CH_2Cl_2); ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 4. 97 (ddd, 1, H1); 4.37 (dd, 1, $J_{2,3}$ = 10.1 Hz, $J_{1,2}$ = 8.4 Hz, H2); 4.29 (dd, 1, $J_{3,4}$ = 2.0 Hz, H3); 4.15 (ddd, 1,

H4); 2.45 (br, 1, OH); 2.07-2.22 (m, 1, H5); 2.11 (s, 3, OAc); 1.88-2.02 (m, 2, H6 and H6'); 1.58-1.81 (m, 1, H5'). ^{13}C-NMR ($CDCl_3$, 75.4 MHz) 169.8 (C=O); 74.6 (COAc); 69.5 (COH); 61.6 (CHBrCOH); 54.8 (CHBrCOAc); 27.6, 24.8 (CH_2); 21.0 (CH_3). IR (Nujol mull) 3429(br), 1717, 1257, 1034 cm^{-1}. Anal. Calcd. for $C_8H_{12}Br_2O_3$: C, 30.41; H, 3.83. Found: C, 30.29; H, 3.52.

(1*S*)-(-)-*cis*-1-Acetoxy-4-hydroxy-2-cyclohexene, 69. Activated zinc dust (31 g, 470 mmol) was added to a mixture of the dibromides **67** and **68** (19.4 g, 61.4 mmol) dissolved in absolute ethanol (300 mL). The suspension was heated and allowed to reflux for 10 min. After cooling of the mixture in a cold water bath, pyridine (30 mL) was added. The mixture was filtered and the filtrate was concentrated by rotary evaporation. The residue was dissolved in ethyl acetate (120 mL) and washed with satd aq sodium bicarbonate (2 x 120 mL), and brine (120 mL). The ethyl acetate solution was dried over magnesium sulfate and concentrated by rotary evaporation, yielding a light yellow oil, 7.9 g (82%).[48] Vacuum distillation gave 6.0 g (62%): bp 75-77 °C (0.05 torr); R_f = 0.26 (3: 2 hexanes/ethyl acetate); $[\alpha]_D$ = -100° (*c* 1.3, CH_2Cl_2); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 5.98 (dd, 1, $J_{1,2}$ = 2.8 Hz, $J_{2,3}$ = 10.1 Hz, H2); 5.80 (dd, 1, $J_{3,4}$ = 3.4 Hz, H3); 5.19 (m, 1, H1); 4.18 (m, 1, H4); 2.15 (br, 1, OH); 2.06 (s, 3, CH_3); 1.70-1.96 (m, 4, H5, H5', H6, H6'). ^{13}C-NMR ($CDCl_3$, 75.4 MHz) δ 170.7 (C=O); 134.8, 127.8 (CH=CH); 67.2 (COAc); 65.3 (COH); 28.1 (CH_2COAc); 24.9 (CH_2COH); 21.2 (CH_3). IR (neat) 3372(br), 3415(br), 1736, 1245, 1037 cm^{-1} MS (CI, NH_3) m/z 174 ($M+NH_4^+$, 63%); exact mass 174.11306 ($C_8H_{16}NO_3$ requires 174.11302, 0.2 ppm error).

(*S*)-(-)-4-acetoxy-2-cyclohexene-1-one, (-)-70. Chromium trioxide (16.3 g, 163 mmol) was added to a stirred solution of dry pyridine (26.3 mL, 326 mmol) in dry

methylene chloride (380 mL) under nitrogen. After thirty min of stirring at room temperature, the olefinic alcohol **69** (4.24 g, 27.1 mmol) in dry methylene chloride (10 mL) was added to the dark reddish-brown solution. A black tarry substance precipitated after a few minutes. The flask was stoppered with a drying tube and the mixture was stirred for 24 h. The methylene chloride solution was decanted and the residue was extracted with alternating portions of ethyl ether and satd aq sodium bicarbonate (2 x 150 mL, 1 x 250 mL each). All extracts were combined with the methylene chloride solution and shaken. The aqueous phase was removed and extracted once with ethyl ether (1000 mL). The organic extracts were washed with satd aq sodium bicarbonate (4 x 250 mL), 2% sulfuric acid (4 x 250 mL), satd sodium bicarbonate (200 mL) and brine (2 x 200 mL). The resulting organic phase was dried over magnesium sulfate and concentrated by rotary evaporation yielding an oil, 3.52 g (84%). R_f = 0.41 (3: 2 hexanes/ethyl acetate); $[\alpha]_D$ = -137°(*c* 1.6, CH_2Cl_2); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 6.85 (ddd, 1, $J_{2,3}$ = 10.3 Hz, $J_{3,4}$ = 2.8 Hz, $J_{3,5}$ = -1.4 Hz, H3); 6.06 (ddd, 1, $J_{2,4}$ = -1.9 Hz, $J_{2,6}$ = -0.9 Hz, H2); 5.57(dddd, 1, $J_{4,5}$ = 4.8 Hz, $J_{4,5'}$ = 8.7 Hz, H4); 2.28-2.70 and 1.99-2.19 (2m's, 3 + 1, H5, H5', H6, H6'); 2.12 (s, 3, CH_3). ^{13}C-NMR($CDCl_3$, 75.4 MHz) δ 197.7 (**C**=O); 170.2 ($OCOCH_3$); 147.5 (**C**HCOAc); 130.8 (**C**HC=O); 67.7 (**C**OAc); 34.9 (CH_2C=O); 28.6 (CH_2COAc); 20.9 (CH_3). IR (neat) 1741, 1686, 1372, 1236, 1037 cm^{-1}. MS (CI, NH_3) m/z 172 ($M+NH_4^+$, 100%), 155 ($M+H^+$, 10%); exact mass 155.07075 ($C_8H_{11}O_3$ requires 155.07082, 0.4 ppm error).

Enantiomeric Purity of 70. Acetylcholinesterase (4 mg) was added to a stirred suspension of acetoxyketone **70** (322 mg, 2.09 mmol) in aq phosphate buffer (20 mL, 10 mM, pH 7.13). The pH was maintained at 7.13 by automatic addition of NaOH (0.10 N). After 27 h only 0.4 mmol of base had been consumed, thus additional enzyme (CRL, 300 mg) was added. After an additional 41 h a total of 2.1 mmol of base had been added. The reaction mixture was extracted with ethyl

acetate (3 x 150 mL) and the combined extracts were washed with satd aq sodium bicarbonate (20 mL), water (20 mL), and brine (2 x 20 mL). The organic phase was dried over magnesium sulfate and concentrated by rotary evaporation yielding crude alcohol, 150 mg (64%): R_f = 0.07 (3: 2 hexanes/ethyl acetate). This alcohol was treated with Mosher's acid chloride using a standard procedure.[49] The resulting ester was purified by column chromatography on silica gel eluted with 9:1 hexanes/ethyl acetate; R_f = 0.41 (3: 2 hexanes/ethyl acetate). A racemic sample of **70** was also treated in the same manner.

Absolute configuration of 70. Acetylcholinesterase (3.6 mg, 0.57 units) was added to a stirred mixture of acetoxy ketone, **70** (300 mg, 1.95 mmol) and aq phosphate buffer (20 mL, 10 mM, pH 7). Aliquots of a 0.107 N NaOH solution were added automatically to maintain the pH of the mixture at 7.01. Due to the slow rate of the hydrolysis, a large amount (700 mg) of CRL was added in three portions over a period of 6 days. The reaction was stopped at 93% conversion. The reaction mixture was extracted with ethyl acetate (3 x 200 mL). Each organic extract was washed with satd aq sodium bicarbonate (5 mL), water (2 x 5 mL), and brine (2 x 5 mL). The extracts were combined, dried over magnesium sulfate, and concentrated by rotary evaporation yielding 169 mg (77%); R_f = 0.07 (3:2 hexanes/ethyl acetate). Without further purification of the alcohol, the benzoate derivative was prepared. Benzoyl chloride (340 μL, 2.92 mmol) was added to a solution of alcohol (164 mg, 1.46 mmol) in pyridine (3 mL, 37 mmol). The mixture was stirred at room temperature for 70 min at which time TLC analysis showed no remaining alcohol. The reaction mixture was added to a separatory funnel containing 0.5 M H_2SO_4 (74 mL) and ethyl ether (50 mL). After vigorous shaking, additional ethyl ether (150 mL) was added. The aqueous phase was discarded and the organic phase was washed with satd aq sodium bicarbonate (20 mL), brine (2 x 20 mL), dried over magnesium sulfate, and concentrated by rotary

evaporation. Purification by flash chromatography (85:15 hexanes/ethyl acetate) yielded the benzoate as an oil, 184 mg (58%): R_f = 0.46 (3:2 hexanes/ethyl acetate); $[\alpha]_D$ = -197° (*c* 1.9, CH_2Cl_2); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 7.41-8.09 (m, 5, aromatic); 6.98 (ddd, 1, $J_{2,3}$ = 10.3 Hz, $J_{3,4}$ = 2.8 Hz, $J_{3,5}$ = -1.3 Hz, H3); 6.11 (ddd, 1, $J_{2,4}$ = -1.9 Hz, $J_{2,6}$ = -0.8 Hz); 5.82 (m, 1, H4); 2.40-2.77 and 2.15-2.34 (2m's, 3+1, H5, H5', H6, H6'). ^{13}C-NMR($CDCl_3$, 75.4 MHz) δ 197.8 (**C**=O); 165.7 (O**C**OCH$_3$); 147.6 (**C**HCOAc); 133.4 (*p*-**C**H); 131.0 (**C**HC=O); 129.7, 128.5 (*o*- and *m*-**C**H); 129.5 (**C**COOR); 68.2 (**C**OAc); 35.0 (**C**H$_2$C=O); 28.8 (**C**H$_2$COAc). UV (CH_3OH): 228 nm (ε 14200 M^{-1} cm^{-1}), 271 nm (ε 554 M^{-1} cm^{-1}), IR (neat), 3050, 2960. 1722, 1683, 1452, 1270, 1113, 710 cm^{-1}. MS (CI, NH_3) m/z 217 ($M+H^+$, 100%); exact mass 217.08646 ($C_{13}H_{13}O_3$ requires 217.08647, 0.0 ppm error). The CD spectrum was obtained using a 4.4 x 10^{-5} M solution of the benzoate in CH_3OH in a 0.1 cm cell using a Jasco 500C spectropolarimeter. A total of 10 scans were made from 250 to 185 nm.

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References and Notes

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29. It was difficult to decide which side was larger for **37**, **38**, **50** and **62**. Compounds **37** and **50** contain a small substituent near the carbinol carbon on one side and a larger substituent further away on the opposite side. Compound **37** was drawn as shown arbitrarily. For **50**, the larger acetoxy substituent is three carbons away from the carbinol carbon and is judged to be less important

than the CH_3 at the 2-position. The unsaturated portion of **38** and the cyclopropyl portion of **62** were judged to be smaller using three-dimensional models. Several other bicyclic structures appear symmetrical as drawn, but three-dimensional models clearly show the right side to be larger.

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Chapter 3

Following the success of the empirical rule for secondary alcohols described in the previous chapter, we endeavoured to determine whether this rule could be extended to include primary alcohol substrates. We chose to use lipase from *Pseudomonas cepacia* as the biocatalyst for this project because initial surveys showed that it was the most promising of the serine esterases for reactions involving primary alcohols.

This chapter describes how a similar but opposite rule is valid for primary alcohols. A reason for this opposite enantiopreference is suggested, using both experimental and molecular modeling techniques.

Note: The text of this article has been reformatted in accordance with the Thesis Preparation Guidelines. A photocopy of the published version is included in the appendices.

Enantiopreference of Lipase from *Pseudomonas cepacia* toward Primary Alcohols.

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Abstract: We propose an empirical rule that predicts which enantiomer of a primary alcohol reacts faster in reactions catalyzed by lipase from *Pseudomonas cepacia* (PCL). This rule, based on the size of the substituents at the stereocenter, shows an 89% reliability (correct for 54 of 61 examples). This rule is not reliable for primary alcohols that have an oxygen atom attached to the stereocenter; we excluded these alcohols from the tally above. Surprisingly, the sense of enantiopreference of PCL toward primary alcohols is opposite to its enantiopreference toward secondary alcohols. That is, the –OH of secondary alcohols and the $-CH_2OH$ of primary alcohols point in opposite directions. We suggest, however, that this opposite orientation does not imply a different position of the substituents in the active site of the lipase. Instead, PCL accommodates the extra CH_2 in primary alcohols as a kink between the stereocenter and the oxygen which allows a similar position of the alcohol oxygen in both. We tried to increase the enantioselectivity of PCL toward primary alcohols by increasing the difference in the size of the substituents, but did not find a consistent increase in enantioselectivity. We suggest that high enantioselectivity toward primary alcohols requires not only a significant difference in the size of the substituents, but also control of the conformation along the C(1)-C(2) bond.

Introduction

Organic chemists have embraced lipases and esterases as enantioselective catalysts for synthetic applications because they combine broad substrate specificity with high enantioselectivity.[1] One current goal of organic chemists is to map the specificity of these enzymes. This mapping identifies both efficiently resolved substrates and the structural features important for their enantiorecognition, allowing chemists to more rationally design resolutions.

Previous mapping of the specificity of lipase from *Pseudomonas cepacia*[2] (PCL, Amano Lipase P) established a simple rule that predicts its enantiopreference toward secondary alcohols, Figure 1a.[3,4,5,6] This rule predicts which enantiomer reacts faster based on the sizes of the substituents at the stereocenter. The same rule holds for ten other hydrolases whose specificities have been mapped: lipase from *Candida rugosa*,[3] lipase from *Pseudomonas* sp.,[7] lipase from *P. aeruginosa*,[8] *Rhizomucor miehei*,[9] lipase from *Arthrobacter* sp.,[10] porcine pancreatic lipase,[11] pancreatic cholesterol esterase,[3] *Mucor* esterase,[11] cultures of *Rhizopus nigricans*,[12] and cultures of *B. subtilus* var. *Niger*.[13] This rule suggests that these hydrolases distinguish between enantiomers based on the size of the substituents. Consistent with this suggestion, researchers have increased the enantioselectivity of lipase-catalyzed reactions of secondary alcohols by increasing the difference in size of the two substituents.[3,5,14]

Recent X-ray crystal structures of transition state analogs bound to lipase from *Candida rugosa* showed that this rule is a good description of the alcohol binding pocket.[15] This lipase contains a large hydrophobic binding pocket that binds the larger substituent of a secondary alcohol and a smaller pocket that binds the medium substituent. Conserved structural elements create this binding crevice, especially the pocket for the medium substituent. For this reason, other lipases probably contain similar alcohol binding sites.

Several groups have tried to extend this rule for secondary alcohols to include primary alcohols, but these efforts have been only partially successful. Naemura *et al.* used a rule based on the size of the substituents to account for the enantiopreference of lipase from *Pseudomonas* sp. toward two primary alcohols[16] and Carrea *et al.*[17] used a similar rule to account for the enantiopreference of PCL toward nine primary alcohols. However, Xie *et al.*[18] proposed an enantiomeric rule for the enantiopreference of PCL toward two primary alcohols without an oxygen at the stereocenter and another rule based on two primary alcohols with an oxygen at the stereocenter.[19] Researchers encountered similar difficulties with porcine pancreatic lipase (PPL) and also proposed enantiomeric rules.[20]

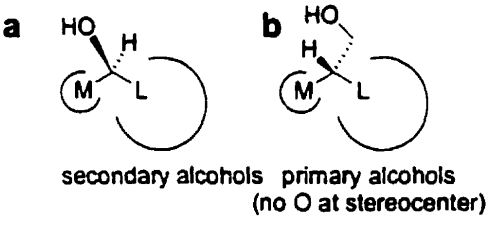

Figure 1. Empirical rules that summarize the enantiopreference of *P. cepacia* lipase (PCL) toward chiral alcohols. (**a**) Shape of the favored enantiomer of secondary alcohols. M represents a medium substituent, e.g. CH_3, while L represents a large substituent, e.g., Ph. (**b**) Shape of the favored enantiomer of primary alcohols. This rule is reliable only when the stereocenter lacks an oxygen atom. Note that PCL shows an opposite enantiopreference toward primary and secondary alcohols.

In this paper, we examine the reported enantiopreference of PCL and propose an empirical rule that summarizes its enantiopreference toward primary alcohols. Although the rule is opposite to the one for secondary alcohols, we suggest that the large and medium substituents adopt a similar position in both. We also attempt, unsuccessfully, to increase the enantioselectivity of PCL toward primary alcohols by increasing the difference in size of the two substituents at the stereocenter.

Results and Discussion

Enantiopreference of PCL toward primary alcohols. Following the suggestion of Xie *et al.*,[18] we divided the primary alcohols into those with an oxygen at the stereocenter and those without an oxygen at the stereocenter. Table 1 and Chart 1 summarize the reported enantioselectivities of PCL toward primary alcohols that lack an oxygen at the stereocenter. The enantiomers shown in Chart 1 are those predicted to react faster by the empirical rule in Figure 1b: the $-CH_2OH$ points into the page and the larger substituent lies on the right. The notation '(ent)' in the Table marks those examples where PCL favored the enantiomer opposite to the one predicted. This list includes only primary alcohols with a tertiary stereocenter, that is, $R^1R^2CHCH_2OH$. The list further includes only reactions catalyzed by PCL from Amano (P, P30, PS, LPL-80, LPL-200S) and SAM-2 from Fluka,[2] and only those substrates for which the enantiomeric ratio[21], E, was >2. The list includes esterifications and transesterifications of primary alcohols as well as hydrolyses of esters of primary alcohols.

The rule in Figure 1b predicts the absolute configuration of the favored enantiomer for 49 out of 54 substrates, 91% accuracy. We will discuss seven more examples in this paper, five of which follow the rule giving an accuracy of 54 out of 61 or 89%. The examples include acyclic, cyclic, and bicyclic alcohols with a wide range of functional groups in the substituents. Approximately 30% of the substrates (16 of the 54) showed excellent enantioselectivities of E >50. The five exceptions to the empirical rule are 4-methyl-1, 3-propanediol (structure **1** where R = CH_2OH), two aziridines, structure **9**, and two cyclohexenone derivatives, structure **18**. In spite of these exceptions, the empirical rule is reliable for most primary alcohols without an oxygen at the stereocenter. The rule in Figure 1b is similar to the rule proposed by Xie *et al.*[18] for two primary alcohols. Although our rule is opposite to the one suggested by Carrea *et al.*,[17] we excluded their examples because they all contained an oxygen at the stereocenter.

Table 1. Enantioselectivity of Lipase from *Pseudomonas cepacia* toward Primary Alcohols without an Oxygen at the Stereocenter.[a]

Struc	R or Ar	E^b	ref	Struc	R or Ar	E^b	ref
1	$CH_2CH_2CH_3$	5.9	*c*	**5**	$CH_2(CH_2)_2CH_3$	7.3	*c*
1	$CH_2(CH_2)_2CH_3$	8.7	*c*	**6**	2,4-Cl_2-C_6H_3	~10	*s*
1	$CH_2(CH_2)_4CH_3$	5.7	*c*	**6**	*N*-Cbz-piperid-4-yl	~7	*t*
1	$CH_2(CH_2)_6CH_3$	9.9, >20	*c, d*	**6**	NHC(O)-1-naphthyl	~25	*u*
1	$CH_2CH_2CH(CH_3)_2$	11	*c*	**6**	$CH_2CH(OEt)_2$	>50	*v*
1	*trans*-CH_2CH=$CHCH_3$	13	*c*	**6**	$(CH_2)_3CHMe(CH_2)_3$i-Pr	13	*w*
1	CH_2CH=CH_2	9.7	*c*	**7**	3,4-OCH_2O-C_6H_3	> ~50	*x*
1	CH_2Ph	20	*e*	**7**	3,4-$(MeO)_2$-C_6H_3	> ~50	*x*
1	CH_2(2-thiophene)	>24->50	*f*	**7**	3,4,5-$(MeO)_3$-C_6H_2	20	*x*
1	CH_2CH_2SPh	21	*g*	**7**	4-Ph-C_6H_4	> ~50	*x*
1	$CH_2CH_2SO_2Ph$	21	*g*	**7**	Ph	>30 ->50	*j, x, y*
1	$CH_2OC(O)Pr$	~28	*h*	**7**	1-naphthyl	13, > ~50	*x, y*
1	NHC(O)OEt	8.6	*i*	**8**		11	*z*
1	CH_2OH	4 (ent)	*j*	**9**	$C(O)OCH_2Ph$	~40 (ent)	*aa*
1	CH_2OAc	21 - >50	*k, l, m*	**9**	tosyl	>~50 (ent)	*aa*
1	CH_2CH_2OH	~4	*n*	**10**		5	*bb*
1	$CH_2CH_2OCH_2Ph$	~20	*n*	**11**		~13	*cc*
1	CH_2O-TBDMS	>50	*m*	**12**		>50	*dd,ee*
1	CH_2O-TBDPS	>50	*k, m*	**13**		~4	*dd*
1	CH_2OCH_2Ph	12 - 35	*m*	**14**		~3	*ff*
1	$CH_2OC(O)Ph$	9 - 35	*m*	**15**		14	*gg*
1	$CH_2CH(CH_3)CH_2OH$	> ~50	*j*	**16**		4	*hh*
2		2 - >40	*o*	**17**		>50	*ii*
3	4-MeO-C_6H_4	7.5 - 40	*p*	**18**	CH_3	14 (ent)	*ii*
4	Ph	3.3	*q*	**18**	CH_2OCH_3	>50 (ent)	*ii*
5	CH_2OAc	~16	*r*	**19**		>50	*jj*
5	NHC(O)OEt	8.9	*i*	**20**		~2	*kk*

[a]All reactions refer to the hydrolysis of the acetate ester in aqueous solution at room temperature, using lipase from *Pseudomonas cepacia* as defined in reference 2, unless otherwise noted. When another ester was hydrolyzed, it is identified; when the alcohol was esterified, the acylating reagent is identified. Only examples that give E >2 are listed. The structures are shown in Chart 1. [b]E, the enantioselectivity, is calculated as in reference [21], except in the case of asymmetric syntheses where it is the ratio of the preferred enantiomer to the least preferred enantiomer. [c]Vinyl acetate: Barth, S.; Effenberger, F. *Tetrahedron: Asymmetry* **1993**, *4*, 823-833. [d]Vinyl acetate , PFL (Fluka): Ferraboschi, P.; Grisenti, P. Manzocchi, A.; Santaniello, E. *J. Chem. Soc., Perkin Trans. 1* **1992**, 1159-1161. [e]Vinyl acetate: Delinck, D. L.; Margolin, A. L. *Tetrahedron Lett.* **1990**, *31*, 6797-6798. [f] Vinyl acetate: Nordin, O.; Hedenström, E.; Högberg, H. E. *Tetrahedron: Asymmetry* **1994**, *5*, 785-788. Bracher, F.; Papke, T. *Tetrahedron: Asymmetry* **1994**, *5*, 1653-1656. [g]Vinyl acetate, PFL (Fluka): Ferraboschi, P.; Grisenti, P.; Manzocchi, A.; Santaniello, E. *J. Org. Chem.* **1990**, *55*, 6214-6216. [h]Butyrate: Wirz, B.; Schmid, R.; Walther, W. *Biocatalysis* **1990**, *3*, 159-167. [i]Francalanci, F.; Cesti, P.; Cabri, W.; Bianchi, D.; Martinengo, T.; Foà, M. *J. Org. Chem.* **1987**, *52*, 5079-5082. [j]Vinyl acetate : Tsuji, K.; Terao, Y.; Achiwa, K. *Tetrahedron Lett.* **1989**, *30*, 6189-6192. [k]Vinyl acetate, PFL (Fluka): Santaniello, E.; Ferraboschi, P.; Grisenti, P. *Tetrahedron Lett.* **1990**, *31*, 5657-5660. TBDPS = *tert*-butyldiphenylsilyl. [l]Xie, Z.-F.; Suemune, H.; Sakai, K. *Tetrahedron: Asymmetry* **1993**, *4*, 973-980. [m]Vinyl acetate, PFL (Fluka): Grisenti, P.; Ferraboschi, P.; Manzocchi,

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Chart 1. Structures for Table 1 and Figure 4

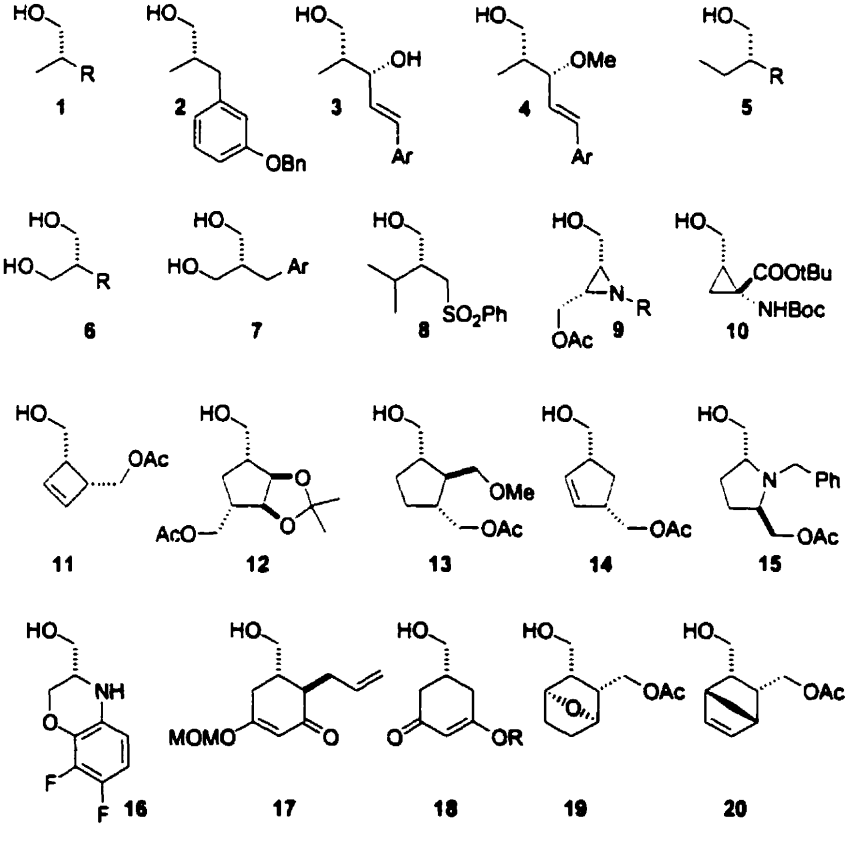


Table 2 and Chart 2 summarize the reported enantioselectivities of PCL toward primary alcohols that have an oxygen at the stereocenter. All structures show the enantiomer predicted to react faster, while the notation '(ent)' marks the exceptions in the Table. The large number of exceptions shows that the empirical rule is not reliable for this group of substrates. The empirical rule predicts the enantiopreference for only 10 of the 27 examples, corresponding to 37% accuracy which is similar to that expected by chance. We excluded substrate **25** ($R = CH_3$) from the tally because both substituents are similar in size. Approximately 30% of the substrates (8 of 27) showed excellent enantioselectivities (E >50); all of these are exceptions to the empirical rule. Thus, the empirical rule in Figure 1b is not reliable when the primary alcohols have an oxygen at the stereocenter. An opposite rule would be slightly more reliable, but still only a slight improvement over guessing.

A similar division of primary alcohols into two groups, those with and without oxygens at the stereocenter, may also resolve the dilemma of enantiomeric rules in the case of PPL. Our preliminary survey found 41 examples of PPL-catalyzed resolutions of primary alcohols without an oxygen at the stereocenter. Twenty-seven examples fit the rule in Figure 1b, four did not; we excluded ten substrates because the sizes of the substituents were too similar. Thus, the reliability was 27/31 or 87% for those with substituents that differed in size. This degree of reliability is similar to that for PCL. We also found 10 examples of PPL-catalyzed resolutions of primary alcohols which have an oxygen at the stereocenter. Three examples fit the rule, six did not; we excluded one substrate. Thus, as with PCL, the rule is not reliable for primary alcohols that have an oxygen at the stereocenter.

Chart 2. Structures for Table 2 and Figure 4

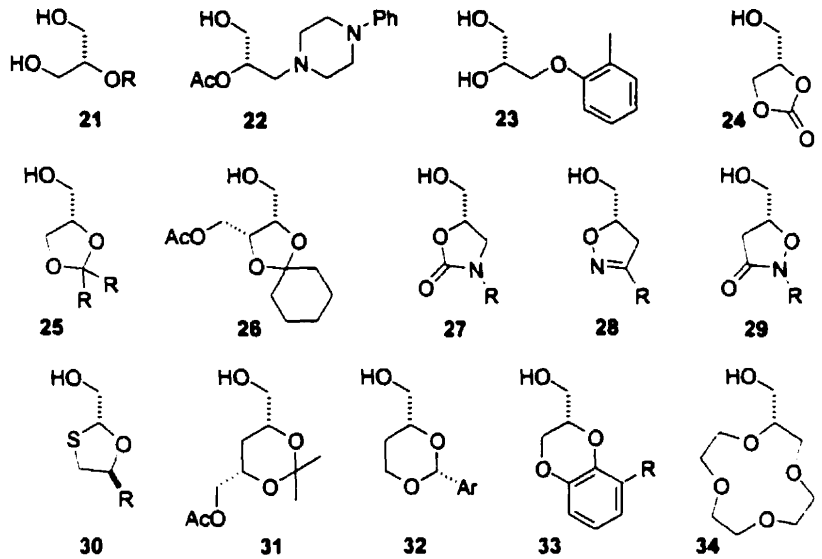


Table 2. Enantioselectivity of Lipase from *Pseudomonas cepacia* toward Primary Alcohols with an Oxygen at the Stereocenter.[a]

Struc	R or Ar	E^b	ref	Struc	R or Ar	E^b	ref
21	CH_3	~6 (ent)	*c*	**27**	$CH(CH_3)_2$	31 - >50 (ent)	*i, m*
21	CH_2CH_3	~20 (ent)	*c*	**28**	Br	6 - >50 (ent)	*n, o, p*
21	CH_2Ph	~20 - 28 (ent)	*c, d*	**28**	OCH_3	14 (ent)	*o*
22		>50 (ent)	*e*	**28**	OCH_2Ph	4 (ent)	*o*
23		4	*f*	**28**	CH_2OH	8 (ent)	*o*
24		15	*g*	**29**	CH_3	3	*o, p*
25	CH_3	4 - 9	*h, i, j*	**29**	Ph	41 (ent)	*o, p*
25	CH_2CH_3	2-6	*i, j*	**30**	1-(5-fluorocytosine)	12	*q*
25	Ph	3 - 8	*i, j, k*	**31**		~50 (ent)	*r*
25	$-(CH_2)_5-$	3	*j*	**32**	Ph	5 - >50 (ent)	*s*
25	CH(CH3)2	23	*i*	**32**	4-OMe-C_6H_4	21 - >50 (ent)	*s*
25	CH_2Ph	9	*i*	**33**	H	8 - 16 (ent)	*t, u*
26		>50 (ent)	*l*	**33**	OCH_3	28 (ent)	*t*
27	$C(CH_3)_3$	>50 (ent)	*m*	**34**		3 (ent)	*v*

[a]All reactions refer to the hydrolysis of the acetate ester in aqueous solution at room temperature, using lipase from *Pseudomonas cepacia* as defined in reference 2, unless otherwise noted. When another ester was hydrolyzed, it is identified; when the alcohol was esterified, the acylating reagent is identified. Only examples that give E >2 are listed. The structures are shown in Chart 2. [b]E, the enantioselectivity, is calculated as in reference 21, except in the case of meso compounds where it is the ratio of the favored to unfavored enantiomers. [c]Vinyl acetate or phenyl acetate: Terao, Y.; Murata, M.; Achiwa, K.; Nishio, T.; Akamtsu, M.; Kamimura, M. *Tetrahedron Lett.* **1988**, *29*, 5173-5176; vinyl

acetate: Murata, M.; Terao, Y.; Achiwa, K.; Nishio, T.; Seto, K. *Chem. Pharm. Bull.* **1989**, *37*, 2670-2672. [d]Breitgoff, D.; Laumen, K.; Schneider, M. P. *J. Chem. Soc., Chem. Commun.* **1986**, 1523-1524; vinyl stearate: Baba, N.; Yoneda, K.; Tahara, S.; Iwasa, J.; Kaneko, T.; Matsuo, M. *J. Chem. Soc., Chem. Commun.* **1990**, 1281-1282; vinyl stearate: Baba, N.; Tahara, S.; Yoneda, K.; Iwasa, J. *Chem. Express* **1991**, *6*, 423-426; Wirz, B.; Schmid, R.; Foricher, J. *Tetrahedron: Asymmetry* **1992**, *3*, 137-142. [e]Transesterification with *n*-propanol as nucleophile: Bianchi, D.; Bosetti, A.; Cesti, P.; Golini, P. *Tetrahedron Lett.* **1992**, *33*, 3231-3234. [f]Vinyl acetate: Theil, F.; Weidner, J.; Ballschuh, S.; Kunath, A.; Schick, H. *J. Org. Chem.* **1994**, *59*, 388-393. [g]Pallavicini, M.; Valoti, E.; Villa, L.; Piccolo, O. *J. Org. Chem.* **1994**, *59*, 1751-1754. [h]Benzoate hydrolysis in 25% DMSO: Bosetti, A.; Bianchi, D.; Cesti, P.; Golini, P. *Biocatalysis* **1994**, *9*, 71-77. [i]Succinic anhydride: Terao, Y.; Tsuji, K.; Murata, M.; Achiwa, K.; Nishio, T.; Watanabe, N.; Seto, K. *Chem. Pharm. Bull.* **1989**, *37*, 1653-1655. [j]Butanoate: Partali, V.; Melbye, A. G.; Alvik, T.; Anthonsen, T. *Tetrahedron: Asymmetry* **1992**, *3*, 65-72. [k]Bellemare, M.-J.; Kazlauskas, R. J., unpublished results. [l]In a water/isopropyl ether emulsion: Gais, H.-J.; Hemmerle, H.; Kossek, S. *Synthesis* **1992**, 169-173. [m]Acetic anhydride: Bianchi, D.; Cesti, P.; Battistel, E. *J. Org. Chem.* **1988**, *53*, 5531-5534. [n]Trifluoroethyl butanoate: Secundo, F.; Riva, S.; Carrea, G. *Tetrahedron: Asymmetry* **1992**, *3*, 267-280. [o]Butyrate: Carrea, G.; De Amici, M.; De Micheli, C.; Liverani, P.; Carnielli, M.; Riva, S. *Tetrahedron: Asymmetry* **1993**, *4*, 1063-1072. [p]Butyrate: De Amici, M.; Magri, P.; De Micheli, C.; Cateni, F.; Bovara, R.; Carrea, G.; Riva, S.; Casalone, G. *J. Org. Chem.* **1992**, *57*, 2825-2829. [q]Butyrate: Hoong, L. K.; Strange, L. E.; Liotta, D. C.; Koszalka, G. W.; Burns, C. L.; Schinazi, R. F. *J. Org. Chem.* **1992**, *57*, 5563-5565. [r]Xie, Z.-F.; Suemune, H.; Sakai, K. *Tetrahedron: Asymmetry* **1993**, *4*, 973-980. [s]Vinyl acetate: Herradón, B. *Tetrahedron: Asymmetry* **1992**, *3*, 209-212; *J. Org. Chem.* **1994**, *59*, 2891-2893; Herradón, B.; Valverde, S. *Tetrahedron: Asymmetry* **1994**, *5*, 1479-1500. [t]Acetic anhydride: Ennis, M. D.; Old, D. W. *Tetrahedron Lett.* **1992**, *33*, 6283-6286. These authors also resolved a similar compound, flesinoxan, but its absolute configuration was tentatively assigned: *Tetrahedron Lett.* **1992**, *33*, 6287-6290. [u]Vinyl acetate: Mauleón, D.; Lobato, C.; Carganico, G. *J. Heterocyclic Chem.* **1994**, *31*, 57-59. [v]Tsukube, H.; Betchaku, A.; Hiyama, Y.; Itoh, T. *J. Chem. Soc., Chem. Commun.* **1992**, 1751-1752; *J. Org. Chem.* **1994**, *59*, 7014-7018. We chose the enantiomer shown because a CH_2 is larger than an oxygen.

Do the Medium and Large Substituents of Primary and Secondary Alcohols Bind to the Same Regions of PCL? We propose two hypotheses to explain how PCL can have an opposite enantiopreference for primary and secondary alcohols, Figure 2a. Hypothesis 1 proposes that the large and medium substituents of primary alcohols bind in the same L and M pockets as the substituents of secondary alcohols. The CH_2OH group must point to the back to place the oxygen in a position similar to that for secondary alcohols. Thus, the opposite enantiopreference for primary and secondary alcohols would stem from accommodating the extra CH_2 in primary alcohols as a kink between the O and the stereocenter.

Hypothesis 2 proposes that the substituents of primary and secondary alcohols bind to different regions of PCL. In particular, both the large and medium substituents of primary alcohols bind in the L pocket, while a hydrogen binds in the M pocket. According to this hypothesis, PCL accommodates the added CH_2 in

a primary alcohol as a turn that places the stereocenter of primary alcohols into the L pocket. Stereoselectivity within the L pocket would determine the enantioselectivity of PCL toward primary alcohols. Vanmiddlesworth and Sih[22] proposed that stereoselectivity within the L pocket of a reductase in yeast can influence the diastereoselectivity of reduction reactions.

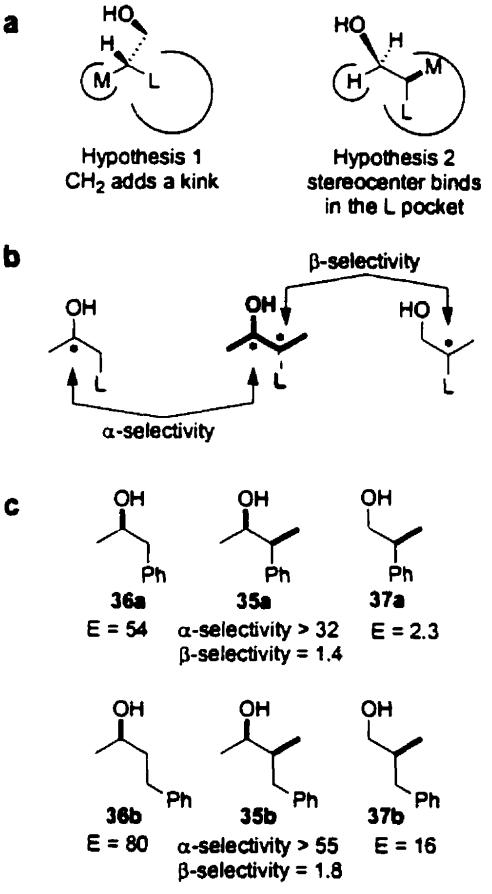


Figure 2. Orientation of the medium and large substituents of primary alcohols in the active site of PCL. (**a**) Two possible orientations of the medium and large substituents can account for the opposite enantiopreference of PCL toward primary and secondary alcohols. In hypothesis 1, PCL accommodates the extra CH_2 group in a primary alcohols as a kink between the stereocenter and the oxygen. The CH_2 group points into the plane of the paper to place the oxygen in a position similar to that in secondary alcohols. In hypothesis 2, PCL accommodates the extra CH_2 group in a primary alcohols as a turn that places both the medium and large substituents into the L pocket. According to this hypothesis, enantioselectivity toward primary alcohols comes from details within the L pocket. (**b**) Measuring the stereoselectivity of the L pocket using a substrate with two stereocenters. Hypothesis 2 predicts that stereoselectivity within the L pocket (β-selectivity) will be similar to the enantioselectivity of PCL toward the corresponding primary alcohol. Hypothesis 1 predicts no relationship between the two selectivities. (**c**) For two examples, the stereoselectivity within the L pocket was lower than the enantioselectivity for the corresponding primary alcohols. These experimental results favor hypothesis 1. Experimental data for the selectivities are in Scheme 1 and Table 3.

To distinguish between these two hypotheses, we measured the stereoselectivity of the L pocket in PCL and compared it to the enantioselectivity of PCL toward primary alcohols, Figure 2b. Hypothesis 1 predicts no relationship between the stereoselectivity of the L pocket and the enantioselectivity of PCL toward primary alcohols. Hypothesis 2 predicts that the stereoselectivity within the L pocket is the same as the enantioselectivity of PCL toward primary alcohols. As detailed below, we found that the stereoselectivity of the L pocket of PCL was significantly lower that the enantioselectivity of PCL toward primary alcohols, thus, we favor hypothesis 1.

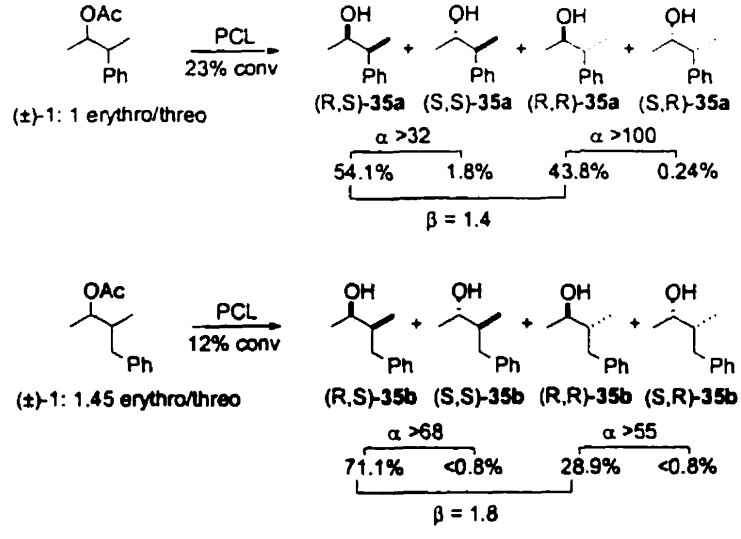
To measure the stereoselectivity of the L pocket in PCL, we measured the stereoselectivity of PCL toward acetates of **35a** and **35b**, Scheme 1. Binding these secondary alcohols to PCL as suggested by the rule in Figure 1a places the β-stereocenter into the L pocket.[23] Thus, the stereoselectivity of PCL toward the β-stereocenter of **35a** and **35b** corresponds to the stereoselectivity of the L pocket.

Scheme 1 summarizes the experimental results for the PCL-catalyzed hydrolysis of the acetate esters of **35a** and **35b**. We prepared alcohols **35a** and **35b** and their acetates as mixtures of diastereomers following literature procedures. After hydrolysis, we determined the relative amounts of each isomer using gas chromatography of the (*S*)-acetyl lactic acid derivatives for **35a** and of the free alcohols for **35b**. To identify the diastereomers, we prepared authentic samples of pure *erythro*-**35a**, and *threo*-**35b** using literature procedures. We confirmed this assignment for **35b** by comparing the ^{1}H-NMR spectrum of the mixture to the ^{1}H-NMR of a known (4: 1) mixture of *erythro*- and *threo*-**35b**.[24]

The absolute configurations of the preferred *erythro*- and *threo*-**35a** were (2*R*, 3*R*) and (2*R*, 3*S*), respectively, based on the negative rotations of the tosyl derivatives. The preferred enantiomer of *threo*-**35b** was (2*R*, 3*S*), by chemical correlation to the ketone derivative, (3*S*)-3-methyl-4-phenyl-2-butanone. The rule

for secondary alcohols, Figure 1a, predicted the favored configuration at the secondary alcohol for all three of these substrates.[23] On this basis, we assigned the (2*R*, 3*R*) configuration to the favored enantiomer for *erythro*-**35b**.

Scheme 1



To calculate the α- and β-selectivities, the stereoselectivities at the α and β stereocenters, we used the approach developed by Sih to measure the enantioselectivity of an enzyme.[25] Equation 1 relates the selectivity of the enzyme to experimentally measured quantities: A_0 and B_0 are the initial amounts of each isomer in the starting material and A and B are the amounts remaining after hydrolysis.

$$Selectivity = \frac{\ln(A/A_0)}{\ln(B/B_0)} \qquad (1)$$

We calculated the α-selectivity either from the relative amounts of the (2*R*, 3*S*) and (2*S*, 3*S*) isomers, α > 32 for **35a**, α > 68 for **35b**, or from the relative amounts of the (2*R*, 3*R*) and (2*S*, 3*R*) isomers, α >100 for **35a**, α > 55 for **35b**. The α-selectivity was high for each pair of isomers and the favored enantiomer was the one predicted by the secondary alcohol rule. These results suggest that the **35a**-

acetate and the **35b**-acetate bound to PCL in the same manner as other secondary alcohols and that the β-stereocenters bound in the L pocket.

We calculated the β-selectivity from the selectivity between the favored 2*R* isomers, (2*R*, 3*S*) and (2*R*, 3*R*), to be β = 1.4 for **35a** and β = 1.8 for **35b**. We did not calculate the β-selectivity from the pair of minor isomers because these 'incorrect' secondary alcohols may bind to PCL in a manner that does not place the β-stereocenter in the L pocket.

To interpret these results, we also measured the enantioselectivity of PCL toward the corresponding secondary alcohols, **36a** and **36b**, and the corresponding primary alcohols, **37a** and **37b** (**37b** is the same as **1** where R = CH_2Ph, Table 1), Table 3. As expected, PCL favored the (*R*)-enantiomer of secondary alcohols **36a** and **36b** with high selectivity, E = 54 and 80, respectively. Also as expected, PCL favored the (*S*)-enantiomer of primary alcohols **37a** and **37b**. The enantioselectivity was low toward **37a**, E = 2.3, and moderate toward **37b**, E = 16. These enantioselectivities are consistent with those measured previously by others for the corresponding esterification reaction, E = 1.3 for **37a**[26] and E = 20 for **37b** (Table 1, structure **1** where R = CH_2Ph).

A summary of these selectivities, Figure 2c, shows a low selectivity in the L pocket of PCL (1.4 and 1.8),[27] while the enantioselectivity toward primary alcohols can be either low or moderate (2.3 and 16). This different selectivity is inconsistent with hypothesis 2 and thus favors hypothesis 1. However, we caution that the addition of a methyl group at the α carbon may inhibit optimal binding of the 'primary alcohol portion' within the large pocket and therefore yield an incorrect measure of the selectivity of the L pocket. With this caution in mind, our results nevertheless favor hypothesis 1. Thus, we propose that the medium and large substituent of primary alcohols bind in the same M and L pockets as the sub-

stituents of a secondary alcohol. The CH_2-kink between the stereocenter and the oxygen causes the reverse orientation of the OH and the CH_2OH in Figure 1.

Table 3. Hydrolysis of Acetates of Primary and Secondary Alcohols Catalyzed by Lipase from *Pseudomonas cepacia*.

Structure	%conv	%ee (product-OH)	abs. config. (product-OH)	E
36a			R	54[a]
36b	34	96[b]	R[c]	80
37a	14	38[d]	S[e]	2.3
37b	44	79[f]	S[g]	16
38	40	71[h]	R[i]	10
39	47	60[d] (SM)[j]	S (SM)[k]	9
40	43	63[l]	R[m]	7
41	49	12[d] (SM)[j]	1S, 2R (SM)[n]	1.4
42	46	34[d]	1R, 2S[o]	2.0
43	41	23[g](SM)[j]	1R, 2S (SM)[p]	2.4
44	85	84[q]	nd[r]	12

[a]Bianchi, D.; Cesti, P.; Battistel, E. *J. Org. Chem.* **1988**, *53*. 5531-5534. Nishio, T.; Kamimura,M.; Murata, M.; Terao, Y.; Achiwa, K. *J. Biochem. Tokyo* **1989**, *105*, 510-512: Esterification with acetic anhydride in organic solvent. [b]Determined by GC of the (*S*)-acetyl lactate derivative using a OV-1701 column. [c]$[\alpha]_D$ = -17.8 (1.8, $CHCl_3$) (lit. (*R*)-(-): Hayashi, T.; Okamoto, Y.; Kabeta, K.; Hagihara, T.; Kumada, M. *J. Org. Chem.* **1984**, *49*, 4224-4226). [d]Determined by GC using a Chiraldex G-TA column. [e]$[\alpha]_D$ = -10.4 (neat) (lit. (*R*)-(-) and (*S*)-(+): Bianchi, D; Cesti, P.; Battistel, E. *J. Org. Chem.* **1988**, *53*, 5531-5534. Bernstein, H.; Whitemore, C. *J. Am. Chem. Soc.* **1939**, *61*, 1326). [f]Determined by GC of the trifluoroacetate derivative using a Chiraldex G-TA column. [g]The product from the PCL-catalyzed esterification of **37a** was identified as the (*S*)-enantiomer (Delinck, D. L.; Margolin, A. L. *Tetrahedron Lett.* **1990**, *31*, 6797-6798). [h]Determined by HPLC using a Chiralcel OD column. [i]$[\alpha]_D$ = -12.1 (0.43, C_6H_6), $[\alpha]_D$ = -19.1 (0.43, $CHCl_3$) (lit. (*R*)-(-): Menicagli, R.; Piccolo, O.; Lardicci, L.; Wis, M. L. *Tetrahedron* **1979**, *35*, 1301-1306. Sonnet, P. E.; Heath, R. R. *J. Chromatogr.* **1985**, *321*, 127-136). [j]Determined for the unreacted acetyl ester. [k]Determined by comparison of the gas chromatogram (Chiraldex G-TA) of the unreacted acetyl ester with that of the acetyl ester of (*S*)-(+)-**39** obtained from authentic (*S*)-(+)-2-amino-1-butanol. [l]Determined by ^1H NMR of the acetyl ester derivative in the presence of (+)-Eu(hfc)3. [m]Determined by comparison of the ^1H NMR spectrum (in the presence of (+)-Eu(hfc)3) of the acetyl ester of the product with that of the acetyl ester of (*S*)-(+)-**40** obtained from authentic (*S*)-(+)-2-amino-1-butanol. [n]Determined by comparison of the gas chromatogram (Chiraldex G-TA) of the unreacted acetyl ester with that of monoacetate **42** obtained from the PCL- catalyzed hydrolysis of the diacetate. [o]$[\alpha]_D$ = -9.04 (3.54, $CHCl_3$) (lit. (1*R*,2*S*)-(-): Alder, U.; Breitgoff, D.; Klein, P.; Laumen, K. E.; Schneider, M. P. *Tetrahedron Lett.* **1989**, *30*, 1793-1796. Laumen, K.; Schneider, M. *Terahedron Lett.* **1985**, *26*, 2073-2076). [p]Determined by comparison of the ^1H NMR spectrum (in the presence of (+)-Eu(hfc)3) of *cis*-(1*R*,2*S*)-1-acetoxymethyl-2-benzoyloxymethylcyclohexane, obtained by benzoylation of monoacetate (1*R*,2*S*)-**42**, to the spectrum of the remaining starting material. The preferred product is therefore *cis*-(1R,2S)-1-benzoyloxymethyl-2-hydroxymethylcyclohexane. [q]Determined by ^1H NMR of the Mosher ester. [r]The absolute configuration was not determined.

Further support for the notion that PCL accommodates the extra CH_2 of primary alcohols as a kink comes from an overlay of models of primary and secondary alcohols, Figure 3. We overlaid minimized structures of the fast- and slow-reacting enantiomers of a primary alcohol acetate, (*S*)- and (*R*)-2-methyl-3-phenylpropyl acetate, onto the fast-reacting enantiomer of the corresponding secondary alcohol acetate, (*R*)-2-acetoxy-1-phenylpropane. In spite of their opposite configurations, the fast-reacting enantiomers overlay more closely at the reaction center, especially at the alcohol oxygen and at the carbonyl group of the ester. This overlay supports the notion that the medium and large substituents of both primary and secondary alcohols can bind in the same M and L pockets, yet show opposite enantiopreference. Other researchers have also noted a reversal in enantiopreference when a CH_2 group is inserted between the stereocenter and the oxygen of other alcohols. For example, the enantiopreference of the Katsuki-Sharpless epoxidation reverses for allylic and homoallylic alcohols. [28]

This explanation is also consistent with the structure of the active site of lipases and their likely mechanism. Researchers believe that the histidine of the catalytic triad protonates the oxygen of the leaving alcohol.[15] For this reason the alcohol oxygen must adopt a similar position in both structures. Furthermore, modelling suggests that the $-CH_2OH$ of a primary alcohol would disrupt the orientation of the catalytic histidine if it pointed in the same direction as the –OH of a secondary alcohol.

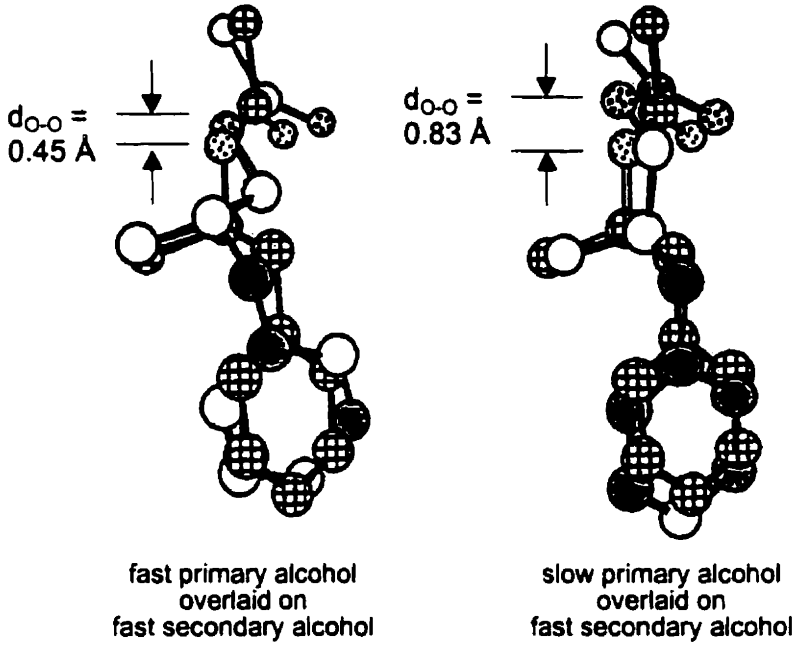


Figure 3. Overlay of the minimized structures of the fast-reacting (*S*)- and slow-reacting (*R*)-enantiomers of the primary alcohol acetate, 2-methyl-3-phenylpropyl acetate, (white/gray carbons) onto the fast-reacting (*R*)-enantiomer of the secondary alcohol acetate, 2-acetoxy-1-phenylpropane, (crosshatched carbons). All oxygen atoms are speckled. In spite of the opposite configuration, the (*S*)-primary alcohol acetate mimics the shape of (*R*)-secondary alcohol acetate better than does the (*R*)-primary alcohol acetate. Note the closer overlap of the alcohol oxygens and the carbonyl groups of the acetates. Models were minimized and overlaid using Chem 3-D. Hydrogen atoms are hidden for clarity.

Increasing Enantioselectivity by Increasing the Size of the Large Substituent. The empirical rule suggests that PCL uses the sizes of the substituents to distinguish between enantiomers. Researchers might enhance enantioselectivity by increasing the difference in size of the substituents. Indeed, this strategy was successful for secondary alcohols.[3, 5, 14] However, we demonstrate below that this strategy was rarely successful for primary alcohols.

The first group are primary alcohols without an oxygen at the stereocenter, Figure 4a and Table 3. The first example, **37a** vs. **38**, showed reversed enantioselectivity when we increase the size of the large substituent. PCL-catalyzed hydrolysis of the acetate ester of **37a**, 2-phenylpropyl acetate, (L = Ph, M = CH_3) showed low enantioselectivity, E = 2.3, favoring the enantiomer predicted by the empirical rule. Replacing the phenyl with the larger naphthyl group in **38** increased the enantioselectivity to E = 10, but also reversed the enantiopreference. Taking into account the reversal, the enantioselectivity decreased by a factor of 23.

The next example showed a slight decrease in enantioselectivity when we increased the size of the large substituent. PCL-catalyzed hydrolysis of the acetate ester of **39** showed an enantioselectivity of 9. Replacing the acetyl protective group with the larger phthalimido group (compound **40**) decreased the enantioselectivity by a factor of 1.3 to E = 7. For both **39** and **40**, PCL favored the enantiomer predicted by the empirical rule.

The third example showed a modest increase in enantioselectivity when we increased the size of one substituent. PCL-catalyzed hydrolysis of racemic *cis*-1-acetoxymethyl-2-(hydroxymethyl)cyclohexane, **41**-acetate, showed low enantioselectivity, E = 1.4, in favor of the (1*R*, 2*S*)-enantiomer of the substrate, which is opposite to the one predicted by the empirical rule. Increasing the size of the CH_2OH substituent to CH_2OAc (**42**-acetate) or CH_2OBz (**43**-acetate) increased the enantioselectivity to E = 2 and E = 2.4. The favored enantiomer for the products, *cis*-1-(acetoxymethyl)-2-(hydroxymethyl)cyclohexane and *cis*-1-[(benzoyloxy)-methyl]-2-(hydroxymethyl)cyclohexane, was (1*R*, 2*S*), as predicted by the empirical rule. Taking into account the reversal, the enantioselectivity increased by modest factors of 2.8 and 3.4, respectively.

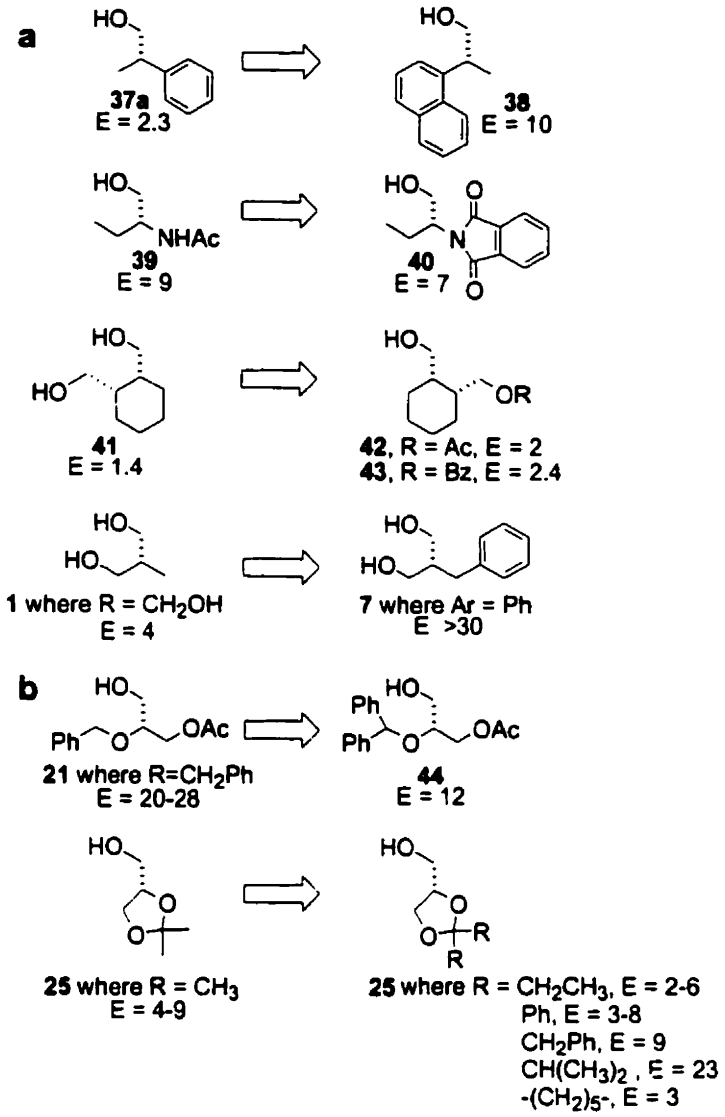


Figure 4. Changes in the enantioselectivity of PCL-catalyzed hydrolyses as the size of one substituents was increased. (**a**) Four examples where the primary alcohol does not have an oxygen at the stereocenter. The first example showed a reversal in enantioselectivity, the second a small decrease, the third a small increase, and the fourth large increase. Data for the first three examples are in Table 3; the fourth comes from the literature. (**b**) Two examples where the primary alcohol has an oxygen at the stereocenter. The first example showed a modest decrease in enantioselectivity, the second example, taken from the literature, showed only small changes in enantioselectivity, except where R = $CH(CH_3)_2$.

The last example in Figure 4a comes from the literature and represents a dramatic increase in enantioselectivity. Tsuji *et al.*[29] reported a low enantioselectivity for the esterification of alcohol **1** where R = CH_2OH, E = 4 in favor of the *pro-R* hydroxyl group. The empirical rule predicts the opposite enantiopreference (M = CH_3, L = CH_2OH). When Tsuji *et al.* replaced the methyl with a larger substituent, CH_2Ph (compound **7** where Ar = Ph in Table 1), the enantioselectivity increased to E >30. Since the CH_2OH is now the medium substituent and CH_2Ph is the large substituent, the enantiopreference now agrees with the empirical rule. Other workers also reported increased enantioselectivity when they replaced the methyl by five other CH_2-aryl groups, see Table 1. This last example is the only one where increasing the size of the substituent dramatically increased the enantioselectivity.

For primary alcohols that contain an oxygen at the stereocenter, increasing the size of the large substituent also did not consistently increase the enantioselectivity, Figure 4b. The hydrolysis of the diacetate of **21** (R = CH_2Ph) showed an enantioselectivity of 20-24 (see Table 2). When we increased the size of the substituent to R = $CHPh_2$ (acetyl ester of **44**), the enantioselectivity decreased by a factor of 2 to E = 12, Table 3. The second example in Figure 4b comes from the literature. The enantioselectivity of PCL toward the glycerol acetal, substructure **25**, changed little as researchers replaced the methyl with ethyl, phenyl, benzyl, or a cyclohexyl acetal; E ranged from 3 to 9, Table 2. An isopropyl substituent increased enantioselectivity, E = 23. Carrea *et al.* also noted that changes in the size of the substituent did not correlate with changes in enantioselectivity for substructures **28** and **29**.[17]

To summarize the effect on enantioselectivity of increasing the difference in the size of the substituents, we found no consistent behavior. Increasing the difference in size may increase, decrease or have no effect on enantioselectivity. We believe the flexibility of primary alcohols accounts for these observations.

Flexibility along the C(1)-C(2) bond allows both enantiomers of primary alcohols to adopt conformations with similar positions of the large and medium substituents, Figure 5. For one enantiomer, a partially eclipsed orientation along the C(1)-C(2) bond orients the large substituent upward and the medium substituent downward. For the other enantiomer, a staggered orientation along the C(1)-C(2) bond also orients the large substituent upward and the medium substituent downward. To distinguish between enantiomers of primary alcohols, PCL must not only distinguish between the large and medium substituents, but also discriminate between the two possible reactive conformations. This explanation may also explain why we needed to exclude primary alcohols with an oxygen at the stereocenter. The oxygen at the stereocenter stabilizes a *gauche* orientation of the oxygen at the stereocenter and the alcohol oxygen due to the *gauche* effect.[30] This stabilization may change the favored orientation along the C(1)-C(2) bond and thereby change the enantiopreference.

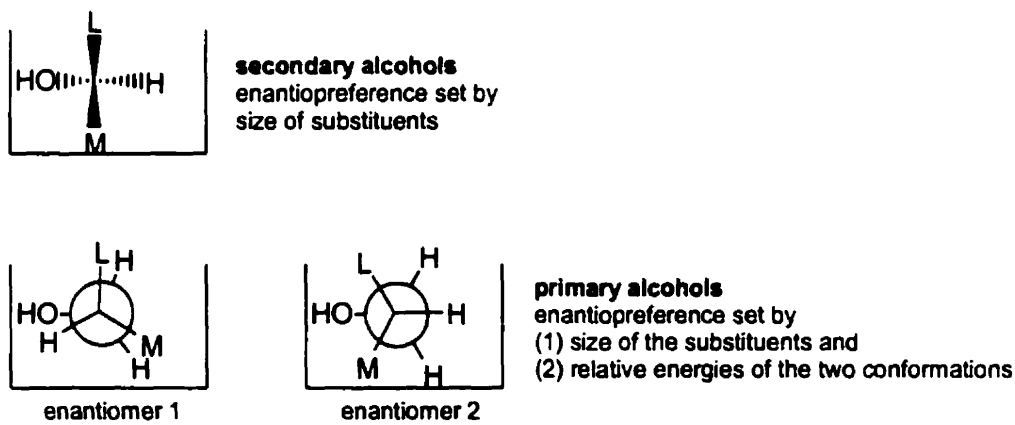


Figure 5. Discrimination between enantiomers of primary alcohols is more difficult than discrimination between enantiomers of secondary alcohols. Flexibility along the C(1)-C(2) bond of primary alcohols allows both enantiomers to adopt conformations with similar orientations of medium and large substituents. For example, enantiomer 1 adopts an upward orientation of the large substituent and a downward orientation of the medium substituent in the eclipsed conformation. Enantiomer 2 adopts a similar orientation in the staggered conformation. To distinguish between enantiomers of primary alcohols, PCL must distinguish between different conformations along the C(1)-C(2) bond in addition to distinguishing between the substituents. The conformation above is only an example. We do not know the conformation of primary alcohols in the active site of PCL.

Experimental Section

General. Lipase from *Pseudomonas cepacia* (PS30 and LPL-200S) was purchased from Amano International Enzyme Co. (Troy, VI). Unless otherwise noted, organic starting materials were purchased from Aldrich Chemical Co. 4-Phenyl-2-butanol was purchased from Janssen Chimica and *cis*-2,3-epoxybutane from Lancaster. 2-(1-Naphthyl)-1-propanol was prepared from 1-napthylacetic acid as described by Sonnet and Heath.[31] *N,O*-Diacetyl-2-amino-1-butanol, **39**, was prepared by a known method.[32]

(±)-*threo/erythro*-3-Phenyl-2-butanol, 35a, was prepared by the nucleophilic addition of methyl magnesium iodide to 2-phenylpropionaldehyde, as described by Overberger *et al.*[33] The ^{1}H NMR of the product agreed with that reported previously.[34] A portion of the product (1 g) was purified by medium pressure chromatography (200 g silica gel, 60% pentane/37% chloroform/3% ethyl acetate, 2.1 min/20 mL fraction). Pure threo alcohol (66.7 mg) and pure erythro alcohol (50.3 mg) were obtained, as well as mixed fractions. Mixed fractions were combined to form a sample (~400 mg) of 50/50 threo/erythro. The threo and erythro isomers can be separated by tlc when eluted three times with 60% pentane/37% chloroform/3% ethyl acetate: on a 7.5 cm plate, Rf = 0.49 (threo) and Rf = 0.40 (erythro). The diastereomers were identified by comparison of the Rf's and ^{1}H NMR of the mixture to that of the pure erythro alcohol prepared as described below.

(±)-*erythro*-3-Phenyl-2-butanol, (±)-*erythro*-35a, was prepared by reaction of phenyllithium with *trans*-2,3-epoxybutane according to a literature procedure.[35] The only change in the procedure was the use of a stock solution of phenyllithium (1.8 M in 70: 30 cyclohexane/ether) instead of preparing the reagent *in situ*. This method was not used for large scale preparation due to low yields (34%). Rf = 0.40 (60% pentane/37% chloroform/3% ethyl acetate). ^{1}H NMR ($CDCl_3$, 250 MHz) δ

7.17-7.35 (m, 5), 3.88 (apparent quintet, 1, J_{app} ~ 6.2 Hz), 2.73 (apparent quintet, 1, J_{app} ~ 6.7 Hz), 1.55 (br s, 1), 1.32 and 1.08 (two d, 3 + 3, J = 6.6 Hz and J = 5.8 Hz).

Acetyl Esters. Acetic anhydride (1.5 equiv), DMAP (0.05 equiv) and anhydrous sodium carbonate (1.5 equiv) were added to a stirred solution of alcohol in ethyl acetate overnight at ambient temperature. The reaction mixture was then diluted with ethyl acetate, washed with water and brine, and the organic extracts dried ($MgSO_4$) and evaporated to afford the pure acetyl ester.

(±)-*threo*-2-Acetoxy-3-phenylbutane, Acetyl Ester of *threo*-35a has been previously prepared.[36] ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.18-7.34 (m, 5, aromatic), 5.09 (apparent quintet, 1, J_{app} = 7 Hz), 2.93 (apparent quintet, 1, J_{app} = 7 Hz), 1.92 (s, 3), 1.28 (d, 3, $J_{H4,H3}$ = 7.3 Hz), 1.16 (d, 3, $J_{H1,H2}$ = 6.6 Hz).

(±)-*erythro*-2-Acetoxy-3-phenylbutane, Acetyl Ester of *erythro*-35a has been previously prepared.[36] ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.16-7.34 (m, 5), 5.03 (dq, 1, $J_{H2,H1}$ = 6.2 Hz), 2.85 (apparent quintet, 1, J_{app} = 7.3 Hz), 2.06 (s, 3), 1.28 (d, 3, $J_{H4,H3}$ = 7.3 Hz), 1.05 (d, 3, $J_{H1,H2}$ = 6.2 Hz).

(±)-*threo/erythro*-3-Methyl-4-phenyl-2-butanol, (±)-*threo/erythro*-35b, was prepared in three steps following a literature procedure.[33,37] Reduction of α-methyl-*trans*-cinnamaldehyde with lithium aluminum hydride yielded 3-phenyl-2-methyl-1-propanol, (±)-**37b**. Swern oxidation followed by the addition of methyl magnesium iodide yielded (±)-*threo/erythro*-**35b**. R_f = 0.26 (4: 1 cyclohexane/ethyl acetate). The ^{1}H NMR of this diasteromeric mixture agrees with that reported in the literature.[38]

(±)-*threo*-3-Methyl-4-phenyl-2-butanol, (±)-*threo*-35b.[39] A solution of benzyl bromide (2.5 mL, 21.0 mmol) in ether (15 mL) was slowly added to magnesium turnings (1 g, 41.1 mmol) under nitrogen. The reaction flask was kept in an ice bath until complete addition and then the reaction mixture was stirred at room

temperature for 1 h. The flask was again cooled and *cis*-2,3-epoxybutane (0.8 mL, 9.2 mmol) in ether (5 mL) was added dropwise. After complete addition, the mixture was refluxed for 2 h. Excess Grignard was quenched by the careful addition of saturated NH_4Cl. Water was added and the slurry was filtered into a separatory funnel, rinsing with ethyl acetate. The filtrate was washed with 0.5 N HCl, saturated aqueous $NaHCO_3$, H_2O, and brine, dried over Na_2SO_4, filtered, and evaporated *in vacuo*. Purification by flash column chromatography (9: 1 pentane/ethyl acetate) yielded the threo alcohol (125 mg, 8%): ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.17-7.33 (m, 5), 3.70 (br apparent quintet, 1, J_{app} ~ 6 Hz), 2.88 (dd, 1, $^2J_{H4\text{-}H4'}$ = 13.3 Hz, $J_{H4\text{-}H3}$ = 4.8 Hz), 2.35 (dd, 1, $^2J_{H4\text{-}H4'}$ = 13.3 Hz, $J_{H4'\text{-}H3}$ = 9.3 Hz), 1.83 (m, 1), 1.37 (br s, 1), 1.21 (d, 3, $J_{H1\text{-}H2}$ = 6.3 Hz), 0.83 (d, 3, $J_{H3\text{-}CH_3\text{-}3}$ = 6.8 Hz).

(±)-*threo/erythro*-2-Acetoxy-3-methyl-4-phenylbutane, Acetyl Ester of (±)-*threo/erythro*-35b has been previously prepared.[40] ^{1}H NMR ($CDCl_3$, 250 MHz) (resonances of the two diastereomers overlap except for the OAc and CH_3-3 resonances) δ 7.09-7.33 (m, 10), 4.76-4.95 (m, 2), 2.72-2.89 (m, 2), 2.20-2.43 (m, 2), 1.82-2.05 (m, 2), 2.05 (s, 3) 2.03 (s, 3), 1.22 (d, 6, $J_{H1,H2}$ = 6.3 Hz), 0.88 (d, 3, $J_{CH_3\text{-}3,H3}$ = 6.9 Hz), 0.83 (d, 3, $J_{CH_3\text{-}3,H3}$ = 6.8 Hz).

(±)-*threo*-2-Acetoxy-3-methyl-4-phenylbutane, Acetyl Ester of (±)-*threo*-35b has been previously prepared.[40] ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.12-7.29 (m, 5), 4.82 (apparent quintet, 1, $J_{H2,H1}$ = 6.3 Hz, $J_{H2,H3}$ = 6.3 Hz), 2.80 (dd, 1, $^2J_{H4,H4'}$ = -13.4 Hz, $J_{H4,H3}$ = 4.7 Hz), 2.28 (dd, 1, $^2J_{H4',H4}$ = -13.4 Hz, $J_{H4',H3}$ = 9.6 Hz), 2.01 (s, 3), 1.89-2.10 (m, 1), 1.20 (d, 3, $J_{H1,H2}$ = 6.3 Hz), 0.81 (d, 3, $J_{CH_3\text{-}3,H3}$ = 6.8 Hz).

(±)-2-Phthalimido-1-butanol, 40, was prepared using a standard method for the protection of amino acids.[41] Phthalic anhydride (1.5 g, 10.1 mmol) and triethylamine (0.6 mL, 4.2 mmol) were added to a solution of (±)-2-amino-1-butanol (500 mg, 5.6 mmol) in toluene (10 mL) in a reaction vessel equipped with a condenser and Dean-Stark trap. After refluxing overnight, the reaction mixture

was cooled to room temperature and extracted twice with ethyl acetate (30 + 20 mL). The combined organic extracts were washed with saturated aqueous $NaHCO_3$ (2 x 50 mL), H_2O (50 mL), and brine (50 mL), and dried over anhydrous $NaSO_4$. The crude residue obtained upon evaporation of the solvent was purified by flash column chromatography (7: 3 pentane/ethyl acetate) yielding 2-phthalimido-1-butanol as an oil (723 mg, 59%): Rf = 0.20 (7: 3 pentane/ethyl acetate); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.79-7.90 (m, 2), 7.68-7.79 (m, 2), 4.29 (m, 1), 4.08 (m, 1), 3.89 (m, 1), 2.73 (br d, 1), 1.92 (apparent decatet, 2), 0.94 (t, 3, J = 7.4 Hz).

1-Acetoxy-2-phthalimidobutane, Acetyl Ester of 40. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.79-7.90 (m, 2), 7.68-7.79 (m, 2), 4.31-4.58 (overlapping m, 3), 1.71-2.22 (overlapping m, 2), 1.97 (s, 3), 0.92 (t, 3, J = 7.4 Hz). Exact mass 261.0999 ($C_{14}H_{15}O_4N$ requires 261.1001, -0.8 ppm error).

***cis*-1-Acetoxymethyl-2-benzoyloxymethylcyclohexane, Acetyl Ester of 43**. 4-dimethylaminopyridine (5.4 mg, 0.044 mmol) and benzoic acid (108 mg, 0.88 mmol) were added to a solution of *cis*-1-acetoxymethyl-2-hydroxymethylcyclohexane (83 mg, 0.44 mmol) in dichloromethane (5 mL). The mixture was cooled in a cold water bath and dicyclohexylcarbodiimide (100 mg, 0.48 mmol) was added. After stirring at room temperature for 3 days, the solvent was removed by rotary evaporation. The residue, taken up in ethyl ether, was filtered and the filtrate was washed with 0.5 N HCl, saturated aqueous $NaHCO_3$, H_2O, and brine, dried over Na_2SO_4, filtered, and evaporated *in vacuo*. The crude product was purified by flash column chromatography (gradient from 9: 1 to 1: 1 pentane/ethyl acetate), yielding *cis*-1-acetoxymethyl-2-benzoyloxymethylcyclohexane as an oil (68 mg, 53%): Rf = 0.68 (7: 3 pentane/ethyl acetate); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.98-8.08 (m, 2), 7.38- 7.62 (m, 3), 4.31 and 4.14 (two d, 2 + 2, J = 7.1 Hz), 2.06-2.32 (m, 2), 1.99 (s, 3), 1.33-1.72 (m, 8). Exact mass 290.1511 ($C_{17}H_{22}O_4$ requires 290.1518, -2.4 ppm error).

2-*O*-Diphenylmethylglycerol Diacetate, Acetyl Ester of 44. *t*-Butyldimethylsilylchloride (10 g, 66mmol) was slowly added to a stirred solution of 1,3-dihydroxyacetone dimer (3 g, 17 mmol) and imidazole (9 g, 133 mmol) in dimethylformamide (22 mL) under nitrogen. The mixture was cooled in a cold water bath until the initial exothermic reaction was over. After stirring at room temperature for 3 h, the reaction mixture was poured into H_2O (800 mL) and extracted with ethyl ether (2 x 700 mL). Each ether extract was washed with H_2O (2 x 800 mL) and dried over $MgSO_4$. The combined ether extracts were filtered and evaporated *in vacuo*, yielding 1,3-di(*t*-butyldimethylsiloxy)acetone as a clear colourless oil, 10.45 g (98%): ^{1}H NMR ($CDCl_3$, 200 MHz) δ 4.41 (s, 4), 0.91 (s, 18), 0.08 (s, 12). Sodium borohydride (237 mg, 6.26 mmol) was added to a stirred solution of the 1,3-di(*t*-butyldimethylsilyloxy)acetone (2 g, 6.28 mmol) in dry methanol (40 mL) at 4 °C under nitrogen. After stirring for 30 min, the reaction mixture was quenched with 5% aqueous CH_3COOH (15 mL) and H_2O (150 mL). The mixture was extracted with ethyl acetate (800 mL) and the organic phase was washed with 5% aqueous CH_3COOH (2 x 200 mL), saturated aqueous $NaHCO_3$ (2 x 250 mL), H_2O (200 mL), and brine (300 mL), and dried over $MgSO_4$. The solvent was evaporated to yield the alcohol as a clear colourless oil, 1.98 g (98.5%): Rf = 0.38 (9: 1 hexane/ethyl acetate). Diphenyldiazomethane[42] (333 mg, 1.71 mmol) was added to a stirred solution of the alcohol (250 mg, 0.78 mmol) in dry acetonitrile (10 mL). After refluxing overnight, additional diphenyldiazomethane (150 mg, 0.77 mmol) was added and the mixture was again refluxed overnight. The reaction mixture was cooled in a refrigerator for 3 h and the filtrate obtained after filtration of the resulting white precipitate was concentrated *in vacuo*. The crude oil was purified by flash column chromatography (98: 2 hexane/ethylacetate), yielding 1,3-di-*O*-(*t*-butyldimethylsilyl)-2-*O*-diphenylmethyl-glycerol. (340 mg, 90%): Rf = 0.65 (9: 1 hexane/ethylacetate). ^{1}H

NMR ($CDCl_3$, 200 MHz) δ 7.21-7.39 (m, 10), 5.77 (s, 1), 3.49-3.78 (m, 5), 0.87 (s, 18), 0.01 (s, 12). Tetrabutylammonium fluoride (1 N in THF, 0.80 mL, 0.80 mmol) was added to a stirred solution of the pure 1,3-di-*O*-(*t*-butyldimethylsilyl)-2-*O*-diphenylmethyl glycerol (130 mg, 0.27 mmol) in THF (3 mL). After stirring for 1 h at room temperature, the reaction mixture was extracted with ethyl acetate (90 mL),washed with H_2O (2 x 5 mL) and brine (2 x 40 mL), and dried over $MgSO_4$. Evaporation of the solvent yielded the crude 1,3-diol (156 mg): Rf = 0.18 (1: 1 hexane/ethyl acetate). Anhydrous sodium carbonate (84 mg, 0.80 mmol), 4-dimethylaminopyridine (3.3 mg, 0.027 mmol), and acetic anhydride (0.075 mL, 0.80 mmol) were added to a solution of the crude diol (150 mg) in ethyl acetate (2 mL). The mixture was stirred at room temperature for 18 h and then extracted with ethyl acetate (50 mL). The organic phase was washed with H_2O (4 x 10 mL) and brine (2 x 10 mL), dried over $MgSO_4$, and evaporated *in vacuo*. The crude residue was purified by flash column chromatography (9: 1 hexane/ethyl acetate), 2-*O*-diphenylmethylglycerol diacetate as an oil (70 mg, 77% over two steps). Rf = 0.37 (7: 3 hexanes/ethyl acetate). ^1H NMR ($CDCl_3$, 200 MHz) δ 7.22-7.38 (m, 10), 5.61 (s, 1), 4.21 (m, 4, J_{AB} = 16.5 Hz), 3.86 (m, 1, J_{AX} = 11.6 Hz, J_{BX} = 5.1 Hz), 2.02 (s, 6). Exact mass 342.1471 ($C_{20}H_{22}O_5$ requires 342.1467, 1.1 ppm error).

General Procedure for PCL-Catalyzed Hydrolyses. A rapidly stirred suspension of substrate dissolved in a small amount of ether and phosphate buffer (10 mM, pH 7) containing lipase from *Pseudomonas cepacia* was maintained at pH 7.0 by automatic titration with NaOH (0.1 N) using a Radiometer RTS 822 pHstat. The reaction was stopped at the desired conversion and the mixture was extracted once with 9: 1 ether/ethanol and three times with ether. The combined organic extracts were washed with saturated aqueous $NaHCO_3$, water, and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The starting esters and product alcohols were separated by flash chromatography.

PCL-Catalyzed Hydrolysis of (±)-*threo,erythro*-2-Acetoxy-3-phenyl-butane, Acetyl Ester of 35a. As a pHstat was not available for this hydrolysis reaction, a series of 25 mL-Erlenmeyer flasks containing 2-acetoxy-3-phenylbutane (16 mg, 0.083 mmol, a racemic 1: 1 mixture of threo/erythro isomers), ethyl ether (0.3 mL), sodium phosphate buffer, (7 mL, 0.1 M, pH 7) and lipase from *Pseudomonas cepacia* (40 mg) were shaken in an incubator at 29-32°C. After 2-3 hrs, the contents of one flask were transferred to a test tube and extracted with ether (5 x 2 mL), mixing with a vortex mixer. Persistent emulsions were broken by centrifugation. The ethereal extracts were dried over Na_2SO_4 and filtered, then analyzed by GC to determine the conversion. The extract was then concentrated to 2 mL. The acetyl lactate derivative was subsequently made to determine the optical and diastereomeric purities of the product alcohols by gas chromatography.

PCL-Catalyzed Hydrolyses of (±)-1-Acetoxy-2-phenylpropane, Acetyl Ester of 37a. Test tubes containing (±)-l-acetoxy-2-phenylpropane (20 mg, 0.112 mmol), ether (0. 1 mL), sodium phosphate buffer (0.5 mL, 0.1 M, pH 7), PCL (2 mg) were shaken in an incubator at 29-32 °C. At various time intervals, the entire contents of one test tube were extracted with ether (4 x 2 mL) using a vortex mixer to mix the two phases. Persistent emulsions were broken by centrifugation. The combined ether extracts were analyzed by GC.

Trifluoroacetyl Esters.[43] Trifluoroacetic anhydride (0.2 mL) was added to a solution of alcohol (1-5 mg) in dichloromethane (0.5 mL). Esterification was complete after 30 min and solvent and excess reagent were evaporated in a stream of nitrogen. The residue was dissolved in ethyl ether for analysis by gas chromatography.

Enantiomeric Purity. Gas Chromatography of Acetyl Lactate Derivatives.[44] (*S*)-Acetyl lactic acid chloride (4 drops) was added to the alcohol (10-15 mg)

dissolved in anhydrous ether (2 mL). The mixture was cooled in a cold water bath (5 °C) and pyridine (3 drops) was added. The mixture was stirred for 10-45 minutes and then stirred at room temperature for 1-3 hours. The reaction mixture was washed three times with 0.5 N HCl, twice with saturated aqueous $NaHCO_3$, H_2O, and brine, and dried over Na_2SO_4. This ethereal solution of (*S*)-*O*-acetyl lactyl esters was analyzed by GC using an SE30 or OV-1701 capillary column. The enantiomeric excess values obtained were corrected to account for the optical purity of the derivatizing agent (97.5% ee). ((*S*)-acetyllactyl)-4-phenyl-2-butanol: OV-1701 column, 150 °C, α = 1.06, 21.0 min (*R*), 22.2 min (*S*). *threo*-((*S*)-acetyllactyl)-3-phenyl-2-butanol: SE30 column, 10 min at 160°C then gradient from 160-220 °C at 3 °C/min, α = 1.03, 22.0 min (2*R*, 3*S*), 22.6 min (2*S*, 3*R*). *erythro*-((*S*)-acetyllactyl)-3-phenyl-2-butanol: SE30 column, 10 min at 160 °C then gradient from 160-220 °C at 3 °C/min, α = 1.02, 22.8 min (2*R*, 3*R*), 23.3 min (2*S*, 3*S*).

Enantiomeric Purity. Gas Chromatography Using a Chiral Stationary Phase. The alcohol, acetyl ester, or trifluoroacetyl ester was dissolved in ether or ethyl acetate and analyzed by GC using a Chiraldex G-TA30 capillary column (Astec, Inc., Whippany, NJ). 1-acetoxy-2-phenylpropane: 90 °C, α = 1.03, 36.0 (*S*), 37.0 min (*R*). 2-phenylpropanol: 90 °C, α = 1.06, 39.3 min (*S*), 41.6 min (*R*). 2-methyl-3-phenylpropanol: 90 °C, α = 1.01, 46.4 min (*S*), 47.3 min (*R*). 1-trifluoroacetoxy-2-methyl-3-phenylpropane: 70 °C, α = 1.03, 49.9 min (*R*), 51.2 min (*S*). *erythro*-3-methyl-4-phenyl-2-butanol: 100 °C, α = 1.02, 28.9 min (2*S*, 3*S*), 29.6 min (2*R*, 3*R*). *threo*-3-methyl-4-phenyl-2-butanol: 100 °C, α = 1.02, 31.6 min (2*S*, 3*R*), 32.4 min (2*R*, 3*S*). *threo* and *erythro*-2-acetoxy-3-methyl-4-phenylbutane: 100 °C, 37.5 min (2*S*, 3*S*), 38.3 min (2*S*, 3*R*), 40.5 min (2*R*, 3*S* and 2*R*, 3*R*). *N,O*-diacetyl-2-amino-1-butanol: 120 °C, α = 1.03, 33.9 min (*S*), 34.9 min (*R*).

Enantiomeric Purity. ^{1}H-NMR. (1) The racemic acetyl ester was dissolved in an NMR tube and the ^{1}H NMR spectrum was obtained using a 200 MHz spectrometer. Solid tris[(3-heptafluoropropylhydroxymethylene)-(+)-camphorato]-europium(III), (+)-Eu(hfc)$_3$, was added portion-wise until baseline separation of the acetate signals was obtined. The number of equivalents of shift reagent necessary to obtain baseline separation of peaks was then added to the sample for which the enantiomeric purity was to be determined. (2) The alcohol was treated with Mosher's acid chloride using a standard procedure[45] and the resulting ester was analyzed by 500 MHz ^{1}H NMR spectroscopy.

Enantiomeric Purity. HPLC Using a Chiral Stationary Phase. A sample of the alcohol and acetyl ester dissolved in the eluting solvent was analyzed by HPLC using a Chiralcel OD column. 2-(1-Naphthyl)-1-propanol (99: 1 hexane/isopropanol, 0.5 mL/min, α = 1.26, 18.4 min (*S*), 23.2 min (*R*).

Absolute Configurations. 35a: The tosyl derivatives of the separated threo and erythro alcohols were prepared as previously described and their optical rotary powers were compared to literature values. Erythro tosylate: $[\alpha]_D$ = -14.8 (5.64, benzene), lit.[36] (*R,R*): $[\alpha]_D$ = -17.41. Threo tosylate: $[\alpha]_D$ = -12.05 (4.73, benzene), lit.[36] (*R,S*): $[\alpha]_D$ = -16.89.

35b: The alcohol obtained from the PCL-catalyzed hydrolysis of *threo*-2-acetoxy-3-methyl-4-phenylbutane was oxidized as follows. Jones' reagent (~0.1 mL) was added dropwise to the alcohol (18 mg) in acetone (5 mL) at 0°C until the orange colour persisted. After stirring for 30 min, 2-propanol was added until the mixture was green. After an additional 10 min, sodium hydroxide (1 M) was added until pH 7 was reached. The mixture was filtered and the solvent was evaporated *in vacuo* from the filtrate. The residue was dissolved in ethyl acetate, washed twice with both water and brine, and dried over $MgSO_4$. The crude product obtained upon evaporation of the solvent was purified on a preparative tlc plate (95: 5

pentane/ethyl acetate), Rf = 0.26. The ketone obtained was identified as the S enantiomer, $[\alpha]_D$ +4.9 (c 4.1, ethanol), lit.[46] S-(+), therefore since the starting material was the acetyl ester of *threo*-**35b**, the preferred product is the (2*R*, 3*S*) isomer. By comparison of the GC chromatogram (Chiraldex-GTA 30) of the product obtained from hydrolysis of the threo ester to the chromatogram of the products obtained from the hydrolysis of the racemic threo/erythro mixture, it was determined that the preferred product of the latter hydrolysis was also the (2*R*, 3*S*) isomer.

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References and Notes

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2. Amano Pharmaceuticals Co. sells several different types of lipase from *Pseudomonas cepacia*: lipase PS, lipase P, LPL-80, LPL-200S, and SAM-II. All of these types come from the same microorganism but differ in the purification method and the types of stabilizers used. Lipase P or PS is the industrial grade which contains diatomaceous earth, dextran, and $CaCl_2$. LPL-80 and LPL-200S are diagnostic grades that contain glycine. SAM-II differs from lipase P or PS only in the purification method. This type is also sold by Fluka under the name of lipase from *Pseudomonas fluorescens*, SAM-2, because this microorganism was only recently reclassified as *Pseudomonas cepacia*. We thank Dr. Yoshihiko Hirose (Amano Pharmaceuticals Co.) for explaining these differences.
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$$\frac{\upsilon_A}{\upsilon_B} = \frac{V_A/K_A}{V_B/K_B} \bullet \frac{A}{B}$$

The ratio of the specificity constants measures the inherent selectivity of the enzyme for a pair of competing substrates. Integration of the above equation yields equation 1 in the text. See reference 21.

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shows that, in some cases, PCL can show high β-selectivity. Nevertheless, the β -selectivity for the simpler structures such as **36a** and **36b** is low.

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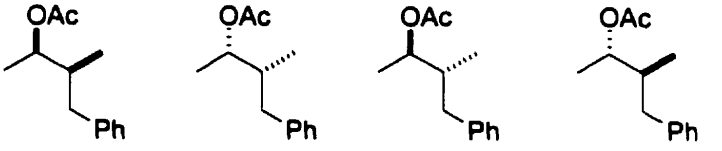
Appendix I

Shown is a sample calculation for the stereoselectivities at the α and β stereocenters for a diastereomeric mixture of enantiomers: enantiomers A and B are diastereomers of C and D.



	A	B	C	D
percent of each isomer in starting material:	29.6	29.6	20.4	20.4
percent of each isomer in remaining starting material after hydrolysis:	21.2	29.5	17.0	20.3

β-selectivity = $\ln(A/A_o) / \ln(C/C_o) = \ln(21.2/29.6) / \ln(17.0/20.4) = 1.8$

α-selectivity = $\ln(A/A_o) / \ln(D/D_o) = \ln(21.2/29.6) / \ln(20.3/20.4) = 68$
or
α-selectivity = $\ln(C/C_o) / \ln(B/B_o) = \ln(17.0/20.4) / \ln(29.5/29.6) = 54$

diastereosectivity = $\ln[(A + B)/(A_o + B_o)] / \ln[(C + D)/(C_o + D_o)]$
$= \ln[(21.2 + 29.5)/(29.6 + 29.6)] / \ln[(17.0 + 20.3) / (20.4 + 20.4)]$
$= 1.7$

Chapter 4

Since all lipases and esterases that had been studied thus far showed the same enantiopreference towards secondary alcohols and isosteric primary amines[*] of the type NH_2CHRR', it was obviously of great interest to find an enzyme which demonstrates an opposite enantiopreference. Consequently, both enantiomers of a given molecule would be obtainable by simply choosing a different enzyme.

Fitzpatrick and Klibanov[†] proposed, on the basis of five examples, that the serine protease, subtilisin, shows an enantiopreference opposite to that of lipases toward secondary alcohols. In this chapter, we review the enantiopreference of subtilisin towards secondary alcohols and confirm that subtilisin does indeed prefer the opposite enantiomer of secondary alcohols. In addition, we investigated the enantiopreference of subtilisin toward isosteric primary amines and found that subtilisin also prefers the opposite enantiomer of primary amines. The regioselectivities of lipases and subtilisin were also compared and found to be opposite. We propose an explanation for the contrasting selectivities of lipases and subtilisin based on the x-ray structures of the two types of hydrolases.

Reprinted from **The Journal of Molecular Catalysis B: Enzymatic**, Vol. 3, Romas J. Kazlauskas and Alexandra N. E. Weissfloch, "A structure-based rationalization of the enantiopreference of subtilisin toward secondary alcohols and isosteric primary amines", 65-72.

Note: The text of this article has been reformatted in accordance with the Thesis Preparation Guidelines. A photocopy of the published version is included in the appendices.

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A Structure-Based Rationalization of the Enantiopreference of Subtilisin toward Secondary Alcohols and Isosteric Primary Amines.

Romas J. Kazlauskas* and Alexandra N. E. Weissfloch

Abstract: Lipases favor one enantiomer of secondary alcohols (HOCHRR') and isosteric primary amines (NH_2CHRR'), while subtilisin favors the other enantiomer. In both cases, simple rules based on the size of the substituents at the stereocenter predict which enantiomer reacts faster. Thus, lipases and subtilisin are a pair of complementary enantioselective reagents for organic synthesis. The success of these rules suggests that these hydrolases distinguish between enantiomers primarily by the size of the substituents. Previously, we proposed a molecular mechanism for the enantiopreference of lipases based on the X-ray crystal structure of transition state analogs bound to a lipase. Here we suggest that a similar mechanism can also account for the opposite enantiopreference of subtilisin. The catalytic machinery (catalytic triad plus the oxyanion-stabilizing residues) in lipases is approximately the mirror image of that in subtilisin. In both hydrolases, the protein fold, as it assembles the catalytic machinery, also creates a restricted pocket for one substituent in the substrate ('M' or medium-sized). However, the catalytic His residue lies on opposite sides of this pocket in the two hydrolases. We propose that enantioselection arises from 1) the limited size of this pocket, 2) and a required hydrogen bond between the catalytic His and the oxygen or nitrogen of the alcohol or amine. This mechanism for enantioselection differs from that proposed by Derewenda and Wei who focussed on which carbonyl face in the ester or amide is attacked. Lipases and subtilisin indeed attack opposite faces, but we propose that this difference does not set the enantiopreference toward secondary alcohols.

Introduction

Synthetic chemists often use proteases and lipases as enantio- and regioselective reagents.[1,2] To simplify the use of these reagents, chemists developed rules, or generalizations, about their selectivity. For example, many researchers proposed rules to predict which enantiomer of a secondary alcohol reacts faster in lipase- and esterase-catalyzed reactions. A simple rule, Figure 1, looks only at the relative sizes of the substituents, but some rules also include polarity or specific size restrictions for the two substituents. These rules have helped chemists use lipases as synthetic reagents since they suggest that lipases discriminate between enantiomers mostly by the sizes of the substituents. For example, resolutions of secondary alcohols where both 'L' and 'M' have similar sizes are rarely efficient, and chemical modifications that increase the difference in size often result in increased enantioselectivity. Recently, Smidt *et al.* suggested that a similar rule can also account for the enantiopreference of a lipase toward the isosteric primary amines of the type NH_2CHRR'.[3]

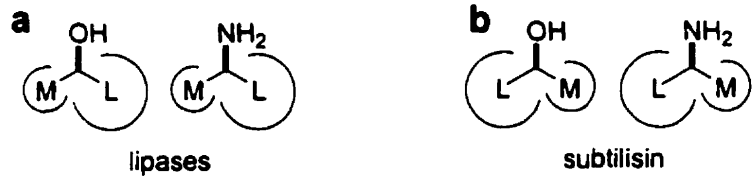

Figure 1. Empirical rules that predict the enantiopreferences of lipases and subtilisins toward secondary alcohols and primary amines of the type NH_2CHRR'. (**a**) Lipases favor the enantiomer with the shape shown where L is a large substituent such as phenyl and M is a medium substituent such as methyl. This rule applies to all lipases and esterases whose substrate specificity has been mapped: thirteen lipases for secondary alcohols and three lipases for amines. Figure 2 summarizes the amines tested. (**b**) Subtilisin has an opposite enantiopreference to lipases. Fitzpatrick and Klibanov proposed the rule shown for five secondary alcohols. These and other examples to support the rule for subtilisin are collected in Figures 3, 4, and 5.

Using X-ray crystallography, Cygler *et al.* identified how the enantiomers of menthol, a typical secondary alcohol, bind to lipase from *Candida rugosa*[4] in the transition state. The alcohol binding site resembled the rule in Figure 1. It

contained a large hydrophobic binding site open to the solvent for the large substituent and a restricted region for the medium-sized substituent. Importantly, the catalytic machinery (Ser-His-Glu triad and the oxyanion-stabilizing residues) and the loops that orient this machimery created the pocket for the medium substituent. The catalytic His residue made a hydrogen bond to the menthol oxygen of the fast-reacting enantiomer, but could not reach this oxygen in the slow-reacting enantiomer because the oxygen pointed away from the His residue. Cyger *et al.* proposed that this lack of a hydrogen bond accounted for the slower reaction.

The X-ray crystal structures of other lipases and esterases showed that, in spite of little similiarity in amino acid sequence, they all fold similarly.[5] This protein fold, named the α/β-hydrolase fold, arranges the catalytic machinery similarly in all lipases and esterases. This similarity allowed a simple rationalization for why lipases and esterases show the same enantiopreference toward secondary alcohols and isosteric primary amines: the similar catalytic machinery restricts the size of the medium pocket in all lipases and esterases. In addition, the catalytic His lies on the same side of the alcohol binding pocket .

Subtilisin, a alkaline serine protease, contains catalytic machinery that is the approximate mirror image of that in α/β-hydrolases.[5] Fitzpatrick and Klibanov found that subtilisin favored the enantiomer opposite to the one favored by lipases. On the basis of five secondary alcohols, they proposed a rule for the enantiopreference of subtilisin opposite to the one for lipases.[6] In this paper, we review the enantiopreference of subtilisin toward secondary alcohols and isosteric primary amines and confirm that its enantiopreference is opposite to that of lipases and esterases. In addition, we show that lipases and subtilisin also have opposite regioselectivity. To rationalize this opposite selelctivity, we show how the enantioselection mechanism proposed for lipases can also account for the enantiopreference of subtilisin.

Results

Enantiopreference of lipases toward primary amines. Researchers only recently resolved amines using lipases and have examined the substrate specificity of only three lipases, Figure 2. Lipase B from *Candida antartica* (CAL-B) is the most popular[7,8,9,10,11,12] , although lipase from *Pseudomonas cepacia* (PCL)[12], and lipase from *Pseudomonas aeruginosa* (PAL)[13] also show high enantioselectivity. Figure 2 omits several efficiently-resolved amines because the authors did not establish their absolute configurations[12]. Smidt *et al.*[3] proposed extending the secondary alcohol rule to primary amines for CAL-B and indeed all of the amines in Figure 2 fit this rule. Thus, as with secondary alcohols, the rule in Figure 1a reliably predicts which enantiomer of primary amines reacts faster in lipase-catalyzed reactions.

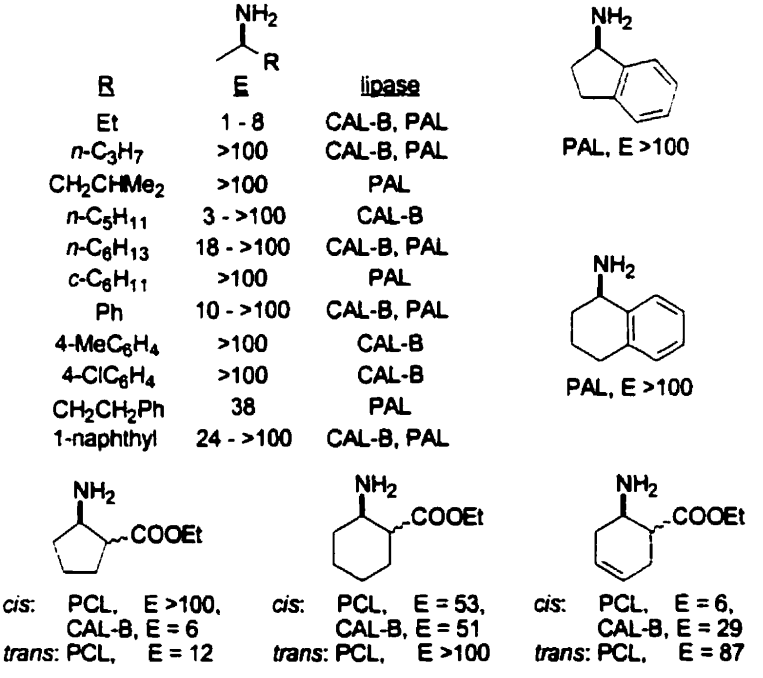

Figure 2. Enantiopreference of lipases toward primary amines of the type NH_2CHRR'. Lipases favored acylation of the enantiomer shown or hydrolysis of the corresponding amide. CAL-B: acylation or hydrolysis using lipase B from *Candida antarctica*; PCL: acylation using lipase from *Pseudomonas cepacia* with either trifluoroethyl acetate or trifluoroethyl chloroacetate; PAL: acylation using lipase from *Pseudomonas aeruginosa*. All twenty two examples fit the rule in Figure 1a. For references, see text.

Enantiopreference of subtilisin toward secondary alcohols and primary amines. Figure 3 summarizes the stereoselectivity of subtilisin toward alcohols and amines. For the thirteen secondary alcohols [6,14,15,16,17-20], eleven follow the rule in Figure 1b, two do not (3-quinuclidol[18] and one of the two reactive hydroxyls in the inositol derivative[19]), giving an overall accuracy of 85%. A possible rationalization for the 3-quinuclidol exception is that solvation of the nitrogen increases the effective size of that substituent. Both substituents in the 1,4-diacetoxy-2-cyclohexene[20] are similar in size so this substrate was excluded from the tally.

For primary amines, all thirteen examples[21,22,23] fit the rule in Figure 1b. To resolve these amines researchers used subtilisin to catalyze the acylation with trifluoroethyl butyrate or the alkoxycarbonylation with diallyl carbonate. Thus, simple rules based on the size of the substituents predict the enantiopreference of subtilisin toward secondary alcohols and primary amines. However, the favored enantiomer is opposite of the one favored by lipases.

To further emphasize the opposite enantiopreference of lipases and subtilisins, Figure 4 compares four enantioselective reactions where researchers tested both subtilisin and lipases. In all four cases, lipases and subtilisin showed an opposite enantioselectivity. In the cyclohexanols the opposite selectivity refers to similar molecules[22,24], while in the 1,3-oxathiolane and inositol derivatives it refers to enantiomers. One of the subtilisin-catalyzed acylations in the inositol is an exception to the rule[18]. For *meso*-1,4-diacetoxy-2-cyclohexene, subtilisin and most lipases catalyzed hydrolysis of opposite acetates, although the enantioselectivity is low and the substituents have similar sizes[19]. Figure 4 omits two examples. First, subtilisin and CRL showed an opposite enantioselectivity toward (±)-α-methylbenzylamine in the reaction with (±)-ethyl 2-chloropropionate[25]. The sense of enantiopreference was as predicted in Figure 1, but the additional stereocenter

in the chloropropionate complicates the interpretation. Second, subtilisin and lipases CRL, PPL, and CE showed high, but opposite, enantioselectivity in the hydrolysis of chloral acetyl methyl acetal - $Cl_3CC(OAc)OMe$, but the absolute configuration was not established[26].

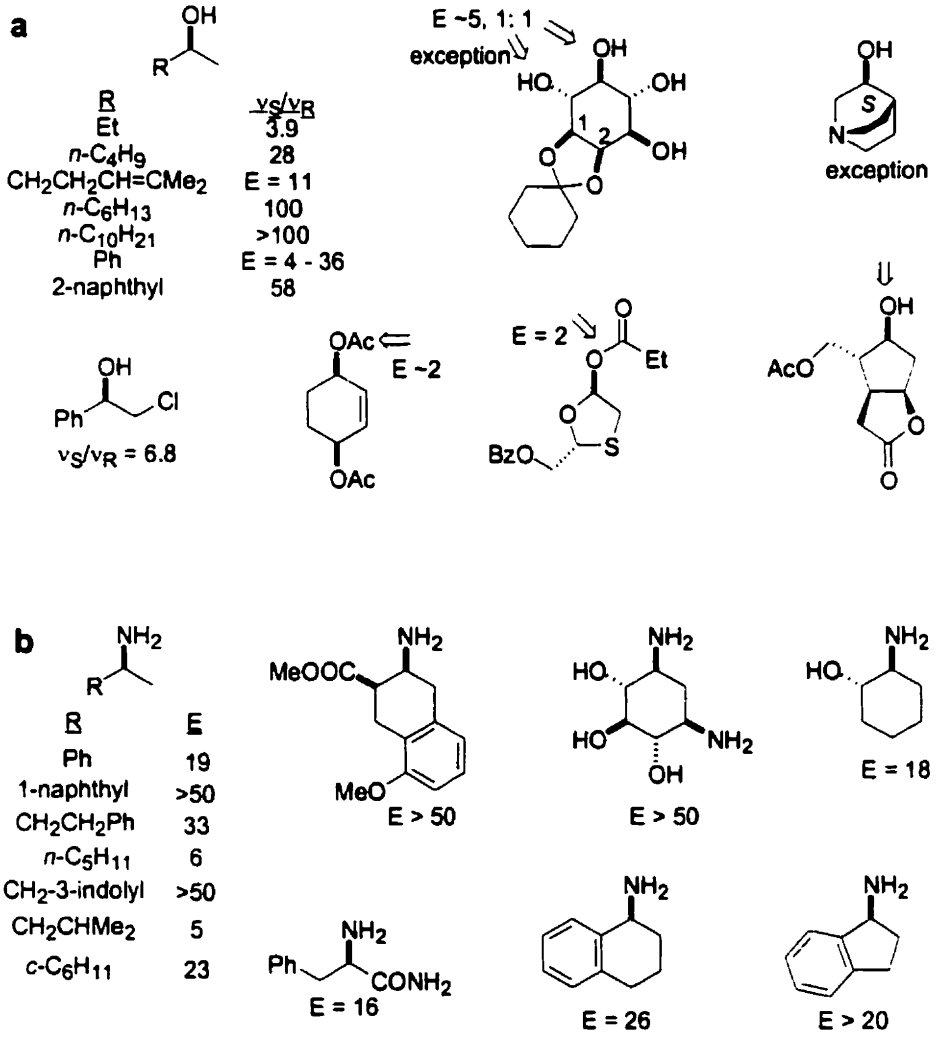

Figure 3. Enantiopreference of subtilisins (Carlsberg or BPN') toward secondary alcohols and isosteric primary amines. (**a**) Fast-reacting enantiomer in the acylation of the alcohol or in the hydrolysis of the corresponding ester. Some researchers estimated the enantioselectivity by measuring the initial rate of reaction of the two enantiomers separately. In these cases, the relative rates, v_S/v_R, are given. For examples without an E value, there was insufficient information to calculate it. Eleven of the thirteen alcohols fit the rule for an overall accuracy of 85%. The two exceptions to the rule are marked 'exception'. (**b**) Fast-reacting enantiomer of primary amines of the type NH_2CHRR' in acylation with trifluoroethyl butyrate or alkoxycarbonylation with diallyl carbonate. All thirteen examples fit the rule in Figure 1b.

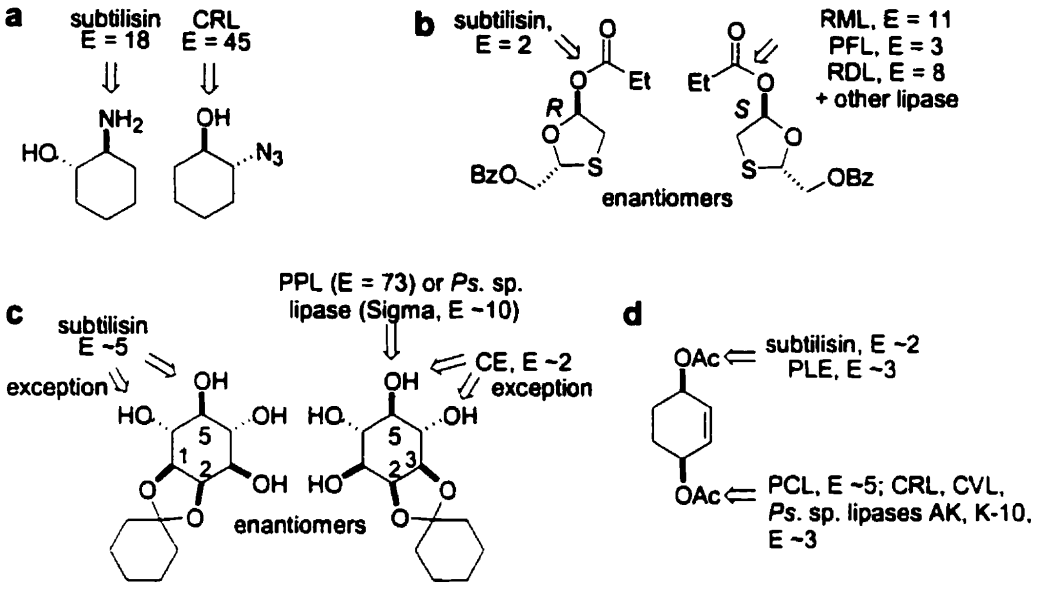

Figure 4. Four examples of opposite enantioselectivity of lipases and subtilisin in the same or similar molecules. (**a**) Subtilisin catalyzed the allyloxycarbonylation of the amino group at the (*S*)-stereocenter, while in a similar molecule, CRL catalyzed the enantioselective hydrolysis of the butyrate ester of the (*R*)-alcohol. (**b**) Subtilisin catalyzed the hydrolysis of the 2*R* propionate, while lipases catalyzed hydrolysis of the 2*S* propionate. (**c**) Subtilisin catalyzed the acetylation of the 5-OH in one enantiomer of the protected *myo*-inositol, while PPL, lipase from *Pseudomonas* sp. (Sigma), and cholesterol esterase (CE) catalyzed the acetylation of the 5-OH in the other enantiomer. Subtilisin and CE also catalyzed acetylation of the 6-OH. (**d**) Subtilisin and PLE favored hydrolysis of the acetoxy group at the (*R*)-stereocenter, while five lipases favored hydrolysis of the acetoxy group at the (*S*)-stereocenter. The rules in Figure 1 predict the reaction in **a**, **b** and the 5-OH in **c**. The substituents in **d** are too similar in size to make predictions. The acetylation of the 6-OH in **c** is an exception to the rules. Abbreviations: lipase from *Candida rugosa*, CRL; lipase from *Pseudomonas cepacia*, PCL; lipase from *Chromobacterium viscosum*, CVL; lipase from two different *Pseudomonas* species, lipase AK and lipase K-10; pig liver esterase, PLE; pig pancreatic lipase, PPL.

This opposite stereoselectivity also extends to the regioselectivity of lipases and subtilisin. Subtilisin and lipases showed opposite regioselectivity toward the secondary alcohol positions in castanospermine, Figure 5a[27], anhydro-sugar derivative, Figure 5b[28], steroids, Figure 5c[29], and quinic acid derivatives, Figure 5d[30].

Note that the stereoselectivity of subtilisin toward alcohols and amines is often lower than that of lipases. For subtilisin, like other proteases, the binding of

the acyl chain (the S_1 binding site[31]) dominates the structural selectivity, while the alcohol binding site is shallow compared to the alcohol binding site in lipases. For synthetic applications, subtilisin usually shows higher enantioselectivity toward chiral acids than toward chiral amines and alcohols.

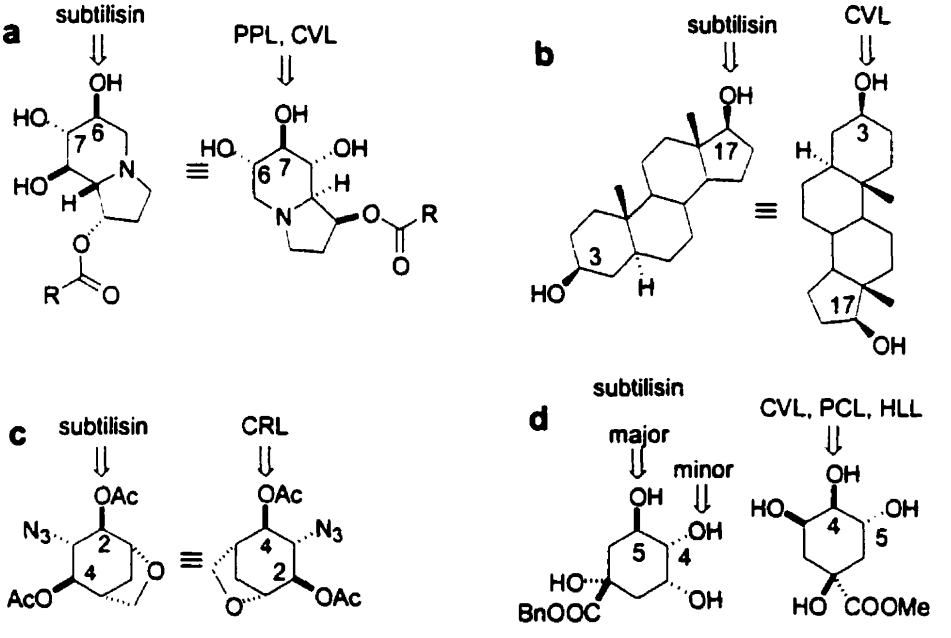

Figure 5. Four examples of opposite regioselectivity of lipases and subtilisin (**a**) Subtilisin favored acylation of the 6-position of 1-*O*-acylcastanospermine by as much as >20:1, while lipases porcine pancreatic lipase (PPL) and lipase from *Chromobacterium viscosum* (CVL) favored acylation of the 7-position by as much as 10:1. (**b**) Subtilisin catalyzed acylation of only the 17-OH in 5α-androstane-3β,17β-diol, while CVL catalyzed the acylation of only the 3-OH. (**c**) Subtilisin catalyzed hydrolysis of the acetate at the 2-position, while lipase from *Candida rugosa* (CRL) catalyzed hydrolysis at the 4-position. (**d**) Lipase from *Pseudomonas cepacia* (PCL), lipase from *Humicola lanuginosa* (HLL), and CVL catalyzed acylation of only the 4-OH of benzyl quinate with trifluoroethyl butanoate, while subtilisin catalyzed the acylation methyl quinate at both 5-OH and 4-OH (1.8:1).

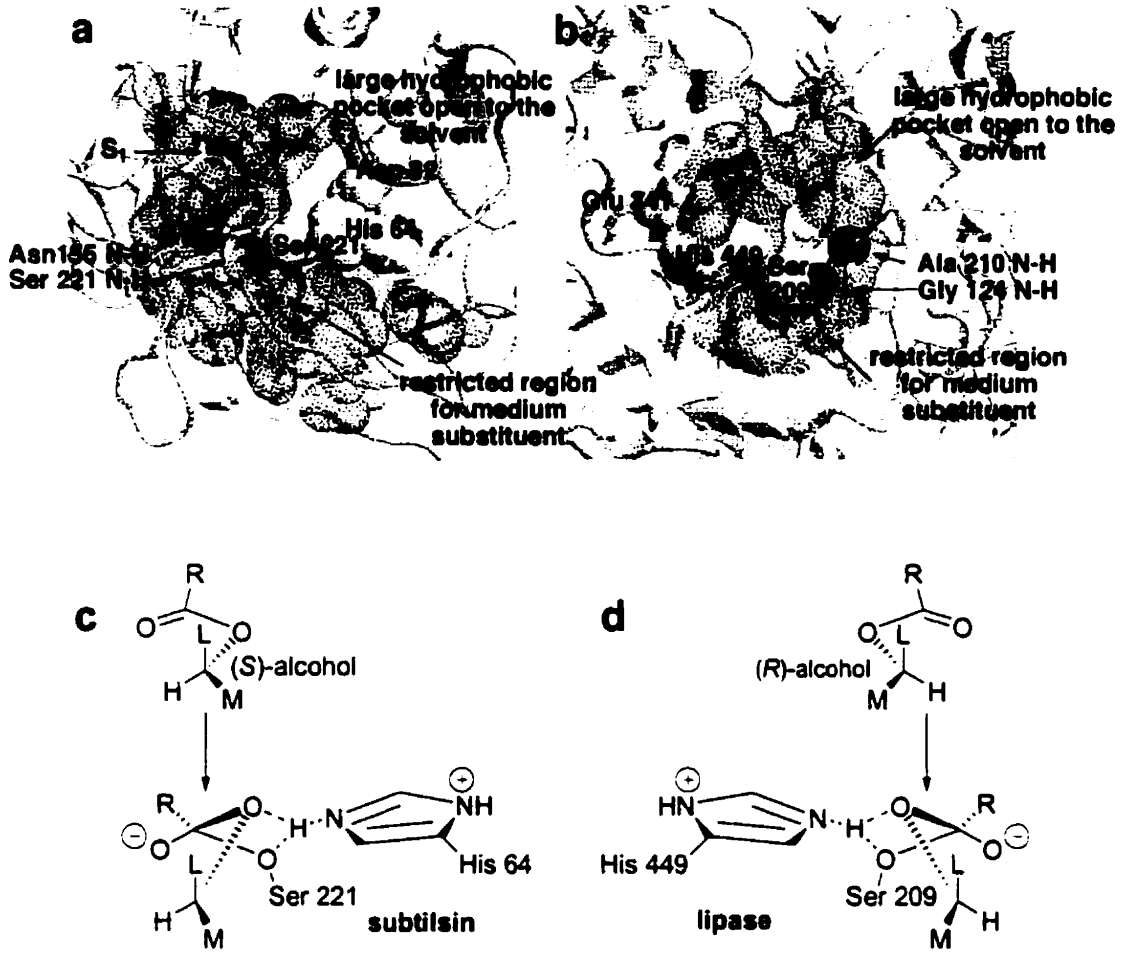

Figure 6. Structures of subtilisin Calsberg and lipase from *Candida rugosa*. (**a**) X-Ray crystal structure of subtilisin showing the catalytic machinery (Ser 221, His 64, Asp 32, and the N-H's of Asn 155 and Ser 221) and a portion of the proposed substrate binding site. The acyl chain binds in the region marked S1. The alcohol binding site has not been identified by X-ray crystallography, but the most likely region for the alcohol binding is suggested above. (**b**) X-Ray crystal structure of the open form of lipase from *Candida rugosa* showing the catalytic machinery (Ser 209, His 449, Glu 341, and the N-H's of Ala 210 and Gly 123) and the proposed alcohol binding site. The two regions of the alcohol binding site were identified by X-ray crystallography of menthol derivatives bound in the active site [4]. (**c**) and (**d**) Proposed structures of the tetrahedral intermediates in the hydrolysis of the favored secondary alcohol esters. The orientation is similar to that of the crystal structures above. Diagrams in a and b were drawn using Rasmac v2.6 [32] using entries 1sbc and 1crl from the Brookhaven protein data bank[33].

Opposite chirality of the catalytic machinery in lipases and subtilisin. X-ray structures of lipases show a serine protease-like catalytic machinery consisting of a Ser-His-Asp triad and an oxyanion hole[5]. However, the chirality of the catalytic machinery is opposite in serine proteases and lipases. For example, Figure 6 compares the structures of subtilisin Carlsberg[34] and lipase from *Candida rugosa*[35]. Because of this difference, lipases and subtilisin attack the opposite faces of the carbonyl and form enantiomeric tetrahedral intermediates.[36] Consistent with this notion, Bjorkling *et al.*[37] found that opposite enantiomers of ethyl *p*-nitrophenyl hexylphosphonate, which has the stereocenter at the phosphorus, inhibited lipases and chymotrypsin (The catalytic machinery of chymotrypsin and subtilisin are superimposable.) However, the opposite face of attack can not explain why the hydrolases have an opposite enantiopreference toward stereocenters farther from the reaction center, such as the stereocenters in secondary alcohols and isosteric primary amines.

Discussion

One criticism of enzymes as enantio- and regioselective catalysts is that only one enantiomer of the enzyme is available. The obvious, but impractical, solution is to create an enzyme from D-amino acids. However, this paper shows that for lipase-catalyzed reactions of secondary alcohols and primary amines, subtilisin is a readily-available catalyst with opposite enantio- and regioselectivity. This complementary behavior may simplify the use of these catalysts for synthesis and make it more rational. The experimental results cited in this paper are for subtilisin BPN' and subtilisin Carlsberg, but other subtilisin-like serine proteases (subtilases) have similar structures[38] and should show a similar enantiopreference.

One disadvantage of subtilisin is that its enantioselectivity is often lower than that of lipases. It may be possible, either by protein engineering or directed evolution to increase the enantioselectivity of subtilisin.

Derewenda and Wei's proposal for the molecular basis of enantiopreference considered only which face of the carbonyl was attacked[39], that is, only the absolute configuration of the catalytic machinery. They stated that 'the reactivity of specific esters of secondary alcohols should be easily predicted from the relative solvent accessibilities of the *Re* and *Si* faces of the respective enantiomers'. However, neither they nor others showed that the two faces differ in their solvent accessibility. In addition, their proposal does not explain why lipases differ in the degree of enantioselectivity toward the same substrate. Neither the face of attack nor the relative solvent accessibility changes in these cases.

In contrast, our proposal for the molecular basis of the enantiopreference of lipases and subtilisin focuses on the protein fold. This fold both sets the absolute configuration of the catalytic machinery and creates a restricted pocket for one substituent in the substrate. Both the α/β-hydrolase fold for lipases and the

subtilase fold for subtilisin create such a pocket, but the opposite absolute configuration of the catalytic machinery places the catalytic His on opposite sides of this pocket. For this reason serine proteases and lipases require opposite chirality in the alcohol for efficient catalysis. Differences in the detailed shape of this pocket explain the different enantioselectivity of different lipases toward the same substrate.

The two proposals differ in their extrapolation to other serine hydrolases. Derewenda and Wei's proposal predicts that all serine proteases will have the same enantiopreference because the absolute configuration of their catalytic machinery is the same. On the other hand, our proposal cannot extrapolate to other serine hydrolases because they have different protein folds. Other protein folds may create a different pocket or none at all. For example, trypsin-like serine proteases, such as chymotrypsin, may have the same, an opposite, or no enantiopreference. Currently, there not enough information about the enantioselectivity of chymotrypsin or other serine hydrolases toward secondary alcohols or isosteric primary amines to test these predictions.

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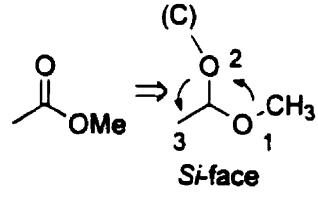
36. Lipases attack the *Re* face of an ester, while subtilisin attacks the *Si* face of an ester. According to Hanson's nomenclature, the face with the clockwise ranking of the three substituents is the *Re*-face; the counterclockwise ranking gives the *Si*-face. For example, the *Si*-face of methyl acetate below is turned toward the reader. To rank the substituents, the carbon-oxygen double bond is replaced by a single bond and a phantom carbon atom '(C)' is added to the oxygen. The ester oxygen ranks higher than the carbonyl oxygen because the

ester oxygen is attached to a real carbon atom. Unfortunately, researchers have sometimes named the faces of esters incorrectly. Note that replacing the OMe with NHMe gives the opposite designation for the face.



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Chapter 5

In the previous chapters, we explored the enantiopreferences of lipases and subtilisin with the goal of making them more approachable as reagents in the domain of synthetic organic chemistry. The work described in this chapter gives an example of the value of hydrolases in the preparation of chiral molecules.

The goal of this project was to prepare both enantiomers of a useful C_2-symmetric synthon bicyclo[2.2.1]heptan-2,5-dione in optically pure form and in large quantities. To our knowledge, only the racemic dione had previously been prepared.

There were three enzymatic approaches for making the enantiomerically pure dione: 1) resolution of the dione; 2) resolution of a dione derivative such as the diol or ketoalcohol; and, 3) resolution or asymmetric synthesis of a dione precursor. The first method had not been tested and the second method had given poor results[1]. We chose the third approach.

Esters of the dione precursor, *endo*-2-norbornenol, had been resolved by CRL, but not with sufficiently high enantioselectivity (E = 15)[2]. However, the ester of its derivative, 5,6-epoxy-*endo*-norbornan-2-ol, was known to be resolved with high enantioselectivity (E ~ 100) by hydrolysis catalyzed by lipase from *Candida rugosa*.[2,3] The preferred product in both cases is that predicted by the secondary alcohol rule proposed in chapter 2. In addition, the increase in enantioselectivity can be accounted for by the rule for secondary alcohols; the difference in size of the substituents is increased when the epoxide is formed from the olefin.

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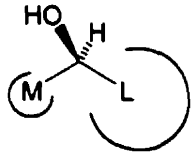
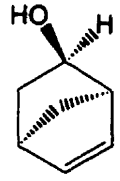
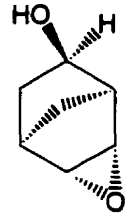


preferred enantiomer of lipases



olefin E=15



epoxide E~100

Based on this, the synthetic scheme proposed involved the resolution the epoxy norbornanyl ester, followed by a regioselective opening of the epoxide and subsequent oxidation to give the dione.

The following research was primarily surpervised by Prof. R. Azerad and the majority of the experiments were carried out in the Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, URA 400 at the Université René Descarte in Paris, France.

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Chemoenzymatic Access to Enantiomeric Bicyclo[2.2.1]Heptan-2,5-Diones.

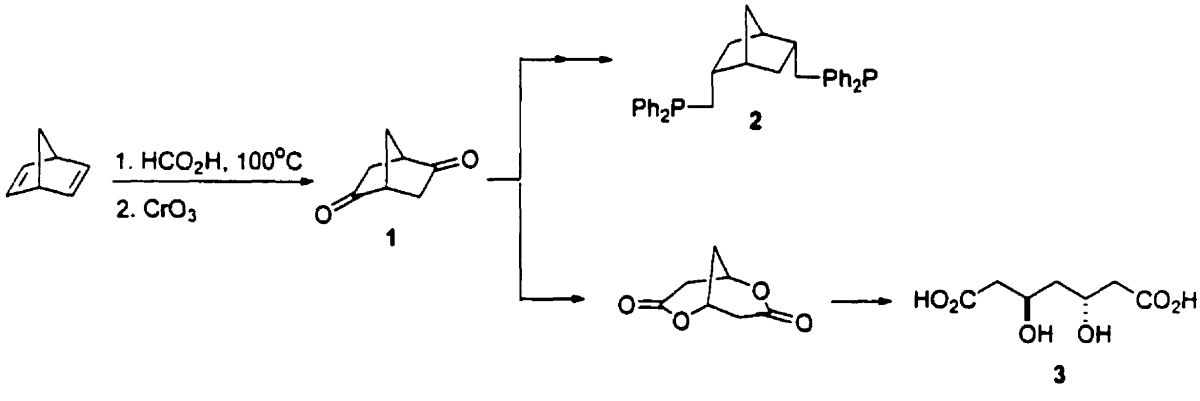
Alexandra Weissfloch and Robert Azerad*

Abstract: A practical integrated process, combining an enzymatic resolution step with a few chemical transformations, is described for the synthesis of (1*R*,4*R*)- and (1*S*,4*S*)-bicyclo[2.2.1]heptan-2,5-diones **1** of high enantiomeric purity, starting from a standard mixture of (±)-*endo*- and *exo*-2-acetoxy-5-norbornene.

Introduction

(±)-Bicyclo[2.2.1]heptan-2,5-dione **1**, which is readily obtained by the addition of formic acid to norbornadiene, followed by Jones oxidation of the resulting diformate esters,[1] has been used as a rigid template for the elaboration of a diphosphine ligand **2**.[2] Dione **1** is also the starting material for the preparation of *anti*-3,5-dihydroxy-heptan-1,7-dicarboxylic acid **3**,[3] a C_2-symmetric synthon possessing two chiral centers, which has been used in the preparation of the lactone rings of avermectins or milbemycins (Scheme I). In view of this, it would be of great use to have a simple preparative access to the pure dione enantiomers which, to our present knowledge, does not yet exist. A recent paper[4] does, however, describe an asymmetric bis-hydrosilylation of norbornadiene which leads to one of the corresponding *exo,exo*-diol diacetate enantiomers, with high optical purity.

Scheme I



We herein report the synthesis of diones of high enantiomeric purity, starting from a commercial mixture of norbornenol acetates, which involves very few steps, the key one being an enzymatic resolution method.

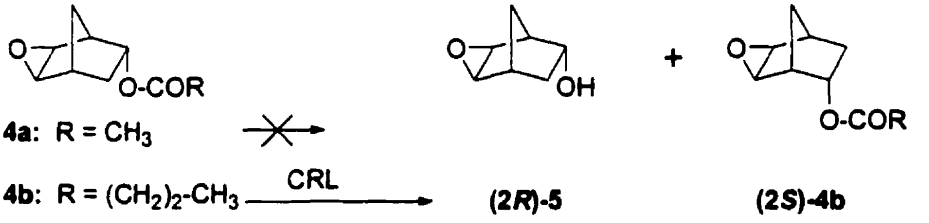
Results and Discussion

Our first attempts, which involved the direct enzymatic resolution of mono- and diesters derived from the racemic diol mixture obtained by the formylation of norbomadiene,[1] were unsuccessful, in agreement with known structural models elaborated for the hydrolysis of such bicyclic esters catalyzed by lipase from *Candida rugosa (= Candida cylindracea)*[5] and recent results obtained with other enzymes.[6-8] An enzymatic hydrolysis of *endo*-2-norbornenyl esters,[9,10] having an enantioselectivity coefficient (E)[11] of about 15, was not enantioselective enough to be of preparative use. Moreover, the recovery of the exceedingly volatile products[10] was difficult and the subsequent formylation of the norbornenyl ester was, to our surprise, unsuccessful.

For these reasons, we turned to another strategy for the introduction of the second oxygen atom using previously described reactions, the *exo*-epoxidation of an *endo*-2-hydroxy-5-norbornene derivative followed by a regioselective reductive opening of the epoxide ring to give an *endo,exo*-2,5-norbornanediol.[12] We were also aware that the enzymatic resolution of a 5,6-epoxy-*endo*-norbornan-2-yl ester,[10] carried out on an analytical scale, was highly effective (E~100) and thus appeared particularly adapted to our purpose.

Analytical Enzymatic Hydrolyses and Transesterifications. Epoxidation of the commercial 2-acetoxy-5-norbornene (a mixture of racemic *endo*- and *exo*-isomers, approximately 8:2) employing magnesium monoperoxyphthalate hexahydrate[13] in ethanol-water afforded, in high yield, the crude epoxide which, upon crystallization, yielded the pure *endo*-epoxyacetate isomer **4a** (about 50% minimal yield). As previously described, this ester was recovered unchanged, when submitted to hydrolysis with lipase from *Candida rugosa,* even for prolonged

incubation times. Conversely, the corresponding butyric ester **4b**, prepared by mild alkaline hydrolysis of **4a** followed by esterification with butyric anhydride, was a good substrate for the same enzyme and was easily resolved (E ~ 92) on a 400 mg-scale, affording, (2*R*)-epoxyalcohol **5** and (2*S*)-epoxyester **4b** of high optical purity (Scheme II). Absolute configurations were attributed from the known stereoselectivity of lipase from *Candida rugosa* in this series[5] and confirmed by comparison of the optical rotation of the resulting epoxyalcohol with the epoxidation product of the previously described, corresponding (1*R*,2*R*,4*R*)-*endo*-norbornenol.[9,10]

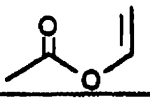
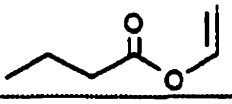
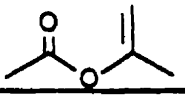
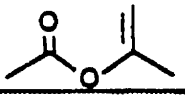
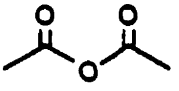
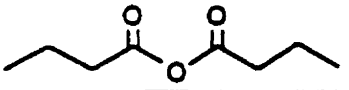


Scheme II

However, the need for a preliminary exchange of the ester group could be eliminated by working with the same lipase in a transesterification reaction, using the racemic alcohol **5** as a substrate in an anhydrous organic solvent.[14] A preliminary screening for a convenient acyl group donor in various organic solvents was effected, the principal results of which are given in Table 1. The most striking outcome is that, unexpectedly, acetyl donors (entries 1, 3, 4 and 5) are effective donating reagents, although they are systematically less effective than butyryl donors. Moreover, the enantioselectivities measured using vinyl or isopropenyl acetate were higher or comparable to those measured using vinyl butyrate (entry 2). In contrast, other donors such as anhydrides (entries 5 and 6) or esters (entry 7), result in lower enantioselectivities. In the case of isopropenyl

acetate, the replacement of toluene by chloroform (entry 4) produced a dramatic effect on the rate and enantioselectivity of the esterification reaction.

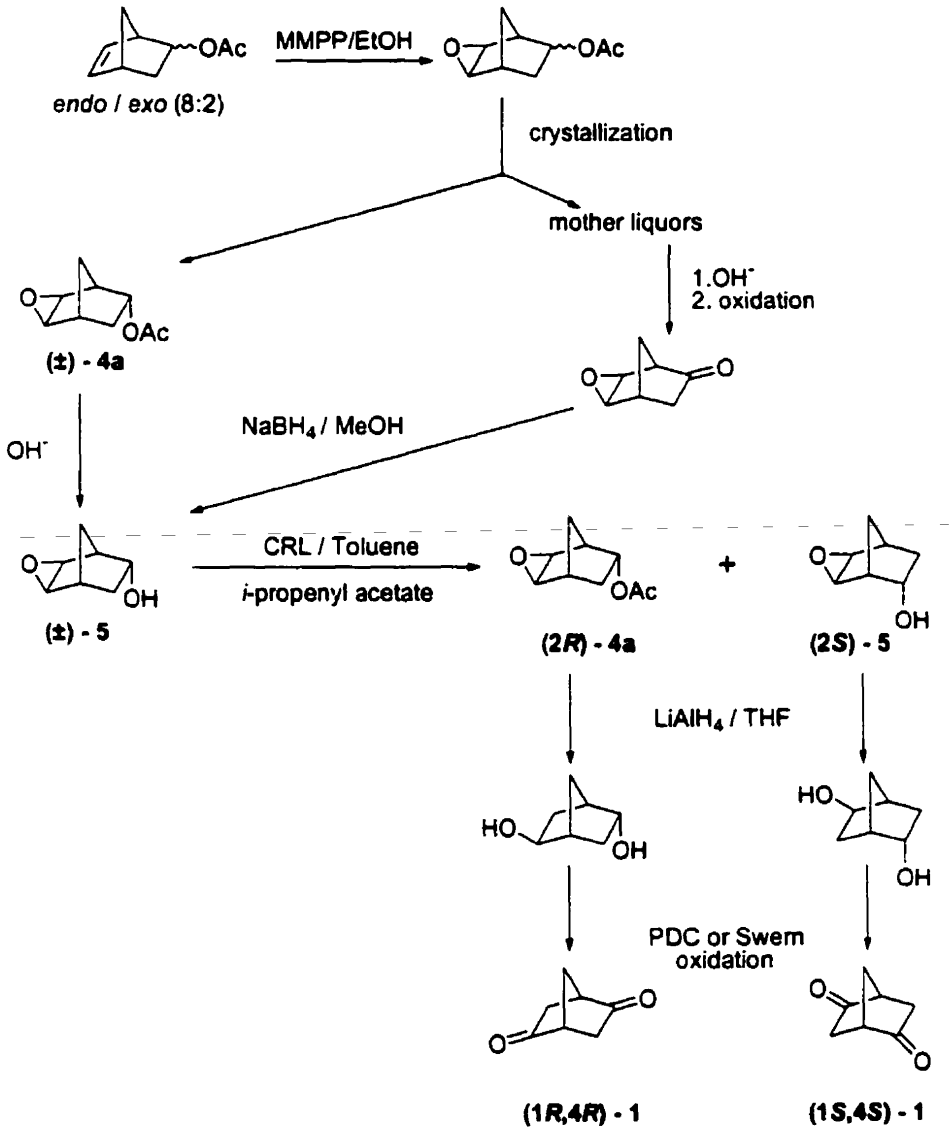
Table 1. Enantioselective esterification[a] of (±)-2-*endo*-hydroxy-5,6-norbornane by lipase from *C. rugosa* in the presence of various acyl donors and solvents.

	Acylating agent (mol/ mol of substrate)		Solvent	Time (hours)	% conversion	E values[b]
1		(2)	Toluene	66	30	50-90
2		(3)	Toluene	3	46	75
3		(4)	Toluene	66	47	200-500
4		(4)	$CHCl_3$	216	14	12
5		(1)	Toluene	120	15	1.3
6		(1)	Toluene	1.5	40	3.6
7	Tributyrin	(used as solvent)		66	37	12

[a]To the substrate (50 mg) in anhydrous solvent (5 mL) were added 4 Å molecular sieves (50 mg), acyl donor, and lipase from *C. rugosa* (10-20 mg). The suspensions were incubated with shaking at 30°C.

[b]calculated from %conversion, determined by GC of the reaction mixture (Dbwax column, 160°C) or from enantiomeric excesses of substrate and product.[11] *e.e*'s were determined either by GC of acetate or trifluoroacetate esters on a Chiraldex G-TA 30 capillary column (110°C) or by HPLC of benzoate esters on a Chiralpak AD column (see Experimental Section).

Preparative Aspects. Under the best conditions (entry 3), starting from 3 g of (±)-epoxy alcohol **5**, it was possible to obtain, in a two-stage operation,[9] 1.6 g (40%) of (2*R*)-epoxyacetate **4a** (96% e.e.) and 1.2 g (40%) of (2*S*)-epoxyalcohol **5** (>98% e.e.). Each product was then reduced with lithium aluminum hydride in terahydrofuran[12] (65-75% yield) and the crystallized[15] *endo*,*exo*-2,5-norbornane-diols were submitted to pyridinium dichromate or Swern oxidation,[16] affording the enantiomeric (1*S*,4*S*)- and (1*R*,4*R*)-bicyclo[2.2.1]heptan-2,5-diones (about 70% yield) in high optical purity (≥ 96% e.e.).



Scheme III

An integrated process, which includes the recycling of unused (±)-*exo*- and *endo*-2-acetoxy-5,6-epoxynorbornanes present in the mother liquors of the epoxyacetate recrystallization, has been designed: mild alkaline hydrolysis, followed by oxidation to epoxynorbornanone and reduction with sodium borohydride in methanol[10] will afford exclusively (≥ 95%) the *endo*-epoxynorbornanol **5**, which could again be used in the enzymatic transesterification procedure. The entire synthetic process, described in Scheme III, is currently being conducted on a multigram scale in our laboratory, and will be reported in due course.

Experimental Section

General. Melting points are uncorrected. ^{1}H and ^{13}C-NMR spectra were recorded on a WM250 Bruker spectrometer at 250 and 62.9 MHz respectively. The residual protons in $CDCl_3$ or pyridine-d_5 were used as reference peaks, with assigned 7.25 and 8.71 ppm chemical shifts, respectively. Signal assignment was aided by 2D ^{1}H homonuclear shift correlated (COSY 45) spectra and ^{13}C distortionless enhanced polarization (DEPT 135) experiments. Optical rotations were measured in 1 dm or 0. 1 dm cells using a Perkin Elmer 241 spectropolarimeter. Gas chromatography was performed on Varian 3700 or Shimadzu G-8A instruments equipped with flame ionization detectors and Shimadzu C-R3A or C-R6A integrating recorders. OV-1701 (Flexibond™, 0.20 mm x 15 m, Pierce Chem. Co.) or Durabondwax (0.32 mm x 30 m, J&W Scientific, Inc.) capillary columns were routinely employed to monitor enzyme reactions, whereas a Chiraldex G-TA capillary column (0.25 mm x 30 m, Astec) was used to determine optical purities. Mass spectrometric analyses (MS) were carried out by electronic impact (EI) on a Hewlett Packard 5972 GC-MS instrument. High resolution mass spectra (HRMS) were supplied by Université P. et M. Curie (Paris). Flash column chromatography was carried out using Merck 60 silica gel (230-400 mesh). Merck $60F_{254}$ precoated glass plates were used for thin layer chromatography. High pressure liquid chromatography was performed using a Chromatem 380 pump, equipped with a Pye-Unicam LC-UV detector, a Shimadzu C-R3A integrating recorder, and a Chiralpack AD column (0.46 x 25 cm, Daicel Chem. Ind.). Lipase from *Candida cylindracea* (*C.rugosa*, E.C.3.1.1.3) was purchased from Sigma Chemical Co. (St Louis, USA).

Determination of enantiomeric excess. Enantiomeric excesses of 2-*endo*-acetoxy-5,6-epoxynorbornanes (Figure 1) and bicyclo[2.2.1]heptan-2,5-diones

(Figure 2) were determined directly by GC on a Chiraldex G-TA30 capillary column at 110°C. Enantiomeric excesses of 2-*endo*-hydroxy-5,6-epoxy-norbornanes were determined by GC of their acetate or trifluoroacetate esters on the same column at 110 or 90°C, respectively. In some cases, enantiomeric excesses of 2-*endo*-hydroxy-5,6-epoxynorbornanes were determined by HPLC of their benzoyl esters on a Chiralpak AD column with hexane-isopropanol (95:5) as solvent (flow rate: 0.5 mL/min, detection at 250 nm).

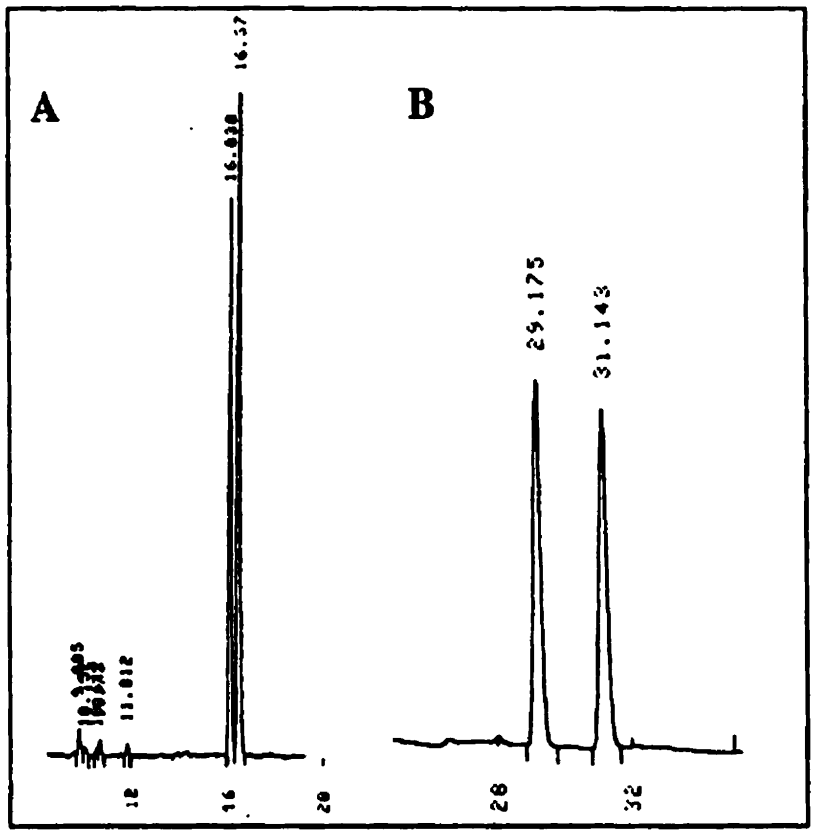


Figure 1. GC analytical separation of enantiomeric *endo*-2-hydroxy-5,6-epoxynorbornane esters on Chiraldex G-TA (see Experimental Section): **A**, trifluoroacetyl esters; **B**, acetyl esters.

Preparation of acetyl esters for GC analysis. To the alcohol (~10 mg) dissolved in ethyl acetate was added 4-dimethylaminopyridine (0.05 eq.), sodium carbonate (1.5 eq.), and acetic anhydride (1.5 eq.). The mixture was stirred for 18 hours, then

washed with water and brine. The organic phase was dried over anhydrous sodium sulfate, filtered, evaporated *in vacuo*, and analyzed without further purification.

Preparation of trifluoroacetyl esters for GC analysis. To the alcohol (1-5 mg) in dichloromethane (0.5 mL) was added trifluoroacetic anhydride (0.2 mL). After stirring for 30 min, the solvent and excess anhydride were evaporated under a stream of nitrogen and the residue was analyzed without further purification.

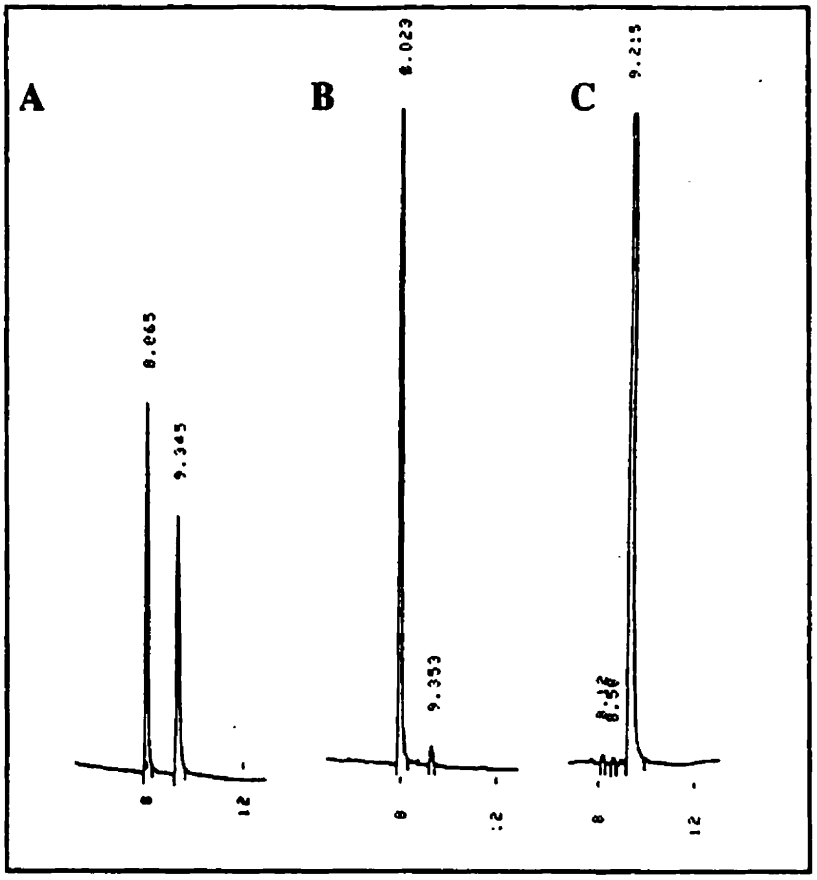


Figure 2. GC analytical separation of enantiomeric bicyclo[2.2.1]heptan-2,5-diones on Chiraldex G-TA (see Experimental Section): **A**, racemic mixture; **B**, 1*S*,4*S*; **C**, 1*R*,4*R*.

Preparation of benzoyl esters for HPLC analysis. Dicyclohexylcarbodiimide (1.1 eq.) was added to a mixture of 4-dimethylaminopyridine (0.1 eq.), benzoic acid (2 eq.) and alcohol in dichloromethane, cooled in an ice-water bath. After

stirring for 30 min, the reaction mixture was warmed to room temperature and stirred for 30 h. Dicyclohexylurea was removed by filtration and the filtrate was washed twice with 1 N HCl, saturated sodium bicarbonate, water and brine. The organic phase was dried over anhydrous sodium sulfate, filtered and evaporated *in vacuo*. The residue was analyzed without further purification.

(±)-2-*endo*-acetoxy-5,6-epoxynorbornane (4a). A solution of magnesium monoperoxyphthalate hexahydrate (375 g, 0.76 mol) in water (2.6 L) was added to (±)-2-*endo*/*exo*-acetoxy-5-norbornene (99 g, 0.650 mol) in absolute ethanol (2 L). The mixture was stirred at room temperature for 3 days. Ethanol, along with a portion of water, was evaporated *in vacuo* and the residue (1.2 L) was divided into two parts. Each portion was extracted with ether (1 L), washed with aqueous saturated sodium bicarbonate (3 x 300 mL), 20% aqueous sodium bisulfite (2 x 300 mL), water (300 mL), and brine (500 mL), and dried over sodium sulfate. The two aqueous phases obtained after the first ether extraction were combined, extracted again with ether (1 L), and washed as above. Evaporation of the solvent from the combined ethereal phases yielded a mixture of the *exo* and *endo* isomers as a slightly yellow oil (80.9 g, 70%). Three crystallizations from ether-hexane yielded the *endo* isomer (54.3 g, 50%, >99% *endo*-isomer by GC). R_f 0.25 (cyclohexane-ethyl acetate, 8:2). Mp 53.5-54°C (lit.[12]: 53-54°C). HRMS for $C_9H_{12}O_3$, calc. 168.078642, found 168.078657. MS (EI): 168(1), 150(1) $[M\text{-}H_2O]^+$, 140(3), 138(3), 126(9) $[M\text{-}CH_2CO]^+$, 108(10), 97(11), 82(81), 43(100).

^{1}H NMR ($CDCl_3$, 250 MHz), δ ppm, *J* Hz: 5.04 (1H, ddd, $J_{2\text{-}3exo}$ = 8.8, $J_{1\text{-}2}$= 4.4, $J_{2\text{-}3endo}$= 3, H-2), 3.33 (1H, br.d, $J_{5\text{-}6}$= 3.6, H-6), 3.23 (1H, br.d, $J_{5\text{-}6}$= 3.6, H-5), 2.75 (1H, dm, $J_{1\text{-}2}$= 4.4, H-1), 2.49 (1H, dm, $J_{4\text{-}3exo}$= 4.4, H-4), 2.07 (1H, ddd, $J_{3exo\text{-}3endo}$= 13.5, $J_{2\text{-}3exo}$= 8.8, $J_{3exo\text{-}4}$= 4.4, H-3 *exo*), 2.02 (3H, s, CH_3CO), 1.34 (1H, dm, $J_{7\text{-}7'}$=

10.2, H-7), 1.07 (1H, dm, $J_{3exo\text{-}3endo}$= 13.5, $J_{2\text{-}3endo}$= 3, H-3 *endo*), 0.78 (1H, dm, $J_{7\text{-}7'}$= 10.2, H-7').

^{13}C NMR ($CDCl_3$, 62.9 MHz), δ ppm: 170.28 (CO), 75.97 (CH, C-2), 50.36, 47.66 (CH, C-5 and C-6), 39.99, 36.44 (CH, C-1 and C-4), 32.56, 24.26 (CH_2, C-3 and C-7), 20.45 (CH_3).

The mother liquors were concentrated *in vacuo,* yielding a yellow oil (25.7 g) containing 35% *endo*-isomer.

(±)-2-*endo*-butyroxy-5,6-epoxynorbornane (4b). To a solution of (±)-2-endo-butyroxy-5-norbornene (900 mg, 5 mmol) in ethanol (15 mL) was added magnesium monoperoxyphthalate hexahydrate (3 g, 6.06 mmol) dissolved in water (20 mL). The mixture was stirred at room temperature for 48 hours. The solvents were evaporated *in vacuo* and the residue dissolved in ether (100 mL). The ethereal solution was washed with aqueous saturated sodium bicarbonate (2 x 50 mL), 20% aqueous sodium bisulfite (6 x 50 mL), saturated sodium bicarbonate (2 x 50 mL), water, and brine, dried over sodium sulfate, and evaporated. The crude product was purified by flash chromatography (hexane-ethyl acetate, 95:5 to 9: 1), yielding the epoxyester as a colorless oil (819 mg, 83%). R_f 0.38 (cyclohexane-ethyl acetate, 8:2). MS (EI): 168(2) $[M\text{-}CO]^+$, 140(5) $[M\text{-}CH_2CH_2CO]^+$, 125(4) $[M\text{-}CH_3CH_2CH_2CO]^+$, 107(5), 97(8), 81(81), 71(100).

^{1}H NMR ($CDCl_3$, 250 MHz), δ ppm, *J* Hz: 5.01 (1H, ddd, $J_{2\text{-}3exo}$= 9, $J_{1\text{-}2}$= 4, $J_{2\text{-}3endo}$= 3, H-2), 3.32 (1H, br.d, $J_{5\text{-}6}$= 3.6, H-6), 3.23 (1H, br.d, $J_{5\text{-}6}$= 3.6, H-5), 2.75 (1H, dm, $J_{1\text{-}2}$= 4, H-1), 2.49 (1H, dm, $J_{4\text{-}3exo}$= 4, H-4), 2.25 (2H, t, *J* = 7.3, CH_2CO), 2.07 (1H, ddd, $J_{3exo\text{-}3endo}$= 13.2, $J_{2\text{-}3exo}$= 9, $J_{3exo\text{-}4}$= 4, H-3 *exo*), 1.62 (2H, sextet, *J* = 7.3, CH_2CH_3), 1.33 (1H, dm, $J_{7\text{-}7'}$= 10.2, H-7), 1.06 (1H, dm, $J_{3exo\text{-}3endo}$= 13-2, $J_{2\text{-}3endo}$= 3, H-3 *endo*), 0.93 (3H, t, *J* = 7.3, CH_3CH_2), 0.79 (1H, br.d, $J_{7\text{-}7'}$= 10.2, H-7').

Enzymatic hydrolysis and resolution of (±)-2-*endo*-butyroxy-5,6-epoxynorbornane (4b). To (±)-2-*endo*-butyroxy-5,6-epoxynorbornane **4b** (388 mg, 1.98 mmol) dissolved in 0.1 M, pH 7 sodium phosphate buffer-acetone (9:1, 150 mL), was added lipase from *C. rugosa* (39 mg). The mixture was orbitally shaken at 27°C for 3 h (39% conversion). The reaction mixture was saturated with sodium chloride and ethyl acetate (100 mL) was added. After stirring vigorously for 5 min, the phases were separated and the aqueous layer was extracted again with ethyl acetate (6 x 100 mL). The combined organic extracts were washed with aqueous saturated sodium bicarbonate and brine, dried over anhydrous sodium sulfate and evaporated *in vacuo*. Purification of the crude product by flash chromatography (hexane-ethyl acetate, 6:4 to 1:1) yielded pure alcohol (2R)-**5** (83 mg, 33%), $[\alpha]_D^{21}$ + 42.4° (c 1.2, $CHCl_3$) (lit.[10]: + 46.5°), 96% e.e., and ester (2S)-**4b** (232 mg, 60%). The isolated butyrate was resubmitted to hydrolysis under the same conditions. After 6 h (57% total conversion), the reaction mixture was worked up as before. Purification of the crude product yielded alcohol (2R)-**5** (16.2 mg, 6%), and butyrate (2S)-**4b** (179 mg, 46%), $[\alpha]_D^{21}$ -13.8° (c 3.9, $CHCl_3$) (lit.[10]: - 13.4°), >99% e.e.

(±)-2-*endo*-hydroxy-5,6-epoxynorbornane (5). To a solution of (±)-*endo*-2-acetoxy-5,6-epoxynorbornane (48.7 g, 0.29 mol) in ethanol (400 mL), cooled in a cold water bath, was slowly added 2 N sodium hydroxide (175 mL, 0.348 mol). The mixture was stirred for 85 min, after which time the reaction was quenched with glacial acetic acid (3.3 mL, 0.06 mol). Ethanol and water were removed by rotatory evaporation and the residue was extracted with ethyl acetate (1.4 L). The organic phase was washed with 0.5 N HCl (100 mL), saturated aqueous sodium bicarbonate (200 mL), and brine (2 x 200 mL), dried over anhydrous sodium sulfate, and evaporated *in vacuo* to yield a pale yellow solid (35.1 g, 96%). The

crude product was crystallized from ethyl acetate-hexane to give the pure epoxyalcohol as white crystals (25.3 g, 70%). The remaining product was purified by flash chromatography (pentane-ethyl acetate, 5:5 to 3:7) yielding additional pure epoxy alcohol (4.4 g, 11%). R_f 0.23 (pentane-ethyl acetate, 5:5). Mp 190-192°C, sealed tube (lit.: 160-162°C,[12] 170-172°C[10]). HRMS for $C_7H_{10}O_2$, calc. 126.068078, found 126.068107. MS (EI): 126(1), 125(1.5), 107(2.5), 95(4), 81(100).

^{1}H NMR ($CDCl_3$, 250 MHz), δ ppm, *J* Hz: 4.38 (1H, m, H-2), 3.44 (1H, br.d, $J_{5\text{-}6}$= 3.7, H-6), 3.27 (1H, br.d, $J_{5\text{-}6}$= 3.7, H-5), 2.60 (1H, m, H-1), 2.45 (1H, m, H-4), 1.99 (1H, ddd, $J_{3exo\text{-}3endo}$= 13.2, $J_{2\text{-}3exo}$= 9, $J_{3exo\text{-}4}$= 4, H-3 *exo*), 1.55 (1H, br.s, OH), 1.27 (1H, dm, $J_{7\text{-}7'}$= 10.2, H-7), 1.00 (1H, dt, $J_{3exo\text{-}3endo}$= 13.2, $J_{2\text{-}3endo}$= 3, H-3 *endo*), 0.74 (1H, br.d, $J_{7\text{-}7'}$= 10.2, H-7').

^{13}C NMR ($CDCl_3$, 62.9 MHz), δ ppm: 74.13 (CH, C-2), 51.47, 48.98 (CH, C-5 and C-6), 42.36, 37.33 (CH, C-1 and C-4), 35.08, 25.15 (CH_2, C-3 and C-7).

Preparative enzymatic resolution of (±)-2-*endo*-hydroxy-5,6-epoxynorbornane (5). Isopropenyl acetate (10.3 mL, 93.5 mmol) was added to (±)-2-*endo*-5,6-epoxy-norbornane (3 g, 23.8 mmol) dissolved in toluene (340 mL). Lipase from *C. rugosa* (1 g) was added and the flask was orbitally shaken at 30°C. After 14 h an additional 700 mg of lipase was added and after another 8 h, 550 mg of lipase was added. After a total of 25 h, the reaction was stopped by filtration of the mixture through glass fiber paper. The product and remaining substrate were separated by medium pressure liquid chromatography (200 g silica gel, Merck 60H, cyclohexane-ethyl acetate 1:1, followed by cyclohexane-ethyl acetate 2:8 once the first alcohol fraction was detected). The solvent was evaporated to yield the alcohol (1.84 g, 77 % e.e.) and the acetate (1.59 g, 40%, 95.5 % e.e.), $[\alpha]_D^{21}$ + 7.4° (c 1, $CHCl_3$). The alcohol was resubmitted to esterification under the same

conditions using 4 g lipase. The reaction was stopped as before after a total of 20 h (corresponding to a 58 % total conversion). Purification by flash column chromatography (cyclohexane-ethyl acetate, 1:1) yielded the remaining alcohol substrate (1.2 g, 40%, >98% e.e.), $[\alpha]_D^{21}$ -46.7° (c 0.85, $CHCl_3$), and the acetate product (0.44 g, 60% e.e.).

Reduction of 2-*endo*-acetoxy-5,6-epoxynorbornane (4a) to 2,5-dihydroxy-norbonane. Dry tetrahydrofuran (80 mL) was added dropwise to lithium aluminum hydride (2.4 g, 56.7 mmol) under nitrogen. After complete addition, the suspension was refluxed for 1.25 h. After cooling the mixture to room temperature, the flask was placed in a cold water bath and 2-*endo*-acetoxy-5,6-epoxynorbornane(1.52 g, 9.04 mmol) in tetrahydrofuran (6 mL) was added dropwise. The dropping funnel was rinsed with tetrahydrofuran (5 mL) and the mixture was heated to a reflux for 3.75 h. The flask was cooled in an ice/water bath and water was carefully added dropwise (2.4 mL), followed by aqueous 15% w/w sodium hydroxide (2.4 mL) and finally water (7.2 mL). The mixture was stirred for 20 min and then filtered, rinsing with tetrahydrofuran and ethyl acetate. The filtrate was dried over anhydrous sodium sulfate and evaporation of the solvent yielded a white solid. Recrystallization from ether-dichloromethane yielded the pure diol as white crystals (614 mg, 53%). Medium pressure liquid chromatography (200g silica, dichloromethane-isopropanol, 9:1) of the residue obtained from evaporation of the mother liquor yielded additonal pure diol (272 mg, 23%). R_f 0.16 (dichloromethane-methanol, 9:1). Mp 180-182°C (sealed tube). $[\alpha]_D^{21}$ + 2.8° (c 2.34, MeOH), $[\alpha]_{578}$ +2.9°, $[\alpha]_{546}$ + 3.2°, $[\alpha]_{436}$ + 4.2°. HRMS for $C_7H_{12}O_2$, calc.128.083728, found 128.083713. MS (EI): 128(2) $[M]^+$, 110(19) $[M-H_2O]^+$, 95(33), 81(24), 66(100).

^{1}H NMR (pyridine-d_5, 250 MHz), δ ppm, *J* Hz: 6.04 (1H, d, *J* = 3.3, *endo*-OH on C-2), 5.99 (1H, d, *J* = 3.3, *exo*-OH on C-5), 4.38 (1H, m, $J_{2\text{-}3exo}$= 10, $J_{2\text{-}3}$=$J_{2\text{-}OH}$= 3.3, $J_{2\text{-}1}$= 1.3, H-2), 4.22 (1H, m, H-5), 2.92 (1H, ddd, $J_{6endo\text{-}6exo}$= 13, $J_{6endo\text{-}5}$= 7, *J* = 2, H-6 *endo*), 2.40 (1H, br.t, $w_{1/2}$= 10, H-1), 2.33 (1H, br.d, *J* = 5, H-4), 2.06-1.93 (2H, m, *J* = 5, H-3*exo* and H-7), 1.63 (1H, dm, $J_{6exo\text{-}6endo}$= 13, H-6 *exo*), 1.31 (1H, br.d, $J_{7\text{-}7'}$= 10, H-7'), 1.01 (1H, dt, $J_{3exo\text{-}3endo}$= 13, $J_{2\text{-}3endo}$= 3.3, H-3 *endo*).

^{13}C NMR ($CDCl_3$, 62.9 MHz), δ ppm: 73.70, 70.62 (CH, C-2 and C-5), 44.40, 41.21 (CH, C-1 and C-4), 34.31, 33.03, 32.26 (CH_2, C-3, C-6, and C-7).

Reduction of 2-*endo*-hydroxy-5,6-epoxynorbornane (5) to 2,5-dihydroxy-norbornane. The above procedure was used for the reduction of the epoxy alcohol (1.17g, 9.27 mmol) with the exception that only 4 eq. of lithium aluminum hydride were used. Recrystallization yielded the diol as white crystals (355 mg, 30%) and chromatography of the mother liquor yielded additional pure diol (411 mg, 34%). $[\alpha]_D^{21}$ - 4. 1° (c 2.25, MeOH), $[\alpha]_{578}$ - 4.2°, $[\alpha]_{546}$ - 4.5°, $[\alpha]_{436}$ - 5.6°.

Oxidation of 2,5-dihydroxynorbornane to enantiomeric bicyclo[2.2.1]heptan-2,5-diones (1).

(i) pyridinium dichromate oxidation. Diol (706 mg, 5.51 mmol) was dissolved in *N,N*-dimethylformamide (100 mL), pyridinium dichromate (7.05 g, 18.7 mmol) was added and the mixture was stirred under nitrogen for 3 h. Aqueous saturated sodium bicarbonate (100 mL) was added to the reaction mixture and it was shaken vigorously. Dichloromethane (300 mL) was added and the organic phase was washed with aqueous saturated sodium bicarbonate (4 x 100 mL), 0.5 N HCl (100 mL), saturated sodium bicarbonate (100 mL), water (2 x 200 mL), and brine (200 mL), and dried over sodium sulfate. The solvent was evaporated *in vacuo* yielding 501 mg of dione (73%, 95% pure by GC).

(ii) Swern oxidation. Freshly distilled oxalyl chloride (0.45 mL, 4.8 mmol) in dry dichloromethane (8 mL) was added dropwise to a solution of dry dimethylsulfoxide (0.83 mL, 10.9 mmol) in dry dichloromethane (5 mL), under nitrogen at -78°C. After stirring for 30 min, 2,5-dihydroxynorbornane (300 mg, 2.34 mmol) in dichloromethane (4 mL) and dimethylsulfoxide (0.6 mL) was added dropwise. After stirring for 3 hours, triethylamine (3 mL, 21.8 mmol) was slowly added. The reaction mixture was allowed to warm to room temperature, then stirred for an additional hour. Water (10 mL) was added dropwise, the reaction mixture was diluted with dichloromethane and the organic phase was washed with 0.5 N HCl, aqueous saturated sodium bicarbonate, and brine. The solvent was evaporated *in vacuo* yielding 200 mg of pure dione (70%). MS (EI): 124(100) $[M]^+$, 95(21), 82(23), 67(87).

^{1}H NMR ($CDCl_3$, 250 MHz), δ ppm, *J* Hz: 2.97 (2H, m, X signal of an ABX system, H-1 and H-4), 2.36 (2H, dm, A signal of an ABX system, J_{AB} = 19, H-3 *exo* and H-6 *exo*), 2.13 (2H, dm, B signal of an ABX system, J_{AB} = 19, H-3 *endo* and H-6 *endo*), 2.08 (2H, m, H-7 and H-7').

^{13}C NMR ($CDCl_3$, 62.9 MHz), δ ppm: 212.04 (CO, C-2 and C-5), 48.48 (CH, C-1 and C-4), 38.80 (CH_2, C-6 and C-3), 36.27 (CH_2, C-7).

(1*S*, 4*S*)-1: mp 140-141°C. $[\alpha]_D^{21}$ –4.5° (c 2.44, EtOH), $[\alpha]_{578}$ –4.5°, $[\alpha]_{546}$ –4.5°, $[\alpha]_{436}$ +26.6°, $[\alpha]_{363}$ +187°; e.e. = 99%. HRMS for $C_7H_8O_2$, calc. 124.052408, found 124.052383.

(1*R*, 4*R*)-1: mp 139-140°C. $[\alpha]_D^{21}$ +5.0° (c 2.0, EtOH), $[\alpha]_{578}$ +5.0°, $[\alpha]_{546}$ +4.0°, $[\alpha]_{436}$ –25.5°, $[\alpha]_{363}$ –179.5°; e.e. = 96%. HRMS for $C_7H_8O_2$, calc. 124.052408, found 124.052383.

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Final Conclusions and Summary

The purpose of the work in this thesis was to shed light into the domain of biocatalysis with the goal of helping organic chemists produce chiral materials with greater efficiency and higher optical purity.

As there are over a hundred commercially available hydrolases, chemists need help in choosing the best hydrolase for their problem. Empirical rules or models that summarize earlier results serve as good guidelines and help organic chemists identify the substrate features that are important for good enantioselectivity.

We have proposed simple empirical substrate rules that predict the enantiopreference of various hydrolases towards alcohols and primary amines. These rules are based solely on the relative size of the substituents at the stereocenter of the substrate; one being larger than the other. Recent three-dimensional crystal structures of the hydrolases support these rules because they show the presence of both a large pocket and medium pocket in the substrate binding site. Although these rules do not give a quantitative estimation of the enantioselectivity, they reliably predict the absolute configuration of the favored product. Their accuracy can be summarized as follows:

Secondary alcohols:	CRL: correct for 51/55 cyclic substrates PCL: correct for 63/64 substrates CE: correct for 14/15 substrates Subtilisin: correct for 11/13 substrates
Primary alcohols:	PCL: correct for 54/61 substrates lacking an oxygen atom directly attached to the stereocenter PPL: correct for 27/31 substrates lacking an oxygen atom directly attached to the stereocenter
Primary amines:	CAL-B, PAL, PCL: correct for 18/18 substrates Subtilisin: correct for 13/13 substrates

Many box-type models exist that are specific to a given enzyme. These too focus largely on the size of the substituents, but they often give more details as to the shape and polar or non-polar characteristics of the substituents. Similarly to the empirical substrate rules, they all have a larger side and a smaller side. Although the box-type models provide more information, the simple empirical substrate rules still remain very useful because they are more general and very easy to apply. For example, although there have been two detailed box-type models proposed for PCL, these models are not the same and therefore are difficult for the organic chemist to use.

PCL exhibits an opposite enantiopreference towards primary and secondary alcohols. Based on studies carried out with substrates having two stereocenters, we proposed that tne substituents of both primary and secondary alcohols are positioned in the same way in the substrate binding site. The opposite enantiopreference is accounted for by the following: because of the extra carbon atom between the stereocenter and the alcohol oxygen, the opposite enantiomer must bind to allow the primary alcohol oxygen to be placed in a similar position to the secondary alcohol oxygen in the active site. This proposal was made before the availability of a three-dimensional crystal structure of PCL. Although crystal structures have since been obtained and molecular modeling studies carried out, the two ensuing explanations for the opposite enantiopreference are contradictory (see introduction, section 1.5). The proposal in this work agrees with one and disagrees with the other. Perhaps a comparison of X-ray structures with covalently-bound transition state analogues of primary and secondary alcohols will give a definitive answer.

In Chapter 4, we accounted for the opposite enantioselectivity of lipases and subtilisins towards secondary alcohols and primary amines. The proposal is based on the fact that the active sites of these two classes of hydrolases are approximate

mirror images: the large and medium substituents bind in the same respective pockets but the opposite enantiomer must bind to be more catalytically productive.

Based on these rules, we developed a method to increase the enantioselectivity of lipases towards secondary alcohols. This methodology involves increasing the difference in size of the substituents at the stereocenter. The lipase can then discriminate more easily between the two enantiomers. We successfully applied this method to the preparation of useful chiral synthons with high optical purity. Unfortunately, however, this method cannot be applied to primary alcohols. It has not yet been tested on primary amines.

We have designed a synthetic scheme that combines a few chemical transformations with a key enzymatic resolution step for the preparation of both enantiomers of a useful bicyclic dione. During this work we observed an interesting example of how a lipase's activity can be vastly different in organic solvent: CRL does not catalyze the hydrolysis of the given acetate but does catalyze the reverse reaction, acetylation, in organic solvent. Despite all the research conducted to study the effects of organic solvents on hydrolase, there is still no clear explanation for this phenomenon.

Contributions to Knowledge

- We proposed a very useful, simple, and reliable empirical rule that predicts the enantioselectivity of three hydrolases towards secondary alcohols and their esters. It is based solely on the difference in size of the substituents at the stereocenter. The utility of this rule has been proven by the numerous times that it has been applied and quoted by other researchers (over 200 citations). We feel as though it set the groundwork for further research in this field.

- We proposed a similar rule to predict the enantioselectivity of lipase from *Pseudomonas cepacia* towards primary alcohols and their esters. It is very reliable for primary alcohols that lack an oxygen at the stereocenter. We give a possible explanation for the reason that *Pseudomonas cepacia* prefers the opposite enantiomers of primary and secondary alcohols. This is based on both experimental and computer modeling results. This work has also been quoted many times, with over 43 citations.

- We have proposed a rule that predicts the enantiopreference of subtilisin towards primary amines. This rule is opposite to the rule for lipases. We also reviewed the enantiopreference of lipases towards primary amines and of subtilisin towards secondary alcohols, in order to determine the reliability of previously proposed rules. In addition, we show that subtilisin has a regioselectivity towards secondary alcohols that is opposite to that of lipases.

- We give a structure-based rationalization to explain the opposite enantiopreference of lipases and subtilisin towards secondary alcohols and primary amines.

- We developed a method by which to improve the enantioselectivity of hydrolases, on the basis of the rule for secondary alcohols. It is applied by simply increasing the difference in size of the two substituents at the stereocenter. The method was successfully applied to the synthesis of two useful chiral synthons, (*R*)-(+)-*tert*-butyl lactate and (*S*)-(-)-4-acetoxy-2-cyclohexen-1-one. Other researchers have since used this method successfully.[1]

- To our knowledge, we prepared the first sample of enantiomerically-pure (*S*)-(-)-4-acetoxy-2-cyclohexene-1-one. This was done on a preparative scale. Our method of preparing this chiral synthon has been employed by at least two other groups of researchers for use as a precursor in the synthesis of several optically-pure key intermediates.[2]

- To our knowledge, we carried out the first synthesis of optically-pure (1*R*,4*R*)- and (1*S*,2*S*)-bicyclo[2.2.1]heptan-2,5-diones, useful C_2-symmetric synthons. This was accomplished by an efficient chemoenzymatic route that involves a lipase-catalyzed transesterification step.

- I feel that my overall contribution to knowledge is that I have participated in research has helped to make hydrolases less intimidating and more approachable to synthetic chemists, as well as having helped to increase our understanding of the process by which enzymes distinguish between enantiomers.

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[1] See section 1.7 of the general introduction for references.

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Glossary of Symbols and Abbreviations

Å	angstrom
$[\alpha]_D$	specific rotation at the sodium D line wavelength
Ac	acetyl
ACE	acetylcholinesterase
anal	analytical
app	apparent
Ar	aryl
bp	boiling point
br	broad (spectral line width)
Bz	benzyl
c	centi (10^{-2})
c	conversion
c	concentration (for rotation)
C	*Candida*
°C	degrees Celcius
CAL-B	lipase B from *Candida antartica*
calcd (calc)	calculated
CD	circular dichroism
CE	cholesterol esterase
CI	chemical ionization
conv	conversion
COSY	two-dimensional nuclear magnetic resonance correlation spectroscopy
CRL	lipase from *Candida rugosa*
CVL	lipase from *Chromobacterium viscosum*

d	doublet (NMR)
d	deci (10^{-1})
δ	chemical shift
Da	Dalton
DEPT	distortionless enhancement by polarization transfer
dm	doublet of multiplet (NMR)
DMAP	4-dimethylaminopyridine
DMSO	dimethyl sulfoxide
E	enantiomeric ratio
E	enzyme
EI	electron ionization
ee	enantiomeric excess
ent	enantiomer
eq	equation
equiv / eq	equivalent(s)
EtOH	ethanol
FAB	fast atom bombardment
g	gram(s)
GC	gas chromatography
h	hour(s)
hfc	3-(heptafluoropropylhydroxymethylene)-*d*-camphorato
HLL	lipase from *Humicola lanuginosa*
HPLC	high preformance liquid chromatography
HRMS	high resolution mass spectrometry
Hz	hertz
hv	light
IR	infrared spectroscopy

J	coupling constant (NMR)
k	kilo (10^3)
K_M	Michaelis-Menten constant
k_{cat}	enzyme turnover number
L	large
L	liter(s)
l	length
LC	liquid chromatography
lit	literature
m	meter(s)
m	milli (10^{-3})
m	multiplet (NMR)
m	meta
μ	micro (10^{-6})
M	medium
M	molar
M	*Mucor*
MeOH	methanol
min	minute(s)
MML	lipase from *Mucor meihei*
mol	mole(s)
mp	melting point
MS	mass spectrometry
m/z	mass-to-charge ratio
n	nano (10^{-9})
N	normal

na	not available
nd	not determined
NMR	nuclear magnetic resonance
%	percent
o	ortho
OAc	acetate
p	*para*
P	Pseudomonas
P	product
PAL	lipase from *Pseudomonas aeruginosa*
PCC	pyridinium chlorochromate
PCL	*Pseudomonas cepacia*
PDB	Protein Databank
pH	negative logarithm (base 10) of the hydrogen ion concentration
Ph	phenyl
pKa	negative logarithm of equilibrium constant for association
PLE	porcine liver esterase
ppm	parts per million
PPL	porcine pancreatic lipase
py	pyridine
q	quartet (NMR)
R	substituent
R_f	retention factor
ref	reference
s	singlet (NMR)

S	small
s	substrate
satd	saturated
sec	second(s)
SM	starting material
sp	species
struc	structure
t	triplet (NMR)
T_d	tetrahedral intermediate
THF	tetrahydrofuran
TLC	thin layer chromatography
UV	ultraviolet spectroscopy
v/v	volume to volume ratio

Appendices

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while becoming labyrinths ourselves."

Salvador Dàli

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Dear Dr Weissfloch

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July 30, 1999

To Whom It May Concern:

I hereby give permisssion to Alexandra Weissfloch to include the following articles, which I coauthored with her, in her Ph.D. thesis.

> Weissfloch, A. N. E.; Kazlauskas, R. J. (1995), Enantiopreference of lipase from *Pseudomonas cepacia* toward primary alcohols, *J. Org. Chem.*, **60**, 6959-6969.
>
> Kazlauskas, R. J., Weissfloch, A. N. E. (1997), A structure-based rationalization of the enantiopreference of subtilisin toward secondary alcohols and isosteric primary amines, *J. Mol. Catal. B Enz.*, **3**, 65-72.

In addition, and acting on behalf of Louis Cuccia and Aviva Rappaport as well as myself, I give permission to Alexandra Weissfloch to include the following article, which we coauthored together, in her Ph.D. thesis.

> Kazlauskas, R. J.; Weissfloch, A. N. E.; Rappaport, A. T.; Cuccia, L. A. (1991), A rule to predict which enantiomer of a secondary acohol reacts faster in reactions catalyzed by cholesterol esterase, lipase from *Pseudomonas cepacia*, and lipase from *Candida rugosa*, *J. Org. Chem.*, **56**, 2656-2665.

Sincerely,

Romas Kazlauskas
Associate Professor of Chemistry

Robert AZERAD

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Mss Alexandra WEISSFLOCH
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Paris, 28 July 1999

Dear Mss Weissfloch,

As you requested, I accept and authorize you to include in your thesis our paper entitled:
- "Chemoenzymatic access to enantiomeric bicyclo[2.2.1]heptane-2,5-diones" by A. Weissfloch and R. Azerad, published in *Bioorganic and Medicinal Chemistry* (1994) **2**, 493-500.

Best regards and wishes

Yours sincerely

R. Azerad

Reprinted from The Journal of Organic Chemistry, 1991, Vol. 56.

A Rule To Predict Which Enantiomer of a Secondary Alcohol Reacts Faster in Reactions Catalyzed by Cholesterol Esterase, Lipase from *Pseudomonas cepacia*, and Lipase from *Candida rugosa*[1]

Romas J. Kazlauskas,* Alexandra N. E. Weissfloch, Aviva T. Rappaport, and Louis A. Cuccia

McGill University, Department of Chemistry, 801 Sherbrooke St. W., Montréal, Québec H3A 2K6, Canada

Received October 2, 1990

The enantioselectivity of the title enzymes for more than 130 esters of secondary alcohols is correlated by a rule based on the sizes of the substituents at the stereocenter. This rule predicts which enantiomer of a racemic secondary alcohol reacts faster for 14 of 15 substrates of cholesterol esterase (CE), 63 of 64 substrates of lipase from *Pseudomonas cepacia* (PCL), and 51 of 55 cyclic substrates of lipase from *Candida rugosa* (CRL). The enantioselectivity of CRL for acyclic secondary alcohols is not reliably predicted by this rule. This rule implies that the most efficiently resolved substrates are those having substituents which differ significantly in size. This hypothesis was used to design syntheses of two chiral synthons: esters of (*R*)-lactic acid and (*S*)-(−)-4-acetoxy-2-cyclohexen-1-one, **70**. As predicted, the acetate group of the methyl ester of lactyl acetate was hydrolyzed by PCL with low enantioselectivity because the two substituents, CH_3 and $C(O)OCH_3$, are similar in size. To improve the enantioselectivity, the methyl ester was replaced by a *tert*-butyl ester. The acetate group of the *tert*-butyl ester of lactyl acetate was hydrolyzed with high enantioselectivity ($E > 50$). Enantiomerically pure (*R*)-(+)-*tert*-butyl lactate (>98% ee, 6.4 g) was prepared by kinetic resolution. For the second example, low enantioselectivity ($E < 3$) was observed in the hydrolysis of *cis*-1,4-diacetoxycyclohex-2-ene, a meso substrate where the two substituents, CH_2CH_2 and CH=CH, are similar in size. To improve enantioselectivity, the size of the CH=CH substituent was increased by addition of Br_2. The new substrate was hydrolyzed with high enantioselectivity ($E > 65$) using either CE or CRL. Enantiomerically pure **70** (98% ee) was obtained after removal of the bromines with zinc and oxidation with CrO_3/pyridine.

Microorganism- and enzyme-catalyzed syntheses and resolutions are among the best methods for the preparation of enantiomerically pure compounds.[2] Enantioselective hydrolyses and transesterifications are especially useful because they are efficient, can be carried out on a large scale, and apply to a wide range of substrates. Unfortunately, there is little X-ray structural information available for the esterases and lipases that are used for these preparations;[3] thus, the appropriate enzyme is usually chosen by screening.

In order to streamline screening, substrate models have been developed using substrate selectivity data. Some models attempt to define the shape and hydrophobic character of the active site and are used to predict which new substrates will be transformed by the enzyme and whether the reaction will be enantioselective. In most cases, this type of model is only accurate for substrates that are similar to those already tested. For example, a model for PLE[4] predicts its selectivity with α,β-substituted carboxylic acids,[5] and a model for CRL predicts its selectivity for bicyclo[2.2.1]heptanols and bicyclo[2.2.2]octanols.[6] In some cases, more general models which define the sizes of hydrophobic pockets near the active site have been developed. These can be used to predict reactivity for a wider range of substrates.[7]

The simplest models for enzyme selectivity, more accurately referred to as rules, predict only which enantiomer reacts faster, usually based on either the size or hydrophobicity of the substituents at the stereocenter. The earliest example of such a rule is Prelog's rule which predicts the enantioselectivity of the reduction of ketones by the yeast *Culvaria lunata* based on the size of the two substituents at the carbonyl (Figure 1).[8] The advantage of this rule is that it applies to a wider range of substrates, but the disadvantage is that there are exceptions to this rule. Nevertheless, this rule is sufficiently reliable to be used for the determination of absolute configurations[9] and has been used to redesign substrates in order to improve the enantioselectivity.[10]

A rule similar to Prelog's rule has been proposed for a hydrolysis catalyzed by a yeast. For 47 esters of racemic secondary alcohols, a rule based on the sizes of the substituents at the stereocenter predicts which enantiomer is hydrolyzed faster in cultures of the yeast *Rhizopus nigrigans*.[11] This rule has been used to determine the ab-

(1) *Candida rugosa* was previously classified as *Candida cylindracea* and many workers still use the older name (*Catalogue of Fungi/Yeasts*, 17th ed.; American Type Culture Collection: Washington, 1987). Lipase from *Pseudomonas cepacia* is Amano Lipase P, PS, or P30. The microbe from which this enzyme is prepared was previously classified as *Pseudomonas fluorescens*, but now has been reclassified as *P. cepacia* (Amano Pharmaceutical Co., Nagoya, Japan, personal communication). This paper was presented in part at the Chemical Institute of Canada Congress, Halifax, NS, July 1990, Abstract 881 and the American Chemical Society National Meeting, Washington, DC, August 1990, Abstract ORG 226.

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(4) Abbreviations for enzymes used in this paper: ACE, acetylcholinesterase from electric eel; CE, bovine cholesterol esterase; CRL, lipase from *Candida rugosa*; MML, lipase from *Mucor meihei*; PCL, Amano P = lipase from *Pseudomonas cepacia*; PLE, porcine liver esterase.

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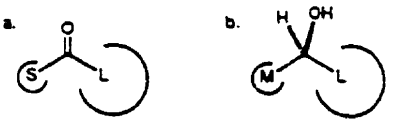

Figure 1. (a) Prelog's rule predicts that the yeast *Culvaria lunata* preferentially adds H_2 to the front side of ketones having the shape indicated. (b) An extension of Prelog's rule to hydrolases. For esters of secondary alcohols, the enantiomer shown reacts faster with CE, PCL, and CRL than the other enantiomer.

solute configuration of secondary alcohols.[12] However, this microorganism has not been used for large scale synthesis.

Similar rules have been proposed for two commercially available lipases: *Mucor meihei*[13] (MML) and *Arthrobacter*.[14] It is not yet clear how useful these rules will be because they are based on only six substrates for the *Mucor* enzyme and only two substrates for the *Arthrobacter* enzyme. A "two-site model" that has been recently proposed for cyclic substrates of PCL also appears similar to the rule discussed herein.[15]

This paper proposes a rule based on the sizes of the substituents for three hydrolases (Figure 1). This rule is supported by 14 out of 15 substrates for CE, 63 out of 64 substrates for PCL, and 51 out of 55 cyclic substrates for CRL and has an accuracy of ≥93%. This rule is the first attempt to predict the enantioselectivity of CE. For PCL, this rule is similar to the "two-site model" for cyclic substrates,[14] but the evidence presented in this paper shows that this rule accounts for the enantioselectivity of acyclic secondary alcohols as well. For CRL, this rule is the first general rule and complements the model for bicyclic substrates.[6]

Besides correlating a large amount of experimental data, this rule also suggests a strategy for improving the efficiency of resolutions catalyzed by these enzymes: secondary alcohols having substituents which differ significantly in size should be more efficiently resolved than secondary alcohols having substituents which are similar in size. This hypothesis was used to design the preparations of enantiomerically pure *tert*-butyl lactate and (*S*)-(−)-4-acetoxy-2-cyclohexen-1-one.

Lactate esters are used as chiral starting materials and chiral auxiliaries. For example, lactate esters were used in the preparation of (*S*)-2-arylpropionic acids, a class of nonsteroidal antiinflammatory drugs,[16] α-*N*-hydroxy amino acids,[17] chiral enolates,[18] and chiral auxiliaries for an enantioselective Diels–Alder reaction.[19] Polylactides containing interpenetrating networks of poly-(*R*)-lactide and poly-(*S*)-lactide are stronger than those containing racemic chains.[20]

Although enantiomerically pure (*S*)-(+)-lactic acid is readily available, the unnatural enantiomer, (*R*)-(−), remains expensive. Enantiomerically pure (*R*)-lactic acid can be prepared by D-lactate dehydrogenase catalyzed reduction of pyruvate,[21] fermentation, or microbial destruction of the *S* enantiomer.[22] The resolution of lactate esters using PCL described in this paper yields material of high enantiomeric purity and is inexpensive and simple to carry out. An enzyme-catalyzed resolution of lactate esters has been reported previously using an enzyme from a similar microorganism.[23]

The second example is an enantioselective synthesis of (*S*)-(−)-4-acetoxy-2-cyclohexen-1-one, a new compound that should also be useful for synthesis. The analogous (4*S*)-*tert*-butyldimethylsiloxy derivative has been prepared in six steps from quinic acid[24] and has been used for the synthesis of the cholesterol-lowering drugs ML 236A and compactin.[25] The procedure described below for the acetate derivative is simpler. Since the analogous cyclopentenone, 4-acetoxy-2-cyclopenten-1-one, has been widely used in synthesis,[26] the six-membered analog should also find many uses.

Results

A Survey of Enantioselectivity for Esters of Secondary Alcohols. Pancreatic CE catalyzed the hydrolysis of the acetates of the secondary alcohols listed in Table I. The measured values of enantiomeric excess and percent conversion were used to calculate the enantioselectivity, *E*, which indicates the degree to which the enzyme prefers one enantiomer over the other.[27] Several examples from the literature are also included in Table I. Chart I indicates the structure of the fast-reacting enantiomer. The chart and tables are arranged so that the larger group is always on the right side as predicted by the rule in Figure 1b. CE showed no enantioselectivity toward **40**, but for the other 14 examples the rule correctly predicts which enantiomer reacts faster. The overall accuracy of the rule for CE is 14 of 15 substrates or 93%; the single exception, the *tert*-butyl ester of lactyl acetate, will be introduced and discussed below.

Enantioselectivity data for PCL was gathered from the literature (Table II). This list includes all secondary alcohols prepared using lipase P from Amano Pharmaceutical. Reactions using other lipases from *Pseudomonas* (e.g. AK, K-10, or SAM-II) are not included. Patent literature is also not included. The reaction conditions used for the examples listed in Table II include both hydrolyses in aqueous solution as well as transesterifications and esterifications in organic solvents. The structures of the substrates in Table II include acyclic secondary alcohols and cyclic secondary alcohols in rings ranging from four- to seven-membered. For 63 of the 64 substrates, the rule in Figure 1 correctly predicts the fast-reacting enantiomer. The single exception, indicated by "(ent)" in the enantioselectivity column, was one of five substrates which

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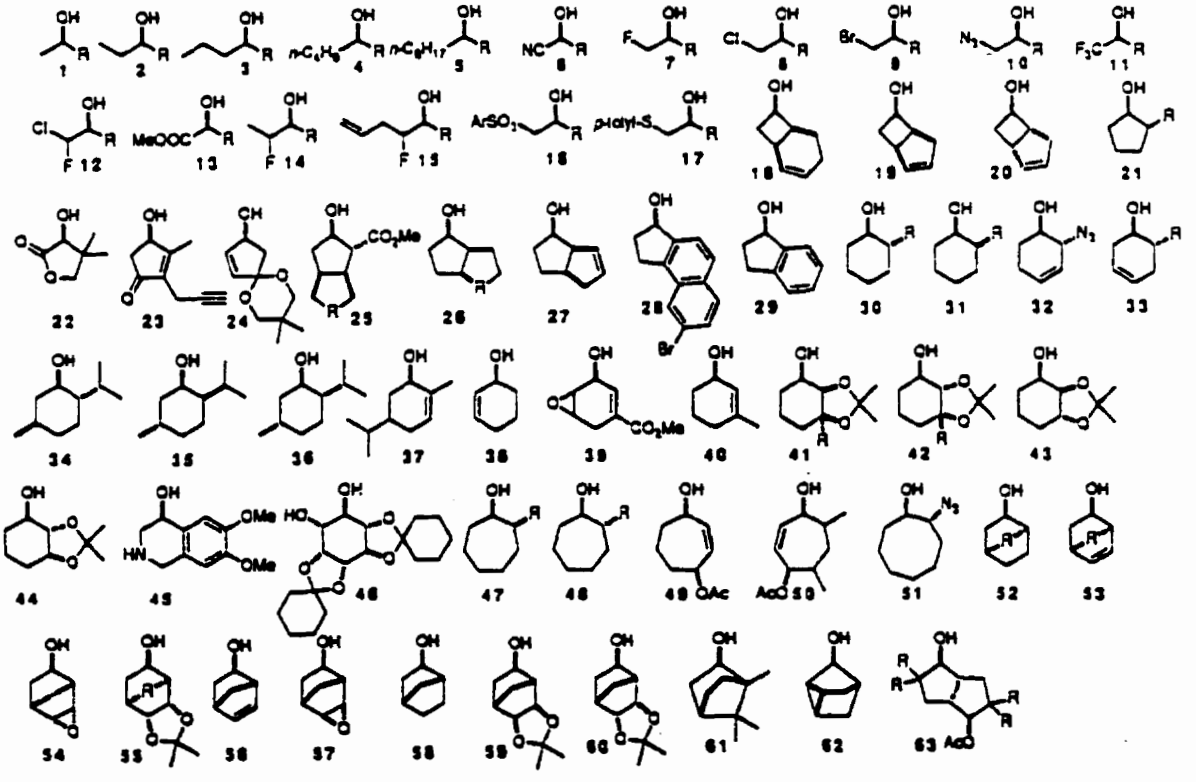
Table I. Enantioselectivity of Bovine Pancreatic Cholesterol Esterase for Acetates of Secondary Alcohols

structure	R =	initial rate[a] (units/mg)	convn (%)	enantiomeric excess (%)[b]	*E*, enantioselectivity
1	Ph	0.31	43	89	8.8[c]
1	n-C_6H_{13}	0.36	46	35	2.6[d]
1	$(CH_3)_2CH{=}C(CH_3)_2$	0.50	46	45	4.3[e]
2	Ph	0.12	34	6.2	4.6[f]
28		0.02	-	-	2.9[g]
29		0.56	29	65	6.3[g]
30	CH_3	0.32	50	94	>100[h,i]
30	OAc	0.20	44	56	10[j]
30	OH	0.59	48	59	6.8[j]
31	CH_3	0.59	53	46	4.4[h,k]
34		0.21	44	96	>100[g]
39	cis	0.04	-	-	14[l]
39	trans	0.07	-	-	37[l]
40		0.60	50	0	1
46		-	-	-	37[m]

[a] For hydrolysis of the corresponding acetate ester unless otherwise noted. Unit = μmol of ester hydrolyzed/min. [b] The enantiomeric purity of the product alcohol was determined by derivatizing with isopropyl isocyanate and separating the enantiomers by capillary GC using an XE-60-(*S*)-valine-(*S*)-α-phenylethylamide column (Chromopak). [c] The product alcohol showed $[\alpha]_D$ (obsd) = +40.5° (*c* 1.2, hexane) indicating the *R* configuration: lit. *S*-(+), Klyne, W.; Buckingham, J. *Atlas of Stereochemistry*; Chapman and Hall: London, 1974; p 26. [d] Absolute configuration was determined by comparison to an authentic sample. [e] The product alcohol showed $[\alpha]_D$ (obsd) = -5.3° (*c* 2.2, hexane) indicating the *R* configuration: lit. *S*-(+), $[\alpha]_D$ = +16.1° (neat): Jacques, J.; Gros, C.; Bourcier, S. *Absolute Configuration of 6000 Selected Compounds with One Asymmetric Carbon Atom*; G. Thieme: Stuttgart, 1977. Levene, P. A.; Haller, H. L. *J. Biol. Chem.* **1929**, *83*, 177–183. [f] The product alcohol showed a positive rotation (hexane) indicating the *R* configuration: lit. *S*, $[\alpha]_D$ = -39° (neat): MacLeod, R.; Welch, F. J.; Mosher, H. S. *J. Am. Chem. Soc.* **1960**, *82*, 876–880. [g] Kazlauskas, R. *J. J. Am. Chem. Soc.* **1989**, *111*, 4953–4959. [h] The enantiomeric purity was determined by formation of the Mosher's ester and integration of the ^{1}H NMR signals for the ring CH_3 groups: Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543–2549. [i] The product alcohol showed $[\alpha]_D$ (obsd) = -8.7° (*c* 1.2, CH_2Cl_2) indicating the *R* configuration: lit. *R*-(-), Beard, C.; Djerassi, C.; Elliott, T.; Tao, R. C. C. *J. Am. Chem. Soc.* **1962**, *84*, 874–875. [j] Hydrolysis was caried out in aqueous solution saturated with sodium chloride. The % ee refers to unreacted starting material and was determined by HPLC of the benzoate derivative on a Chiralpak OT (Daicel, New York): Caron, G.; Kazlauskas, R. J., unpublished results. [k] The product alcohol showed $[\alpha]_D$ (obsd) = -34° (*c* 1.8, hexane) indicating the *R* configuration: lit. *R*-(-), Beard, C.; Djerassi, C.; Elliott, T.; Tao, R. C. C. *J. Am. Chem. Soc.* **1962**, *84*, 874–875. [l] As the butyrate ester at 0–6 °C: Pawlak, J. L.; Berchtold, G. A. *J. Org. Chem.* **1987**, *52*, 1765–1771. [m] Liu, Y.-C.; Chen, C.-S. *Tetrahedron Lett.* **1989**, *30*, 1617–1620.

Chart I. Structures for Tables I–III



showed low enantioselectivity, $E \leq 3$. Thus, for PCL the rule predicts which enantiomer reacts faster with 98% accuracy.

Enantioselectivity data for CRL was also gathered from the literature (Table III). The *Candida* list includes results using enzyme either from Sigma Chemical Co. or

Table II. Enantioselectivity of Lipase from *Pseudomonas cepacia* (Amano P) for Esters of Secondary Alcohols

structure	R =	E	ref	structure	R =	E	ref
1	Ph, CH_2Ph	>50	*a*	12	Ph, CH_2Ph, CH_2CH_2Ph	23 to >50	*k*
1	n-C_6H_{13}, n-$C_{10}H_{21}$	~12	*a*	13	$CH(CH_3)$-1-(2,6-dithiane)	>50	*l*
1	CH_2CN	29	*b*	13	*syn*-CH(Ar)(SAr')	>50	*m*
1	CH_2COOEt	~8	*c*	21	*cis*-COOEt, OAc; *trans*-COOEt, OAc	>50	*n*
1	1-(2,6-dithiane), 1-(2,5-dithiolane), CH_2-1-(2,6-dithiane), CH_2-1-(2,5-dithiolane)	>50	*d*	21	*trans*-N_3	>50	*o*
				23		>50	*h*
1	CH_2-1(2,6-dioxane)	2	*d*	24		>50	*p*
2	Ph	>50	*a*	25	C=O, $C{=}CH_2$, HC=CH	>50	*n*
2	$CH(N_3)C_2H_5$ (threo, erythro)	>50	*e*	25	1-(2,5-dioxolane)	3	*n*
3	$CH(N_3)CH_2CH_2CH_3$ (erythro)	>50	*e*	26	C=O, $C{=}CH_2$, 1-(2,5-dioxolane)	>50	*q*
4	CH_2N_3	2	*e*	27		>50	*r*
4	CH_2OSO_2Ar	25	*f*	30	COOEt, OAc, CN, C_6H_5	>50	*n*, *o*, *s*
5	*syn*-CH(Cl)CH_2COOEt	50	*g*	30	OH	17	*t*
6	*p*-C_6H_4OPh	>50	*h*	31	OAc	8	*n*
6	Ph, CH_2CH_2Ph, CH=CHPh	18 to >50	*b*	31	COOEt	>50	*n*
8	Ph, 3,4-$(MeO)_2C_6H_3$	>50	*i*	33	OAc	>50	*u*
8	CH_2OSO_2Ar	24	*f*	45		17	*v*
9	2-naphthyl, 4-BrC_6H_4, 4-$MeOC_6H_4$	>50	*i*	47	OAc	1	*n*
9	1-(3-bromo-5-isoxazolyl)	20	*j*	47	CO_2Me	>50	*n*
10	Ph	>50	*e*	48	CO_2Me,OAc	>50	*n*
10	n-C_6H_{13}	2 (ent)	*e*				

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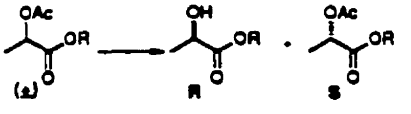
from Meito Sangyo (Lipase MY or OF-360); however, examples where the absolute configuration of the product is uncertain were not included,[28] nor were examples from patents. The reaction conditions again include both hydrolyses in aqueous solution as well as transesterifications and esterifications in organic solvents.

For acyclic substrates of CRL the rule in Figure 1 does *not* reliably predict which enantiomer reacts faster. Only for about half, 14 of the 31 acyclic substrates, is the fast-reacting enantiomer predicted correctly; this proportion is close to that expected for random guesses. A large fraction of the acyclic substrates (12 of 31) showed low enantioselectivity, $E \leq 3$. Thus, the rule is not useful for acyclic substrates of CRL. Generalizations about this rule given below do *not* include acyclic substrates of CRL, but do include acyclic substrates of CE and PCL.

For cyclic substrates of CRL the rule reliably predicts which enantiomer reacts faster. These substrates include secondary alcohols in four- to eight-membered rings as well as secondary alcohols in bicyclic substrates. The rule predicts correctly for 51 of the 55 substrates where CRL was enantioselective, an accuracy of 93%. Three exceptions, indicated by "(ent)" as before, were among the 13 cyclic substrates which showed low enantioselectivity, $E \leq 3$; one exception, **22**, showed moderate enantioselectivity, $E = 8$.[29]

Scheme I. Kinetic Resolution of Esters of Lactyl Acetate



Thus, the rule predicts which enantiomer of a secondary alcohol reacts faster for ≥93% of both the cyclic and acyclic substrates of CE and PCL and the cyclic substrates of CRL. None of the substrates that react contrary to the rule show enantioselectivity greater than eight and therefore none of the exceptions are synthetically useful.

This generalization implies that a substrate having substituents which differ significantly in size should be resolved with higher enantioselectivity than a substrate where the two substituents are similar in size. The data in Tables I–III suggests that this generalization is valid;

(28) Chênevert, R.; Desjardins, M.; Gagnon, R. *Chem. Lett.* **1990**, 33–34. Abramowicz, D. A.; Keese, C. R. *Biotechnol. Bioengineer.* **1989**, *33*, 146–156. Takano, S.; Inomata, K.; Ogasawara, K. *J. Chem. Soc., Chem. Commun.* **1989**, 271–272.

(29) It was difficult to decide which side was larger for **37**, **38**, **50**, and **62**. Compounds **37** and **50** contain a small substituent near the carbinol carbon on one side and a larger substituent further away on the opposite side. Compound **37** was drawn as shown arbitrarily. For **50**, the larger acetoxy substituent is three carbons away from the carbinol carbon and is judged to be less important than the CH_3 at the 2-position. The unsaturated portion of **38** and the cyclopropyl portion of **62** were judged to be smaller using three-dimensional models. Several other bicyclic structures appear symmetrical as drawn, but three-dimensional models clearly show the right side to be larger.

Table III. Enantioselectivity of Lipase from *Candida rugosa* for Acetates of Secondary Alcohols

structure	R	E	ref	structure	R	E	ref
1	C_2H_5	34	a	33	N_3	>50	o
1	n-C_6H_{13}	1.6	b	34		~10 to >50	q, s
1	2-furyl	5	c	35		>60	q
1	$CH_2(OCH_3)_2$	2.5	d	36		27	q
2	$CH(N_3)C_2H_5$ (threo, erythro)	12	e	37	trans	1	t
3	*erythro*-$CH(N_3)CH_2CH_2CH_3$	18	e	37	cis	25	t
4	CH_2N_3	2 (ent)	e	38		1.2	t
6	Ph	8 (ent)	f	39	cis, trans	6	u
6	p-C_6H_4OPh	12 (ent)	g	40		3.4	d
7	CH_2S-p-tolyl	1.3 (ent)	h	41	H, CH_3	>50	v
8	Ph	2	f	42	H	2	v
10	Ph	13	e	42	CH_3	26	v
10	n-C_6H_{13}	2	e	43		>50	v
10	t-C_4H_9	>50 (ent)	e	44		>50	v
11	CH=CHPh (*E*, *Z*)	>50 (ent)	i	45		20	w
11	$CH=CH(CH_2)_5CH_3$(*E*, *Z*)	~16 (ent)	i	46	N_3	30	o
12	Ph	6	j	49		2.5	x
12	CH_2Ph, CH_2CH_2Ph	~3 (ent)	j	50	cis, trans	>50	z
13	Ph	>50 (ent)	f	51		>50	o
13	*anti*-$CH(CH_3)$-3-indolyl	3-40	k	52	CH_2	11	y
14	CH_2S-p-tolyl (syn, anti)	1.3 (ent)	h	52	CHCOOR', $CHOCH_2Ph$	3-10	y
15	CH_2S-p-tolyl (syn, anti)	1.2-2.1 (ent)	h	53	CH_2	35	y
16	Ph	>50 (ent)	l	53	$CHCO_2Me$, $C(OMe)_2$	~10	y
17	$CFPhCH_3$ (syn, anti)	6.5	h	53	O	>50	y
18		17	m	54		>50	y
19		>40	n	55	CH_2	22	y
20		1.2 (ent)	n	55	O	>50	y
21	*trans*-N_3	1.4	o	56		1	y
22		8 (ent)	f	57		1.4 (ent)	y
27		>50	p	58		1.5	y
30	CH_3, C_2H_5, i-C_3H_7, t-C_4H_9	>50	q	59		4	y
30	CN, NO_2, N_3	40 to >50	o	60		1.8	y
30	OAc, OH	2, 1 (ent)	r	61		>10	z
31	CH_3, i-C_3H_7	24	q	62		10	aa
32		20	o	63	H, CH_3	15	bb

[a] Cambou, B.; Klibanov, A. M. *Biotechnol. Bioeng.* 1984, *26*, 1449–1454. [b] Gerlach, D.; Missel, C.; Schreier, P. *Z. Lebensm. Unters. Forsch.* 1988, *186*, 315–318. Esterification with n-$C_{11}H_{23}COOH$ in heptane. [c] Drueckhammer, D. G.; Barbas, C. F., III; Nozaki, K.; Wong, C.-H. *J. Org. Chem.* 1988, *53*, 1607–1611. [d] Wang, Y.-F.; Lalonde, J. J.; Momongan, M.; Bergbreiter, D. E.; Wong, C.-H. *J. Am. Chem. Soc.* 1988, *110*, 7200–7205. Acetylation with vinyl acetate. [e] Foelsche, E.; Hickel, A.; Hönig, H.; Seufer-Wasserthal, P. *J. Org. Chem.* 1990, *55*, 1749–1753. Hydrolysis of butyrates. [f] Bevinakatti, H. S.; Banerji, A. A.; Newadkar, R. V. *J. Org. Chem.* 1989, *54*, 2453–2455. Bevinakatti, H. S.; Newadkar, R. V. *Biotechnol. Lett.* 1989, *11*, 785–788. Transesterification with 1-butanol in isopropyl ether. [g] Hirohara, H.; Mitsuda, S.; Ando, E.; Komaki, R. In *Biocatalysis in Organic Synthesis*; Tramper, J., van der Plas, H. C., Linko, P., Eds.; Elsevier: Amsterdam, 1985; 119–134. [h] Bucciarelli, M.; Forni, A.; Moretti, I.; Prati, F.; Torre, G.; Reanati, G.; Bravo, P. *Tetrahedron* 1989, *45*, 7505–7514. [i] Kitazume, T.; Lin, J. T.; Yamazaki, T. *J. Fluorine Chem.* 1989, *43*, 177–187. [j] Yamazaki, T.; Ichikawa, S.; Kitazume, T. *J. Chem. Soc., Chem. commun.* 1989, 253–255. [k] Akita, H.; Enoki, Y.; Yamada, H.; Oishi, T. *Chem. Pharm. Bull.* 1989, *37*, 2876–2878. Hydrolysis in water-saturated benzene, enzyme was immobilized on Celite. [l] Chen, C.-S.; Liu, Y.-C. *Tetrahedron Lett.* 1989, *30*, 7165–7168. Transesterification of the butyrate ester in hexane. [m] Cotterill, I. C.; Finch, H.; Reynolds, D. P.; Roberts, S. M.; Rzepa, H. S.; Short, K. M.; A. M. Z.; Wallis, C. J.; Williams, D. J. *J. Chem. Soc., Chem. Commun.* 1988, 470–472. [n] Cotterill, I. C.; MacFarlane, E. L. A.; Roberts, S. M. *J. Chem. Soc., Perkin Trans. 1* 1988, 3387–3389. [o] Hönig, H.; Seufer-Wasserthal, P.; Fülöp, F. *J. Chem. Soc., Perkin Trans. 1* 1989, 2341–2345. Faber, K.; Hönig, H.; Seufer-Wasserthal, P. *Tetrahedron Lett.* 1988, 29, 1903–1904. Hydrolysis of butyrate ester. [p] Klempier, N.; Faber, K.; Griengl, H. *Synthesis* 1989, 933–934. [q] Legrand, G.; Secchi, M.; Buono, G.; Baratti, J.; Triantaphylides, C. *Tetrahedron Lett.* 1985, *26*, 1857–1860. Esterification in hexane or heptane with n-$C_{11}H_{23}COOH$. [r] Caron, G.; Kazlauskas, R. J., unpublished results. [s] Koshino, S.; Sonomoto, K.; Tanaka, A.; Fukui, S. *J. Biotechnol.* 1985, *2*, 47–57. Esterification with 5-phenylpentanoic acid in water-saturated isooctane. [t] Oritani, T.; Yamashita, K. *Agric. Biol. Chem.* 1989, *44*, 2637–2642. [u] Pawlak, J. L.; Berchtold, G. A. *J. Org. Chem.* 1987, *52*, 1765–1771. Hydrolysis of butyrate ester. [v] Dumortier, L.; Van der Eycken, J.; Vandewalle, M. *Tetrahedron Lett.* 1989, *30*, 3201–3204. [w] Hoshino, O.; Itoh, K.; Umezawa, B.; Akita, H.; Oishi, T *Tetrahedron Lett.* 1988, 29, 567–568. Hydrolysis in water-saturated isooctane. [x] Pearson, A. J.; Lai, Y.-S.; Lu, W.; Pinkerton, A. A. *J. Org. Chem.* 1989, *54*, 3882–3893. [y] Eichberger, G.; Penn, G.; Faber, K.; Griengl, H. *Tetrahedron Lett.* 1986, *27*, 2843–2844. Oberhauser, T.; Bodenteich, M.; Faber, K.; Penn, G.; Griengl, H. *Tetrahedron* 1987, *43*, 3931–2944. Saf, R.; Faber, K.; Penn, G.; Griengl, H. *Tetrahedron* 1988, *44*, 389–392. Königsberger, K.; Faber, K.; Marschner, C.; Penn, G.; Baumgartner, P.; Griengl, H. *Tetrahedron* 1989, *45*, 673–680. [z] Sonomoto, K.; Tanaka, A. *Ann. N. Y. Acad. Sci.* 1988, *542*, 235–239. Esterification with 5-phenylpentanoic acid in water-saturated isooctane. [aa] Hirose, Y.; Anzai, M.; Saitoh, M.; Naemura, K.; Chikamatsu, H. *Chem. Lett.* 1989, 1939–1942. [bb] Naemura, K.; Matsumura, T.; Komatsu, M.; Hirose, Y.; Chikamatsu, H. *J. Chem. Soc., Chem. Commun.* 1988, 239–241.

however, it is difficult to test this hypothesis quantitatively. Nevertheless, we used this hypothesis to redesign substrates that could be efficiently resolved by these enzymes.

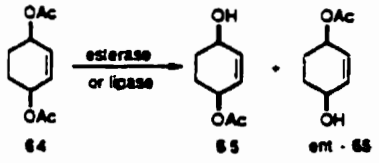
Kinetic Resolution of Lactate Esters. To resolve lactate esters we increased the size of the ester group until we found a substrate that was hydrolyzed with high enantioselectivity (Scheme I, Table IV). Lactyl acetate, R = H, was not a substrate for the three enzymes tested: activity <0.002 units/mg. The methyl ester of lactyl acetate, R = CH_3, was hydrolyzed with the *R* enantiomer reacting faster, but with very low enantioselectivity: the remaining starting material showed <32% ee at ~45% conversion. Accurate determination of the enantioselectivity was complicated by competing hydrolysis of the methyl ester which accounted for 55–80% of the disappearance of the starting material. The *tert*-butyl ester of lactyl acetate, R = *tert*-butyl, still showed low enantioselectivity with CE and CRL, but showed excellent enantioselectivity with PCL, $E > 50$. The lack of any increase in enantioselectivity for CRL is not surprising because the rule is not reliable for acyclic substrates of CRL. The unexpected result was that the enantioselectivity of CE was opposite of that predicted by the rule. The reason for this reversal is not known; this is the first substrate of CE

Table IV. Enantioselectivity of the Hydrolysis of Esters of Lactyl Acetate

	methyl ester				*tert*-butyl ester				
enzyme	rate[a]	% C[b]	% ee[c]	(R/S)[d]	rate[a]	% C[b]	% ee[c]	(R/S)[d]	E[e]
CE	0.18	42	8	R	0.06	32	21	S	3
CRL	0.30	49	32	R	0.01	40	26	R	4.4
PCL	0.01	40	24	R	0.35	50	89	R	51
PCL[f]					0.35	47	>98[g]	R	>200

[a] Initial activity of the enzyme in units/mg solid. Units = μmol of ester hydrolyzed/min. [b] The conversion refers to the amount of starting material consumed. This value was determined by ^{1}H NMR after extraction of the reaction mixture with ethyl ether. [c] Enantiomeric excess of the recovered starting material was determined by ^{1}H NMR in the presence of Eu(hfc)$_3$. [d] The absolute configuration of the preferred enantiomer was determined from the rotation of the recovered starting material: *R*-(−), Ito, S.; Kasai, M.; Ziffer, H.; Silverton, J. V. *Can. J. Chem.* 1987, *65*, 574–582. [e] Enantioselectivity, see ref 27 for details. [f] Hydrolysis of esters of lactyl butyrate. [g] Enantiomeric excess of the product.

Scheme II. Enzyme-Catalyzed Hydrolysis of 64 Showed Poor Enantioselectivity



that does not fit the rule in Figure 1. Even with this exception, the accuracy of the rule remains high: 14/15 substrates, 93%. The increase in enantioselectivity with PCL is consistent with the rule; indeed another lipase from *Pseudomonas* sp. (lipase K-10 from Amano) has been reported to show excellent enantioselectivity when R = *tert*-butyl.[23] Thus, increasing the size of the R group in Scheme I resulted in a substrate which was more efficiently resolved.

A preparative-scale resolution of *tert*-butyl lactate was carried out using the butyrate instead of the acetate to simplify separation of the product alcohol and unreacted butyrate by fractional distillation. This change from acetate to butyrate also resulted in a further increase in enantioselectivity of PCL to >200. Hydrolysis of racemic *tert*-butyl ester of lactyl butyrate (50 g) yielded the unnatural enantiomer, (*R*)-(+)-*tert*-butyl lactate (6.4 g), with >98% ee after distillation.

Enantioselective Synthesis of (*S*)-(−)-4-Acetoxy-2-cyclohexen-1-one (70). The five-membered 4-acetoxy-2-cyclopenten-1-one is a useful chiral starting material that can be prepared by an enzyme-catalyzed hydrolysis.[30] Acetylcholinesterase selectively hydrolyzes the *R* acetate in *cis*-1,4-diacetoxy-2-cyclopentene and the resulting olefinic alcohol is oxidized to the enone. An attempt to prepare the corresponding six-membered compound by an analogous enzyme-catalyzed synthesis was not successful because the acetate was removed with only low to moderate selectivity (0–72% ee, Scheme II, Table V).

The rule suggests a possible reason for this difficulty. In the cyclopentene case, the substituents at the stereocenter—CH_2CHOAc and CH=CH—differ in size and can be distinguished by the enzyme; however, in the cyclohexene case, the substituents—CH=CH and CH_2CH_2—are too similar in size to be distinguished by the enzyme. To increase the selectivity, bromine was added across the double bond of **64** to increase the difference in size of the substituents (Scheme III).

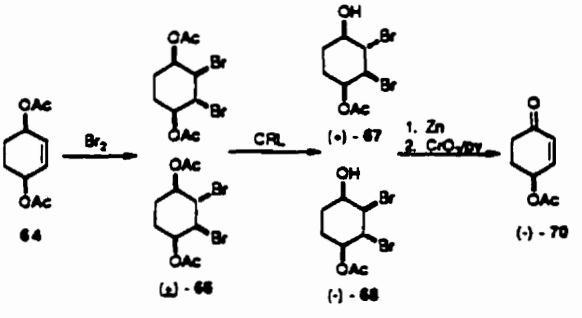
The highest yields for the addition of bromine to **64** were obtained using reaction conditions that favor free radical intermediates (CS_2, −78 °C, *hν*). Addition of bromine under conditions which favor ionic intermediates (polar solvents, dark) resulted in a mixture of products which may have resulted from intramolecular attack of an acetate on the bromonium ion intermediate. The addition of bromine to **64** yielded the trans-dibromide, **66**, identified from coupling constant of 9.3 Hz for the ^{1}H NMR signals for the hydrogens at CHBr (δ 4.36, 4.27), indicating an axial–axial arrangement.[31] For the cis-dibromide a coupling

Table V. Enantioselective Hydrolysis of *cis*-1,4-Diacetoxycyclohexane Derivatives

enzyme[a]	substrate	rate[b]	product	% ee[c]
CE	**64**	0.6	**65**	32
CRL	**64**	4.1[d]	ent-**65**	41
PCL	**64**	0.03	ent-**65**	72
PLE	**64**	18.2[d]	**65**	62
ACE	**64**	18	**65** + ent-**65**	0
MML	**64**	0.0004	ent-**65**	14
CE	(±)-**66**	3.9	(+)-**67**, (−)-**68**	>97, >97
CRL	(±)-**66**	0.4[d]	(+)-**67**, (−)-**68**	>97, >97
PLE	(±)-**66**	4.2[d]	(+)-**67**, (−)-**68**	>97, >97
PCL	(±)-**66**	0.002	(+)-**67**, **68** + diol	14[e]
ACE	(±)-**66**	0.57	(+)-**67**, (−)-**68** + diol[f]	85,[e] >95[g]
MML	(±)-**66**	0.00005	(+)-**67**, **68** + diol[h]	87[e]

[a] ACE = acetylcholinesterase from electric eel, MML = lipase from *Mucor meihei*. [b] Initial activity of the enzyme in units/mg solid. Unit = μmol of ester hydrolyzed/min. [c] Determined by ^{1}H NMR in the presence of Eu(hfc)$_3$. For the hydrolysis of (±)-**66**, the enantiomeric purity was determined for both **67** and **68** after separation by flash chromatography. The ^{1}H NMR signals for the acetyl methyl group of the two enantiomers of **67** or of **68** are separated by 0.4 ppm in the presence of approximately 1.5 equiv of Eu(hfc)$_3$. With this excellent separation even 1.5 mol % of the other enantiomer can be detected as shown by a deliberate addition of racemate to an enantiomerically pure sample. [d] In units/mg protein. [e] For **67**. [f] The ratio of isolated **68**:**67**:diol was approximately 1:4:8 after 0.9 equiv of base had been consumed. [g] For **68**. [h] After 0.7 equiv of base had been consumed, the major product was diol. Only traces of **68** were observed.

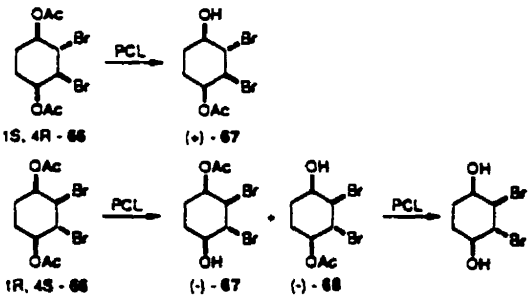
Scheme III. Preparation of 70 via the Dibromide Derivative



(30) Deardorff, D. R.; Matthews, A. J.; McMeekin, D. S.; Craney, C. L. *Tetrahedron Lett.* **1986**, *27*, 1255–1256. Wang, Y.-F.; Chen, C.-S.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1984**, *106*, 3695–3696.

(31) Cooper, J. W. *Spectroscopic Techniques for Organic Chemists*; Wiley: New York, 1980; p 81.

Scheme IV. Hydrolysis of Racemic 66 Catalyzed by PCL



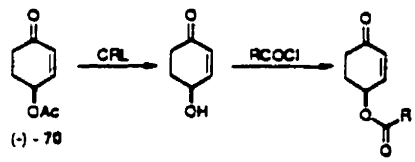
constant of 2–3 Hz would be expected. This trans addition of bromine converted, **64**, a meso compound, into a pair of enantiomers, (±)-**66**. The rule predicts selective hydrolysis of the *R* acetate in both enantiomers yielding the two enantiomerically pure diastereomers: **67** and **68**. Note that the two *R* acetates that are predicted to be hydrolyzed are diastereotopic: one *R* acetate is cis to an adjacent bromine whereas the other *R* acetate is trans.

With CE, CRL, or PLE as catalysts, the hydrolysis of (±)-**66** slowed and stopped after half of the acetates had been hydrolyzed. Analysis of the reaction mixture by TLC showed no remaining starting material, equal amounts of **67** and **68**, and traces of diol which resulted from hydrolysis of both acetates. The two products were enantiomerically pure as shown by ^{1}H NMR in the presence of Eu(hfc)$_3$ (Table V). The opposite sign of rotation of (+)-**67** and (−)-**68** is presumably caused by the differing orientation of the adjacent bromine. Thus, for these three enzymes the hydrolysis of (±)-**66** proceeded as predicted by the rule.

When PCL was used as the catalyst, the reaction did not slow appreciably after half of the acetates had been hydrolyzed. The products isolated from a reaction stopped after half of the acetates had been hydrolyzed were unreacted starting material, (+)-**67** having low enantiomeric excess, a small amount of **68**, and a small amount of diol. Several other enzymes were also screened as possible catalysts, Table V. MML and ACE[4] showed results similar to PCL. Formation of these products can be accounted for by the different reactions of the two enantiomers of **66** as shown in Scheme IV. For (1*S*,4*R*)-**66**, removal of the *R* acetate is predicted by the rule. This acetate is also the more chemically reactive one because it is oriented trans to the adjacent bromine. Hydrolysis occurs as predicted and yields (+)-**67**. For the other enantiomer, (1*R*,4*S*)-**66**, removal of the *R* acetate is also predicted by the rule; however, this acetate is the less chemically reactive one because it is oriented cis to the adjacent bromine. Hydrolysis of both acetates is observed: hydrolysis of the *R* acetate yields (−)-**68**, hydrolysis of the *S* acetate yields (−)-**67**. This last product accounts for the low enantiomeric excess of the isolated **67**. Hydrolysis of both acetates yields the diol. Thus, the rule only partly accounts for the PCL-catalyzed hydrolysis of (±)-**66** due to the differences in chemical reactivity of the acetates caused by the differing orientation of the adjacent bromine. The rule in Figure 1 is too simple to include such effects.

Of the three enzymes which showed excellent selectivity, CRL was chosen for the preparative-scale reaction because it is the least expensive on a unit basis. Hydrolysis of 26 g of (±)-**66** catalyzed by CRL yielded 20 g of enantiomerically pure (+)-**67** and (−)-**68** in 82% yield as cocrystalline diastereomers. To complete the synthesis of **70**, the bromines were removed using zinc dust (82–90% yield) and the resulting olefinic alcohol was oxidized to the enone, **70**, with chromium trioxide/pyridine (84% yield). Other

Scheme V. Preparation of Derivatives of 70



methods of oxidation either gave lower yields (MnO_2, 62%) or gave side products (Swern, PCC/NaOAc, DMSO/Ac_2O, DMSO/Ac_2O/py/CF_3COOH).

The enantiomeric purity of **70** was 98% as shown by ^{1}H and ^{19}F NMR of the Mosher's ester derivative. This derivative was prepared by removal of the acetyl group either by CRL-catalyzed or base-promoted hydrolysis followed by reaction with the acid chloride as shown in Scheme V. As a control, a racemic sample of **70** was also hydrolyzed using CRL and derivatized with the Mosher's acid chloride.[32] For the racemic sample, the diastereomers could be distinguished by ^{1}H NMR (two well-separated multiplets for the proton at the carbinol carbon centered at δ 6.85) or by ^{19}F NMR (two multiplets centered at δ −71.95). The ester derived from enantiomerically pure **70** showed ~1% of the minor diastereomer by either method corresponding to 98% ee. Deliberate addition of material derived from racemic **70** confirmed that the small peaks were due to the other diastereomer.

Determination of the Absolute Configuration of 70. The absolute configuration of **70** was established to be (*S*)-(−) using the exiton chirality method.[33] The acetyl group of **70** was replaced with a benzoyl group as shown in Scheme V. The acetyl group was removed by an enzyme-catalyzed hydrolysis[31] and the product alcohol was treated with benzoyl chloride. The circular dichroism spectrum of the resulting benzoate showed a split Cotton effect, negative at 227 nm ($\Delta\epsilon$ = −5.7) and positive at 192 nm ($\Delta\epsilon$ = +4.0). This splitting indicates a left-handed screw sense between the benzoate and the enone chromophores, i.e. *S*. This assignment of absolute configuration is consistent with the expected enantioselectivity of the three enzymes and with previous assignments for a cyclopentenone[34] and a substituted cyclohexenone.[35]

Discussion

The major advantage of this rule is its simplicity. It is straightforward to use and correlates a large amount of experimental data because it applies to a wide range of substrates. It further suggests a strategy for improving the efficiency of resolutions: to increase the difference in size of the substituents at the stereocenter. Two tests of this strategy were successful because an efficiently resolved substrate/enzyme combination was found for both examples. The strategy of adding a large group to one side of a molecule resulted in a substrate that was efficiently

(32) The enzymic hydrolysis of **70** showed no enantiospecificity; nevertheless, the hydrolysis was continued to completion to assure that no enhancement of enantiomeric purity occurred at this step. Consistent with this notion, the Mosher's ester derived from racemic **70** using CRL-catalyzed hydrolysis showed equal amounts of each diastereomer. The enzyme-catalyzed reaction was preferred because the reaction showed none of the side products observed by TLC during base-promoted hydrolysis.

(33) Harada, H.; Nakanishi, K. *Circular Dichroic Spectroscopy: Exiton Coupling in Organic Stereochemistry*; University Science Books: New York, 1983.

(34) Tanaka, T. Kurosumi, S. Toru, T.; Miura, S. Kobayashi, M.; Ishimoto, S. *Tetrahedron* **1976**, *32*, 1713–1718.

(35) Koreeda, M.; Weiss, G.; Nakanishi, K. *J. Am. Chem. Soc.* **1973**, *95*, 239–240.

resolved by a least one of the enzymes. Thus, screening a substrate with a large group as one of the substituents at the stereocenter appears to be more reliable than screening a substrate having substituents of similar size at the stereocenter.

The major disadvantage of this rule is that it does not account for subtleties in the selectivities of these enzymes. For example, it does not rationalize why only PCL and not the other two enzymes showed increased enantioselectivity for the *tert*-butyl ester of lactyl acetate as compared to the methyl ester. The rule also does not predict the effect of additional stereocenters, for example, the cis vs trans oriented bromine at C_2 of **66**. The sensitivity of PCL to this orientation rendered this enzyme unsuitable for the preparation of **70**, whereas the other two enzymes which were not sensitive to this orientation were suitable.

In spite of this disadvantage, a general rule such as that proposed in Figure 1 may be the most reasonable way to describe the active site of these enzymes. Structural data suggests that large conformational changes are required before the substrate can bind to the active site in pancreatic lipase and lipase from *Mucor meihei*.[3] Due to this flexibility it may never be possible to define an exact size and shape for the substrate binding region of lipases because this region may change for each substrate. Consistent with this notion are reports that the enantioselectivity and conformation of CRL change upon treatment with bile salt and organic solvent[36] or with dextromethorphan.[37]

A second reason to use a general rule is that these enzymes may not be homogeneous. Sequencing of the gene for CRL showed several nonidentical DNA sequences which code for this enzyme,[38] thus it is likely that the commercial enzymes are a mixture of isozymes. The enantioselectivities of isozymes of PLE were similar, but not identical;[39] a similar situation may hold for the isozymes of CRL. This heterogeneity may frustrate attempts to precisely define the size and shape of the active site, thus a general rule may be the most accurate way of describing the commercial catalyst.

A third reason for using rules and models is that even when the X-ray crystal structure of an enzyme is known, models are often used to predict enantioselectivity because they are simpler to use. For example, a high resolution X-ray crystal structure has been determined for alcohol dehydrogenase from horse liver, yet a cubic space model is usually used to predict its enantioselectivity.[40] Further, it remains difficult to predict which binding interactions are most important in an enzyme-substrate complex, thus it may remain difficult to predict enantioselectivity for an untested substrate even when the X-ray crystal structure is known. For example, the origin of the high selectivity for transfer of the *pro*-4*S* hydrogen of NADH catalyzed by lactate dehydrogenase ($>10^6$:1) is difficult to explain from the known crystal structure.[41] These rules and models may be used, along with X-ray crystal structures and molecular modeling, to determine which interactions are most important in determining the enantioselectivity of these enzymes.

(36) Wu, S.-H.; Guo, Z.-W.; Sih, C. J. *J. Am. Chem. Soc.* **1990**, *112*, 1990–1995.
(37) Guo, Z.-W.; Sih, C. J. *J. Am. Chem. Soc.* **1989**, *111*, 6836–6841.
(38) Kawaguchi, Y.; Honda, H.; Taniguchi-Morimura, J.; Iwasaki, S. *Nature* **1989**, *341*, 164–166.
(39) Lam, L. P. K.; Brown, C. M.; De Jeso, B.; Lym, L.; Toone, E. J.; Jones, J. B. *J. Am. Chem. Soc.* **1988**, *110*, 4409–4411.
(40) Jones, J. B. Jacovac, I. J. *Can. J. Chem.* **1982**, *60*, 19–28.
(41) LaReau, R. D.; Anderson, V. E. *J. Biol. Chem.* **1989**, *264*, 15338–15343.

Experimental Section

General. Cholesterol esterase (bovine, 0.2 units/mg solid with 0.1 M cholesterol acetate in ethyl ether as substrate) was purchased from Genzyme Corp., Boston, MA. Lipase from *C. rugosa* (L-1754, 0.2 units/mg solid using olive oil), porcine liver esterase (E-3128, 240 units/mg protein using ethyl butyrate), and acetyl cholinesterase (electric eel, C-3389, 28 units/mg solid with acetyl choline) were purchased from Sigma Chemical Co., St. Louis, MO. Lipase from *P. cepacia* (lipase P30, 0.06 units/mg solid using olive oil) and lipase from *M. meihei* (lipase MAP-10, 0.05 units/mg solid using olive oil) were purchased from Amano International Enzyme Co., Troy, VI. Activated MnO_2 (Aldrich) was heated at 120 °C for 24 h before use. Zinc dust (60 g) was activated by stirring for 1 min with 2% aqueous HCl. The dust was collected by vacuum filtration and washed with 120 mL of the following: 1 × 2% HCl, 2 × 95% ethanol, 1 × anhydrous ethyl ether. Elemental analyses were done by Guelph Laboratories, ON.

Enzyme-Catalyzed Hydrolyses. A rapidly stirred suspension of substrate (1 mmol) in phosphate buffer (10 mM, 10 mL) containing enzyme (0.2–50 mg) was maintained at pH 7.0 by automatic titration with NaOH (0.1 N) using a Radiometer RTS 822 pHstat. Crystalline substrates were first dissolved in ethyl ether (10 mL). Sodium taurocholate (30 mg) was added to hydrolyses where CE was used as the catalyst. The rate of consumption of sodium hydroxide over the first 5% of the reaction was used to calculate the initial rates listed in Tables I, IV, and V. The reaction was stopped after the consumption of base indicated 20–50% conversion and the mixture was extracted four times with ethyl acetate. The combined extracts were washed with saturated aqueous sodium bicarbonate, water, and brine, dried over magnesium sulfate, and concentrated. The starting ester and alcohol were separated by flash chromatography, and the enantiomeric purity was determined.

Determination of Enantiomeric Purity by Gas Chromatography. Alcohol (4 μL), isopropyl isocyanate (300 μL), and dichloromethane (300 μL) were heated at 100 °C for 1.5 h in a tightly sealed glass vial. The completeness of the reaction was checked by TLC. Solvent and excess reagent were evaporated in a stream of nitrogen, and the residue was diluted to 1 mL with dichloromethane and analyzed by gas chromatography using an XE-60-(*S*)-valine-(*S*)-α-phenylethylamide capillary column.

Determination of Enantiomeric Purity by ^{1}H NMR. A 5-mg sample of the ester was dissolved in 0.5 mL of $CDCl_3$ in an NMR tube, and the ^{1}H NMR spectrum was obtained using a Varian XL-200 NMR spectrometer. Solid tris[(3-heptafluoropropylhydroxymethylene)-(+)-camphorato]europium(III), Eu(hfc)$_3$, was added in four portions, and the spectra were obtained. A total of more than 1.3 equiv of shift reagent was added to each sample.

Acetyl Esters. Two to three equivalents of acetyl chloride was added dropwise to a stirred solution of alcohol in pyridine. Solid alcohols were dissolved in a mixture of ethyl ether and pyridine. Acetylation was complete after 10 min to 2 h as shown by TLC. The reaction mixture was washed twice with 10% sodium bicarbonate and once with water. If only one layer formed upon washing, the product was extracted into ether. The organic layer was dried with magnesium sulfate and concentrated by rotary evaporation. The esters were purified either by distillation or by flash chromatography.

(±)-Lactyl acetate was prepared using a literature procedure,[42] but substituting toluene for benzene. A mixture of racemic lactic acid (120 mL of 85% purity, 1.1 mol), glacial acetic acid (640 mL, 11.2 mol), toluene (80 mL), and concd sulfuric acid (0.40 mL) was refluxed with the continuous removal of distillate with a Dean–Stark trap until a ^{1}H NMR spectra of the distillate showed that no more water was present. Approximately 1 L of solution was removed during 40 h; acetic acid (~600 mL) and toluene (~100 mL) were periodically added to the reaction to replace what was removed. The reaction mixture was neutralized with sodium acetate (1.6 g) and distilled under vacuum yielding 86 g (59%): bp 35–37 °C (0.2 Torr) [lit.[43] bp 127 °C (11 Torr)]; ^{1}H NMR

(42) Filachione, E. M.; Langel, J. H.; Fisher, C. H. *J. Am. Chem. Soc.* **1944**, *66*, 494–496.
(43) Windholz, M., Ed. *The Merck Index*, 9th ed., Merck & Co. Inc.: Nuttley, 1976; p 10.

($CDCl_3$, 200 MHz) δ 10.6 (s, 1), 5.10 (q, 1, J = 7.1 Hz), 2.14 (s, 3), 1.53 (d, 3, J = 7.1 Hz).

(±)-Methyl Ester of Lactyl Acetate. A suspension of potassium methoxide (3.8 g, 54 mmol) in dry ethyl ether (100 mL) containing (±)-2-acetoxypropionyl chloride[44] (7.3 g, 50 mmol) was stirred for 72 h. The reaction mixture was washed twice with saturated sodium bicarbonate solution, the organic layer was collected, and the aqueous phase was extracted with ether (2 × 200 mL). The combined organic layers were dried with anhydrous magnesium sulfate, concentrated by rotary evaporation, and distilled, giving 1.6 g (22%): bp 60–64 °C (~4 Torr) [lit.[45] bp 64 °C (9.8 Torr)]; ^{1}H NMR (neat, 60 MHz) δ 4.8 (q, 1, J = 7 Hz), 3.5 (s, 3), 1.8 (s, 3), 1.2 (d, 3, J = 7 Hz).

(±)-*tert*-Butyl Ester of Lactyl Acetate. A two-phase mixture of (±)-lactyl acetate (66 g, 0.5 mol), liquified isobutylene (120 mL, 1.5 mol), ethyl ether (85 mL), and concd sulfuric acid (4 mL) in a 500-mL pressure bottle was sealed with a rubber stopper wired securely like the cork of a champagne bottle and stirred for 7 h at room temperature until a single phase formed. The bottle was chilled in an ice–salt water bath or dry ice–acetone bath and opened, and the contents were slowly added to a saturated phosphate buffer (300 mL, pH 7). The pH of the buffer was maintained between 7 and 8 throughout the addition with a concd sodium hyroxide solution. The combined ether extracts were dried over anhydrous potassium carbonate and filtered into a round-bottomed flask that had been washed with a sodium hydroxide solution and rinsed with water to ensure the removal of trace acid. The ether and excess isobutylene were evaporated under vacuum and the resulting clear, slightly yellow oil was distilled giving 81 g (86%): bp 95–100 °C (~1 Torr); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 4.64 (q, 1, J = 7.1 Hz), 1.84 (s, 3), 1.21 (s, 9), 1.18 (d, 3, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 169.4, 169.3 (C=O), 81.0 ($C(CH_3)_3$), 68.5 (CH), 27.3 ($C(CH_3)_3$), 20.0 (CH_3C=O), 16.3 (CH_3CH).

(±)-*tert*-Butyl Ester of Lactyl Butyrate. Butyryl chloride (244 mL, 2.36 mol) was added dropwise to stirred lactic acid (125 g of 85% purity, 1.18 mol). After addition was complete, the mixture was stirred overnight at room temperature. A ^{1}H NMR spectrum of the reaction mixture indicated no remaining lactic acid. Excess butyryl chloride and butyric acid were removed by vacuum distillation leaving crude lactyl butyrate, 156 g (82%). A portion of this material (125 g, 0.78 mol) was treated with isobutylene (187 mL, 2.34 mol) as described above for the acetate derivative. Distillation yielded 51 g (36%): bp 94 °C (~2 Torr); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 4.95 (q, 1, J = 7 Hz), 2.35 (t, 2, J = 7 Hz), 1.68 (m, 2), 1.45 (s + d, 9 + 3), 0.95 (t, 3, J = 7 Hz); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 173.6, 170.7 (C=O), 82.2 ($C(CH_3)_3$), 69.2 (CH), 36.1 ($OC(O)CH_2$), 28.1 ($C(CH_3)_3$), 18.5 ($OC(O)CH_2CH_2$), 17.1 (CH_3CH), 13.8 ($CH_2CH_2CH_3$).

(*R*)-(+)-*tert*-Butyl Lactate. A suspension of racemic *tert*-butyl ester of lactyl butyrate (50 g, 0.23 mol) in phosphate buffer (400 mL, 0.1 M, pH 7.0) containing PCL (1.0 g) was stirred at room temperature. The pH was maintained between 6.9 and 7.1 by automatic addition of NaOH (0.5 M). After 22 h, 215 mL of base had been added, indicating 47% conversion. The suspension was saturated with sodium chloride and extracted with ethyl ether (4 × 750 mL). The combined extracts were dried over magnesium sulfate and concentrated by rotary evaporation to yield an oil, 42 g. Distillation yielded (*R*)-(+)-*tert*-butyl lactate, 6.4 g (38% of theoretical yield): bp 51–54 °C (4 Torr). [lit.[46] bp 45–47 °C (9 Torr)]; oil which solidifies, mp 35–37.5 °C; $[\alpha]_D$ = +7.98 (*c* 1.7 CH_2Cl_2) [lit.[46] $[\alpha]^{20}_D$ = +9.48 (neat, *l* = 1)]; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 4.62 (q, 1, J = 7 Hz), 2.9 (s, br), 1.49 (s, 9), 1.38 (d, 3, J = 7 Hz); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 175.8 (C=O), 82.6 ($C(CH_3)_3$), 67.2 (CH), 28.2 ($C(CH_3)_3$), 20.7 (CH_3CH); >98% ee by ^{1}H NMR with Eu(hfc)$_3$ on the acetyl derivative. The limit of detection was determined by deliberate addition of racemic *tert*-butyl ester of lactyl acetate to the NMR tube.

***cis*-1,4-Diacetoxy-2-cyclohexene, 64,** was prepared using Bäckvall's method[47] with the following changes. The acetic acid solution was heated to dissolve the palladium diacetate, then cooled prior to the addition of the other reagents. After the reaction completed, the solution was filtered through Whatman no. 41 paper on a Büchner funnel prior to extraction with pentane to minimize the formation of an emulsion.

(±)-1α,4α-Diacetoxy-2β,3α-dibromocyclohexane, 66. A solution of **64** (5.0 g, 25 mmol) in CS_2 (120 mL) was cooled to −78 °C in an acetone/dry ice bath and irradiated with a Phillips 150-W reflector flood lamp placed 35 cm from the reaction mixture. A solution of Br_2 in CS_2 (3.9 M, 8.8 mL, 34 mmol) was added to the stirred reaction mixture in one portion. After 50 min, TLC showed the presence of a small amount of starting material; however, longer reaction time did not result in its disappearance. The reaction mixture was diluted with cold chloroform (−20 °C, 700 mL) and subsequently washed with saturated aqueous Na_2SO_3 (2 × 70 mL), water (70 mL), and brine (2 × 80 mL). The reaction mixture must remain cold until after the first washing with Na_2SO_3. The organic phase was dried over magnesium sulfate and concentrated by rotary evaporation. Recrystallization (ether/hexanes) yielded white crystals, 7.35 g (81%): mp 71.5–72 °C; R_f = 0.3 (4:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 5.32 (m, 1, H4), 5.01 (ddd, 1, H1), 4.36 (dd, 1, $J_{1,2}$ = 9.0 Hz, $J_{2,3}$ = 9.3 Hz, H2), 4.27 (dd, 1, $J_{3,4}$ = 2.7 Hz, H3), 2.15 (s, 3, CH_3), 2.11 (s, 3, CH_3), 2.20–2.00 and 1.70–1.85 (2 m, 4, H5, H5′, H6, H6′); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 169.7, 169.6 (C=O); 73.9, 70.7 (CHOAc), 54.1 (CHBr), 26.1, 25.3 (CH_2), 21.0, 20.9 (CH_3); IR (Nujol mull) 1751, 1731, 1257, 1231, 1024 cm^{-1}; MS (CI, NH_3) m/z 374 (M + NH_4^+, 46); exact mass 373.96018 ($C_{10}H_{18}Br_2NO_4$ requires 373.96026, 2 ppm error). Anal. Calcd for $C_{10}H_{14}Br_2O_4$: C, 33.55; H, 3.94. Found: C, 33.14; H, 3.84.

(1*S*)-(+)-1α-Acetoxy-2α,3β-dibromo-4α-hydroxycyclohexane, (+)-67, and (1*S*)-(−)-1α-Acetoxy-2β,3α-dibromo-4α-hydroxycyclohexane, (−)-68. Lipase from *C. rugosa* (15 g) was added to a stirred mixture of dibromo diacetate, (±)-**66** (26 g, 73 mmol), aqueous phosphate buffer (260 mL, 0.1 M, pH 7.00), and ethyl ether (10 mL). Aliquots of a 0.5 M NaOH solution were added as required to maintain the pH of the mixture between 6.95 and 7.05. After 3 days a total of 1 equiv (73 mmol) of base had been added. The mixture was extracted with ethyl acetate (4 × 750 mL), and the combined extracts were washed with saturated aqueous sodium bicarbonate (600 mL), and brine (600 mL). The organic phase was dried over magnesium sulfate and concentrated by rotary evaporation. Recrystallization of the crude residue from 115 mL of CH_2Cl_2/hexanes (35:65 v/v) yielded cocrystalline diastereomers **67** and **68**, 18.7 g (82%): mp 97–104 °C. A sample of the diastereomers was separated by flash chromatography (4:1 hexanes/ethyl acetate). (+)-**67**: mp 125.5 °C; R_f = 0.34 (3:2 hexanes/ethyl acetate); >97% ee by ^{1}H NMR with Eu(hfc)$_3$; $[\alpha]_D$ = +141° (*c* 1.6, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 5.31 (m, 1, H1), 4.29 (dd, 1, $J_{2,3}$ = 10.8 Hz, $J_{3,4}$ = 8.4 Hz, H3), 4.19 (dd, 1, $J_{1,2}$ = 2.6 Hz, H2), 3.79 (m, 1, H4), 2.66 (br, 1, OH), 2.15 (s, 3, OAc), 1.97–2.17 (m, 2, H5, H6), 1.65–1.88 (m, 2, H5′ and H6′); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 169.6 (C=O), 74.4 (COAc), 71.6 (COH), 63.0 (CHBrCHOH), 54.6 (CHBrCHOAc), 27.0, 26.6 (CH_2), 20.9 (CH_3); IR (Nujol mull) 3409 (br), 1728, 1260, 1072 cm^{-1}. Anal. Calcd for $C_8H_{12}Br_2O_3$: C, 30.41; H, 3.83. Found: C, 30.03; H, 3.54. (−)-**68**: mp 122 °C; R_f = 0.48 (3:2 hexanes/ethyl acetate); >97% ee by ^{1}H NMR with Eu(hfc)$_3$; $[\alpha]_D$ = −125° (*c* 1.6, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 4.97 (ddd, 1, H1), 4.37 (dd, 1, $J_{2,3}$ = 10.1 Hz, $J_{1,2}$ = 8.4 Hz, H2), 4.29 (dd, 1, $J_{3,4}$ = 2.0 Hz, H3), 4.15 (ddd, 1, H4), 2.45 (br, 1, OH), 2.07–2.22 (m, 1, H5), 2.11 (s, 3, OAc), 1.88–2.02 (m, 2, H6 and H6′), 1.58–1.81 (m, 1, H5′); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 169.8 (C=O), 74.6 (COAc), 69.5 (COH), 61.6 (CHBrCOH), 54.8 (CHBrCOAc), 27.6, 24.8 (CH_2), 21.0 (CH_3); IR (Nujol mull) 3429 (br), 1717, 1257, 1034 cm^{-1}. Anal. Calcd for $C_8H_{12}Br_2O_3$: C, 30.41; H, 3.83. Found: C, 30.29; H, 3.52.

(1*S*)-(−)-*cis*-1-Acetoxy-4-hydroxy-2-cyclohexene, 69. Activated zinc dust (31 g, 470 mmol) was added to a mixture of the dibromides **67** and **68** (19.4 g, 61.4 mmol) dissolved in absolute ethanol (300 mL). The suspension was heated and allowed to reflux for 10 min. After cooling of the mixture in a cold water

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bath, pyridine (30 mL) was added. The mixture was filtered, and the filtrate was concentrated by rotary evaporation. The residue was dissolved in ethyl acetate (120 mL) and washed with saturated aqueous sodium bicarbonate (2 × 120 mL) and brine (120 mL). The ethyl acetate solution was dried over magnesium sulfate and concentrated by rotary evaporation, yielding a light yellow oil, 7.9 g (82%).[48] Vacuum distillation gave 6.0 g (62%): bp 75–77 °C (0.05 Torr); R_f = 0.26 (3:2 hexanes/ethyl acetate); $[\alpha]_D$ = −100° (c 1.3, CH_2Cl_2); ^1H NMR ($CDCl_3$, 200 MHz) δ 5.98 (dd, 1, $J_{1,2}$ = 2.8 Hz, $J_{2,3}$ = 10.1 Hz, H2), 5.80 (dd, 1, $J_{3,4}$ = 3.4 Hz, H3), 5.19 (m, 1, H1), 4.18 (m, 1, H4), 2.15 (br, 1, OH), 2.06 (s, 3, CH_3), 1.70–1.96 (m, 4, H5, H5′, H6, H6′); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 170.7 (C=O), 134.8, 127.8 (*CH*=*CH*), 67.2 (COAc), 65.3 (COH), 28.1 (CH_2COAc), 24.9 (CH_2COH), 21.2 (CH_3); IR (neat) 3372 (br), 3415 (br), 1736, 1245, 1037 cm^{-1}; MS (CI, NH_3) *m/z* 174 (M + NH_4^+, 63%); exact mass 174.11306 ($C_8H_{16}NO_3$ requires 174.11302, 0.2 ppm error).

(*S*)-(−)-4-Acetoxy-2-cyclohexen-1-one, (−)-70. Chromium trioxide (16.3 g, 163 mmol) was added to a stirred solution of dry pyridine (26.3 mL, 326 mmol) in dry methylene chloride (380 mL) under nitrogen. After 30 min of stirring at room temperature, the olefinic alcohol 69 (4.24 g, 27.1 mmol) in dry methylene chloride (10 mL) was added to the dark reddish-brown solution. A black tarry substance precipitated after a few minutes. The flask was stoppered with a drying tube, and the mixture was stirred for 24 h. The methylene chloride solution was decanted, and the residue was extracted with alternating portions of ethyl ether and saturated aqueous sodium bicarbonate (2 × 150 mL, 1 × 250 mL each). All extracts were combined with the methylene chloride solution and shaken. The aqueous phase was removed and extracted once with ethyl ether (1000 mL). The organic extracts were washed with saturated aqueous sodium bicarbonate (4 × 250 mL), 2% sulfuric acid (4 × 250 mL), saturated sodium bicarbonate (200 mL), and brine (2 × 200 mL). The resulting organic phase was dried over magnesium sulfate and concentrated by rotary evaporation yielding an oil, 3.52 g (84%): R_f = 0.41 (3:2 hexanes/ethyl acetate); $[\alpha]_D$ = −137° (c 1.6, CH_2Cl_2); ^1H NMR ($CDCl_3$, 200 MHz) δ 6.85 (ddd, 1, $J_{2,3}$ = 10.3 Hz, $J_{3,4}$ = 2.8 Hz, $J_{3,5}$ = −1.4 Hz, H3), 6.06 (ddd, 1, $J_{2,4}$ = −1.9 Hz, $J_{2,6}$ = −0.9 Hz, H2), 5.57 (dddd, 1, $J_{4,5}$ = 4.8 Hz, $J_{4,5'}$ = 8.7 Hz, H4), 2.28–2.70 and 1.99–2.19 (2 m, 3 + 1, H5, H5′, H6, H6′), 2.12 (s, 3, CH_3); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 197.7 (C=O), 170.2 ($OCOCH_3$), 147.5 (*CH*COAc), 130.8 (*CH*C=O), 67.7 (COAc), 34.9 (CH_2C=O), 28.6 (CH_2COAc), 20.9 (CH_3); IR (neat) 1741, 1686, 1372, 1236, 1037 cm^{-1}. MS (CI, NH_3) *m/z* 172 (M + NH_4^+, 100), 155 (M + H^+, 10); exact mass 155.07075 ($C_8H_{11}O_3$ requires 155.07082, 0.4 ppm error).

Enantiomeric Purity of 70. Acetylcholinesterase (4 mg) was added to a stirred suspension of acetoxy ketone 70 (322 mg, 2.09 mmol) in aqueous phosphate buffer (20 mL, 10 mM, pH 7.13). The pH was maintained at 7.13 by automatic addition of NaOH (0.10 N). After 27 h only 0.4 mmol of base had been consumed, thus additional enzyme (CRL, 300 mg) was added. After an additional 41 h a total of 2.1 mmol of base had been added. The reaction mixture was extracted with ethyl acetate (3 × 150 mL), and the combined extracts were washed with saturated aqueous bicarbonate (20 mL), water (20 mL), and brine (2 × 20 mL). The organic phase was dried over magnesium sulfate and concentrated by rotary evaporation yielding crude alcohol, 150 mg (64%): R_f = 0.07 (3:2 hexanes/ethyl acetate). This alcohol was treated with Mosher's acid chloride using a standard procedure.[49] The resulting ester was purified by column chromatography on silica gel eluted with 9:1 hexanes/ethyl acetate; R_f = 0.41 (3:2 hexanes/ethyl acetate). A racemic sample of 70 was also treated in the same manner.

Absolute Configuration of 70. Acetyl cholinesterase (3.6 mg, 0.57 units) was added to a stirred mixture of acetoxy ketone 70 (300 mg, 1.95 mmol) and aqueous phosphate buffer (20 mL, 10 mM, pH 7). Aliquots of a 0.107 N NaOH solution were added automatically to maintain the pH of the mixture at 7.01. Due to the slow rate of the hydrolysis, a large amount (700 mg) of CRL was added in three portions over a period of 6 days. The reaction was stopped at 93% conversion. The reaction mixture was extracted with ethyl acetate (3 × 200 mL). Each organic extract was washed with saturated aqueous sodium bicarbonate (5 mL), water (2 × 5 mL), and brine (2 × 5 mL). The extracts were combined, dried over magnesium sulfate, and concentrated by rotary evaporation, yielding 169 mg (77%); R_f = 0.07 (3:2 hexanes/ethyl acetate). Without further purification of the alcohol, the benzoate derivative was prepared. Benzoyl chloride (340 μL, 2.92 mmol) was added to a solution of alcohol (164 mg 1.46 mmol) in pyridine (3 mL, 37 mmol). The mixture was stirred at room temperature for 70 min at which time TLC analysis showed no remaining alcohol. The reaction mixture was added to a separatory funnel containing 0.5 M H_2SO_4 (74 mL) and ethyl ether (50 mL). After vigorous shaking, additional ethyl ether (150 mL) was added. The aqueous phase was discarded, and the organic phase was washed with saturated aqueous sodium bicarbonate (20 mL) and brine (2 × 20 mL), dried over magnesium sulfate, and concentrated by rotary evaporation. Purification by flash chromatography (85:15 hexanes/ethyl acetate) yielded the benzoate as an oil, 184 mg (58%): R_f = 0.46 (3:2 hexanes/ethyl acetate); $[\alpha]_D$ = −197° (c 1.9, CH_2Cl_2); ^1H NMR ($CDCl_3$, 200 MHz) δ 7.41–8.09 (m, 5, aromatic), 6.98 (ddd, 1, $J_{2,3}$ = 10.3 Hz, $J_{3,4}$ = 2.8 Hz, $J_{3,5}$ = −1.3 Hz, H3), 6.11 (ddd, 1, $J_{2,4}$ = −1.9 Hz, $J_{2,6}$ = −0.8 Hz), 5.82 (m, 1, H4), 2.40–2.77 and 2.15–2.34 (2 m, 3 + 1, H5, H5′, H6, H6′); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 197.8 (C=O), 165.7 (OCOPh), 147.6 (*CH*COBz), 133.4 (*p*-CH), 131.0 (*CH*C=O), 129.7, 128.5 (*o*- and *m*-CH), 129.5 (*C*COOR), 68.2 (*C*OBz), 35.0 (CH_2C=O), 28.8 (CH_2COBz); UV (CH_3OH) 228 nm (ε 14 200 M^{-1} cm^{-1}), 271 nm (ε 554 M^{-1} cm^{-1}); IR (neat) 3050, 2960, 1722, 1683, 1452, 1270, 1113, 710 cm^{-1}; MS (CI, NH_3) *m/z* 217 (M + H^+, 100); exact mass 217.08646 ($C_{13}H_{13}O_3$ requires 217.08647, 0.0 ppm error). The CD spectrum was obtained using a 4.4 × 10^{-5} M solution of the benzoate in CH_3OH in a 0.1-cm cell using a JASCO 500C spectropolarimeter. A total of 10 scans were made from 250 to 185 nm.

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Enantiopreference of Lipase from *Pseudomonas cepacia* toward Primary Alcohols

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We propose an empirical rule that predicts which enantiomer of a primary alcohol reacts faster in reactions catalyzed by lipase from *Pseudomonas cepacia* (PCL). This rule, based on the size of the substituents at the stereocenter, shows an 89% reliability (correct for 54 of 61 examples). This rule is not reliable for primary alcohols that have an oxygen atom attached to the stereocenter; we excluded these alcohols from the tally above. Surprisingly, the sense of enantiopreference of PCL toward primary alcohols is opposite to its enantiopreference toward secondary alcohols. That is, the OH of secondary alcohols and the CH_2OH of primary alcohols point in opposite directions. We suggest, however, that this opposite orientation does not imply a different position of the substituents in the active site of the lipase. Instead, PCL accommodates the extra CH_2 in primary alcohols as a kink between the stereocenter and the oxygen which allows a similar position of the alcohol oxygen in both. We tried to increase the enantioselectivity of PCL toward primary alcohols by increasing the difference in the size of the substituents but did not find a consistent increase in enantioselectivity. We suggest that high enantioselectivity toward primary alcohols requires not only a significant difference in the size of the substituents, but also control of the conformation along the C(1)–C(2) bond.

Introduction

Organic chemists have embraced lipases and esterases as enantioselective catalysts for synthetic applications because they combine broad substrate specificity with high enantioselectivity.[1] One current goal of organic chemists is to map the specificity of these enzymes. This mapping identifies both efficiently resolved substrates and the structural features important for their enantiorecognition, allowing chemists to more rationally design resolutions.

Previous mapping of the specificity of lipase from *Pseudomonas cepacia*[2] (PCL, Amano Lipase P) established a simple rule that predicts its enantiopreference toward secondary alcohols, Figure 1a.[3-6] This rule predicts which enantiomer reacts faster based on the sizes of the substituents at the stereocenter. The same rule holds for ten other hydrolases whose specificities have been mapped: lipase from *Candida rugosa*,[3] lipase from *Pseudomonas* sp.,[7] lipase from *P. aeruginosa*,[8] *Rhizomucor miehei*,[9] lipase from *Arthrobacter* sp.,[10] porcine pancreatic lipase,[11] pancreatic cholesterol esterase,[3] *Mucor* esterase,[11] cultures of *Rhizopus nigricans*,[12] and cultures of *B. subtilus* var. *Niger*.[13] This rule suggests

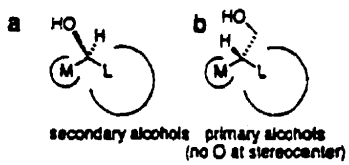

Figure 1. Empirical rules that summarize the enantiopreference of *P. cepacia* lipase (PCL) toward chiral alcohols. (a) Shape of the favored enantiomer of secondary alcohols. M represents a medium substituent, e.g. CH_3, while L represents a large substituent, e.g., Ph. (b) Shape of the favored enantiomer of primary alcohols. This rule for primary alcohols is reliable only when the stereocenter lacks an oxygen atom. Note that PCL shows an opposite enantiopreference toward primary and secondary alcohols.

that these hydrolases distinguish between enantiomers based on the size of the substituents. Consistent with this suggestion, researchers have increased the enantioselectivity of lipase-catalyzed reactions of secondary alcohols by increasing the difference in size of the two substituents.[3,5,14]

Recent X-ray crystal structures of transition state analogs bound to lipase from *Candida rugosa* showed that this rule is a good description of the alcohol binding

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(2) Amano Pharmaceuticals Co. sells several different types of lipase from *P. cepacia*: lipase PS, lipase P, LPL-80, LPL-200S, and SAM-II. All of these types come from the same microorganism but differ in the purification method and the types of stabilizers used. Lipase P or PS is the industrial grade which contains diatomaceous earth, dextran, and $CaCl_2$. LPL-80 and LPL-200S are diagnostic grades that contain glycine. SAM-II differs from lipase P or PS only in the purification method. This type is also sold by Fluka under the name of lipase from *Pseudomonas fluorescens*, SAM-2, because this microorganism was only recently reclassified as *Pseudomonas cepacia*. We thank Dr. Yoshihiko Hirose (Amano Pharmaceuticals Co.) for explaining these differences.

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pocket.[15] This lipase contains a large hydrophobic binding pocket that binds the larger substituent of a secondary alcohol and a smaller pocket that binds the medium substituent. Conserved structural elements create this binding crevice, especially the pocket for the medium substituent. For this reason, other lipases probably contain similar alcohol binding sites.

Several groups have tried to extend this rule for secondary alcohols to include primary alcohols, but these efforts have been only partially successful. Naemura *et al.* used a rule based on the size of the substituents to account for the enantiopreference of lipase from *Pseudomonas* sp. toward two primary alcohols,[16] and Carrea *et al.*[17] used a similar rule to account for the enantiopreference of PCL toward nine primary alcohols. However, Xie *et al.*[18] proposed an enantiomeric rule for the enantiopreference of PCL toward two primary alcohols without an oxygen at the stereocenter and another rule based on two primary alcohols with an oxygen at the stereocenter.[19] Researchers encountered similar difficulties with porcine pancreatic lipase (PPL) and also proposed enantiomeric rules.[20]

In this paper, we examine the reported enantiopreference of PCL and propose an empirical rule that summarizes its enantiopreference toward primary alcohols. Although the rule is opposite to the one for secondary alcohols, we suggest that the large and medium substituents adopt a similar position in both. We also attempt, unsuccessfully, to increase the enantioselectivity of PCL toward primary alcohols by increasing the difference in size of the two substituents at the stereocenter.

Results and Discussion

Enantiopreference of PCL toward Primary Alcohols. Following the suggestion of Xie *et al.*,[18] we divided the primary alcohols into those with an oxygen at the stereocenter and those without an oxygen at the stereocenter. Table 1 and Chart 1 summarize the reported enantioselectivities of PCL toward primary alcohols that lack an oxygen at the stereocenter. The enantiomers shown in Chart 1 are those predicted to react faster by the empirical rule in Figure 1b: the CH_2OH points into the page and the larger substituent lies on the right. The notation "(ent)" in Table 1 marks those examples where PCL favored the enantiomer opposite to the one predicted. This list includes only primary alcohols with a tertiary stereocenter, that is, $R^1R^2CHCH_2OH$. The list further includes only reactions catalyzed by PCL from Amano (P, P30, PS, LPL-80, LPL-200S) and SAM-2 from Fluka,[2] and only those substrates for which the enantiomeric ratio,[21] E, was >2. The list includes esterifications and transesterifications of primary alcohols as well as hydrolyses of esters of primary alcohols.

The rule in Figure 1b predicts the absolute configuration of the favored enantiomer for 49 out of 54 substrates, 91% accuracy. We will discuss seven more examples in this paper, five of which follow the rule giving an accuracy of 54 out of 61 or 89%. The examples include acyclic, cyclic, and bicyclic alcohols with a wide range of functional groups in the substituents. Approximately 30% of the substrates (16 of the 54) showed excellent enantioselectivities of $E > 50$. The five exceptions to the empirical rule are 2-methyl-1,3-propanediol (structure 1 where $R = CH_2OH$), two aziridines, structure 9, and two cyclohexenone derivatives, structure 18. In spite of these exceptions, the empirical rule is reliable for most primary alcohols without an oxygen at the stereocenter. The rule in Figure 1b is similar to the rule proposed by Xie *et al.*[18] for two primary alcohols. Although our rule is opposite to the one suggested by Carrea *et al.*,[17] we excluded their examples because they all contained an oxygen at the stereocenter.

Table 2 and Chart 2 summarize the reported enantioselectivities of PCL toward primary alcohols that have an oxygen at the stereocenter. All structures show the enantiomer predicted to react faster, while the notation "(ent)" marks the exceptions in Table 2. The large number of exceptions shows that the empirical rule is not reliable for this group of substrates. The empirical rule predicts the enantiopreference for only 10 of the 27 examples, corresponding to 37% accuracy which is similar to that expected by chance. We excluded substrate 25 ($R = CH_3$) from the tally because both substituents are similar in size. Approximately 30% of the substrates (8 of 27) showed excellent enantioselectivities ($E > 50$); all of these are exceptions to the empirical rule. Thus, the empirical rule in Figure 1b is not reliable when the primary alcohols have an oxygen at the stereocenter. An opposite rule would be slightly more reliable, but still only a slight improvement over guessing.

A similar division of primary alcohols into two groups, those with and without oxygens at the stereocenter, may also resolve the dilemma of enantiomeric rules in the case of PPL. Our preliminary survey found 41 examples of PPL-catalyzed resolutions of primary alcohols without an oxygen at the stereocenter. Twenty-seven examples fit the rule in Figure 1b and four did not; we excluded ten substrates because the sizes of the substituents were too similar. Thus, the reliability was 27/31 or 87% for those with substituents that differed in size. This degree of reliability is similar to that for PCL. We also found 10 examples of PPL-catalyzed resolutions of primary alcohols which have an oxygen at the stereocenter. Three examples fit the rule and six did not; we excluded one substrate. Thus, as with PCL, the rule is not reliable for primary alcohols that have an oxygen at the stereocenter.

Do the Medium and Large Substituents of Primary and Secondary Alcohols Bind to the Same Regions of PCL? We propose two hypotheses to explain how PCL can have an opposite enantiopreference for primary and secondary alcohols, Figure 2a. Hypothesis 1 proposes that the large and medium substituents of primary alcohols bind in the same L and M pockets as

(15) Cygler, M.; Grochulski, P.; Kazlauskas, R. J.; Schrag, J. D.; Bouthillier, F.; Rubin, B.; Serreqi, A. N.; Gupta, A. K. *J. Am. Chem. Soc.* **1994**, *116*, 3180–3186.

(16) Naemura, K.; Fukuda, R.; Takahashi, N.; Konishi, M.; Hirose, Y.; Tobe, Y. *Tetrahedron: Asymmetry* **1993**, *4*, 911–918.

(17) Carrea, G.; De Amici, M.; De Micheli, C.; Liverani, P.; Carnielli, M.; Riva, S. *Tetrahedron: Asymmetry* **1993**, *4*, 1063–1072.

(18) Xie, Z.-F.; Suemune, H.; Sakai, K. *Tetrahedron: Asymmetry* **1993**, *4*, 973–980. Earlier reports (see reference 4) from this group indicate that PFL is Amano lipase P.

(19) Only two of the three examples supported this suggestion. The original reference for the third example, structure 21 in reference 18, indicated that PCL prefers the enantiomer opposite to the one drawn. Other researchers also noted difficulties in extending the rule for secondary alcohols to primary alcohols. Ferraboschi, P.; Casati, S.; Degrandi, S.; Grisenti, P.; Santaniello, E. *Biocatalysis* **1994**, *10*, 279–288.

(20) Ehrler, J.; Seebach, D. *Liebigs Ann. Chem.* **1990**, 379–388; Wimmer, Z. *Tetrahedron* **1992**, *39*, 8431–8436. Hultin, P. G.; Jones, J. B. *Tetrahedron Lett.* **1992**, *33*, 1399–1402. Guanti, G.; Banfi, L.; Narisano, E. *J. Org. Chem.* **1992**, *57*, 1540–1554.

(21) Chen, C.-S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294–7299

Table 1. Enantioselectivity of Lipase from *Pseudomonas cepacia* toward Primary Alcohols without an Oxygen at the Stereocenter[a]

structure	R or Ar	E[b]	ref	structure	R or Ar	E[b]	ref
1	$CH_2CH_2CH_3$	5.9	*c*	5	$CH_2(CH_2)_2CH_3$	7.3	*c*
1	$CH_2(CH_2)_2CH_3$	8.7	*c*	6	2,4-Cl_2-C_6H_3	~10	*s*
1	$CH_2(CH_2)_4CH_3$	5.7	*c*	6	*N*-Cbz-piperid-4-yl	~7	*t*
1	$CH_2(CH_2)_6CH_3$	9.9, >20	*c,d*	6	NHC(O)-1-naphthyl	~25	*u*
1	$CH_2CH_2CH(CH_3)_2$	11	*c*	6	$CH_2CH(OEt)_2$	>50	*v*
1	*trans*-$CH_2CH{=}CHCH_3$	13	*c*	6	$(CH_2)_2CHMe(CH_2)_2$-*i*-Pr	13	*w*
1	$CH_2CH{=}CHCH_2$	9.7	*c*	7	3,4-OCH_2O-C_6H_3	>~50	*x*
1	CH_2Ph	20	*e*	7	3,4-$(MeO)_2$-C_6H_3	>~50	*x*
1	CH_2(2-thiophene)	>24–>50	*f*	7	3,4,5-$(MeO)_3$-C_6H_2	20	*x*
1	CH_2CH_2SPh	21	*g*	7	4-Ph-C_6H_4	>~50	*x*
1	$CH_2CH_2SO_2Ph$	21	*g*	7	Ph	>30–>50	*j,x,y*
1	$CH_2OC(O)Pr$	~28	*h*	7	1-naphthyl	13, >~50	*x, y*
1	NHC(O)OEt	8.6	*i*	8		11	*z*
1	CH_2OH	4 (ent)	*j*	9	$C(O)OCH_2Ph$	~40 (ent)	*aa*
1	CH_2OAc	2–>50	*k–m*	9	tosyl	>~50 (ent)	*aa*
1	CH_2CH_2OH	~4	*n*	10		5	*bb*
1	$CH_2CH_2OCH_2Ph$	~20	*n*	11		~13	*cc*
1	CH_2O-TBDMS	>50	*m*	12		>50	*dd,ee*
1	CH_2O-TBDPS	>50	*k,m*	13		~4	*dd*
1	CH_2OCH_2Ph	12–35	*m*	14		~3	*ff*
1	$CH_2OC(O)Ph$	9–35	*m*	15		14	*gg*
1	$CH_2CH(CH_3)CH_2OH$	>~50	*j*	16		4	*hh*
2		2–>40	*o*	17		>50	*ii*
3	4-MeO-C_6H_4	7.5–40	*p*	18	CH_3	14 (ent)	*ii*
4	Ph	3.3	*q*	18	CH_2OCH_3	>50 (ent)	*ii*
5	CH_2OAc	~16	*r*	19		>50	*jj*
5	NHC(O)OEt	8.9	*i*	20		~2	*kk*

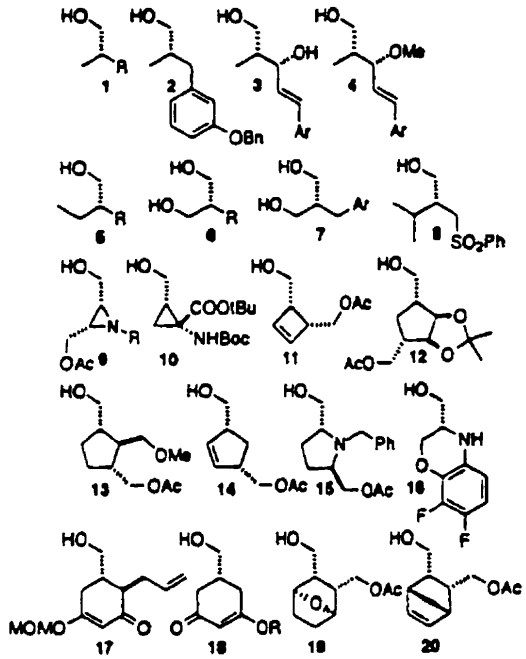
[a] All reactions refer to the hydrolysis of the acetate ester in aqueous solution at room temperature, using lipase from *Pseudomonas cepacia* as defined in reference 2, unless otherwise noted. When another ester was hydrolyzed, it is identified; when the alcohol was esterified, the acylating reagent is identified. Only examples that give $E > 2$ are listed. The structures are shown in Chart 1. [b] E, the enantioselectivity, is calculated as in reference 21, except in the case of asymmetric syntheses where it is the ratio of the preferred enantiomer to the least preferred enantiomer. [c] Vinyl acetate: Barth, S.; Effenberger, F. *Tetrahedron: Asymmetry* **1993**, *4*, 823–833. [d] Vinyl acetate, PFL (Fluka): Ferraboschi, P.; Grisenti, P. Manzocchi, A.; Santaniello, E. *J. Chem. Soc., Perkin Trans. 1* **1992**, 1159–1161. [e] Vinyl acetate: Delinck, D. L.; Margolin, A. L. *Tetrahedron Lett.* **1990**, *31*, 6797–6798. [f] Vinyl acetate: Nordin, O.; Hedenström, E.; Högberg, H. E. *Tetrahedron: Asymmetry* **1994**, *5*, 785–788. Bracher, F.; Papke, T. *Tetrahedron: Asymmetry* **1994**, *5*, 1653–1656. [g] Vinyl acetate, PFL (Fluka): Ferraboschi, P.; Grisenti, P.; Manzocchi, A.; Santaniello, E. *J. Org. Chem.* **1990**, *55*, 6214–6216. [h] Butyrate: Wirz, B.; Schmid, R.; Walther, W. *Biocatalysis* **1990**, *3*, 159–167. [i] Francalanci, F.; Cesti, P.; Cabri, W.; Bianchi, D.; Martinengo, T.; Foà, M. *J. Org. Chem.* **1987**, *52*, 5079–5082. [j] Vinyl acetate: Tsuji, K.; Terao, Y.; Achiwa, K. *Tetrahedron Lett.* **1989**, *30*, 6189–6192. [k] Vinyl acetate, PFL (Fluka): Santaniello, E.; Ferraboschi, P.; Grisenti, P. *Tetrahedron Lett.* **1990**, *31*, 5657–5660. TBDPS = *tert*-butyldiphenylsilyl. [l] Xie, Z.-F.; Suemune, H.; Sakai, K. *Tetrahedron: Asymmetry* **1993**, *4*, 973–980. [m] Vinyl acetate, PFL (Fluka): Grisenti, P.; Ferraboschi, P.; Manzocchi, A.; Santaniello, E. *Tetrahedron* **1992**, *48*, 3827–3834. [n] Vinyl acetate, PFL (Fluka): Grisenti, P.; Ferraboschi, P.; Casati, S.; Santaniello, E. *Tetrahedron: Asymmetry* **1993**, *4*, 997–1006. [o] Ethyl acetate: Bianchi, D.; Cesti, P.; Golini, P.; Spezia, S.; Filippini, L.; Garavaglia, C.; Mirenna, L. *J. Agric. Food Chem.* **1992**, *40*, 1989–1992. [p] Isopropenyl acetate: Akita, H; Umezawa, I.; Nozawa, M.; Nagumo, S. *Tetrahedron: Asymmetry* **1993**, *4*, 757–760. [q] Isopropenyl acetate: Akita, H.; Nozawa, M.; Umezawa, I.; Nagumo, S. *Biocatalysis* **1994**, *9*, 79–87. [r] Gaucher, A.; Ollivier, J.; Marguerite, J.; Paugam, R.; Salaün, J. *Can. J. Chem.* **1994**, *72*, 1312–1327. [s] Ethyl acetate: Bianchi, D.; Cesti, P.; Spezia, S.; Garavaglia, C.; Mirenna, L. *J. Agric. Food Chem.* **1991**, *39*, 197–201. [t] PFL (Fluka): Guanti, G.; Banfi, L.; Brusco, S.; Narisano, E. *Tetrahedron: Asymmetry* **1994**, *5*, 537–540. [u] Vinyl butyrate: Terradas, F.; Teston-Henry, M.; Fitzpatrick, P. A.; Klibanov, A. M. *J. Am. Chem. Soc.* **1993**, *115*, 390–396. [v] Vinyl acetate: Ohsawa, K.; Shiozawa, T.; Achiwa, K.; Terao, Y. *Chem. Pharm. Bull.* **1993**, *41*, 1906–1909. [w] Takabe, K.; Sawada, H.; Satani, T.; Yamada, T.; Katagiri, T.; Yoda, H. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 157–160. [x] Vinyl acetate: Itoh, T.; Chika, J.; Takagi, Y.; Nishiyama, S. *J. Org. Chem.* **1993**, *58*, 5717–5723. [y] Vinyl acetate: Atsumi, S.; Nakano, M.; Koike, Y.; Tanaka, S.; Ohkubo, M.; Yonezawa, T.; Funabashi, H.; Hashimoto, J.; Morishima, H. *Tetrahedron Lett.* **1990**, *31*, 1601–1604. [z] Chloroacetate: Guevel, R.; Paquette, L. A. *Tetrahedron: Asymmetry* **1993**, *4*, 947–956. [aa] Transesterification with 1-butanol: Fuji, K.; Kawabata, T.; Kiryu, Y.; Sugiura, Y.; Taga, T.; Miwa, Y. *Tetrahedron Lett.* **1990**, *31*, 6663–6666. [bb] Vinyl acetate: Burgess, K.; Ho, K.-K. *J. Org. Chem.* **1992**, *57*, 5931–5936. [cc] Harvey, I.; Crout, D. H. G. *Tetrahedron: Asymmetry* **1993**, *4*, 807–812. [dd] Tanaka, M.; Yoshioka, M.; Sakai, K. *Tetrahedron: Asymmetry* **1993**, *4*, 981–996. [ee] PFL (Fluka): Mohar, B.; Stimac, A.; Kobe, J. *Tetrahedron: Asymmetry* **1994**, *5*, 863–878. [ff] PFL (Fluka): Mekrami, M.; Sicsic, S. *Tetrahedron: Asymmetry* **1992**, *3*, 431–436. [gg] In phosphate buffer containing 20% DMSO: Kawanami, Y.; Moriya, H.; Goto, Y. *Chem. Lett.* **1994**, 1161–1162; vinyl acetate: Sibi, M. P.; Lu, J. *Tetrahedron Lett.* **1994**, *35*, 4915–4918. [hh] Sakano, K.; Yokohama, S.; Hayakawa, I.; Atarashi, S.; Kadoya, S. *Agric. Biol. Chem.* **1987**, *51*, 1265–1270. [ii] Vinyl acetate: Miyaoka, H.; Sagawa, S.; Inoue, T;. Nagaoka, H.; Yamada, Y. *Chem. Pharm. Bull.* **1994**, *42*, 405–407. [jj] Patel, R. N.; Liu, M.; Banerjee, A.; Szarka, L. J. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 180–183. [kk] Vinyl acetate: Murata, M.; Ikoma, S.; Achiwa, K. *Chem. Pharm. Bull.* **1990**, *38*, 2329–2331.

the substituents of secondary alcohols. The CH_2OH group must point to the back to place the oxygen in a position similar to that for secondary alcohols. Thus, the opposite enantiopreference for primary and secondary alcohols would stem from accommodating the extra CH_2 in primary alcohols as a kink between the O and the stereocenter.

Hypothesis 2 proposes that the substituents of primary and secondary alcohols bind to different regions of PCL. In particular, both the large and medium substituents of primary alcohols bind in the L pocket, while a hydrogen binds in the M pocket. According to this hypothesis, PCL accommodates the added CH_2 in a primary alcohol as a turn that places the stereocenter of primary alcohols into the L pocket. Stereoselectivity within the L pocket would determine the enantioselectivity of PCL toward primary alcohols. Vanmiddlesworth and Sih[22] proposed that stereoselectivity within the L pocket of a reductase in yeast can influence the diastereoselectivity of reduction reactions.

To distinguish between these two hypotheses, we measured the stereoselectivity of the L pocket in PCL

Chart 1. Structures for Table 1 and Figure 4



and compared it to the enantioselectivity of PCL toward primary alcohols, Figure 2b. Hypothesis 1 predicts no relationship between the stereoselectivity of the L pocket and the enantioselectivity of PCL toward primary alcohols. Hypothesis 2 predicts that the stereoselectivity within the L pocket is the same as the enantioselectivity of PCL toward primary alcohols. As detailed below, we found that the stereoselectivity of the L pocket of PCL was significantly lower that the enantioselectivity of PCL toward primary alcohols; thus, we favor hypothesis 1.

To measure the stereoselectivity of the L pocket in PCL, we measured the stereoselectivity of PCL toward acetates of **35a** and **35b**, Scheme 1. Binding these secondary alcohols to PCL as suggested by the rule in Figure 1a places the β-stereocenter into the L pocket.[23] Thus, the stereoselectivity of PCL toward the β-stereocenter of **35a** and **35b** corresponds to the stereoselectivity of the L pocket.

Scheme 1 summarizes the experimental results for the PCL-catalyzed hydrolysis of the acetate esters of **35a** and **35b**. We prepared alcohols **35a** and **35b** and their acetates as mixtures of diastereomers following literature procedures. After hydrolysis, we determined the relative amounts of each isomer using gas chromatography of the (*S*)-acetyl lactic acid derivatives for **35a** and of the free alcohols for **35b**. To identify the diastereomers, we prepared authentic samples of pure *erythro*-**35a**, and *threo*-**35b** using literature procedures. We confirmed this assignment for **35b** by comparing the ^{1}H-NMR spectrum of the mixture to the ^{1}H-NMR of a known (4:1) mixture of *erythro*- and *threo*-**35b**.[24]

The absolute configurations of the preferred *erythro*- and *threo*-**35a** were (2*R*,3*R*) and (2*R*,3*S*), respectively, based on the negative rotations of the tosyl derivatives. The preferred enantiomer of *threo*-**35b** was (2*R*,3*S*), as determined by chemical correlation to the ketone derivative, (3*S*)-3-methyl-4-phenyl-2-butanone. The rule for secondary alcohols, Figure 1a, predicted the favored configuration at the secondary alcohol for all three of these substrates.[23] On this basis, we assigned the (2*R*,3*R*) configuration to the favored enantiomer for *erythro*-**35b**.

To calculate the α- and β-selectivities, the stereoselectivities at the α and β stereocenters, we used the approach developed by Sih to measure the enantioselectivity of an enzyme.[25] Equation 1 relates the selectivity of the enzyme to experimentally measured quantities: A_0 and B_0 are the initial amounts of each isomer in the starting material and A and B are the amounts remaining after hydrolysis.

$$\text{selectivity} = \frac{\ln(A/A_0)}{\ln(B/B_0)} \qquad (1)$$

We calculated the α-selectivity either from the relative amounts of the (2*R*,3*S*) and (2*S*,3*S*) isomers, $\alpha > 32$ for **35a**, $\alpha > 68$ for **35b**, or from the relative amounts of the (2*R*,3*R*) and (2*S*,3*R*) isomers, $\alpha > 100$ for **35a**, $\alpha > 55$ for **35b**. The α-selectivity was high for each pair of isomers, and the favored enantiomer was the one predicted by the secondary alcohol rule. These results suggest that the **35a**-acetate and the **35b**-acetate bound to PCL in the same manner as other secondary alcohols and that the β-stereocenters bound in the L pocket.

We calculated the β-selectivity from the selectivity between the favored 2*R* isomers, (2*R*,3*S*) and (2*R*,3*R*), to be $\beta = 1.4$ for **35a** and $\beta = 1.8$ for **35b**. We did not calculate the β-selectivity from the pair of minor isomers because these "incorrect" secondary alcohols may bind to PCL in a manner that does not place the β-stereocenter in the L pocket.

To interpret these results, we also measured the enantioselectivity of PCL toward the corresponding secondary alcohols **36a** and **36b**, and the corresponding primary alcohols **37a** and **37b** (**37b** is the same as **1** where R = CH_2Ph, Table 1), Table 3. As expected, PCL favored the (*R*)-enantiomer of secondary alcohols **36a** and **36b** with high selectivity, $E = 54$ and 80, respectively. Also as expected, PCL favored the (*S*)-enantiomer of primary alcohols **37a** and **37b**. The enantioselectivity was low toward **37a**, $E = 2.3$, and moderate toward **37b**, $E = 16$. These enantioselectivities are consistent with those measured previously by others for the corresponding esterification reaction, $E = 1.3$ for **37a**[26] and $E = 20$ for **37b** (Table 1, structure 1 where R = CH_2Ph).

A summary of these selectivities, Figure 2c, shows a low selectivity in the L pocket of PCL (1.4 and 1.8),[27]

(22) Vanmiddlesworth, F.; Sih, C. J. *Biocatalysis* **1987**, *1*, 117–127; see also Shieh, W. R.; Sih, C. J. *Tetrahedron: Asymmetry* **1993**, *4*, 1259–1269.

(23) Our earlier survey (reference 3) showed that this rule shows a 98% reliability (63 of 64 substrates) for PCL-catalyzed reactions of secondary alcohols.

(24) Gammill, R. B.; Nash, S. A.; Bell, L. T.; Watt, W.; Mizsak, S. A.; Scahill, T. A.; Sobieray, D. *Tetrahedron Lett.* **1990**, *31*, 5303–5306. We thank Dr. Gammill for sending us a copy of his ^{1}H-NMR spectrum.

(25) According to this approach, the relative rate of reaction of two competing substrates, v_A/v_B, depends on the concentration of the two substrates, A and B, and on the ratio of the specificity constants, V/K, for the two substrates. The ratio of the specificity constants measures the inherent selectivity of the enzyme for a pair of competing substrates. Integration of the equation yields eq 1 in the text. See reference 21.

$$\frac{v_A}{v_B} = \frac{V_A/K_A}{V_B/K_B} \times \frac{[A]}{[B]}$$

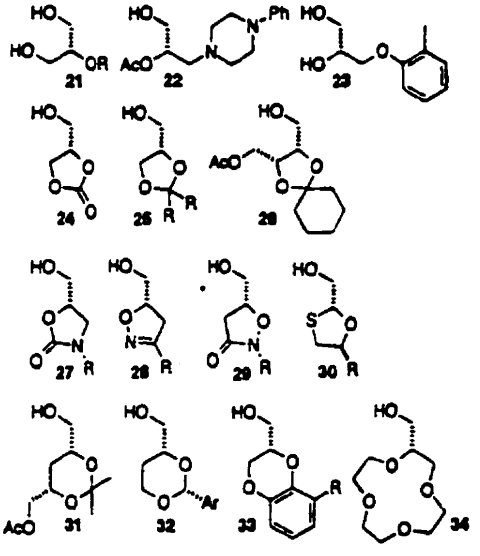
(26) Bianchi, D.; Cesti, P.; Battistel, E. *J. Org. Chem.* **1988**, *53*, 5531–5534.

Table 2. Enantioselectivity of Lipase from *Pseudomonas cepacia* toward Primary Alcohols with an Oxygen at the Stereocenter[a]

structure	R or Ar	E[b]	ref	structure	R or Ar	E[b]	ref
21	CH_3	~6 (ent)	*c*	27	$CH(CH_3)_2$	31->50 (ent)	*i,m*
21	CH_2CH_3	~20 (ent)	*c*	28	Br	6->50 (ent)	*n-p*
21	CH_2Ph	~20-28 (ent)	*c,d*	28	OCH_3	14 (ent)	*o*
22		>50 (ent)	*e*	28	OCH_2Ph	4 (ent)	*o*
23		4	*f*	28	CH_2OH	8 (ent)	*o*
24		15	*g*	29	CH_3	3	*o,p*
25	CH_3	4-9	*h-j*	29	Ph	41 (ent)	*o, p*
25	CH_2CH_3	2-6	*i,j*	30	1-(5-fluorocytosine)	12	*q*
25	Ph	3-8	*i-k*	31		~50 (ent)	*r*
25	$-(CH_2)_6-$	3	*j*	32	Ph	5->50 (ent)	*s*
25	$CH(CH_3)_2$	23	*i*	32	4-OMe-C_6H_4	21->50 (ent)	*s*
25	CH_2Ph	9	*i*	33	H	8-16 (ent)	*t,u*
26		>50 (ent)	*l*	33	OCH_3	28 (ent)	*t*
27	$C(CH_3)_3$	>50 (ent)	*m*	34		3 (ent)	*v*

[a] All reactions refer to the hydrolysis of the acetate ester in aqueous solution at room temperature, using lipase from *Pseudomonas cepacia* as defined in reference 2, unless otherwise noted. When another ester was hydrolyzed, it is identified; when the alcohol was esterified, the acylating reagent is identified. Only examples that give $E > 2$ are listed. The structures are shown in Chart 2. [b] *E*, the enantioselectivity, is calculated as in reference 21, except in the case of meso compounds where it is the ratio of the favored to unfavored enantiomers. [c] Vinyl acetate or phenyl acetate: Terao, Y.; Murata, M.; Achiwa, K.; Nishio, T.; Akamtsu, M.; Kamimura, M. *Tetrahedron Lett.* **1988**, *29*, 5173-5176; vinyl acetate: Murata, M.; Terao, Y.; Achiwa, K.; Nishio, T.; Seto, K. *Chem. Pharm. Bull.* **1989**, *37*, 2670-2672. [d] Breitgoff, D.; Laumen, K.; Schneider, M. P. *J. Chem. Soc., Chem. Commun.* **1986**, 1523-1524; vinyl stearate: Baba, N.; Yoneda, K.; Tahara, S.; Iwasa, J.; Kaneko, T.; Matsuo, M. *J. Chem. Soc., Chem. Commun.* **1990**, 1281-1282; vinyl stearate: Baba, N.; Tahara, S.; Yoneda, K.; Iwasa, *J. Chem. Express* **1991**, *6*, 423-426; Wirz, B.; Schmid, R.; Foricher, J. *Tetrahedron: Asymmetry* **1992**, *3*, 137-142. [e] Transesterification with 1-propanol as nucleophile: Bianchi, D.; Bosetti, A.; Cesti, P.; Golini, P. *Tetrahedron Lett.* **1992**, *33*, 3231-3234. [f] Vinyl acetate: Theil, F.; Weidner, J.; Ballschuh, S.; Kunath, A.; Schick, H. *J. Org. Chem.* **1994**, *59*, 388-393. [g] Pallavicini, M.; Valoti, E.; Villa, L.; Piccolo, O. *J. Org. Chem.* **1994**, *59*, 1751-1754. [h] Benzoate hydrolysis in 25% DMSO: Bosetti, A.; Bianchi, D.; Cesti, P.; Golini, P. *Biocatalysis* **1994**, *9*, 71-77. [i] Succinic anhydride: Terao, Y.; Tsuji, K.; Murata, M.; Achiwa, K.; Nishio, T.; Watanabe, N.; Seto, K. *Chem. Pharm. Bull.* **1989**, *37*, 1653-1655. [j] Butanoate: Partali, V.; Melbye, A. G.; Alvik, T.; Anthonsen, T. *Tetrahedron: Asymmetry* **1992**, *3*, 65-72. [k] Bellemare, M.-J.; Kazlauskas, R. J., unpublished results. [l] In a water/isopropyl ether emulsion: Gais, H.-J.; Hemmerle, H.; Kossek, S. *Synthesis* **1992**, 169-173. [m] Acetic anhydride: Bianchi, D.; Cesti, P.; Battistel, E. *J. Org. Chem.* **1988**, *53*, 5531-5534. [n] Trifluoroethyl butanoate: Secundo, F.; Riva, S.; Carrea, G. *Tetrahedron: Asymmetry* **1992**, *3*, 267-280. [o] Butyrate: Carrea, G.; De Amici, M.; De Micheli, C.; Liverani, P.; Carnielli, M.; Riva, S. *Tetrahedron: Asymmetry* **1993**, *4*, 1063-1072. [p] Butyrate: De Amici, M.; Magri, P.; De Micheli, C.; Cateni, F.; Bovara, R.; Carrea, G.; Riva, S.; Casalone, G. *J. Org. Chem.* **1992**, *57*, 2825-2829. [q] Butyrate: Hoong, L. K.; Strange, L. E.; Liotta, D. C.; Koszalka, G. W.; Burns, C. L.; Schinazi, R. F. *J. Org. Chem.* **1992**, *57*, 5563-5565. [r] Xie, Z.-F.; Suemune, H.; Sakai, K. *Tetrahedron: Asymmetry* **1993**, *4*, 973-980. [s] Vinyl acetate: Herradón, B. *Tetrahedron: Asymmetry* **1992**, *3*, 209-212; *J. Org. Chem.* **1994**, *59*, 2891-2893; Herradón, B.; Valverde, S. *Tetrahedron: Asymmetry* **1994**, *5*, 1479-1500. [t] Acetic anhydride: Ennis, M. D.; Old, D. W. *Tetrahedron Lett.* **1992**, *33*, 6283-6286. These authors also resolved a similar compound, flesinoxan, but its absolute configuration was tentatively assigned: *Tetrahedron Lett.* **1992**, *33*, 6287-6290. [u] Vinyl acetate: Mauleón, D.; Lobato, C.; Carganico, G. *J. Heterocycl. Chem.* **1994**, *31*, 57-59. [v] Tsukube, H.; Betchaku, A.; Hiyama, Y.; Itoh, T. *J. Org. Chem. Soc., Chem. Commun.* **1992**, 1751-1752; *J. Org. Chem.* **1994**, *59*, 7014-7018. We chose the enantiomer shown because a CH_2 is larger than an oxygen.

Chart 2. Structures for Table 2 and Figure 4



while the enantioselectivity toward primary alcohols can be either low or moderate (2.3 and 16). This different selectivity is inconsistent with hypothesis 2 and thus favors hypothesis 1. However, we caution that the addition of a methyl group at the α carbon may inhibit optimal binding of the "primary alcohol portion" within the large pocket and therefore yield an incorrect measure of the selectivity of the L pocket. With this caution in mind, our results nevertheless favor hypothesis 1. Thus, we propose that the medium and large substituent of primary alcohols bind in the same M and L pockets as the substituents of a secondary alcohol. The CH_2-kink between the stereocenter and the oxygen causes the reverse orientation of the OH and the CH_2-OH in Figure 1.

Further support for the notion that PCL accommodates the extra CH_2 of primary alcohols as a kink comes from an overlay of models of primary and secondary alcohols, Figure 3. We overlaid minimized structures of the fast- and slow-reacting enantiomers of a primary alcohol acetate, (*S*)- and (*R*)-2-methyl-3-phenylpropyl acetate, onto the fast-reacting enantiomer of the corresponding secondary alcohol acetate, (*R*)-2-acetoxy-1-phenylpropane. In spite of their opposite configurations, the fast-reacting enantiomers overlay more closely at the reaction center, especially at the alcohol oxygen and at the carbonyl group of the ester. This overlay supports the notion that the medium and large substituents of both primary and secondary alcohols can bind

(27) Barco *et al.* found that the PCL-catalyzed acetylation of the secondary alcohol (*R*)-*N*-benzoyl-3-phenylisoserine methyl ester showed high selectivity for the anti isomer (Barco, A.; Benetti, S.; Derisi, C.; Pollini, G. P.; Romagnoli, R.; Zanirato, V. *Tetrahedron Lett.* **1994**, *35*, 9289-9292). This diastereoselectivity shows that, in some cases, PCL can show high β-selectivity. Nevertheless, the β-selectivity for the simpler structures such as **36a** and **36b** is low.

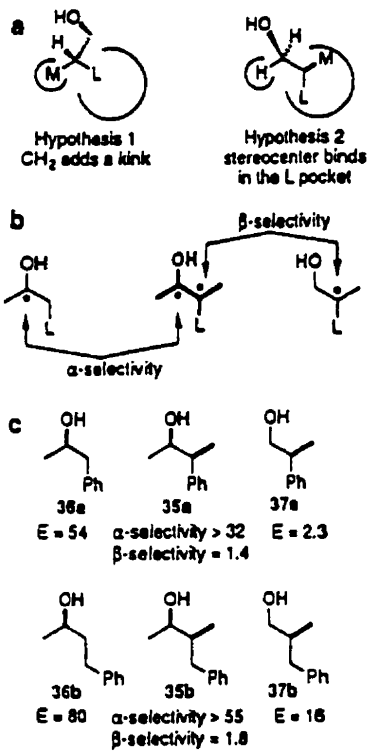


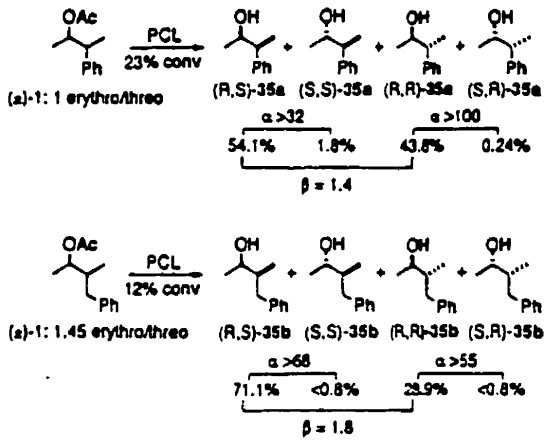
Figure 2. Orientation of the medium and large substituents of primary alcohols in the active site of PCL. (a) Two possible orientations of the medium and large substituents can account for the opposite enantiopreference of PCL toward primary and secondary alcohols. In hypothesis 1, PCL accommodates the extra CH_2 group in primary alcohols as a kink between the stereocenter and the oxygen. The CH_2 group points into the plane of the paper to place the oxygen in a position similar to that in secondary alcohols. In hypothesis 2, PCL accommodates the extra CH_2 group in primary alcohols as a turn that places both the medium and large substituents into the L pocket. According to this hypothesis, enantioselectivity toward primary alcohols comes from details within the L pocket. (b) Measuring the stereoselectivity of the L pocket using a substrate with two stereocenters. Hypothesis 2 predicts that stereoselectivity within the L pocket (β-selectivity) will be similar to the enantioselectivity of PCL toward the corresponding primary alcohol. Hypothesis 1 predicts no relationship between the two selectivities. (c) For two examples, the stereoselectivity within the L pocket was lower than the enantioselectivity for the corresponding primary alcohols. These experimental results favor hypothesis 1. Experimental data for the selectivities are in Scheme 1 and Table 3.

in the same M and L pockets, yet show opposite enantiopreference. Other researchers have also noted a reversal in enantiopreference when a CH_2 group is inserted between the stereocenter and the oxygen of other alcohols. For example, the enantiopreference of the Katsuki–Sharpless epoxidation reverses for allylic and homoallylic alcohols.[28]

This explanation is also consistent with the structure of the active site of lipases and their likely mechanism. Researchers believe that the histidine of the catalytic triad protonates the oxygen of the leaving alcohol.[15] For this reason the alcohol oxygen must adopt a similar position in both structures. Furthermore, modeling

(28) Rossiter, B. E.; Sharpless, K. B. *J. Org. Chem.* **1984**, *49*, 3707–3711.

Scheme 1



suggests that the CH_2OH of a primary alcohol would disrupt the orientation of the catalytic histidine if it pointed in the same direction as the OH of a secondary alcohol.

Increasing Enantioselectivity by Increasing the Size of the Large Substituent. The empirical rule suggests that PCL uses the sizes of the substituents to distinguish between enantiomers. Researchers might enhance enantioselectivity by increasing the difference in size of the substituents. Indeed, this strategy was successful for secondary alcohols.[3,5,14] However, we demonstrate below that this strategy was rarely successful for primary alcohols.

The first group are primary alcohols without an oxygen at the stereocenter, Figure 4a and Table 3. The first example, **37a** vs **38**, showed reversed enantioselectivity when we increase the size of the large substituent. PCL-catalyzed hydrolysis of the acetate ester of **37a**, 2-phenylpropyl acetate (L = Ph, M = CH_3), showed low enantioselectivity, E = 2.3, favoring the enantiomer predicted by the empirical rule. Replacing the phenyl with the larger naphthyl group in **38** increased the enantioselectivity to E = 10, but also reversed the enantiopreference. Taking into account the reversal, the enantioselectivity decreased by a factor of 23.

The next example showed a slight decrease in enantioselectivity when we increased the size of the large substituent. PCL-catalyzed hydrolysis of the acetate ester of **39** showed an enantioselectivity of 9. Replacing the acetyl protective group with the larger phthalimido group (compound **40**) decreased the enantioselectivity by a factor of 1.3 to E = 7. For both **39** and **40**, PCL favored the enantiomer predicted by the empirical rule.

The third example showed a modest increase in enantioselectivity when we increased the size of one substituent. PCL-catalyzed hydrolysis of racemic *cis*-1-acetoxymethyl-2-(hydroxymethyl)cyclohexane, **41**-acetate, showed low enantioselectivity, E = 1.4, in favor of the (1*R*,2*S*)-enantiomer of the substrate, which is opposite to the one predicted by the empirical rule. Increasing the size of the CH_2OH substituent to CH_2OAc (**42**-acetate) or CH_2OBz (**43**-acetate) increased the enantioselectivity to E = 2 and E = 2.4. The favored enantiomer for the products, *cis*-1-(acetoxymethyl)-2-(hydroxymethyl)cyclohexane and *cis*-1-[(benzoyloxy)methyl]-2-(hydroxymethyl)cyclohexane, was (1*R*,2*S*), as predicted by the empirical rule. Taking into account the reversal, the enantiose-

Table 3. Hydrolysis of Acetates of Primary and Secondary Alcohols Catalyzed by Lipase from *Pseudomonas cepacia*

structure	% conv	% ee (product-OH)	abs config (product-OH)	*E*
36a			*R*	54[a]
36b	34	96[b]	*R*[c]	80
37a	14	38[d]	*S*[e]	2.3
37b	44	79[f]	*S*[g]	16
38	40	71[h]	*R*[i]	10
39	47	60[d] (SM)[j]	*S* (SM)[k]	9
40	43	63[l]	*R*[m]	7
41	49	12[d] (SM)[j]	1*S*,2*R* (SM)[n]	1.4
42	46	34[d]	1*R*,2*S*[o]	2.0
43	41	23[d](SM)[j]	1*R*,2*S* (SM)[p]	2.4
44	85	84[q]	nd[r]	12

[a] Bianchi, D.; Cesti, P.; Battistel, E. *J. Org. Chem.* **1988**, *53*, 5531–5534. Nishio, T.; Kamimura, M.; Murata, M.; Terao, Y.; Achiwa, K. *J. Biochem. Tokyo* **1989**, *105*, 510–512: Esterification with acetic anhydride in organic solvent. [b] Determined by GC of the (*S*)-acetyl lactate derivative using a OV-1701 column. [c] $[\alpha]_D = -17.8$ (1.8, $CHCl_3$) (lit. (*R*)-(−): Hayashi, T.; Okamoto, Y.; Kabeta, K.; Hagihara, T.; Kumada, M. *J. Org. Chem.* **1984**, *49*, 4224–4226). [d] Determined by GC using a Chiraldex G-TA column. [e] $[\alpha]_D = -10.4$ (neat) (lit. (*R*)-(−) and (*S*)-(+): Bianchi, D; Cesti, P.; Battistel, E. *J. Org. Chem.* **1988**, *53*, 5531–5534. Bernstein, H.; Whitemore, C. *J. Am. Chem. Soc.* **1939**, *61*, 1326). [f] Determined by GC of the trifluoroacetate derivative using a Chiraldex G-TA column. [g] The product from the PCL-catalyzed esterification of **37a** was identified as the (*S*)-enantiomer (Delinck, D. L.; Margolin, A. L. *Tetrahedron Lett.* **1990**, *31*, 6797–6798). [h] Determined by HPLC using a Chiralcel OD column. [i] $[\alpha]_D = -12.1$ (0.43, C_6H_6), $[\alpha]_D = -19.1$ (0.43, $CHCl_3$) (lit. (*R*)-(−): Menicagli, R.; Piccolo, O.; Lardicci, L.; Wis, M. L. *Tetrahedron* **1979**, *35*, 1301–1306. Sonnet, P. E.; Heath, R. R. *J. Chromatogr.* **1985**, *321*, 127–136). [j] Determined for the unreacted acetyl ester. [k] Determined by comparison of the gas chromatogram (Chiraldex G-TA) of the unreacted acetyl ester with that of the acetyl ester of (*S*)-(+)-**39** obtained from authentic (*S*)-(+)-2-amino-1-butanol. [l] Determined by ^1H NMR of the acetyl ester derivative in the presence of (+)-Eu(hfc)$_3$. [m] Determined by comparison of the ^1H NMR spectrum (in the presence of (+)-Eu(hfc)$_3$) of the acetyl ester of the product with that of the actyl ester of (*S*)-(+)-**40** obtained from authentic (*S*)-(+)-2-amino-1-butanol. [n] Determined by comparison of the gas chromatogram (Chiraldez G-TA) of the unreacted acetyl ester with that of monoacetate **42** obtained from the PCL-catalyzed hydrolysis of the diacetate. [o] $[\alpha]_D = -9.04$ (3.54, $CHCl_3$) (lit. (1*R*,2*S*)-(−): Alder, U.; Breitgoff, D.; Klein, P.; Laumen, K. E.; Schneider, M. P. *Tetrahedron Lett.* **1989**, *30*, 1793–1796. Laumen, K.; Schneider, M. *Terahedron Lett.* **1985**, *26*, 2073–2076). [p] Determined by comparison of the ^1H NMR spectrum (in the presence of (+)-Eu(hfc)$_3$) of *cis*-(1*R*,2*S*)-1-(acetoxymethyl)-2-[(benzoyloxy)methyl]cyclohexane, obtained by benzoylation of monoacetate (1*R*,2*S*)-**42**, to the spectrum of the remaining starting material. The preferred product is therefore *cis*-(1*R*,2*S*)-1-[(benzoyloxy)methyl]-2-(hydroxymethyl)cyclohexane. [q] Determined by ^1H NMR of the Mosher ester. [r] The absolute configuration was not determined.

lectivity increased by modest factors of 2.8 and 3.4, respectively.

The last example in Figure 4a comes from the literature and represents a dramatic increase in enantioselectivity. Tsuji *et al.*[29] reported a low enantioselectivity for the esterification of alcohol **1** where R = CH_2OH, $E = 4$, in favor of the *pro-R* hydroxyl group. The empirical rule predicts the opposite enantiopreference (M = CH_3, L = CH_2OH). When Tsuji *et al.* replaced the methyl with a larger substituent, CH_2Ph (compound **7** where Ar = Ph in Table 1), the enantioselectivity increased to $E > 30$. Since the CH_2OH is now the medium substituent and CH_2Ph is the large substituent, the enantiopreference now agrees with the empirical rule. Other workers also

(29) Tsuji, K.; Terao, Y.; Achiwa, K. *Tetrahedron Lett.* **1989**, *30*, 6189–6192.

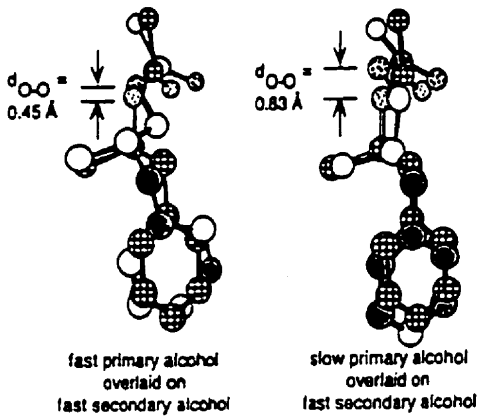


Figure 3. Overlay of the minimized structures of the fast-reacting (*S*)- and slow-reacting (*R*)-enantiomers of the primary alcohol acetate, 2-methyl-3-phenylpropyl acetate (gray carbons) onto the fast-reacting (*R*)-enantiomer of the secondary alcohol acetate, 2-acetoxy-1-phenylpropane (crosshatched carbons). All oxygen atoms are speckled. In spite of the opposite configuration, the (*S*)-primary alcohol acetate mimics the shape of (*R*)-secondary alcohol acetate better than does the (*R*)-primary alcohol acetate. Note the closer overlap of the alcohol oxygens and the carbonyl groups of the acetates. Models were minimized and overlaid using Chem 3-D. Hydrogen atoms are hidden for clarity.

reported increased enantioselectivity when they replaced the methyl by five other CH_2-aryl groups, see Table 1. This last example is the only one where increasing the size of the substituent dramatically increased the enantioselectivity.

For primary alcohols that contain an oxygen at the stereocenter, increasing the size of the large substituent also did not consistently increase the enantioselectivity, Figure 4b. The hydrolysis of the diacetate of **21** (R = CH_2Ph) showed an enantioselectivity of 20–24 (see Table 2). When we increased the size of the substituent to R = $CHPh_2$ (acetyl ester of **44**), the enantioselectivity decreased by a factor of 2 to $E = 12$, Table 3. The second example in Figure 4b comes from the literature. The enantioselectivity of PCL toward the glycerol acetal, substructure **25**, changed little as researchers replaced the methyl with ethyl, phenyl, benzyl, or a cyclohexyl acetal; E ranged from 3 to 9, Table 2. An isopropyl substituent increased enantioselectivity, $E = 23$. Carrea *et al.* also noted that changes in the size of the substituent did not correlate with changes in enantioselectivity for substructures **28** and **29**.[17]

To summarize the effect on enantioselectivity of increasing the difference in the size of the substituents, we found no consistent behavior. Increasing the difference in size may increase, decrease, or have no effect on enantioselectivity. We believe the flexibility of primary alcohols accounts for these observations. Flexibility along the C(1)–C(2) bond allows both enantiomers of primary alcohols to adopt conformations with similar positions of the large and medium substituents, Figure 5. For one enantiomer, a partially eclipsed orientation along the C(1)–C(2) bond orients the large substituent upward and the medium substituent downward. For the other enantiomer, a staggered orientation along the C(1)–C(2) bond also orients the large substituent upward and the medium substituent downward. To distinguish between enantiomers of primary alcohols, PCL must not only distinguish between the large and medium substit-

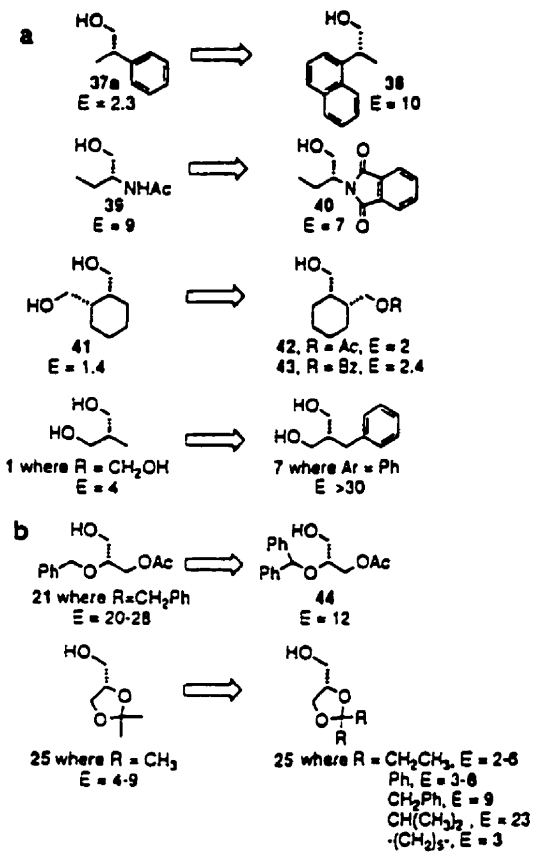


Figure 4. Changes in the enantioselectivity of PCL-catalyzed hydrolyses as the size of one substituent was increased. (a) Four examples where the primary alcohol does not have an oxygen at the stereocenter. The first example showed a reversal in enantioselectivity, the second a small decrease, the third a small increase, and the fourth a large increase. Data for the first three examples are in Table 3; the fourth comes from the literature. (b) Two examples where the primary alcohol has an oxygen at the stereocenter. The first example showed a modest decrease in enantioselectivity, the second example, taken from the literature, showed only small changes in enantioselectivity, except where R = $CH(CH_3)_2$.

uents but also discriminate between the two possible reactive conformations. This explanation may also explain why we needed to exclude primary alcohols with an oxygen at the stereocenter. The oxygen at the stereocenter stabilizes a gauche orientation of the oxygen at the stereocenter and the alcohol oxygen due to the gauche effect.[30] This stabilization may change the favored orientation along the C(1)–C(2) bond and thereby change the enantiopreference.

Experimental Section

General. Lipase from *P. cepacia* (PS30 and LPL-200S) was purchased from Amano International Enzyme Co. (Troy, VI). Unless otherwise noted, organic starting materials were purchased from Aldrich Chemical Co. 4-Phenyl-2-butanol was purchased from Janssen Chimica and *cis*-2,3-epoxybutane from Lancaster. 2-(1-Naphthyl)-1-propanol was prepared from 1-napthylacetic acid as described by Sonnet and Heath.[31] *N,O*-Diacetyl-2-amino-1-butanol, **39**, was prepared by a known method.[32]

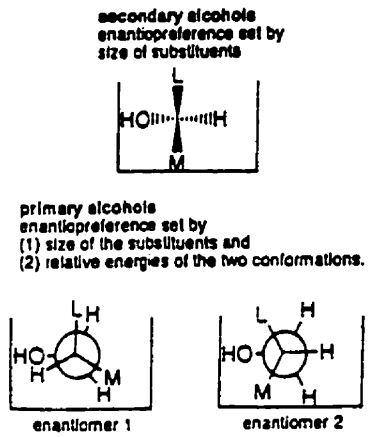


Figure 5. Discrimination between enantiomers of primary alcohols is more difficult than discrimination between enantiomers of secondary alcohols. Flexibility along the C(1)–C(2) bond of primary alcohols allows both enantiomers to adopt conformations with similar orientations of medium and large substituents. For example, enantiomer 1 adopts an upward orientation of the large substituent and a downward orientation of the medium substituent in the eclipsed conformation. Enantiomer 2 adopts a similar orientation in the staggered conformation. To distinguish between enantiomers of primary alcohols, PCL must distinguish between different conformations along the C(1)–C(2) bond in addition to distinguishing between the substituents. The conformations above are only an example. We do not know the conformation of primary alcohols in the active site of PCL.

(±)-*threo/erythro*-3-Phenyl-2-butanol, 35a, was prepared by the nucleophilic addition of methyl magnesium iodide to 2-phenylpropionaldehyde, as described by Overberger *et al.*[33] The ^{1}H NMR of the product agreed with that reported previously.[34] A portion of the product (1 g) was purified by medium pressure chromatography (200 g silica gel, 60% pentane/37% chloroform/3% ethyl acetate, 2.1 min/20 mL fraction). Pure threo alcohol (66.7 mg) and pure erythro alcohol (50.3 mg) were obtained, as well as mixed fractions. Mixed fractions were combined to form a sample (~400 mg) of 50/50 threo/erythro. The threo and erythro isomers can be separated by TLC when eluted three times with 60% pentane/37% chloroform/3% ethyl acetate: on a 7.5 cm plate, R_f = 0.49 (threo) and R_f = 0.40 (erythro). The diastereomers were identified by comparison of the R_f's and ^{1}H NMR of the mixture to that of the pure erythro alcohol prepared as described below.

(±)-*erythro*-3-Phenyl-2-butanol, (±)-*erythro*-35a, was prepared by reaction of phenyllithium with *trans*-2,3-epoxybutane according to a literature procedure.[34] The only change in the procedure was the use of a stock solution of phenyllithium (1.8 M in 70: 30 cyclohexane/ether) instead of preparing the reagent *in situ*. This method was not used for large scale preparation due to low yields (34%). R_f = 0.40 (60% pentane/37% chloroform/3% ethyl acetate). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.17–7.35 (m, 5), 3.88 (apparent quintet, 1, J_{app} ~ 6.2 Hz), 2.73 (apparent quintet, 1, J_{app} ~ 6.7 Hz), 1.55 (br s, 1), 1.32 and 1.08 (two d, 3 + 3, J = 6.6 Hz and J = 5.8 Hz).

Acetyl Esters. Acetic anhydride (1.5 equiv), DMAP (0.05 equiv), and anhydrous sodium carbonate (1.5 equiv) were added to a stirred solution of alcohol in ethyl acetate overnight at ambient temperature. The reaction mixture was then

(30) Wolfe, S. *Acc. Chem. Res.* **1972**, *5*, 102–111.
(31) Sonnet, P. E.; Heath, R. R. *J. Chromatogr.* **1985**, *321*, 127–136.
(32) Bevinakatti, H. S.; Newadkar, R. V. *Tetrahedron: Asymmetry* **1990**, *1*, 583–586.
(33) Overberger, C. G.; Pearce, E. M.; Tanner, D. *J. Am. Chem. Soc.* **1958**, *80*, 1761–1765.
(34) Jones, P. R.; Goller, E. J.; Kauffman, W. J. *J. Org. Chem.* **1971**, *36*, 3311–3315. Kingsbury, C. A.; Thornton, W. B. *J. Org. Chem.* **1966**, *31*, 1000–1004.
(35) Smissman, E. E.; Pazdernik, T. L. *J. Med. Chem.* **1973**, *16*, 14–18.

diluted with ethyl acetate and washed with water and brine, and the organic extracts were dried ($MgSO_4$) and evaporated to afford the pure acetyl ester.

(±)-*threo*-2-Acetoxy-3-phenylbutane, Acetyl Ester of *threo*-35a has been previously prepared.[36] ^1H NMR ($CDCl_3$, 200 MHz) δ 7.18−7.34 (m, 5, aromatic), 5.09 (apparent quintet, 1, J_{app} = 7 Hz), 2.93 (apparent quintet, 1, J_{app} = 7 Hz), 1.92 (s, 3), 1.28 (d, 3, $J_{H4,H3}$ = 7.3 Hz), 1.16 (d, 3, $J_{H1,H2}$ = 6.6 Hz).

(±)-*erythro*-2-Acetoxy-3-phenylbutane, Acetyl Ester of *erythro*-35a has been previously prepared.[36] ^1H NMR ($CDCl_3$, 200 MHz) δ 7.16−7.34 (m, 5), 5.03 (dq, 1, $J_{H2,H1}$ = 6.2 Hz), 2.85 (apparent quintet, 1, J_{app} = 7.3 Hz), 2.06 (s, 3), 1.28 (d, 3, $J_{H4,H3}$ = 7.3 Hz), 1.05 (d, 3, $J_{H1,H2}$ = 6.2 Hz).

(±)-*threo/erythro*-3-Methyl-4-phenyl-2-butanol, (±)-*threo/erythro*-35b, was prepared in three steps following a literature procedure.[37,37] Reduction of α-methyl-*trans*-cinnamaldehyde with lithium aluminum hydride yielded 3-phenyl-2-methyl-1-propanol, (±)-37b. Swern oxidation followed by the addition of methyl magnesium iodide yielded (±)-*threo/erythro*-35b. R_f = 0.26 (4:1 cyclohexane/ethyl acetate). The ^1H NMR of this diasteromeric mixture agrees with that reported in the literature.[38]

(±)-*threo*-3-Methyl-4-phenyl-2-butanol, (±)-*threo*-35b.[39] A solution of benzyl bromide (2.5 mL, 21.0 mmol) in ether (15 mL) was slowly added to magnesium turnings (1 g, 41.1 mmol) under nitrogen. The reaction flask was kept in an ice bath until complete addition, and then the reaction mixture was stirred at room temperature for 1 h. The flask was again cooled, and *cis*-2,3-epoxybutane (0.8 mL, 9.2 mmol) in ether (5 mL) was added dropwise. After complete addition, the mixture was refluxed for 2 h. Excess Grignard was quenched by the careful addition of saturated NH_4Cl. Water was added, and the slurry was filtered into a separatory funnel, rinsing with ethyl acetate. The filtrate was washed with 0.5 N HCl, saturated aqueous $NaHCO_3$, H_2O, and brine, dried over Na_2SO_4, filtered, and evaporated *in vacuo*. Purification by flash column chromatography (9:1 pentane/ethyl acetate) yielded the threo alcohol (125 mg, 8%): ^1H NMR ($CDCl_3$, 200 MHz) δ 7.17−7.33 (m, 5), 3.70 (br apparent quintet, 1, $J_{app} \sim 6$ Hz), 2.88 (dd, 1, $^2J_{H4-H4'}$ = 13.3 Hz, J_{H4-H3} = 4.8 Hz), 2.35 (dd, 1, $^2J_{H4-H4'}$ = 13.3 Hz, $J_{H4'-H3}$ = 9.3 Hz), 1.83 (m, 1), 1.37 (br s, 1), 1.21 (d, 3, J_{H1-H2} = 6.3 Hz), 0.83 (d, 3, J_{H3-CH_3-3} = 6.8 Hz).

(±)-*threo/erythro*-2-Acetoxy-3-methyl-4-phenylbutane, Acetyl Ester of (±)-*threo/erythro*-35b, has been previously prepared.[40] ^1H NMR ($CDCl_3$, 250 MHz) (resonances of the two diastereomers overlap except for the OAc and CH_3-3 resonances) δ 7.09−7.33 (m, 10), 4.76−4.95 (m, 2), 2.72−2.89 (m, 2), 2.20−2.43 (m, 2), 1.82−2.05 (m, 2), 2.05 (s, 3) 2.03 (s, 3), 1.22 (d, 6, $J_{H1,H2}$ = 6.3 Hz), 0.88 (d, 3, $J_{CH_3-3,H3}$ = 6.9 Hz), 0.83 (d, 3, $J_{CH_3-3,H3}$ = 6.8 Hz).

(±)-*threo*-2-Acetoxy-3-methyl-4-phenylbutane, Acetyl Ester of (±)-*threo*-35b, has been previously prepared.[40] ^1H NMR ($CDCl_3$, 250 MHz) δ 7.12−7.29 (m, 5), 4.82 (apparent quintet, 1, $J_{H2,H1}$ = 6.3 Hz, $J_{H2,H3}$ = 6.3 Hz), 2.80 (dd, 1, $^2J_{H4,H4'}$ = −13.4 Hz, $J_{H4,H3}$ = 4.7 Hz), 2.28 (dd, 1, $^2J_{H4',H4}$ = −13.4 Hz, $J_{H4',H3}$ = 9.6 Hz), 2.01 (s, 3), 1.89−2.10 (m, 1), 1.20 (d, 3, $J_{H1,H2}$ = 6.3 Hz), 0.81 (d, 3, $J_{CH_3-3,H3}$ = 6.8 Hz).

(±)-2-Phthalimido-1-butanol, 40, was prepared using a standard method for the protection of amino acids.[41] Phthalic anhydride (1.5 g, 10.1 mmol) and triethylamine (0.6 mL, 4.2 mmol) were added to a solution of (±)-2-amino-1-butanol (500 mg, 5.6 mmol) in toluene (10 mL) in a reaction vessel equipped with a condenser and Dean−Stark trap. After refluxing overnight, the reaction mixture was cooled to room temperature and extracted twice with ethyl acetate (30 + 20 mL). The combined organic extracts were washed with saturated aqueous $NaHCO_3$ (2 × 50 mL), H_2O (50 mL), and brine (50 mL) and dried over anhydrous $NaSO_4$. The crude residue obtained upon evaporation of the solvent was purified by flash column chromatography (7:3 pentane/ethyl acetate) yielding 2-phthalimido-1-butanol as an oil (723 mg, 59%): R_f = 0.20 (7:3 pentane/ethyl acetate); ^1H NMR ($CDCl_3$, 200 MHz) δ 7.79−7.90 (m, 2), 7.68−7.79 (m, 2), 4.29 (m, 1), 4.08 (m, 1), 3.89 (m, 1), 2.73 (br d, 1), 1.92 (apparent decatet, 2), 0.94 (t, 3, J = 7.4 Hz).

1-Acetoxy-2-phthalimidobutane, Acetyl Ester of 40. ^1H NMR ($CDCl_3$, 200 MHz) δ 7.79−7.90 (m, 2), 7.68−7.79 (m, 2), 4.31−4.58 (overlapping m, 3), 1.71−2.22 (overlapping m, 2), 1.97 (s, 3), 0.92 (t, 3, J = 7.4 Hz). Exact mass 261.0999 ($C_{14}H_{15}O_4N$ requires 261.1001, −0.8 ppm error).

***cis*-1-(Acetoxymethyl)-2-[(benzoyloxy)methyl]cyclohexane, Acetyl Ester of 43.** 4-(dimethylamino)pyridine (5.4 mg, 0.044 mmol) and benzoic acid (108 mg, 0.88 mmol) were added to a solution of *cis*-1-(acetoxymethyl)-2-(hydroxymethyl)-cyclohexane (83 mg, 0.44 mmol) in dichloromethane (5 mL). The mixture was cooled in a cold water bath, and dicyclohexylcarbodiimide (100 mg, 0.48 mmol) was added. After stirring at room temperature for 3 days, the solvent was removed by rotary evaporation. The residue, taken up in ethyl ether, was filtered, and the filtrate was washed with 0.5 N HCl, saturated aqueous $NaHCO_3$, H_2O, and brine, dried over Na_2SO_4, filtered, and evaporated *in vacuo*. The crude product was purified by flash column chromatography (gradient from 9:1 to 1:1 pentane/ethyl acetate), yielding *cis*-1-(acetoxymethyl)-2-[(benzoyloxy)methyl]cyclohexane as an oil (68 mg, 53%): R_f = 0.68 (7:3 pentane/ethyl acetate); ^1H NMR ($CDCl_3$, 200 MHz) δ 7.98−8.08 (m, 2), 7.38- 7.62 (m, 3), 4.31 and 4.14 (two d, 2 + 2, J = 7.1 Hz), 2.06−2.32 (m, 2), 1.99 (s, 3), 1.33−1.72 (m, 8). Exact mass 290.1511 ($C_{17}H_{22}O_4$ requires 290.1518, −2.4 ppm error).

2-*O*-(Diphenylmethyl)glycerol Diacetate, Acetyl Ester of 44. *tert*-Butyldimethylsilyl chloride (10 g, 66 mmol) was slowly added to a stirred solution of 1,3-dihydroxyacetone dimer (3 g, 17 mmol) and imidazole (9 g, 133 mmol) in dimethylformamide (22 mL) under nitrogen. The mixture was cooled in a cold water bath until the initial exothermic reaction was over. After stirring at room temperature for 3 h, the reaction mixture was poured into H_2O (800 mL) and extracted with ethyl ether (2 × 700 mL). Each ether extract was washed with H_2O (2 × 800 mL) and dried over $MgSO_4$. The combined ether extracts were filtered and evaporated *in vacuo*, yielding 1,3-bis(*tert*-butyldimethylsiloxy)acetone as a clear colorless oil, 10.45 g (98%): ^1H NMR ($CDCl_3$, 200 MHz) δ 4.41 (s, 4), 0.91 (s, 18), 0.08 (s, 12). Sodium borohydride (237 mg, 6.26 mmol) was added to a stirred solution of the 1,3-bis(*tert*-butyldimethylsilyloxy)acetone (2 g, 6.28 mmol) in dry methanol (40 mL) at 4 °C under nitrogen. After stirring for 30 min, the reaction mixture was quenched with 5% aqueous CH_3COOH (15 mL) and H_2O (150 mL). The mixture was extracted with ethyl acetate (800 mL), and the organic phase was washed with 5% aqueous CH_3COOH (2 × 200 mL), saturated aqueous $NaHCO_3$ (2 × 250 mL), H_2O (200 mL), and brine (300 mL) and dried over $MgSO_4$. The solvent was evaporated to yield the alcohol as a clear colorless oil, 1.98 g (98.5%): R_f = 0.38 (9:1 hexane/ethyl acetate). Diphenyldiazomethane[42] (333 mg, 1.71 mmol) was added to a stirred solution of the alcohol (250 mg, 0.78 mmol) in dry acetonitrile (10 mL). After refluxing overnight, additional diphenyldiazomethane (150 mg, 0.77 mmol) was added and the mixture was again refluxed overnight. The reaction mixture was cooled in a refrigerator for 3 h, and the filtrate obtained after filtration of the resulting white precipitate was concentrated *in vacuo*. The crude oil was purified by flash column chromatography (98:2 hexane/ethyl acetate), yielding 1,3-di-*O*-(*tert*-butyldimethylsilyl)-2-*O*-(diphenylmethyl)glycerol (340 mg, 90%): R_f = 0.65 (9: 1 hexane/ethyl acetate). ^1H NMR ($CDCl_3$, 200 MHz) δ 7.21−7.39 (m, 10), 5.77 (s, 1), 3.49−3.78 (m, 5), 0.87 (s, 18), 0.01 (s, 12). Tetrabutylammonium fluoride (1 N in THF, 0.80 mL, 0.80 mmol) was

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added to a stirred solution of the pure 1,3-di-*O*-(*tert*-butyldimethylsilyl)-2-*O*-(diphenylmethyl)glycerol (130 mg, 0.27 mmol) in THF (3 mL). After stirring for 1 h at room temperature, the reaction mixture was extracted with ethyl acetate (90 mL), washed with H_2O (2 × 5 mL) and brine (2 × 40 mL), and dried over $MgSO_4$. Evaporation of the solvent yielded the crude 1,3-diol (156 mg): R_f = 0.18 (1: 1 hexane/ethyl acetate). Anhydrous sodium carbonate (84 mg, 0.80 mmol), 4-(dimethylamino)-pyridine (3.3 mg, 0.027 mmol), and acetic anhydride (0.075 mL, 0.80 mmol) were added to a solution of the crude diol (150 mg) in ethyl acetate (2 mL). The mixture was stirred at room temperature for 18 h and then extracted with ethyl acetate (50 mL). The organic phase was washed with H_2O (4 × 10 mL) and brine (2 × 10 mL), dried over $MgSO_4$, and evaporated *in vacuo*. The crude residue was purified by flash column chromatography (9:1 hexane/ethyl acetate) to afford 2-*O*-(diphenylmethyl)glycerol diacetate as an oil (70 mg, 77% over two steps). R_f = 0.37 (7:3 hexanes/ethyl acetate). ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.22–7.38 (m, 10), 5.61 (s, 1), 4.21 (m, 4, J_{AB} = 16.5 Hz), 3.86 (m, 1, J_{AX} = 11.6 Hz, J_{BX} = 5.1 Hz), 2.02 (s, 6). Exact mass 342.1471 ($C_{20}H_{22}O_5$ requires 342.1467, 1.1 ppm error).

General Procedure for PCL-Catalyzed Hydrolyses. A rapidly stirred suspension of substrate dissolved in a small amount of ether and phosphate buffer (10 mM, pH 7) containing lipase from *P. cepacia* was maintained at pH 7.0 by automatic titration with NaOH (0.1 N) using a Radiometer RTS 822 pHstat. The reaction was stopped at the desired conversion, and the mixture was extracted once with 9:1 ether/ethanol and three times with ether. The combined organic extracts were washed with saturated aqueous $NaHCO_3$, water, and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The starting esters and product alcohols were separated by flash chromatography.

PCL-Catalyzed Hydrolysis of (±)-*threo,erythro*-2-Acetoxy-3-phenylbutane, Acetyl Ester of 35a. As a pHstat was not available for this hydrolysis reaction, a series of 25 mL-Erlenmeyer flasks containing 2-acetoxy-3-phenylbutane (16 mg, 0.083 mmol, a racemic 1:1 mixture of threo/erythro isomers), ethyl ether (0.3 mL), sodium phosphate buffer (7 mL, 0.1 M, pH 7), and lipase from *P. cepacia* (40 mg) were shaken in an incubator at 29–32 °C. After 2–3 h, the contents of one flask were transferred to a test tube and extracted with ether (5 × 2 mL), mixing with a vortex mixer. Persistent emulsions were broken by centrifugation. The ethereal extracts were dried over Na_2SO_4, filtered, and then analyzed by GC to determine the conversion. The extract was then concentrated to 2 mL. The acetyl lactate derivative was subsequently made to determine the optical and diastereomeric purities of the product alcohols by gas chromatography.

PCL-Catalyzed Hydrolyses of (±)-1-Acetoxy-2-phenylpropane, Acetyl Ester of 37a. Test tubes containing (±)-1-acetoxy-2-phenylpropane (20 mg, 0.112 mmol), ether (0.1 mL), sodium phosphate buffer (0.5 mL, 0.1 M, pH 7), and PCL (2 mg) were shaken in an incubator at 29–32 °C. At various time intervals, the entire contents of one test tube were extracted with ether (4 × 2 mL) using a vortex mixer to mix the two phases. Persistent emulsions were broken by centrifugation. The combined ether extracts were analyzed by GC.

Trifluoroacetyl Esters.[43] Trifluoroacetic anhydride (0.2 mL) was added to a solution of alcohol (1–5 mg) in dichloromethane (0.5 mL). Esterification was complete after 30 min and solvent and excess reagent were evaporated in a stream of nitrogen. The residue was dissolved in ethyl ether for analysis by gas chromatography.

Enantiomeric Purity. Gas Chromatography of Acetyl Lactate Derivatives.[44] (*S*)-*O*-Acetyllactic acid chloride (4 drops) was added to the alcohol (10–15 mg) dissolved in anhydrous ether (2 mL). The mixture was cooled in a cold water bath (5 °C), and pyridine (3 drops) was added. The mixture was stirred for 10–45 min and then stirred at room temperature for 1–3 h. The reaction mixture was washed three times with 0.5 N HCl, twice with saturated aqueous $NaHCO_3$, H_2O, and brine, and dried over Na_2SO_4. This ethereal solution of (*S*)-*O*-acetyllactoyl esters was analyzed by GC using an SE30 or OV-1701 capillary column. The enantiomeric excess values obtained were corrected to account for the optical purity of the derivatizing agent (97.5% ee). ((*S*)-*O*-Acetyllactoyl)-4-phenyl-2-butanol: OV-1701 column, 150 °C, α = 1.06, 21.0 min (*R*), 22.2 min (*S*). *threo*-((*S*)-*O*-Acetyllactoyl)-3-phenyl-2-butanol: SE30 column, 10 min at 160 °C then gradient from 160–220 °C at 3 °C/min, α = 1.03, 22.0 min (2*R*,3*S*), 22.6 min (2*S*,3*R*). *erythro*-((*S*)-*O*-Acetyllactoyl)-3-phenyl-2-butanol: SE30 column, 10 min at 160 °C then gradient from 160–220 °C at 3 °C/min, α = 1.02, 22.8 min (2*R*,3*R*), 23.3 min (2*S*,3*S*).

Enantiomeric Purity. Gas Chromatography Using a Chiral Stationary Phase. The alcohol, acetyl ester, or trifluoroacetyl ester was dissolved in ether or ethyl acetate and analyzed by GC using a Chiraldex G-TA30 capillary column (Astec, Inc., Whippany, NJ). 1-Acetoxy-2-phenylpropane: 90 °C, α = 1.03, 36.0 (*S*), 37.0 min (*R*). 2-Phenylpropanol: 90 °C, α = 1.06, 39.3 min (*S*), 41.6 min (*R*). 2-Methyl-3-phenylpropanol: 90 °C, α = 1.01, 46.4 min (*S*), 47.3 min (*R*). 1-(Trifluoroacetoxy)-2-methyl3-phenyl-propane: 70 °C, α = 1.03, 49.9 min (*R*), 51.2 min (*S*). *erythro*-3-methyl-4-phenyl-2-butanol: 100 °C, α = 1.02, 28.9 min (2*S*,3*S*), 29.6 min (2*R*,3*R*). *threo*-3-methyl-4-phenyl-2-butanol: 100 °C, α = 1.02, 31.6 min (2*S*,3*R*), 32.4 min (2*R*,3*S*). *threo*- and *erythro*-2-acetoxy-3-methyl-4-phenylbutane: 100 °C, 37.5 min (2*S*,3*S*), 38.3 min (2*S*,3*R*), 40.5 min (2*R*,3*S* and 2*R*,3*R*). *N*,*O*-Diacetyl-2-amino-1-butanol: 120 °C, α = 1.03, 33.9 min (*S*), 34.9 min (*R*).

Enantiomeric Purity. ^{1}H-NMR. (1) The racemic acetyl ester was dissolved in an NMR tube, and the ^{1}H NMR spectrum was obtained using a 200 MHz spectrometer. Solid tris[[3-(heptafluoropropyl)hydroxymethylene]-(+)-camphorato]-europium(III), (+)-Eu(hfc)$_3$, was added portionwise until base-line separation of the acetate signals was obtained. The number of equivalents of shift reagent necessary to obtain base-line separation of peaks was then added to the sample for which the enantiomeric purity was to be determined. (2) The alcohol was treated with Mosher's acid chloride using a standard procedure[45] and the resulting ester was analyzed by 500 MHz ^{1}H NMR spectroscopy.

Enantiomeric Purity. HPLC Using a Chiral Stationary Phase. A sample of the alcohol and acetyl ester dissolved in the eluting solvent was analyzed by HPLC using a Chiralcel OD column. 2-(1-Naphthyl)-1-propanol (99: 1 hexane/2-propanol, 0.5 mL/min, α = 1.26, 18.4 min (*S*), 23.2 min (*R*).

Absolute Configurations. 35a: The tosyl derivatives of the separated threo and erythro alcohols were prepared as previously described and their optical rotary powers were compared to literature values. Erythro tosylate: $[\alpha]_D$ = −14.8 (5.64, benzene), lit.[35] (*R*,*R*): $[\alpha]_D$ = −17.41. Threo tosylate: $[\alpha]_D$ = −12.05 (4.73, benzene), lit.[35] (*R*,*S*): $[\alpha]_D$ = −16.89.

35b: The alcohol obtained from the PCL-catalyzed hydrolysis of *threo*-2-acetoxy-3-methyl-4-phenylbutane was oxidized as follows. Jones reagent (~0.1 mL) was added dropwise to the alcohol (18 mg) in acetone (5 mL) at 0 °C until the orange color persisted. After stirring for 30 min, 2-propanol was added until the mixture was green. After an additional 10 min, sodium hydroxide (1 M) was added until pH 7 was reached. The mixture was filtered and the solvent was evaporated *in vacuo* from the filtrate. The residue was dissolved in ethyl acetate, washed twice with both water and brine, and dried over $MgSO_4$. The crude product obtained upon evaporation of the solvent was purified on a preparative TLC plate (95:5 pentane/ethyl acetate), R_f = 0.26. The ketone obtained was identified as the *S* enantiomer, $[\alpha]_D$ +4.9 (*c* 4.1,

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ethanol), lit.[46] *S*-(+), therefore since the starting material was the acetyl ester of *threo*-**35b**, the preferred product is the (2*R*,3*S*) isomer. By comparison of the GC chromatogram (Chiraldex-GTA 30) of the product obtained from hydrolysis of the threo ester to the chromatogram of the products obtained from the hydrolysis of the racemic threo/erythro mixture, it was determined that the preferred product of the latter hydrolysis was also the (2*R*,3*S*) isomer.

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A structure-based rationalization of the enantiopreference of subtilisin toward secondary alcohols and isosteric primary amines

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Abstract

Lipases favor one enantiomer of secondary alcohols (HOCHRR') and isosteric primary amines (NH_2CHRR'), while subtilisin favors the other enantiomer. In both cases, simple rules based on the size of the substituents at the stereocenter predict which enantiomer reacts faster. Thus, lipases and subtilisin are a pair of complementary enantioselective reagents for organic synthesis. The success of these rules suggests that these hydrolases distinguish between enantiomer primarily by the size of the substituents. Previously, we proposed a molecular mechanism for the enantiopreference of lipases based on the X-ray crystal structure of transition state analogs bound to a lipase. Here we suggest that a similar mechanism can also account for the opposite enantiopreference of subtilisin. The catalytic machinery (catalytic triad plus the oxyanion-stabilizing residues) in lipases is approximately the mirror image of that in subtilisin. In both hydrolases, the protein fold, as it assembles the catalytic machinery, also creates a restricted pocket for one substituent in the substrate ('M' or medium-sized). However, the catalytic His residue lies on opposite sides of this pocket in the two hydrolases. We propose that enantioselection arises from (1) the limited size of this pocket, (2) and a required hydrogen bond between the catalytic His and the oxygen or nitrogen of the alcohol or amine. This mechanism for enantioselection differs from that proposed by Derewenda and Wei who focussed on which carbonyl face in the ester or amide is attacked. Lipases and subtilisin indeed attack opposite faces, but we propose that this difference does not set the enantiopreference toward secondary alcohols.

Keywords: Lipase; Subtilisin; Enantioselectivity; Secondary alcohols; Primary amines; Models; Regioselectivity; α/β-Hydrolase; Subtilase

1. Introduction

Synthetic chemists often use proteases and lipases as enantio- and regioselective reagents [1,2]. To simplify the use of these reagents, chemists developed rules, or generalizations, about their selectivity. For example, many researchers proposed rules to predict which enantiomer of a secondary alcohol reacts faster in lipase- and esterase-catalyzed reactions. A simple rule, Fig. 1, looks only at the relative sizes of the substituents, but some rules also include polarity or specific size restrictions for the two substituents. These rules have helped chemists use lipases as synthetic reagents since they suggest that lipases discriminate between enantiomers mostly by the sizes of the substituents. For example, resolutions of secondary alcohols where both 'L' and 'M' have similar sizes are rarely efficient, and chemical modifications that increase the difference in size often result in increased enantioselectivity. Recently, Smidt et

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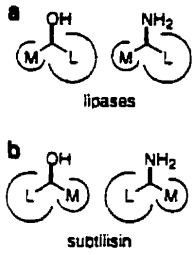


Fig. 1. Empirical rules that predict the enantiopreferences of lipases and subtilisins toward secondary alcohols and primary amines of the type NH_2CHRR'. (a) Lipases favor the enantiomer with the shape shown where L is a large substituent such as phenyl and M is a medium substituent such as methyl. This rule applies to all lipases and esterases whose substrate specificity has been mapped: thirteen lipases for secondary alcohols and three lipases for amines. Fig. 2 summarizes the amines tested. (b) Subtilisin has an opposite enantiopreference to lipases. Fitzpatrick and Klibanov proposed the rule shown for five secondary alcohols. These and other examples to support the rule for subtilisin are collected in Fig. 3Fig. 4Fig. 5.

al. suggested that a similar rule can also account for the enantiopreference of a lipase toward the isosteric primary amines of the type NH_2CHRR' [3].

Using X-ray crystallography, Cygler et al. identified how the enantiomers of menthol, a typical secondary alcohol, bind to lipase from *Candida rugosa* [4] in the transition state. The alcohol binding site resembled the rule in Fig. 1. It contained a large hydrophobic binding site open to the solvent for the large substituent and a restricted region for the medium-sized substituent. Importantly, the catalytic machinery (Ser–His–Glu triad and the oxyanion-stabilizing residues) and the loops that orient this machinery created the pocket for the medium substituent. The catalytic His residue made a hydrogen bond to the menthol oxygen of the fast-reacting enantiomer, but could not reach this oxygen in the slow-reacting enantiomer because the oxygen pointed away from the His residue. Cyger et al. proposed that this lack of a hydrogen bond accounted for the slower reaction.

The X-ray crystal structures of other lipases and esterases showed that, in spite of little similarity in amino acid sequence, they all fold similarly [5]. This protein fold, named the α/β-hydrolase fold, arranges the catalytic machinery similarly in all lipases and esterases. This similarity allowed a simple rationalization for why lipases and esterases show the same enantiopreference toward secondary alcohols and isosteric primary amines: the similar catalytic machinery restricts the size of the medium pocket in all lipases and an esterases. In addition, the catalytic His lies on the same side of the alcohol binding pocket.

Subtilisin, an alkaline serine protease, contains catalytic machinery that is the approximate mirror image of that in α/β-hydrolases [5]. Fitzpatrick and Klibanov found that subtilisin favored the enantiomer opposite to the one favored by lipases. On the basis of five secondary alcohols they proposed a rule for the enantiopreference of subtilisin opposite to the one for lipases [6]. In this paper, we review the enantiopreference of subtilisin toward secondary alcohols and isosteric primary amines and confirm that its enantiopreference is opposite to that of lipases and esterases. In addition, we show that lipases and subtilisin also have opposite regioselectivity. To rationalize this opposite selectivity, we show how the enantioselection mechanism proposed for lipases can also account for the enantiopreference of subtilisin.

2. Results

2.1. Enantiopreference of lipases toward primary amines

Researchers only recently resolved amines using lipases and have examined the substrate specificity of only three lipases, Fig. 2. Lipase B from *Candida antarctica* (CAL-B) is the most popular [7–12], although lipase from *Pseudomonas cepacia* (PCL) [12], and lipase from *Pseudomonas aeruginosa* (PAL) [13] also show high enantioselectivity. Fig. 2 omits several efficiently-resolved amines because the authors did not establish their absolute configurations [12]. Smidt et al. [3] proposed extending

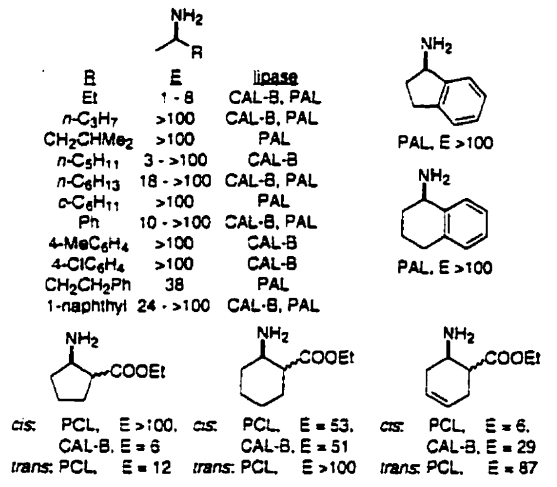


Fig. 2. Enantiopreference of lipases toward primary amines of the type NH_2CHRR'. Lipases favored acylation of the enantiomer shown or hydrolysis of the corresponding amide. CAL-B: acylation or hydrolysis using lipase B from *Candida antarctica*; PCL: acylation using lipase from *Pseudomonas cepacia* with either trifluoroethyl acetate or trifluoroethyl chloroacetate; PAL: acylation using lipase from *Pseudomonas aeruginosa*. All twenty two examples fit the rule in Fig. 1a. For references, see text.

the secondary alcohol rule to primary amines for CAL-B and indeed all of the amines in Fig. 2 fit this rule. Thus, as with secondary alcohols, the rule in Fig. 1a reliably predicts which enantiomer of primary amines reacts faster in lipase-catalyzed reactions.

2.2. Enantiopreference of subtilisin toward secondary alcohols and primary amines

Fig. 3 summarizes the stereoselectivity of subtilisin toward alcohols and amines. For the thirteen secondary alcohols [6,14–20], eleven follow the rule in Fig. 1b, two do not (3-quinuclidol [18] and one of the two reactive hydroxyls in the inositol derivative [19]), giving overall accuracy of 85%. A possible rationalization for the 3-quinuclidol exception is that solvation of the nitrogen increases the effective size of that substituent. Both substituents in the 1,4-diacetoxy-2-cyclohexene [20] are similar in size so this substrate was excluded from the tally.

For primary amines, all thirteen examples [21–23] fit the rule in Fig. 1b. To resolve these amines researchers used subtilisin to catalyze the acylation with trifluoroethyl butyrate or the alkoxycarbonylation with diallyl carbonate. Thus, simple rules based on the size of the substituents predict the enantiopreference of subtilisin toward secondary alcohols and primary amines. However, the favored enantiomer is opposite of the one favored by lipases.

To further emphasize the opposite enantiopreference of lipases and subtilisins, Fig. 4 compares four enantioselective reactions where researchers tested both subtilisin and lipases. In all four cases, lipases and subtilisin showed an opposite enantioselectivity. In the cyclohexanols

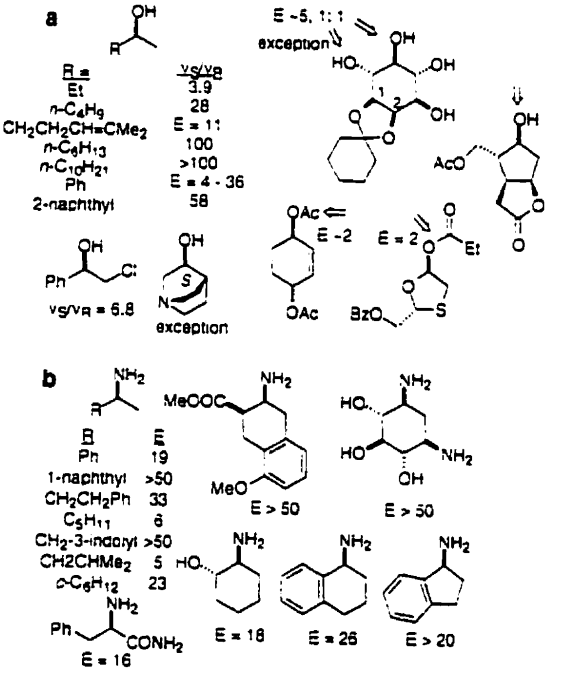


Fig. 3. Enantiopreference of subtilisins (Carlsberg or BPN') toward secondary alcohols and isosteric primary amines. (a) Fast-reacting enantiomer in the acylation of the alcohol or in the hydrolysis of the corresponding ester. Some researchers estimated the enantioselectivity by measuring the initial rate of reaction of the two enantiomers separately. In these cases, the relative rates, v_S / v_R, are given. For examples without an E value, there was insufficient information to calculate it. Eleven of the thirteen alcohols fit the rule for an overall accuracy of 85%. The two exceptions to the rule are marked 'exception'. (b) Fast-reacting enantiomer of primary amines of the type NH_2CHRR' in acylation with trifluoroethyl butyrate or alkoxycarbonylation with diallyl carbonate. All thirteen examples fit the rule in Fig. 1b.

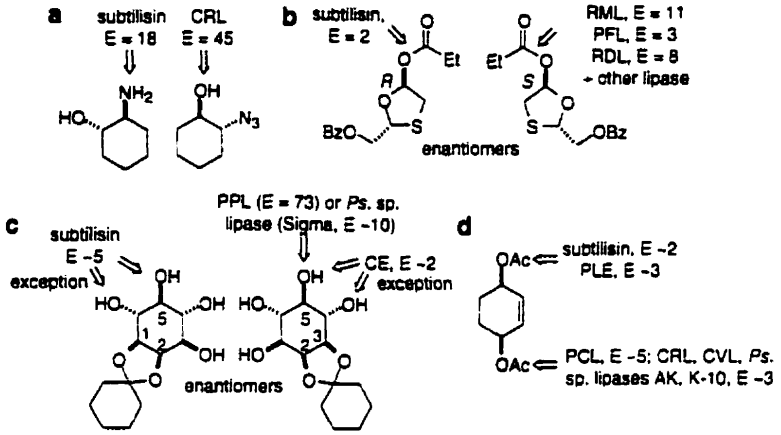


Fig. 4. Four examples of opposite enantioselectivity of lipases and subtilisin in the same or similar molecules. (a) Subtilisin catalyzed the allyloxycarbonylation of the amino group at the (*S*)-stereocenter, while in a similar molecule, CRL catalyzed the enantioselective hydrolysis of the butyrate ester of the (*R*)-alcohol. (b) Subtilisin catalyzed the hydrolysis of the 2*R* propionate, while lipases catalyzed hydrolysis of the 2*S* propionate. (c) Subtilisin catalyzed the acetylation of the 5-OH in one enantiomer of the protected *myo*-inositol, while PPL, lipase from *Pseudomonas* sp. (Sigma), and cholesterol esterase (CE) catalyzed the acetylation of the 5-OH in the other enantiomer. Subtilisin and CE also catalyzed acetylation of the 6-OH. (d) Subtilisin and PLE favored hydrolysis of the acetoxy group at the (*R*)-stereocenter, while five lipases favored hydrolysis of the acetoxy group at the (*S*)-stereocenter. The rules in Fig. 1 predict the reaction in a, b and the 5-OH in c. The substituents in d are too similar in size to make predictions. The acetylation of the 6-OH in c is an exception to the rules. Abbreviations: lipase from *Candida rugosa*, CRL; lipase from *Pseudomonas cepacia*, PCL; lipase from *Chromobacterium viscosum*, CVL; lipase from two different *Pseudomonas* species, lipase AK and lipase K-10; pig liver esterase, PLE; pig pancreatic lipase, PPL.

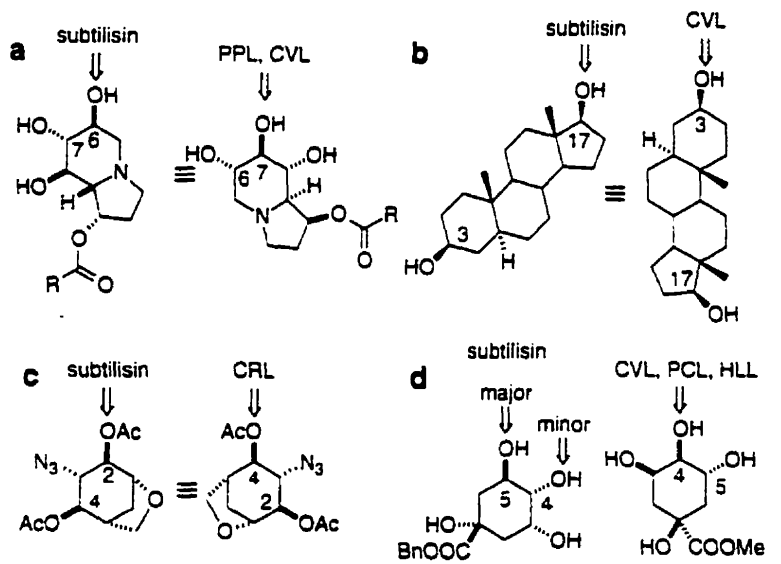


Fig. 5. Four examples of opposite regioselectivity of lipases and subtilisin. (a) Subtilisin favored acylation of the 6-position of 1-*O*-acylcastanospermine by as much as > 20: 1, while lipases porcine pancreatic lipase (PPL) and lipase from *Chromobacterium viscosum* (CVL) favored acylation of the 7-position by as much as 10:1. (b) Subtilisin catalyzed acylation of only the 17-OH in 5α-androstane-3β,17β-diol, while CVL catalyzed the acylation of only the 3-OH. (c) Subtilisin catalyzed hydrolysis of the acetate at the 2-position, while lipase from *Candida rugosa* (CRL) catalyzed hydrolysis at the 4-position. (d) Lipase from *Pseudomonas cepacia* (PCL), lipase from *Humicola lanuginosa* (HLL), and CVL catalyzed acylation of only the 4-OH of benzyl quinate with trifluoroethyl butanoate, while subtilisin catalyzed the acylation methyl quinate at both 5-OH and 4-OH (1.8:1).

the opposite selectivity refers to similar molecules [22,24], while in the 1,3-oxathiolane and inositol derivatives it refers to enantiomers. One of the subtilisin-catalyzed acylation in the inositol is an exception to the rule [18]. For *meso*-1,4-diacetoxy-2-cyclohexene, subtilisin and most lipases catalyzed hydrolysis of opposite acetates, although the enantioselectivity is low and the substituents have similar sizes [19]. Fig. 4 omits two examples. First, subtilisin and CRL showed an opposite enantioselectivity toward (±)-α-methylbenzylamine in the reaction with (±)-ethyl 2-chloropropionate [25]. The sense of enantiopreference was as predicted in Fig. 1, but the additional stereocenter in the chloropropionate complicates the interpretation. Second, subtilisin and lipases CRL, PPL, and CE showed high, but opposite, enantioselectivity in the hydrolysis of chloral acetyl methyl acetal – $Cl_3CC(OAc)OMe$, but the absolute configuration was not established [26].

This opposite stereoselectivity also extends to the regioselectivity of lipases and subtilisin. Subtilisin and lipase showed opposite regioselectivity toward the secondary alcohol positions in castanospermine, Fig. 5a [27], anhydro-sugar derivative, Fig. 5b [28], steroids, Fig. 5c [29], and quinic acid derivatives, Fig. 5d [30].

Note that the stereoselectivity of subtilisin toward alcohols and amines is often lower than that of lipases. For subtilisin, like other proteases, the binding of the acyl chain (the S_1 binding site [31]) dominates the structural selectivity, while the alcohol binding site is shallow compared to the alcohol binding site in lipases. For synthetic applications, subtilisin usually

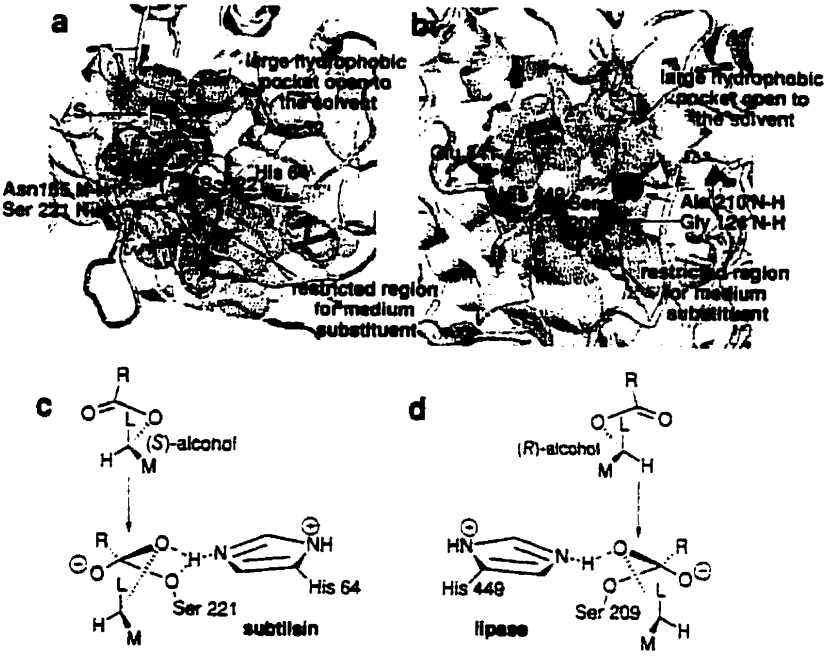


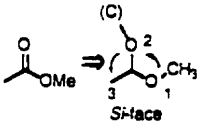
Fig. 6. Structures of subtilisin Calsberg and lipase from *Candida rugosa*. (a) X-ray crystal structure of subtilisin showing the catalytic machinery (Ser 221, His 64, Asp 32, and the N–H's of Asn 155 and Ser 221) and a portion of the proposed substrate binding site. The acyl chain binds in the region marked S1. The alcohol binding site has not been identified by X-ray crystallography, but the most likely region for the alcohol binding is suggested above. (b) X-ray crystal structure of the open form of lipase from *Candida rugosa* showing the catalytic machinery (Ser 209, His 449, Glu 341, and the N–H's of Ala 210 and Gly 123) and the proposed alcohol binding site. The two regions of alcohol binding site were identified by X-ray crystallography of menthol derivatives bound in the active site [4]. (c and d) Proposed structures of the tetrahedral intermediates the hydrolysis of the favored secondary alcohol esters. The orientation is similar to that of the crystal structures above. Diagrams in a and b were drawn using Rasmac v2.6 [32] using entries 1sbc and 1crl from the Brookhaven protein data bank [33].

shows higher enantioselectivity toward chiral acids than toward chiral amines and alcohols.

2.3. Opposite chirality of the catalytic machinery in lipases and subtilisin

X-ray structures of lipases show a serine protease-like catalytic machinery consisting of a Ser–His–Asp triad and an oxyanion hole [5]. However, the chirality of the catalytic machinery is opposite in serine proteases and lipases. For example, Fig. 6 compares the structures of subtilisin Carlsberg [34] and lipase from *Candida rugosa* [35]. Because of this difference, lipases and subtilisin attack the opposite faces of the carbonyl and form enantiomeric tetrahedral intermediates [1]. Consistent with this notion, Bjorkling et al. [37] found that opposite enantiomers of ethyl *p*-nitrophenyl hexylphosphonate, which has the stereocenter at the phosphorus, inhibited lipases and chymotrypsin (The catalytic machinery of chymotrypsin and subtilisin are superimposable.) However, the opposite face of attack can not explain why the hydrolases have an opposite enantiopreference toward stereocenters farther from the reaction center, such as the stereocenters in secondary alcohols and isosteric primary amines.

[1] Lipases attack the *Re* face of an ester, while subtilisin attacks the *Si* face of an ester. According to Hanson's nomenclature, the face with the clockwise ranking of the three substituents is the *Re*-face; the counterclockwise ranking gives the *Si*-face. For example, the *Si*-face of methyl acetate below is turned toward the reader. To rank the substituents, the carbon–oxygen double bond is replaced by a single bond and a phantom carbon atom '(C)' is added to the oxygen. The ester oxygen ranks higher than the carbonyl oxygen because the ester oxygen is attached to a real carbon atom. Unfortunately, researchers have sometimes named the faces of esters incorrectly. Note that replacing the OMe with NHMe gives the opposite designation for the face [36].



Si-face

3. Discussion

One criticism of enzymes as enantio- and regioselective catalysts is that only one enantiomer of the enzyme is available. The obvious, but impractical, solution is to create an enzyme from D-amino acids. However, this paper shows that for lipase-catalyzed reactions of secondary alcohols and primary amines, subtilisin is a readily available catalyst with opposite enantio- and regioselectivity. This complementary behavior may simplify the use of these catalyst for synthesis and make it more rational. The experimental results cited in this paper are for subtilisin BPN′ and subtilisin Carlsberg, but other subtilisin-like serine proteases (subtilases) have similar structures [38] and should show a similar enantiopreference.

One disadvantage of subtilisin is that its enantioselectivity is often lower than that of lipases. It may be possible, either by protein engineering or directed evolution to increase the enantioselectivity of subtilisin.

Derewenda and Wei's proposal for the molecular basis of enantiopreference considered only which face of the carbonyl was attacked [39], that is, only the absolute configuration of the catalytic machinery. They stated that "the reactivity of specific esters of secondary alcohols should be easily predicted from the relative solvent accessibilities of the *re* and *si* faces of the respective enantiomers". However, neither they nor others showed that the two faces differ in their solvent accessibility. In addition, their proposal does not explain why lipases differ in the degree of enantioselectivity toward the same substrate. Neither the face of attack nor the relative solvent accessibility changes in these cases.

In contrast, our proposal for the molecular basis of the enantiopreference of lipases and subtilisin focuses on the protein fold. This fold both sets the absolute configuration of the catalytic machinery and creates a restricted pocket for one substituent in the substrate. Both the α/β-hydrolase fold for lipases and the subtilase

fold for subtilisin create such a pocket, but the opposite absolute configuration of the catalytic machinery places the catalytic His on opposite sides of this pocket. For this reason serine proteases and lipases requires opposite chirality in the alcohol for efficient catalysis. Differences in the detailed shape of this pocket explain the different enantioselectivity of different lipases toward the same substrate.

The two proposals differ in their extrapolation to other serine hydrolases. Derewenda and Wei's proposal predicts that all serine proteases will have the same enantiopreference because the absolute configuration of their catalytic machinery is the same. On the other hand, our proposal cannot extrapolate to other serine hydrolases because they have different protein folds. Other protein folds may creates a different pocket or none at all. For example, trypsin-like serine proteases, such as chymotrypsin, may have the same, opposite, or no enantiopreference. Currently, there is not enough information about the enantioselectivity of chymotrypsin or other serine hydrolases toward secondary alcohols or isosteric primary amines to test these predictions.

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Chemoenzymatic Access to Enantiomeric Bicyclo[2.2.1]Heptan-2,5-Diones

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Abstract—A practical integrated process, combining an enzymatic resolution step with a few chemical transformations, is described for the synthesis of (1*R*,4*R*)- and (1*S*,4*S*)-bicyclo[2.2.1]heptan-2,5-diones 1 of high enantiomeric purity, starting from a standard mixture of (±)-*endo*- and *exo*-2-acetoxy-5-norbornene.

Introduction

(±)-Bicyclo[2.2.1]heptan-2,5-dione 1, which is readily obtained by the addition of formic acid to norbornadiene, followed by Jones oxidation of the resulting diformate esters,[1] has been used as a rigid template for the elaboration of a diphosphine ligand 2.[2] Dione 1 is also the starting material for the preparation of *anti*-3,5-dihydroxy-heptan-1,7-dicarboxylic acid 3,[3] a C_2-symmetric synthon possessing two chiral centers, which has been used in the preparation of the lactone rings of avermectins or milbemycins (Scheme I). In view of this, it would be of great use to have a simple preparative access to the pure dione enantiomers which, to our present knowledge, does not yet exist. A recent paper[4] does, however, describe an asymmetric bis-hydrosilylation of norbornadiene which leads to one of the corresponding *exo,exo*-diol diacetate enantiomers with high optical purity.

We herein report the synthesis of diones of high enantiomeric purity, starting from a commercial mixture of norbornenol acetates, which involves very few steps, the key one being an enzymatic resolution method.

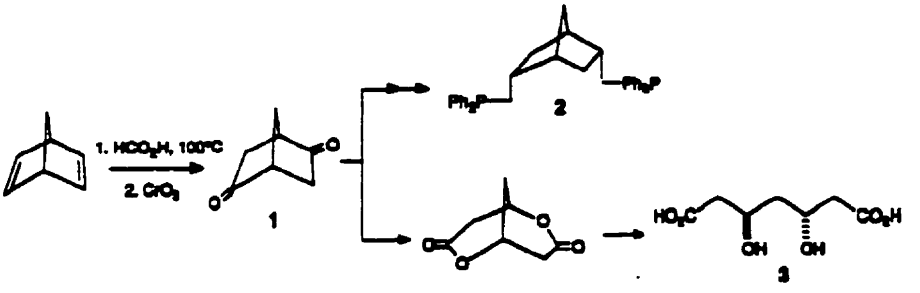
Results and Discussion

Our first attempts, which involved the direct enzymatic resolution of mono- and diesters derived from the racemic diol mixture obtained by the formylation of norbornadiene,[1] were unsuccessful, in agreement with known structural models elaborated for the hydrolysis of such bicyclic esters catalyzed by lipase from *Candida rugosa* (= *Candida cylindracea*)[5] and recent results obtained with other enzymes.[6-8] An enzymatic hydrolysis of *endo*-2-norbornenyl esters,[9,10] having an enantioselectivity coefficient (*E*)[11] of about 15, was not enantioselective enough to be of preparative use. Moreover, the recovery of the exceedingly volatile products[10] was difficult and the subsequent formylation of the norbornenyl ester was, to our surprise, unsuccessful.

For these reasons, we turned to another strategy for the introduction of the second oxygen atom using previously described reactions, the *exo*-epoxidation of an *endo*-2-hydroxy-5-norbornene derivative followed by a regioselective reductive opening of the epoxide ring to give an *endo,exo*-2,5-norbornanediol.[12] We were also aware that the enzymatic resolution of a 5,6-epoxy-*endo*-norbornan-2-yl ester,[10] carried out on an analytical scale, was highly effective (*E* ~ 100) and thus appeared particularly adapted to our purpose.

Analytical enzymatic hydrolyses and transesterifications

Epoxidation of the commercial 2-acetoxy-5-norbornene (a mixture of racemic *endo*- and *exo*-isomers, approximately 8:2) employing magnesium monoperoxyphthalate hexa-

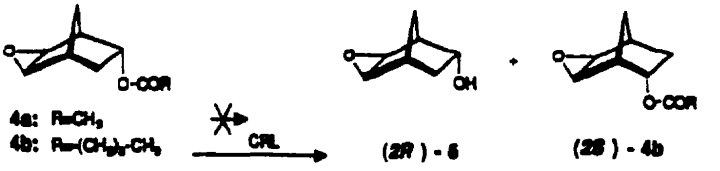


Scheme I.

Keywords: Enzymatic hydrolysis; transesterification; *Candida rugosa* lipase; asymmetric synthon.

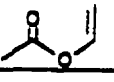
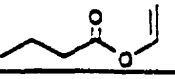
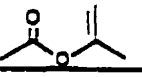
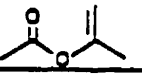
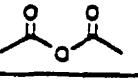
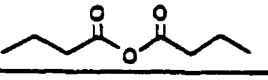
hydrate[13] in ethanol–water afforded, in high yield, the crude epoxide which, upon crystallization, yielded the pure *endo*-epoxyacetate isomer **4a** (about 50 % minimal yield). As previously described,[10] this ester was recovered unchanged when submitted to hydrolysis with lipase from *C. rugosa*, even for prolonged incubation times. Conversely, the corresponding butyric ester **4b**, prepared by mild alkaline hydrolysis of **4a** followed by esterification with butyric anhydride, was a good substrate for the same enzyme and was easily resolved ($E \sim 92$) on a 400 mg-scale, affording (2*R*)-epoxyalcohol **5** and (2*S*)-epoxyester **4b** of high optical purity (Scheme II). Absolute configurations were attributed from the known stereoselectivity of lipase from *C. rugosa* in this series[5] and confirmed by comparison of the optical rotation of the resulting epoxyalcohol with the epoxidation product of the previously described, corresponding (1*R*,2*R*,4*R*)-*endo*-norbornenol.[9,10]

However, the need for a preliminary exchange of the ester group could be eliminated by working with the same lipase in a transesterification reaction, using the racemic alcohol **5** as a substrate in an anhydrous organic solvent.[14] A preliminary screening for a convenient acyl group donor in various organic solvents was effected, the principal results of which are given in Table 1. The most striking outcome is that, unexpectedly, acetyl donors (entries 1, 3, 4 and 5) are effective donating reagents, although they are systematically less effective than butyryl donors. Moreover, the enantioselectivities measured using vinyl or isopropenyl acetate were higher or comparable to those measured using vinyl butyrate (entry 2). In contrast, other donors such as anhydrides (entries 5 and 6) or esters (entry 7), result in lower enantioselectivities. In the case of isopropenyl acetate, the replacement of toluene by chloroform (entry 4) produced a dramatic effect on the rate and enantioselectivity of the esterification reaction.



Scheme II.

Table 1. Enantioselective esterification[a] of (±)-2-*endo*-hydroxy-5,6-epoxynorbornane by lipase from *C. rugosa* in the presence of various acyl donors and solvents.

	Acylating agent (mol/ mol of substrate)		Solvent	Time (hours)	% conversion	E values[b]
1		(2)	Toluene	66	30	50-90
2		(3)	Toluene	3	46	75
3		(4)	Toluene	66	47	200-500
4		(4)	$CHCl_3$	216	14	12
5		(1)	Toluene	120	15	1.3
6		(1)	Toluene	1.5	40	3.6
7	Tributyrin	(used as solvent)		66	37	12

[a] To the substrate (50 mg) in anhydrous solvent (5 mL) were added 4 Å molecular sieves (50 mg), acyl donor, and lipase from *C. rugosa* (10–20 mg). The suspensions were incubated with shaking at 30 °C.

[b] calculated from % conversion, determined by GC of the reaction mixture (DBwax column, 160 °C) or from enantiomeric excesses of substrate and product.[11] *e.e.s* were determined either by GC of acetate or trifluoroacetate esters on a Chiraldex G-TA 30 capillary column (110 °C) or by HPLC of benzoate esters on a Chiralpack AD column (see Experimental Section).

Preparative aspects

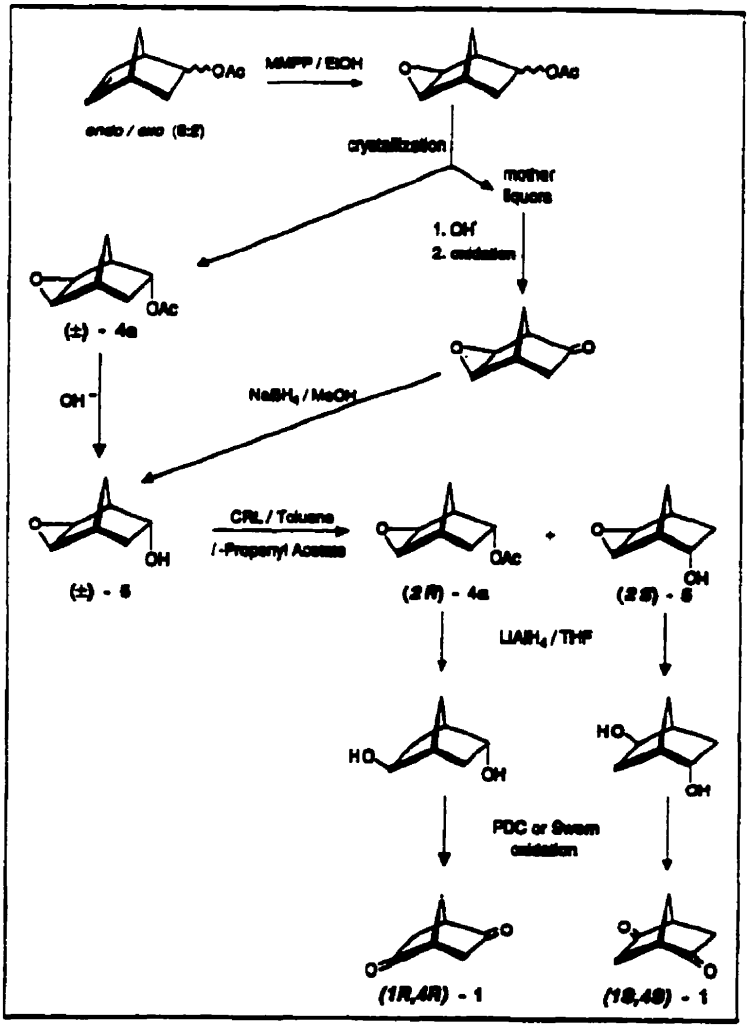
Under the best conditions (entry 3), starting from 3 g of (±)-epoxy alcohol **5**, it was possible to obtain, in a two-stage operation,[9] 1.6 g (40 %) of (2*R*)-epoxyacetate **4b** (96 % *e.e.*) and 1.2 g (40 %) of (2*S*)-epoxyalcohol **5** (> 98 % *e.e.*). Each product was then reduced with lithium aluminum hydride in tetrahydrofuran[12] (65–75 % yield) and the crystallized[15] *endo,exo*-2,5-norbornanediols were submitted to pyridinium dichromate or Swern oxidation,[16] affording the enantiomeric (1*S*,4*S*)- and (1*R*,4*R*)-bicyclo[2.2.1]heptan-2,5-diones (about 70 % yield) in high optical purity (≥ 96 % *e.e.*).

An integrated process, which includes the recycling of unused (±)-*exo*- and *endo*-2-acetoxy-5,6-epoxynorbornanes present in the mother liquors of the epoxyacetate recrystallization, has been designed: mild alkaline hydrolysis, followed by oxidation to epoxynorbornanone and reduction with sodium borohydride in methanol[10] will afford exclusively (≥ 95 %) the *endo*-epoxynorbornanol **5**, which could again be used in the enzymatic transesterification procedure. The entire synthetic process, described in Scheme III, is currently being conducted on a multigram scale in our laboratory, and will be reported in due course.

Experimental Section

General

Melting points are uncorrected. ^1H and ^{13}C-NMR spectra were recorded on a WM250 Bruker spectrometer at 250 and 62.9 MHz respectively. The residual protons in $CDCl_3$ or pyridine-d_5 were used as reference peaks, with assigned 7.25 and 8.71 ppm chemical shifts, respectively. Signal assignment was aided by 2D ^1H homonuclear shift correlated (COSY 45) spectra and ^{13}C distortionless enhanced polarization (DEPT 135) experiments. Optical



Scheme III.

rotations were measured in 1 dm or 0.1 dm cells using a Perkin Elmer 241 spectropolarimeter. Gas chromatography was performed on Varian 3700 or Shimadzu G-8A instruments equipped with flame ionization detectors and Shimadzu C-R3A or C-R6A integrating recorders. OV-1701 (Flexibond™, 0.20 mm x 15 m, Pierce Chem. Co) or Durabondwax (0.32 mm x 30 m, J&W Scientific, Inc.) capillary columns were routinely employed to monitor enzyme reactions, whereas a Chiraldex G-TA capillary column (0.25 mm x 30 m, Astec) was used to determine optical purities. Mass spectrometric analyses (MS) were carried out by electronic impact (EI) on a Hewlett Packard 5972 GC–MS instrument. High resolution mass spectra (HRMS) were supplied by Université P. et M. Curie (Paris). Flash column chromatography was carried out using Merck 60 silica gel (230–400 mesh). Merck $60F_{254}$ precoated glass plates were used for thin layer chromatography. High pressure liquid chromatography was performed using a Chromatem 380 pump, equipped with a Pye–Unicam LC-UV detector, a Shimadzu C-R3A integrating recorder, and a Chiralpack AD column (0.46 x 25 cm, Daicel Chem. Ind.). Lipase from *Candida cylindracea* (*C. rugosa*, E.C.3.1.1.3) was purchased from Sigma Chemical Co. (St Louis, USA).

Determination of enantiomeric excess

Enantiomeric excesses of 2-*endo*-acetoxy-5,6-epoxynorbornanes (Figure 1) and bicyclo[2.2.1]heptan-2,5-diones (Figure 2) were determined directly by GC on a Chiraldex G-TA30 capillary column at 110 °C. Enantiomeric excesses of 2-*endo*-hydroxy-5,6-epoxynorbornanes were determined by GC of their acetate or trifluoroacetate esters on the same column at 110 or 90 °C, respectively. In some cases, enantiomeric excesses of 2-*endo*-hydroxy-5,6-epoxynorbornanes were determined by HPLC of their benzoyl esters on a Chiralpak AD column with hexane–isopropanol (95:5) as solvent (flow rate: 0.5 mL/min, detection at 250 nm).

Preparation of acetyl esters for GC analysis: to the alcohol (~ 10 mg) dissolved in ethyl acetate was added 4-dimethylaminopyridine (0.05 eq.), sodium carbonate (1.5 eq.), and acetic anhydride (1.5 eq.). The mixture was stirred for 18 h, then washed with water and brine. The organic phase was dried over anhydrous sodium sulfate, filtered, evaporated *in vacuo*, and analyzed without further purification.

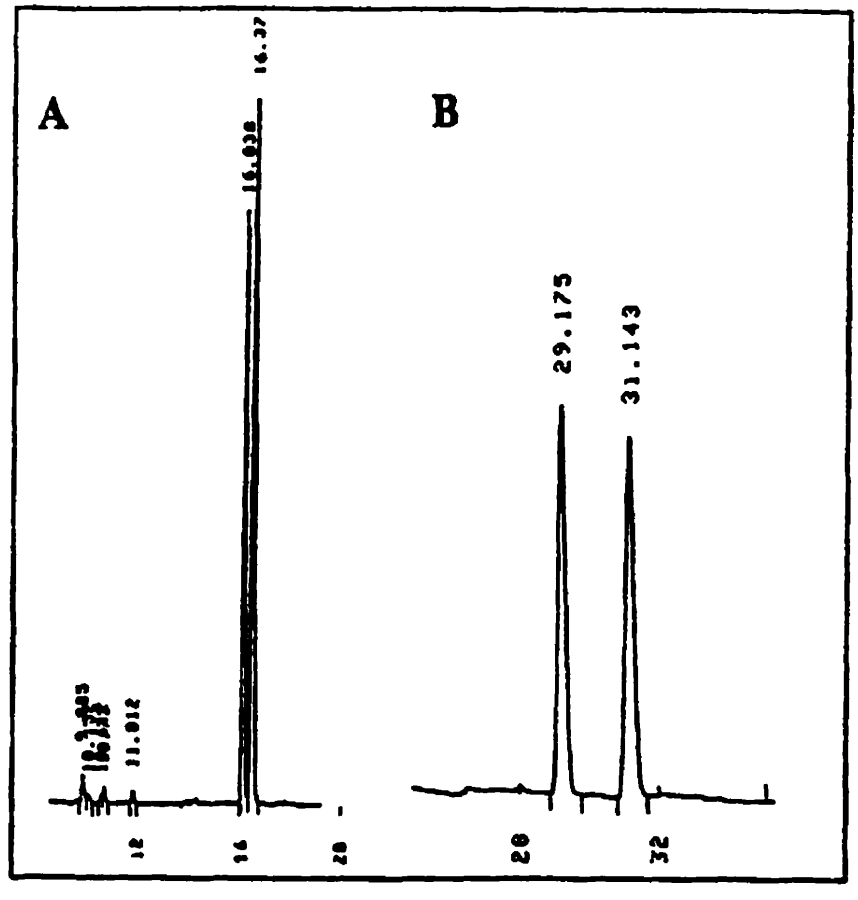


Figure 1. GC analytical separation of enantiomeric *endo*-2-hydroxy-5,6-epoxynorbornane esters on Chiraldex G-TA (see Experimental Section): A, trifluoroacetyl esters; B, acetyl esters.

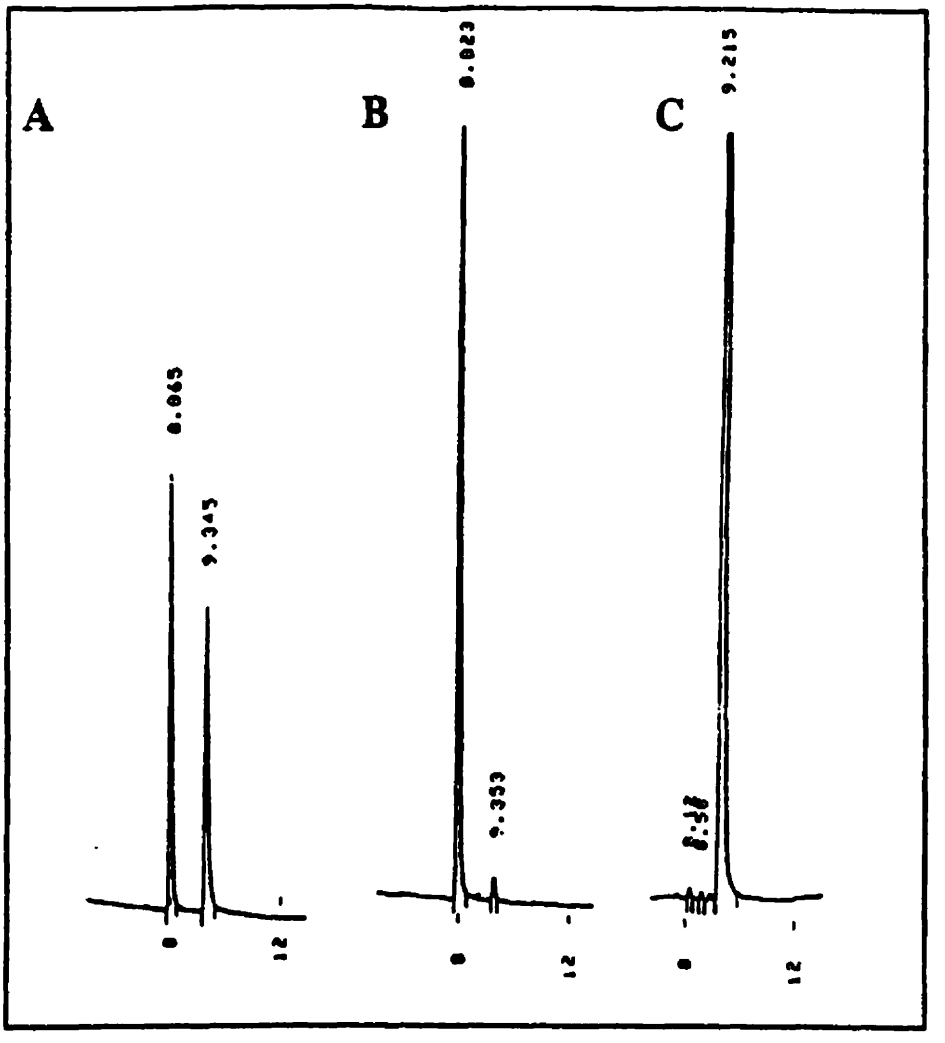


Figure 2. GC analytical separation of enantiomeric bicyclo[2.2.1]heptan-2,5-diones on Chiraldex G-TA (see Experimental Section): A, racemic mixture; B, 1*S*,4*S*; C, 1*R*,4*R*.

Preparation of trifluoroacetyl esters for GC analysis: to the alcohol (1–5 mg) in dichloromethane (0.5 mL) was added trifluoroacetic anhydride (0.2 mL). After stirring for 30 min, the solvent and excess anhydride were evaporated under a stream of nitrogen and the residue was analyzed without further purification.

Preparation of benzoyl esters for HPLC analysis: Dicyclohexylcarbodiimide (1.1 eq.) was added to a mixture of 4-dimethylaminopyridine (0.1 eq.), benzoic acid (2 eq.) and alcohol in dichloromethane, cooled in an ice-water bath. After stirring for 30 min, the reaction mixture was warmed to room temperature and stirred for 30 h. Dicyclohexylurea was removed by filtration and the filtrate was washed twice with 1 N HCl, saturated sodium bicarbonate, water and brine. The organic phase was dried over anhydrous sodium sulfate, filtered and evaporated *in vacuo*. The residue was analyzed without further purification.

(±)-2-endo-acetoxy-5,6-epoxynorbornane (4a)

A solution of magnesium monoperoxyphthalate hexahydrate (375 g, 0.76 mol) in water (2.6 L) was added to (±)-2-*endo*/*exo*-acetoxy-5-norbornene (99 g, 0.650 mol) in absolute ethanol (2 L). The mixture was stirred at room temperature for 3 days. Ethanol, along with a portion of water, was evaporated *in vacuo* and the residue (1.2 L) was divided into two parts. Each portion was extracted with ether (1 L), washed with aqueous saturated sodium bicarbonate (3 x 300 mL), 20 % aqueous sodium bisulfite (2 x 300 mL), water (300 mL), and brine (500 mL), and dried over sodium sulfate. The two aqueous phases obtained after the first ether extraction were combined, extracted again with ether (1 L), and washed as above. Evaporation of the solvent from the combined ethereal phases yielded a mixture of the *exo* and *endo* isomers as a slightly yellow oil (80.9 g, 70 %). Three crystallizations from ether–hexane yielded the *endo* isomer (54.3 g, 50 %, > 99 %

endo-isomer by GC). R_f 0.25 (cyclohexane–ethyl acetate, 8:2). Mp 53.5–54 °C (lit.[12]: 53–54 °C). HRMS for $C_9H_{12}O_3$, calc. 168.078642, found 168.078657. MS (EI): 168(1), 150(1) $[M-H_2O]^+$, 140(3), 138(3), 126(9) $[M-CH_2CO]^+$, 108(10), 97(11), 82(81), 43(100).

^{1}H NMR ($CDCl_3$, 250 MHz), δ ppm, *J* Hz: 5.04 (1H, ddd, J_{2-3exo} = 8.8, J_{1-2} = 4.4, $J_{2-3endo}$ = 3, H-2), 3.33 (1H, br.d, J_{5-6} = 3.6, H-6), 3.23 (1H, br.d, J_{5-6} = 3.6, H-5), 2.75 (1H, dm, J_{1-2} = 4.4, H-1), 2.49 (1H, dm, J_{4-3exo} = 4.4, H-4), 2.07 (1H, ddd, $J_{3exo-3endo}$ = 13.5, J_{2-3exo} = 8.8, J_{3exo-4} = 4.4, H-3 *exo*), 2.02 (3H, s, CH_3CO), 1.34 (1H, dm, $J_{7-7'}$ = 10.2, H-7), 1.07 (1H, dm, $J_{3exo-3endo}$ = 13.5, $J_{2-3endo}$ = 3, H-3 *endo*), 0.78 (1H, dm, $J_{7-7'}$ = 10.2, H-7').

^{13}C NMR ($CDCl_3$, 62.9 MHz), δ ppm: 170.28 (CO), 75.97 (CH, C-2), 50.36, 47.66 (CH, C-5 and C-6), 39.99, 36.44 (CH, C-1 and C-4), 32.56, 24.26 (CH_2, C-3 and C-7), 20.45 (CH_3).

The mother liquors were concentrated *in vacuo*, yielding a yellow oil (25.7 g) containing 35 % *endo*-isomer.

(±)-2-endo-butyroxy-5,6-epoxynorbornane (4b)

To a solution of (±)-2-*endo*-butyroxy-5-norbornene (900 mg, 5 mmol) in ethanol (15 mL) was added magnesium monoperoxyphthalate hexahydrate (3 g, 6.06 mmol) dissolved in water (20 mL). The mixture was stirred at room temperature for 48 h. The solvents were evaporated *in vacuo* and the residue dissolved in ether (100 mL). The ethereal solution was washed with aqueous saturated sodium bicarbonate (2 x 50 mL), 20 % aqueous sodium bisulfite (6 x 50 mL), saturated sodium bicarbonate (2 x 50 mL), water, and brine, dried over sodium sulfate, and evaporated. The crude product was purified by flash chromatography (hexane–ethyl acetate, 95:5 to 9:1), yielding the epoxyester as a colorless oil (819 mg, 83 %). R_f 0.38 (cyclohexane–ethyl acetate, 8:2). MS (EI): 168(2) $[M-CO]^+$, 140(5) $[M-CH_2CH_2CO]^+$, 125(4) $[M-CH_3CH_2CH_2CO]^+$, 107(5), 97(8), 81(81), 71(100).

^{1}H NMR ($CDCl_3$, 250 MHz), δ ppm, *J* Hz: 5.01 (1H, ddd, J_{2-3exo} = 9, J_{1-2} = 4, $J_{2-3endo}$ = 3, H-2), 3.32 (1H, br.d, J_{5-6} = 3.6, H-6), 3.23 (1H, br.d, J_{5-6} = 3.6, H-5), 2.75 (1H, dm, J_{1-2} = 4, H-1), 2.49 (1H, dm, J_{4-3exo} = 4, H-4), 2.25 (2H, t, *J* = 7.3, CH_2CO), 2.07 (1H, ddd, $J_{3exo-3endo}$ = 13.2, J_{2-3exo} = 9, J_{3exo-4} = 4, H-3 *exo*), 1.62 (2H, sextet, *J* = 7.3, CH_2CH_3), 1.33 (1H, dm, $J_{7-7'}$ = 10.2, H-7), 1.06 (1H, dm, $J_{3exo-3endo}$ = 13.2, $J_{2-3endo}$ = 3, H-3 *endo*), 0.93 (3H, t, *J* = 7.3, CH_3CH_2), 0.79 (1H, br.d, $J_{7-7'}$ = 10.2, H-7').

Enzymatic hydrolysis and resolution of (±)-2-endo-butyroxy-5,6-epoxynorbornane (4b)

To (±)-2-*endo*-butyroxy-5,6-epoxynorbornane **4b** (388 mg, 1.98 mmol) dissolved in 0.1 M, pH 7 sodium phosphate buffer–acetone (9:1, 150 mL), was added lipase from *C. rugosa* (39 mg). The mixture was orbitally shaken at 27 °C for 3 h (39 % conversion). The reaction mixture was saturated with sodium chloride and ethyl acetate (100 mL) was added. After stirring vigorously for 5 min, the phases were separated and the aqueous layer was extracted again with ethyl acetate (6 x 100 mL). The combined organic extracts were washed with aqueous saturated sodium bicarbonate and brine, dried over anhydrous sodium sulfate and evaporated *in vacuo*. Purification of the crude product by flash chromatography (hexane–ethyl acetate, 6:4 to 1:1) yielded pure alcohol (2*R*)-**5** (83 mg, 33 %), $[\alpha]_D^{21}$ + 42.4 ° (c 1.2, $CHCl_3$) (lit.[10]: + 46.5 °), 96 % *e.e.*, and ester (2*S*)-**4b** (232 mg, 60 %). The isolated butyrate was resubmitted to hydrolysis under the same conditions. After 6 h (57 % total conversion), the reaction mixture was worked up as before. Purification of the crude product yielded alcohol (2*R*)-**5** (16.2 mg, 6 %), and butyrate (2*S*)-**4b** (179 mg, 46 %), $[\alpha]_D^{21}$ – 13.8 ° (c 3.9, $CHCl_3$) (lit [10]: – 13.4 °), > 99 % *e.e.*

(±)-2-endo-hydroxy-5,6-epoxynorbornane (5)

To a solution of (±)-*endo*-2-acetoxy-5,6-epoxynorbornane (48.7 g, 0.29 mol) in ethanol (400 mL), cooled in a cold water bath, was slowly added 2 N sodium hydroxide (175 mL, 0.348 mol). The mixture was stirred for 85 min, after which time the reaction was quenched with glacial acetic acid (3.3 mL, 0.06 mol). Ethanol and water were removed by rotatory evaporation and the residue was extracted with ethyl acetate (1.4 L). The organic phase was washed with 0.5 N HCl (100 mL), saturated aqueous sodium bicarbonate (200 mL), and brine (2 x 200 mL), dried over anhydrous sodium sulfate, and evaporated *in vacuo* to yield a pale yellow solid (35.1 g, 96 %). The crude product was crystallized from ethyl acetate–hexane to give the pure epoxy alcohol as white crystals (25.3 g, 70 %). The remaining product was purified by flash chromatography (pentane–ethyl acetate, 5:5 to 3:7) yielding additional pure epoxy alcohol (4.4 g, 11 %). R_f 0.23 (pentane–ethyl acetate, 5:5). Mp 190–192 °C, sealed tube (lit.: 160–162 °C,[12] 170–172 °C[10]). HRMS for $C_7H_{10}O_2$, calc. 126.068078, found 126.068107. MS (EI): 126(1), 125(1.5), 107(2.5), 95(4), 81(100).

^{1}H NMR ($CDCl_3$, 250 MHz), δ ppm, *J* Hz: 4.38 (1H, m, H-2), 3.44 (1H, br.d, J_{5-6} = 3.7, H-6), 3.27 (1H, br.d, J_{5-6} = 3.7, H-5), 2.60 (1H, m, H-1), 2.45 (1H, m, H-4), 1.99 (1H, ddd, $J_{3exo-3endo}$ = 13.2, J_{2-3exo} = 9, J_{3exo-4} = 4, H-3 *exo*), 1.55 (1H, br.s, OH), 1.27 (1H, dm, $J_{7-7'}$ = 10.2, H-7), 1.00 (1H, dt, $J_{3exo-3endo}$ = 13.2, $J_{2-3endo}$ = 3, H-3 *endo*), 0.74 (1H, br.d, $J_{7-7'}$ = 10.2, H-7').

^{13}C NMR ($CDCl_3$, 62.9 MHz), δ ppm: 74.13 (CH, C-2), 51.47, 48.98 (CH, C-5 and C-6), 42.36, 37.33 (CH, C-1 and C-4), 35.08, 25.15 (CH_2, C-3 and C-7).

Preparative enzymatic resolution of (±)-2-endo-hydroxy-5,6-epoxynorbornane (5)

Isopropenyl acetate (10.3 mL, 93.5 mmol) was added to (±) 2-*endo*-5,6-epoxynorbornane (3 g, 23.8 mmol) dissolved in toluene (340 mL). Lipase from *C. rugosa* (1

g) was added and the flask was orbitally shaken at 30 °C. After 14 h an additional 700 mg of lipase was added and after another 8 h, 550 mg of lipase was added. After a total of 25 h, the reaction was stopped by filtration of the mixture through glass fiber paper. The product and remaining substrate were separated by medium pressure liquid chromatography (200 g silica gel, Merck 60H, cyclohexane–ethyl acetate 1:1, followed by cyclohexane–ethyl acetate 2:8 once the first alcohol fraction was detected). The solvent was evaporated to yield the alcohol (1.84 g, 77 % *e.e.*) and the acetate (1.59 g, 40 %, 95.5 % *e.e.*), $[\alpha]_D^{21}$ + 7.4 ° (c 1, $CHCl_3$). The alcohol was resubmitted to esterification under the same conditions using 4 g lipase. The reaction was stopped as before after a total of 20 h (corresponding to a 58 % total conversion). Purification by flash column chromatography (cyclohexane–ethyl acetate, 1:1) yielded the remaining alcohol substrate (1.2 g, 40 %, > 98 % *e.e.*), $[\alpha]_D^{21}$ – 46.7 ° (c 0.85, $CHCl_3$), and the acetate product (0.44 g, 60 % *e.e.*).

Reduction of 2-endo-*acetoxy-5,6-epoxynorbornane (4a) to 2,5-dihydroxynorbornane*

Dry tetrahydrofuran (80 mL) was added dropwise to lithium aluminum hydride (2.4 g, 56.7 mmol) under nitrogen. After complete addition, the suspension was refluxed for 1.25 h. After cooling the mixture to room temperature, the flask was placed in a cold water bath and 2-*endo*-acetoxy-5,6-epoxynorbornanol (1.52 g, 9.04 mmol) in tetrahydrofuran (6 mL) was added dropwise. The dropping funnel was rinsed with tetrahydrofuran (5 mL) and the mixture was heated to a reflux for 3.75 h. The flask was cooled in an ice-water bath and water was carefully added dropwise (2.4 mL), followed by aqueous 15 % w/w sodium hydroxide (2.4 mL) and finally water (7.2 mL). The mixture was stirred for 20 min and then filtered, rinsing with tetrahydrofuran and ethyl acetate. The filtrate was dried over anhydrous sodium sulfate and evaporation of the solvent yielded a white solid. Recrystallization from ether–dichloromethane yielded the pure diol as white crystals (614 mg, 53 %). Medium pressure liquid chromatography (200 g silica, dichloromethane–isopropanol, 9:1) of the residue obtained from evaporation of the mother liquor yielded additional pure diol (272 mg, 23 %). R_f 0.16 (dichloromethane–methanol, 9:1). Mp 180–182 °C (sealed tube). $[\alpha]_D^{21}$ + 2.8 ° (c 2.34, MeOH), $[\alpha]_{578}$ + 2.9 °, $[\alpha]_{546}$ + 3.2 °, $[\alpha]_{436}$ + 4.2 °. HRMS for $C_7H_{12}O_2$, calc.128.083728, found 128.083713. MS (EI): 128(2) $[M]^+$, 110(19) $[M-H_2O]^+$, 95(33), 81(24), 66(100).

^{1}H NMR (pyridine-d_5, 250 MHz), δ ppm, *J* Hz: 6.04 (1H, d, J = 3.3, *endo*-OH on C-2), 5.99 (1H, d, J = 3.3, *exo*-OH on C-5), 4.38 (1H, m, J_{2-3exo} = 10, J_{2-3} = J_{2-OH} = 3.3, J_{2-1} = 1.3, H-2), 4.22 (1H, m, H-5), 2.92 (1H, ddd, $J_{6endo-6exo}$ = 13, $J_{6endo-5}$ = 7, J = 2, H-6 *endo*), 2.40 (1H, br.t, $w_{1/2}$ = 10, H-1), 2.33 (1H, br.d, J = 5, H-4), 2.06–1.93 (2H, m, J = 5, H-3$_{exo}$ and H-7), 1.63 (1H, dm, $J_{6exo-6endo}$ = 13, H-6 *exo*), 1.31 (1H, br.d, $J_{7-7'}$ = 10, H-7'), 1.01 (1H, dt, $J_{3exo-3endo}$ = 13, $J_{2-3endo}$ = 3.3, H-3 *endo*).

^{13}C NMR ($CDCl_3$, 62.9 MHz), δ ppm: 73.70, 70.62 (CH, C-2 and C-5), 44.40, 41.21 (CH, C-1 and C-4), 34.31, 33.03, 32.26 (CH_2, C-3, C-6, and C-7).

Reduction of 2-endo-*hydroxy-5,6-epoxynorbornane (5) to 2,5-dihydroxynorbornane*

The above procedure was used for the reduction of the epoxy alcohol (1.17g, 9.27 mmol) with the exception that only 4 eq. of lithium aluminum hydride were used. Recrystallization yielded the diol as white crystals (355 mg, 30 %) and chromatography of the mother liquor yielded additional pure diol (411 mg, 34 %). $[\alpha]_D^{21}$ – 4.1 ° (c 2.25, MeOH), $[\alpha]_{578}$ – 4.2 °, $[\alpha]_{546}$ – 4.5 °, $[\alpha]_{436}$ – 5.6 °.

Oxidation of 2,5-dihydroxynorbornane to enantiomeric bicyclo[2.2.1]heptan-2,5-diones (1)

(i) *Pyridinium dichromate oxidation.* Diol (706 mg, 5.51 mmol) was dissolved in *N,N*-dimethylformamide (100 mL), pyridinium dichromate (7.05 g, 18.7 mmol) was added and the mixture was stirred under nitrogen for 3 h. Aqueous saturated sodium bicarbonate (100 mL) was added to the reaction mixture and it was shaken vigorously. Dichloromethane (300 mL) was added and the organic phase was washed with aqueous saturated sodium bicarbonate (4 x 100 mL), 0.5 N HCl (100 mL), saturated sodium bicarbonate (100 mL), water (2 x 200 mL), and brine (200 mL), and dried over sodium sulfate. The solvent was evaporated *in vacuo* yielding 501 mg of dione (73 %, 95 % pure by GC).

(ii) *Swern oxidation.* Freshly distilled oxalyl chloride (0.45 mL, 4.8 mmol) in dry dichloromethane (8 mL) was added dropwise to a solution of dry dimethylsulfoxide (0.83 mL, 10.9 mmol) in dry dichloromethane (5 mL), under nitrogen at –78 °C. After stirring for 30 min, 2,5-dihydroxynorbornane (300 mg, 2.34 mmol) in dichloromethane (4 mL) and dimethylsulfoxide (0.6 mL) was added dropwise. After stirring for 3 h, triethylamine (3 mL, 21.8 mmol) was slowly added. The reaction mixture was allowed to warm to room temperature, then stirred for an additional hour. Water (10 mL) was added dropwise, the reaction mixture was diluted with dichloromethane and the organic phase was washed with 0.5 N HCl, aqueous saturated sodium bicarbonate, and brine. The solvent was evaporated *in vacuo* yielding 200 mg of pure dione (70 %). MS (EI): 124(100) $[M]^+$, 95(21), 82(23), 67(87).

^{1}H NMR ($CDCl_3$, 250 MHz), δ ppm, *J* Hz: 2.97 (2H, m, X signal of an ABX system, H-1 and H-4), 2.36 (2H, dm, A signal of an ABX system, J_{AB} = 19, H-3 *exo* and H-6 *exo*), 2.13 (2H, dm, B signal of an ABX system, J_{AB} = 19, H-3 *endo* and H-6 *endo*), 2.08 (2H, m, H-7 and H-7').

^{13}C NMR ($CDCl_3$, 62.9 MHz), δ ppm: 212.04 (CO, C-2 and C-5), 48.48 (CH, C-1 and C-4), 38.80 (CH_2, C-6 and C-3), 36.27 (CH_2, C-7).

(1*S*,4*S*)-**1**: mp 140–141 °C. $[\alpha]_D^{21}$ – 4.5 ° (c 2.44, EtOH), $[\alpha]_{578}$ – 4.5 °, $[\alpha]_{546}$ – 2.9 °, $[\alpha]_{436}$ + 26.6 °, $[\alpha]_{363}$ + 187 °; *e.e.* = 99 %. HRMS for $C_7H_8O_2$, calc. 124.052408, found 124.052383.

(1*R*,4*R*)-**1**: mp 139–140 °C. $[\alpha]_D^{21}$ + 5.0 ° (c 2.0, EtOH), $[\alpha]_{578}$ + 5.0 °, $[\alpha]_{546}$ + 4.0 °, $[\alpha]_{436}$ – 25.5 °, $[\alpha]_{363}$ – 179.5 °; *e.e.* = 96 %. HRMS for $C_7H_8O_2$, calc.124.052408, found 124.052383.

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