Screening Methods to Identify Stereoselective Hydrolases for Synthetic Applications

A) Empirical rules to predict the enantiopreference of *Aspergillus niger* lipase.

B) Novel spectrophotometric assays to rapidly measure the activity and stereoselectivity of hydrolases.

by

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For my parents and for Kevin
without whose support this would not have been possible,

&

in memory of D.

"...to know and love one other human being is the root of all wisdom"

Evelyn Waugh

Brideshead Revisited

"My labours on the castle keep were also made harder, and unnecessarily so (unnecessary in that the burrow derived no real benefit from those labours), by the fact that just at the place where, according to my calculations, the castle keep should be, the soil was very loose and sandy and had literally to be hammered and pounded into a firm state to serve as a wall for the beautifully vaulted chamber. But for such tasks, the only tool I possess is my forehead. So I had to run with my forehead thousands and thousands of times, for whole days and nights, against the ground, and I was glad when the blood came, for that was proof that the walls were beginning to harden; and in that way, as everybody must admit, I richly paid for my castle keep."

Franz Kafka
The Burrow

Abstract

Although chemists often exploit the high stereoselectivity of hydrolytic enzymes to produce pure enantiomers, the difficulty in selecting the best hydrolase from hundreds of commercial hydrolases and microorganisms remains a major deterrent to their wider use. Moreover, the emerging ability to create large libraries of recombinant enzymes demands fast and simple selection methods. The current method of selection is time-consuming because it requires carrying out small-scale reactions and measuring enantiomeric purity. This thesis focuses on the development of methods to speed up this selection process.

We first report the development of empirical substrate models for *Aspergillus niger* lipase (ANL) to predict which enantiomer of a racemate reacts faster. For secondary alcohols, a rule based upon the size of the substituents at the stereocentre of a substrate previously proposed for other lipases and esterases works for ANL. Surprisingly, a rule based upon charge rather than size of the substituents can predict the preferred enantiomer of α-amino acids. These qualitative rules aid in the design of new synthetic applications of ANL.

Next, we report the development of a spectrophotometric assay to accurately but rapidly measure the enantioselectivity of a hydrolase towards a target substrate without measuring enantiomeric purity called "quick E". The initial rates of hydrolysis of each enantiomer of a substrate are separately measured relative to a reference compound. The ratio of the two relative rates yields the enantioselectivity. We first developed this method for chromogenic esters. Next, to extend quick E to non-chromogenic esters, we developed a pH indicator assay to quantitatively detect ester hydrolysis. We optimized this method for screening in 96-well plates for speed.

Finally, we apply these spectrophotometric assays to solve a synthetic problem. Dioxolane nucleosides are powerful pharmacological agents used in the treatment of HIV and hepatitis-B virus but their syntheses require expensive and tedious silica gel chromatography to separate mixtures of diastereomers. Using the quick E method, we rapidly identify two hydrolases that produce the desired dioxolane diastereomer in good yield and excellent diastereomeric excess (>98%).

Overall, our screening methods are simpler to perform than traditional methods and require significantly less substrate and hydrolase (μg quantities per measurement) without compromising sensitivity and quantitativeness.

Résumé

Bien que les chimistes exploitent souvent la haute stéréosélectivité d'enzymes hydrolytiques afin de produire des énantiomères purs, la difficulté de choisir la meilleure hydrolase parmi les centaines de microorganismes et d'hydrolases commerciaux reste un obstacle majeur à une plus grande généralisation de leur usage. La méthode usuelle de sélection est longue, requérant en effet la réalisation de réactions à petite échelle ainsi que la mesure de la pureté énantiomérique. Cette thèse est centrée sur le développement de méthodes accélérant ce processus de sélection.

Nous rapportons le développement de modèles empiriques de substrats pour la lipase *Aspergillus niger* (ANL) afin de prévoir quel énantiomère réagira le plus vite. Pour les alcools secondaires, une règle basée sur la taille des substituents du centre chiral du substrat, déjà proposée pour d'autres lipases et estérases, se révèle correcte aussi pour l'ANL. Pour les α-amino acides, une seconde règle basée sur la charge plutôt que sur la taille des substituents permet de prévoir l'énantiomère le plus réactif. Ces règles qualitatives améliorent les capacités de conception d'applications synthétiques d'ANL.

En second lieu, nous décrivons le développement d'une titration spectophotométrique, appelée quick E, quantifiant précisément et rapidement l'énantiosélectivité d'une hydrolase envers un substrat, sans toutefois nécessiter la mesure de la pureté énantiomérique. Les taux initiaux d'hydrolyse de chaque énantiomère sont mesurés relativement à un composé de référence. Le ratio de ces deux taux relatifs détermine l'énantiosélectivité. Nous avons développé cette méthode en premier lieu pour les esters chromogéniques. Ensuite, pour étendre quick E aux esters non-chromogéniques, nous avons mis au point une titration pH-métrique pour détecter quantitativement l'hydrolyse des esters. Pour plus de rapidité, cette technique fut optimisée pour permettre le screening utilisant des plaques à 96 puits.

Enfin, nous avons appliqué ces titrations spectrophotométriques à la résolution d'un problème synthétique. Les nucléosides dioxalane sont de puissants principes pharmacologiques utilisés pour le traitement des virus HIV et de l'hépatite B mais leur synthèse nécessite plusieurs difficiles et coûteuses étapes de purification par chromatographie au gel de silice, afin de séparer les mélanges diastéréomériques. Grâce à la méthode quick E, nous avons rapidement identifié deux hydrolases produisant le

diastéréomère désiré avec de bons rendements et d'excellents excès diastéoréomériques (>98%).

En conclusion, nos méthodes de screening sont plus simples à mettre en oeuvre que les techniques traditionnelles et requièrent de moins grandes quantités de subtrats et d'hydrolase (de l'ordre du microgramme par mesure), sans compromettre la sensibilité et la précision quantitative.

Acknowledgements

There are many people to whom I am grateful for helping me throughout my studies. First, I must offer my sincere thanks to my research supervisor, Dr. Romas Kazlauskas, for allowing me to conduct research in his laboratory, providing funding throughout my degree and sharing in my enthusiasm for developing the quick E screen. I especially thank him for carefully editing our publications and for generously providing the funding for me to attend several conferences. I thank all of my colleagues, past and present, in the Kazlauskas lab and the Chemistry Department for kindly giving their help and expertise in the lab and their companionship outside of it. Financial help in the form of a teaching fellowship from the Chemistry Department of McGill University is also acknowledged.

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There are several people who have been a tremendous personal support for me at McGill University and I am particularly grateful to them. I thank Stephanie Michaud for being a wonderful friend and sympathetic scientist, Stephanie Warner for helping me battle homesickness, Dr. Ross Markwell for his loyal friendship, Dr. Lucy Dickinson for her encouragement, and Meg Steele for sharing her friendship and two great cats during the writing of this thesis.

Since I view my Doctoral degree as the culmination of my education, there are many people who have been instrumental in helping me reach this point and I wish to thank them. I thank Mr. William MacKenzie of Sydney Academy who encouraged me in my studies and helped me gain a much needed scholarship to attend Harvard University; Professor Dudley Herschbach of Harvard University who first encouraged me to be a

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I thank my parents, Melvin and Judith Janes, and my grandparents, Constance Boyd and the late Gerald Boyd, for their support and acceptance of my absence throughout my education, and for helping to ease the financial burden during my studies.

Finally, I must thank my two best friends, Kevin Bunten and Rhonda Moules, who constantly forgave my numerous shortcomings throughout this degree. I could not have completed this degree without their support, patience and love through the good times and bad times.

I hope I have not forgotten to thank anyone.

Thesis Formatting

The following text, concerning the inclusion of manuscripts in a thesis, is reproduced from the "Guidelines for Thesis Preparation".

"As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers that have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

- Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the Thesis Preparation Guidelines with respect to font size, line spacing, and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)
- The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, **connecting texts that provide logical bridges between the different papers are mandatory.**
- The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: a table of contents; an abstract in English and French; an introduction which clearly states the rational and objectives of the research, a comprehensive review of the literature (in addition to that covered in the introduction to each paper); a final conclusion and summary. **Students are not required to organize their references into one comprehensive list in a manuscript-based thesis. They are free to choose whether to make a comprehensive list or to put references after each paper.**
- As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

- In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** This statement should appear in a single section entitled "**Contribution of Authors**" as a preface to the thesis. The supervisor must attest to the accuracy of the statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored material.
- When previously published copyright material is presented in the thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition.
- Irrespective of the internal and external examiners reports, if the oral defence committee feels that the thesis has major omissions with regard to the above guidelines, the candidate may be required to resubmit an amended version of the thesis.
- In no case can a co-author of any component of such a thesis serve as an external examiner for that thesis."

Contribution of Authors

This thesis represents a collection of five manuscripts, three have been already published, and two which are manuscripts to be submitted for publication shortly. All of the work contained in these manuscripts has been completed as part of my research for the degree of Doctor of Philosophy.

Although these manuscripts have co-authors, I have been the first author of each manuscript, and as such, have written the manuscripts in entirety under the supervision of my supervisor, Romas J. Kazlauskas. Dr. Kazlauskas and I carefully edited these manuscripts together. Other co-authors did not write the manuscripts but carefully proofread them.

Each manuscript is presented as a separate chapter (Chapters 2-6) in the thesis since each chapter represents a different problem, requiring its own introduction and discussion. The common theme of these manuscripts/chapters is the development of methods to select active and stereoselective hydrolases for the production of pure stereoisomers. Chapter 1 serves as a general but solid introduction to the current methods and considerations when choosing stereoselective hydrolases for synthetic applications. Chapters 2-6 present the logical and stepwise progression of our research to simplify but accelerate the selection process to find stereoselective hydrolases.

Co-Authorship of Manuscripts

Professor Romas J. Kazlauskas, supervisor throughout my doctoral degree, is a co-author for each manuscript.

CHAPTER 4: Dr. A. Christina Löwendahl, a postdoctoral fellow in our lab, worked as a partner with me throughout the development of a quantitative, colorimetric screen using pH indicators. Christina performed the small-scale resolutions and measured the enantiomeric purities reported in the manuscript.

CHAPTER 6: Mr. Alex Cimpoia, a scientist at BioChem Pharm, Laval, Quebec was our contact person for a collaboration between our lab and BioChem Pharma to explore the use of hydrolytic enzymes in the production of therapeutic agents. Mr. Cimpoia provided the dioxolane compounds, made helpful suggestions and met with us regularly to discuss progress.

Dr. Romas Kazlauskas carried out the computer modeling of enzyme-transition state analog complexes reported in the manuscript.

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Glossary of Frequently Used Symbols and Abbreviations

α	separation factor
ACN	acetonitrile
AIDS	Aquired Immune Deficiency Syndrome
ANL	*Aspergillus niger* lipase
B	buffer
BES	*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulphnic acid
br	broad (in NMR)
BSA	bovine serum albumin
c	conversion
C	Celcius
CALB	*Candida antarctica* lipase B
CI	chemical ionization
CRL	*Candida rugosa* lipase
d	doublet (NMR)
δ	chemical shift
D	diastereoselectivity
Da	Dalton
de	diastereomeric excess
ε	extinction coefficient ($M^{-1}cm^{-1}$)
E	enantiomeric ratio
	enzyme
EI	electron ionization
ee	enantiomeric excess
eq	equation
eV	electron volt
g	gram
h	hour
$HClO_4$	Perchloric acid
hfc	3-(heptaflouropropylhydroxymethylene)-d-camphorato
HBV	Hepatitis B virus
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectroscopy
Hz	hertz
In	indicator
IPA	isopropanol
J	coupling constant (in NMR)
k	kilo (10^3)
k'	capacity factor
K_M	Michaelis-Menten constant
K_S	dissociation constant
k_{cat}	enzyme turnover number
l	pathlength

L	large substituent
	liter
m	meter
μ	micro
M	medium substituent
	molar
MES	2-(N-morpholino)ethanesulphonic acid
min	minute
mol	mole(s)
MS	mass spectroscopy
m/z	mass-to-charge ratio
N	normal
na	not available
nd	not determined
%	percent (parts per hundred)
p	para
P	product
PCL	*Pseudomonas cepacia* lipase
pH	hydrogen ion concentration
PLE	porcine liver esterase
pK_a	negative logarithm of equilibrium constant for association
PNPA	*para*-nitrophenyl acetate
pp	pages
PPL	porcine pancreatic proteases
ppm	parts per million
q	quartet (in NMR)
R	Alkyl or aryl group
R_f	retention factor
R_s	Resolution factor
s	singlet (in NMR)
	small substituent
S	substrate
sec.	seconds
t	triplet (in NMR)
T_d	tetrahedral intermediate
TLC	thin-layer chromatography
TRIS	tris(hydroxymethyl)-aminomethane
U	unit
U/mg	μmol of ester hydrolyzed per minute per mg protein.
v	initial velocity
V_{max}	maximum velocity
w/v	weight-to-volume

Chapter 1
General Introduction

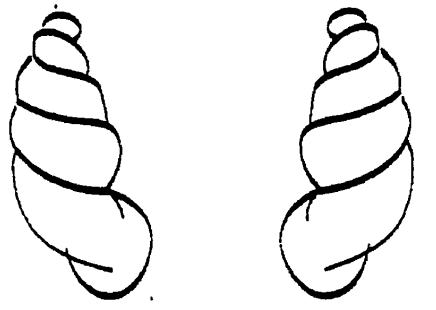
"We thus arrive at the conclusion that the production of single asymmetric compounds, or their isolation from the mixture of their enantiomorphs, is, as Pasteur firmly held, the prerogative of life. Only the living organism with its asymmetric tissues, or the asymmetric products of the living organism, can produce this result. Only asymmetry can beget asymmetry. Is the failure to synthesize single asymmetric compounds without the intervention, either direct or indirect, of life, due to a permanent inability, or merely to a temporary disability which the progress of science may remove?...But even if such an asymmetric force could be discovered – a force which would enable us to synthesize a single enantiomorph – the process would not be free from the intervention of life."

F. R. Jaap
Nature **1898**, *58*, 452-460.

1.1 Chirality in living systems

Chirality has been exquisitely preserved throughout evolution in all living systems on both the microscopic and macroscopic level. For example, evolution has ensured that proteins are composed of *L*-amino acids almost exclusively, while nucleic acids, which carry the genetic code of life, are composed of only *D*-sugars. Similarly, researchers have recently discovered that the internal left-right asymmetry of our internal organ displacement is encoded in our genes and expressed as early as the first few cell divisions of life.[1] Amazingly, snails from the genus *Partula* from the Society Islands in the Pacific Ocean used chirality in their shells as a mechanism to preserve and isolate different species of the same genus on a small isolated island where hybridization was highly likely.[2] Different species of the snails exhibited different coiling of their shells, either right handed or left-handed chirality. When snails of opposite coil chirality mated, fertility was reduced than for that between members with the same coil chirality,

suggesting that chirality acted as a regulatory mechanism to prevent gene flow between the species and, therefore, isolate the species, Figure 1.1.



lefthanded shell coil in *Partula mirabilis*

right handed shell coil in *Partula taeniata*

Figure 1.1 Two species of the now extinct *Partula* snail show opposite coil chirality. Although most snails coil to the right, it is believed that *Partula* snails used chirality as an isolating mechanism between species of the same genus to prevent hybridization on a small island in the Pacific Ocean.

The search for the origins of nature's chirality have long puzzled scientists and many theories have been proposed to account for the deracemization of the prebiotic primordial soup. Some researchers have proposed that chirality resulted from a lack of symmetry in elementary particle interactions while others have suggested that a prebiological event on earth or another planet can account for the origin of chirality in our universe. Some scientists have compared this question to the proverbial dilemma of "which came first - the chicken or the egg?" since chiral structures (such as enzymes and strands of DNA) display great chiral discrimination but they do so because they are made of chiral materials.[3] Despite the mystery of its origins, chirality is ubiquitous in nature and particularly significant to the field of chemistry.

1.2 The importance of chirality on bioactivity

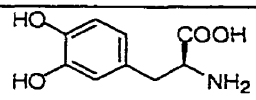
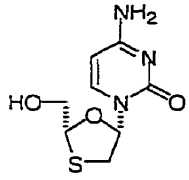
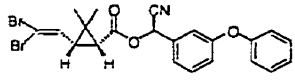
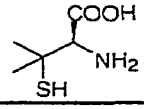
Molecules that are chiral are non-superimposable with their mirror images. The word "chiral" originates from the Latin word *cheir* for hand, since our hands are mirror

images of each other. A molecule and its non-superimposable image are enantiomers of each other and a mixture with equal quantities of enantiomers is called a racemate. Isomers of the same molecule that are not related as mirror images but differ only in their arrangement of atoms in space are diastereomers of each other. Two enantiomers of a molecule have identical physical properties (except for optical rotation) and exact chemical properties (except when in a chiral environment, for example, our bodies) while diastereomers of a molecule usually differ in their chemical and physical properties. A more general term, stereoisomers, includes both enantiomers and diastereomers.[4]

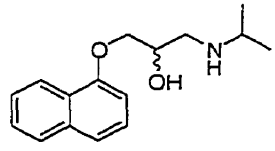
Not surprisingly, chirality is an important consideration in the development of therapeutic agents since enantiomers of molecules act differently in the body, which is a chiral environment. Physiological events in living systems are carried out by refined signaling and message carrying systems. These systems rely upon highly selective and specific interactions between chiral molecules such as hormones, neural transmitters, immunological antibodies; and chiral receptors, such as cell surface receptors. Some researchers believe that these stringent chirality requirements in living systems may have evolved to allow living organism to efficiently use their limited resources.

One of the earliest noted examples of different biological responses to enantiomers of molecules was reported by Piutti in the mid 1880's, noting the sweet versus bland taste of the enantiomers of asparagine.[5] Since then, chemists have increasingly uncovered the relationship between the chirality of a molecule to its biological activity. Several excellent examples in Table 1.1 illustrate the vast differences in bioactivity of different stereoisomers of the same agent.[6]

Table 1.1 Observed biological effect of pharmacological agents as a function of their absolute configurations.

Compound name®	Structure	Absolute Configuration	Observed Biological effect
Dopa		*S* *R*	-Anti-Parkinson agent -Causes side-effects e.g. granulocytopenia
3TC		1'S,4'R 1'R,4'S	-Potent anti-HIV agent, very low toxicity -slightly reduced anti-HIV activity, high toxicity
Deltamethrin		1*R*,3*R*,α*S* 1*S*,3*S*,α*R* and other diastereomers	-Potent insecticide -Inactive or slightly active
Penicillamine		*S* *R*	-Antiarthritic -Mutagen

Not all enantiomer pairs have such distinct differences although most enantiomer pairs will show subtle physiological differences. 3TC, a powerful antiviral agent currently used to treat HIV does not display overt cytotoxicity while its mirror image enantiomer is considerably more cytotoxic and less active towards HIV.[7] Sometimes, one enantiomer is completely inert such as the enantiomer of Deltamethrin; in other cases, racemic mixtures can actually have synergistic effects when taken together. For example, Propranolol, a β-blocking agent, shows an increase in half-life (thus requiring smaller dosage) when taken as a racemate due to suppression of hepatic blood flow.[8]



Propranolol

Thus, despite a close structural relationship, racemates are currently viewed by the pharmaceutical and chemical industry as mixtures of different compounds that must be separately evaluated for potentially different pharmacological activities. The impetus for this view lies in the U. S. Food and Drug administration, which currently urges companies to develop and test new chiral compounds as single enantiomers. Marketing a compound as a single enantiomer typically requires >98% enantiomeric excess (less than 1% of the undesired enantiomer). Yearly reports published in Chemical and Engineering News by the American Chemical Society track developments in the chiral drug industry. In 1998, they reported that of the 500 top-selling drugs worldwide, more than half are single enantiomers.[9] Moreover, one-half of the 1200 drugs in development worldwide in 1997 were being developed as single enantiomers.[10] Controlling absolute stereochemistry remains a key requirement in the development of new bioactive compounds in the pharmaceutical industry as well as in the pesticide, flavor and fragrance, and food industries.

1.3 Methods to produce chiral compounds

Mimicking the exquisite ability of living systems to produce chiral molecules has proven to be a considerable synthetic challenge to the field of chemistry. Professor F. R. Jaap philosophized on the challenges of producing pure 'enantiomorphs" in 1898 as noted in the opening quote of this chapter. Considerable advances have been made over the past 100 years in controlling the absolute stereochemistry of molecules but doing so is still not considered trivial in synthetic chemistry.

Chemical and pharmaceutical companies remain focussed on the production of pure stereoisomers and are continually engaged in developing new methods to produce pure stereoisomers. The major classes of techniques to produce pure stereoisomers can be broadly classified into several fields:

- *Directed chemical reactions, often referred to as asymmetric catalysis.* These processes use chiral catalysts usually composed of a metal complex containing chiral organic ligands to control a metal-mediated process in such a way that one of two enantiomeric products is formed with high preference over the other.[11] Two seminal

developments in this field were titanium-tartrate asymmetric epoxidation of allylic alcohols by Sharpless[12] and Ru(BINAP)-catalyzed directed hydrogenation of functionalized olefins and ketones by Noyori.[13] Recent developments in this field include the development of more generalized catalysts that do not require specific organic coordinating groups such as asymmetric olefin epoxidations using Jacobsen's catalysts.[14] Asymmetric syntheses can also be performed on *meso* or prochiral substrates with enzymes.

- *Difference in reaction kinetics (kinetic resolutions)*. The reaction of racemic mixtures with a chiral agent lead to an accumulation of one of the enantiomers of a compound provided the reaction is stopped prior to completion. The chiral agent can be an enzyme, which is the focus of this thesis, but it can also be a synthetic catalyst, such as the recently developed planar-chiral heterocycles developed by Fu and coworkers for the resolution of chiral secondary alcohols.[15] Unlike asymmetric synthesis, the enantiomeric excess of the reaction products is a function of the degree of conversion so high enantiomeric excess can be sometimes gained at the expense of yield.

- *Crystallization techniques*. Mixtures of stereoisomers are separated physically usually via diastereomer formations with resolving agents.[16]

- *Chromatography processes*. These processes rely on the stereoselective sorption of enantiomers on, or in, chromatographic systems equipped with chiral stationary phases.[17]

- *Stereospecific synthesis from existing chiral precursors*. Chemists can use naturally available chiral compounds such as carbohydrates, amino acids, terpenes and alkaloids as chiral building blocks.

Enzymes are exquisite natural catalysts and remain the standard to which synthetic asymmetric catalysts aspire to reach. Because they are composed of chiral amino acids, they are three-dimensional chiral catalysts that nature has evolved to carry

out highly specialized chemical reactions with high substrate specificity and stereospecificity. Over 2,000 enzymes have been isolated and characterized from a single cell! Each of these enzymes control a specific function that is necessary for life such as cell growth, reproduction, synthesis, metabolism, and energy utilization.

Over the past twenty years it has become increasingly apparent that the natural stereospecificity of enzymes can be exploited to produce chiral molecules *ex vivo*. In particular, researchers have explored the use of hydrolytic enzymes (hydrolases) for kinetic resolutions of racemic ester substrates to produce pure enantiomers. Despite their exquisite biological substrate specificity, hydrolases can display excellent activity and stereoselectivity towards non-natural substrates. Thousands of examples using hydrolases to produce pure stereoisomers of non-natural substrates have appeared in the literature.

This thesis focuses on the use of hydrolases as chiral biocatalysts for the production of pure stereoisomers of synthetically useful compounds.

1.4 The use of hydrolases as chiral biocatalysts

The worldwide sales for industrial enzymes are currently estimated to be US$ 1 billion and at least 75% of these enzymes are hydrolases.[18] In the simplest of terms, they catalyze the hydrolysis and formation of ester and amide bonds. The main industrial applications for hydrolases are in detergents, oil processing, and dairy products, but they have emerged as the most common type of biocatalyst used for the production of chiral building blocks in organic synthesis. Several features that make them attractive as catalysts include:

- A large variety is commercially available.
- They do not require cofactors for activity.
- They can accept a wide variety of substrates, not just their natural substrates.
- They are environmentally friendly.
- They display a wide range of catalytic activities.
- Many important classes of chiral building blocks are substrates for hydrolases, e.g. chiral alcohols and chiral carboxylic acids are easily converted into ester derivatives.

Lipases, esterases and proteases are the three main classes of hydrolytic enzymes used as biocatalysts and their properties have been well documented.[19] Less common members of the hydrolase family include phospholipases, epoxide hydrolases, amidases, and nitrilases. The work in this thesis largely explores the use of the three main classes of hydrolases as chiral biocatalysts because of their low cost, good stability and broad substrate tolerance.

With all hydrolases, a nucleophile in the active site of the enzyme attacks the carbonyl group of the substrate ester. Lipases, esterases and serine proteases use a serine residue in its active site as the nucleophile. With metallo-proteases and aspartyl proteases, the nucleophile is a hydroxyl group generated in the active site. Thiol proteases use a cysteine residue as a nucleophile.

Serine hydrolases contain a catalytic triad that forms a charge relay system, which transfers electrons from the buried side chain of an aspartate/glutamate residue to the serine residue making it a potent nucleophile towards an ester bond. This catalytic triad was first observed in α-chymotrypsin[20] but has since been observed in many other hydrolases, including *Candida rugosa* lipase, Figure 1.2.[21] This mechanism shows the hydrolysis of a butyrate ester. All serine hydrolases largely follow a similar mechanism, which can be described kinetically as an irreversible ping-pong bi-bi reaction.[22] The ester and water are the two substrates and two products, an acid and an alcohol, are released. The first product leaves before the second substrate, water, arrives. The second product is then formed and liberated, leaving the hydrolase in its original state for the next round of catalysis.

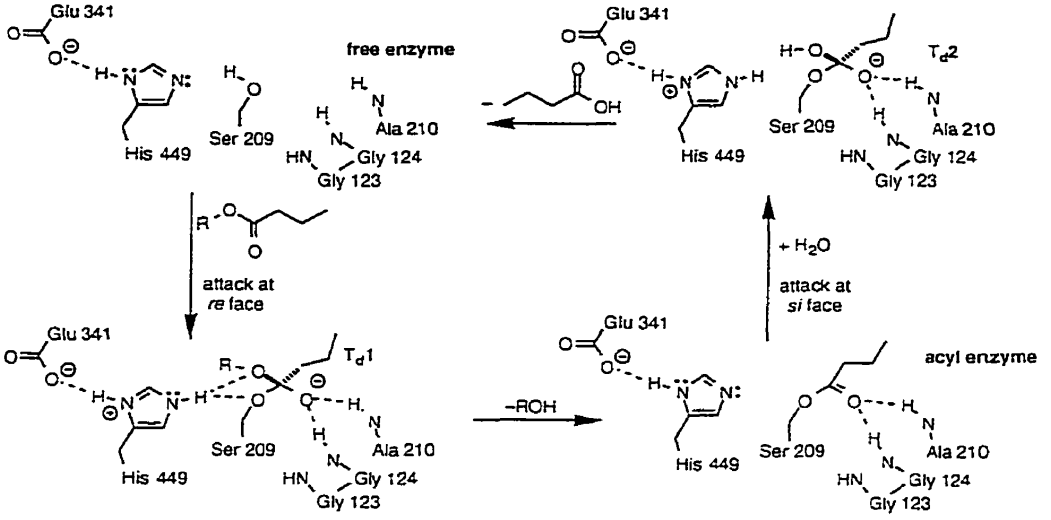


Figure 1.2 Hydrolysis of a butyrate ester catalyzed by lipase from *Candida rugosa*. The catalytic triad is composed of Glu341, His449 and Ser209. The mechanism of hydrolysis involves an acyl enzyme intermediate and two negatively charged tetrahedral intermediates, which are stabilized by residues forming the oxyanion hole.

Due to the chirality of the active site of the hydrolase, one enantiomer of a pair fits in the active site better and is converted to product faster. This difference in rates of reaction is the foundation for hydrolases as chiral biocatalysts. One of the earliest theories to account for this enzyme-substrate fit is the well-known lock and key model proposed at the beginning of this century. Since then, our view of enzymes as rigid three dimensional structures has evolved. We now know that enzymes can modify their conformations to induce a fit for many substrates, yet still display selectivity towards one enantiomer over the other.

1.5 Defining Enantioselectivity

Hein and Neimann first formulated the degree of stereospecificity of an enzyme as the ratio of specificity constants (k_{cat}/K_M) of the (*R*) and (*S*)-enantiomers in α-chymotrypsin catalyzed hydrolysis reactions.[23] The kinetic constants, k_{cat} and K_M, describe the reaction of each enantiomer with the enzyme. This ratio was later formulated by Sih and coworkers as a dimensionless parameter called the enantiomeric ratio, E.[24] This ratio

represents the preference an enzyme has for one enantiomer over the other and is widely accepted as the parameter for the quantification of the intrinsic enantioselectivity of an enzyme. The value of E cannot change unless the values of the kinetic constants change.

The basis for the enantiomeric ratio can be found in kinetic equations that describe enzyme-catalyzed hydrolysis of an ester.

Kinetic equations for enantioselectivity

A simple, irreversible hydrolase-catalyzed single substrate hydrolysis can be described by the Michaelis-Menten mechanism where E is the free enzyme; S is the substrate; ES is the enzyme-substrate complex; P is the product; and k_1, k_{-1} and k_2 are rate constants:[25]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

No chemical changes occur in the first reversible step. The second step is assumed irreversible if [P] is very low, which is the case when the velocity is measured at initial stages of the reaction. Therefore the initial rate equation can be expressed as:

$$v = d[P]/dt = k_2[ES] \qquad \text{Eq. (1.1)}$$

From the steady state assumption, the rate of formation of the ES complex is equal to its rate of destruction which is true if [S] >> [E]. Therefore:

$$+d[ES]/dt = -d[ES]/dt \qquad \text{Eq. (1.2)}$$

The concentration of [ES] can be defined as:

$$k_1[E][S] = (k_2 + k_{-1})[ES] \qquad \text{Eq. (1.3)}$$

During the reaction, the enzyme is either bound to the substrate as an ES complex or free in solution. Therefore:

$$[E]_o = [E] + [ES] \qquad \text{Eq. (1.4)}$$

where $[E]_o$ is the total enzyme concentration and [E] is the free enzyme in solution.

Combining equations 1.3 and 1.4 gives:

$$[ES] = \frac{[E]_o[S]}{\left(\frac{k_2 + k_{-1}}{k_1}\right) + [S]} \qquad \text{Eq. (1.5)}$$

Equation 1.1 now becomes:

$$v = \frac{k_2[E]_o[S]}{\left(\frac{k_2 + k_{-1}}{k_1}\right) + [S]} \qquad \text{Eq. (1.6)}$$

Defining V_{max} as the maximum velocity at saturating substrate concentration ($V_{max} = k_2[E]_o$) and K_M as the substrate concentration at 50% of maximum velocity ($S = K_M$ if $v = V_{max}/2$):

$$\frac{V_{max}}{2} = \frac{V_{max}K_M}{\left(\frac{k_2 + k_{-1}}{k_1}\right) + K_M} \qquad \text{Eq. (1.7)}$$

Therefore:

$$K_M = \frac{k_2 + k_{-1}}{k_1} \qquad \text{Eq. (1.8)}$$

and equation 1.6 becomes:

$$v = \frac{k_2[E]_o[S]}{K_M + [S]} \qquad \text{Eq. (1.9)}$$

This basic equation of enzyme kinetics is called the Michaelis-Menten equation. It is true under steady-state conditions with no product inhibition. In this thesis we only explore reactions that are assumed to follow Michaelis-Menten kinetics.

Defining V_{max} as:

$$k_2[E_o] = V_{max} \qquad \text{Eq. (1.10)}$$

Equation 1.9 can also be expressed as:

$$v = \frac{V_{max}[S]}{K_M + [S]} \qquad \text{Eq. (1.11)}$$

In a kinetic resolution, the (*R*) and (*S*)-enantiomers compete for the enzyme active site where k_{cat} (identical to k_2) is the enzyme turnover number:

$$\begin{array}{l} S \\ + \\ E \\ + \\ R \end{array} \quad \begin{array}{l} \overset{K_{M\,S}}{\rightleftharpoons} \\ \\ \\ \overset{K_{M\,R}}{\rightleftharpoons} \end{array} \quad \begin{array}{l} ES \xrightarrow{k_{cat\,S}} E + P \\ \\ \\ ER \xrightarrow{k_{cat\,R}} E + Q \end{array}$$

Combining Eq. 1.1 and Eq. 1.9 gives:

$$K_M = \frac{[E][S]}{[ES]} \qquad \text{Eq. (1.12)}$$

Substituting Eq. 1.12 into Eq. 1.1 gives:

$$v_S = \left(\frac{k_{cat}}{K_M}\right)_S [E][S] \qquad \text{Eq. (1.13)}$$

$$v_R = \left(\frac{k_{cat}}{K_M}\right)_R [E][R] \qquad \text{Eq. (1.14)}$$

Dividing Eq. 1.13 by Eq. 1.14:

$$\frac{v_S}{v_R} = \frac{\left(\frac{k_{cat}}{K_M}\right)_S [S]}{\left(\frac{k_{cat}}{K_M}\right)_R [R]} \qquad \text{Eq. (1.15)}$$

This ratio is called the enantiomeric ratio, E. In a racemic mixture the initial substrate concentrations are equal, so $[S]$ and $[R]$ cancel each other out. The enantiomeric ratio, E, is expressed by:

$$E = \frac{\left(\frac{k_{cat}}{K_M}\right)_S}{\left(\frac{k_{cat}}{K_M}\right)_R} \qquad \text{Eq. (1.16)}$$

K_M is called the Michaelis constant and approximates the dissociation constant (K_S) of the enzyme-substrate complex when the subsequent step, k_2, is slow, as it is in hydrolase-catalyzed resolutions. It is a direct reflection of substrate binding; lower values indicate better binding. The kinetic constant, k_{cat}, is the apparent first order rate constant for conversion of the enzyme-substrate complex to product.

The enantiomeric ratio serves as a measure of the performance of the hydrolase to produce enantiomerically pure compounds. For synthetic purposes, the general guideline is that enantiomeric ratios of less than 10 are unacceptable for preparative purposes. A hydrolase is regarded as a modest chiral catalyst if the enantioselectivity is between 10-30 and hydrolases with enantioselectivities greater than 30 are considered excellent.

The free energy approach to enantioselectivity

Similarly, the enantioselectivity of a hydrolase can be related to the difference in free energies during a kinetic resolution, Figure 1.3. The (*R*)-enantiomer and (*S*)-enantiomer compete for the active site of the enzyme and each form diastereomeric complexes

through the catalytic serine with the hydrolase. The difference in free energy of the two diastereomeric transition states equals $\Delta\Delta G^{\ddagger}$

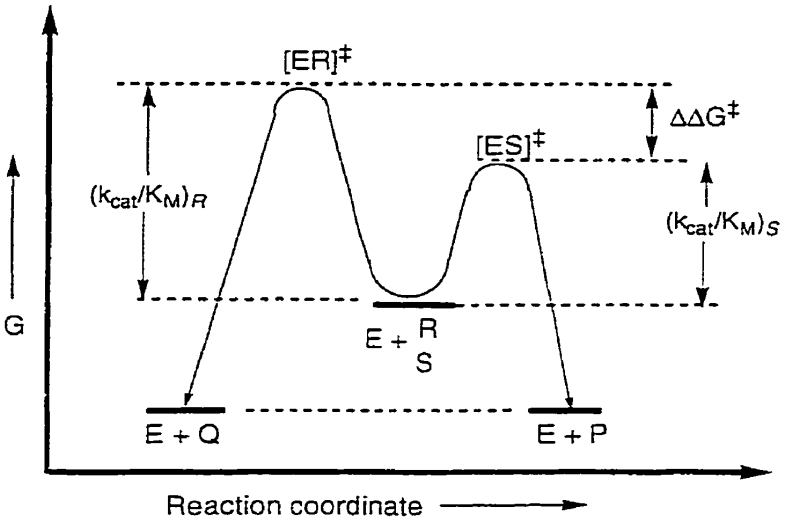


Figure 1.3 Free energy profile of a hypothetical kinetic resolution using a hydrolase that follows Michaelis-Menten kinetics.

According to transition state theory, the enantiomeric ratio, E, is related to the term $\Delta\Delta G^{\ddagger}$ which is the free energy difference of the diastereomeric transition states, Equation 1.17.

$$\Delta\Delta G^{\ddagger} = \Delta G_S^{\ddagger} - \Delta G_R^{\ddagger} = -RT\ln E = -RT\ln\left[\frac{(k_{cat}/K_M)_S}{(k_{cat}/K_M)_R}\right] \qquad \text{Eq. (1.17)}$$

From equation 1.17, we can calculate that a large value for enantioselectivity, E >100, corresponds to a $\Delta\Delta G^{\ddagger}$ of > 2.7 kcal/mol.

1.6 Industrial applications of hydrolases for the production of chiral drugs

Industrial methods that use hydrolases to produce pure stereoisomers are often more selective and cheaper than chemical methods. Three current preparative-scale hydrolase-catalyzed routes to pharmaceuticals and their precursors provide excellent examples.

Lipases are the most commonly used class of hydrolases as biocatalysts and there are numerous excellent examples of their use in the production of optically pure compounds.[26] Patel and coworkers at Bristol-Myers Squibb (New Jersey, USA) used lipase from *Pseudomonas cepacia* to produce (3*R*-*cis*)-3-(acetyloxy)-4-phenyl-2-azetidinone, a sidechain synthon for the anti-cancer drug Taxol, in kilogram quantity, and >99% enantiomeric excess, Figure 1.4.[27]

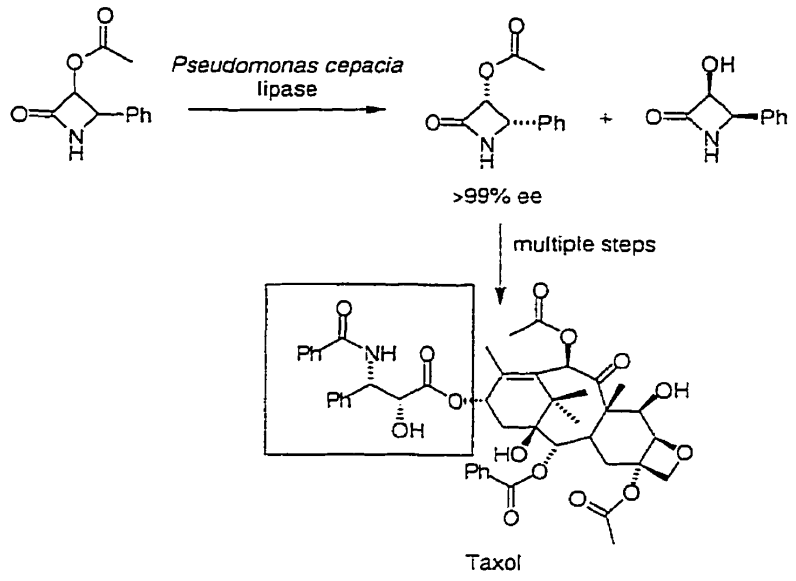


Figure 1.4 Hydrolase-catalyzed resolution of a key intermediate of the anti-cancer drug, Taxol.

In another example, Tanabe Pharmaceutical (Japan) and DSM-Adeno (Netherlands) commercialized a lipase-catalyzed resolution of a key precursor to Diltiazem, a calcium channel blocker for treating high blood pressure, using lipase from *Rhizomucor miehei*[28] or lipase from *Serratia marcescens*[29], Figure 1.5. The unwanted enantiomer was hydrolyzed by both lipases and, conveniently, its acid form spontaneously decarboxylated to yield the desired (2*R*,3*S*)-ester and an aldehyde side product as reaction products. The (2*R*,3*S*)-ester intermediate is used to produce Diltiazem.

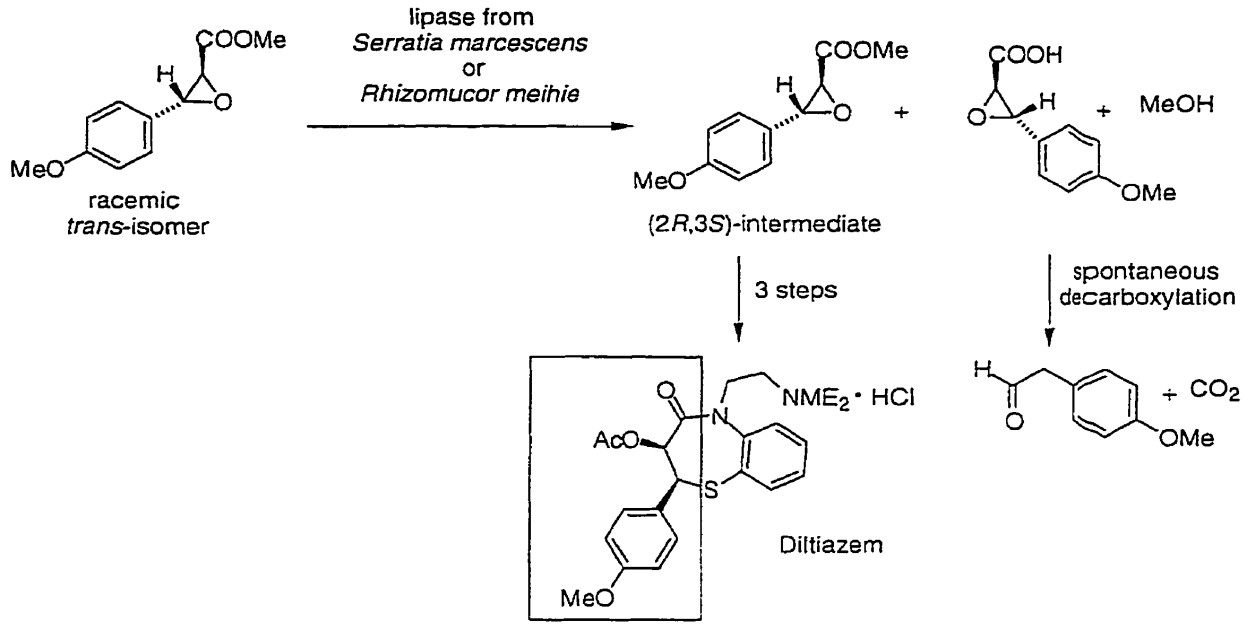


Figure 1.5 Commercial synthesis of Diltiazem by Tanabe Pharmaceutical and DSM-Adeno uses a kinetic resolution catalyzed by lipase from *Rhizomucor miehei* or lipase from *Serratia marcescens*.

Jacobsen and coworkers have also proposed a chemical route to this drug based on an asymmetric epoxidation of a *p*-methoxycinnamate ester using a chiral (salen)Mn complex.[30] The epoxide intermediate is made in high chemical yields and 96% enantiomeric excess but the chemical route requires four synthetic steps to synthesize the starting cinnamate ester. Overall, this chemical method is a less efficient method than the hydrolase-catalyzed route.

In general, it is more cost-efficient to carry out kinetic resolutions on early-stage precursors, but researchers have also used hydrolases to resolve the final drug product. For example, ChiroTech (UK) developed a process to commercially produce (*S*)-Naproxen, a non-steroidal, anti-inflammatory drug, using an esterase of unspecified source, Figure 1.6.[31] Esterases are another major class of hydrolases in the production of pure stereoisomers.[32] In this process, the unwanted enantiomer is continuously racemized using a metal catalyst, so the potential yield for this kinetic resolution is increased from 50% to 100%.

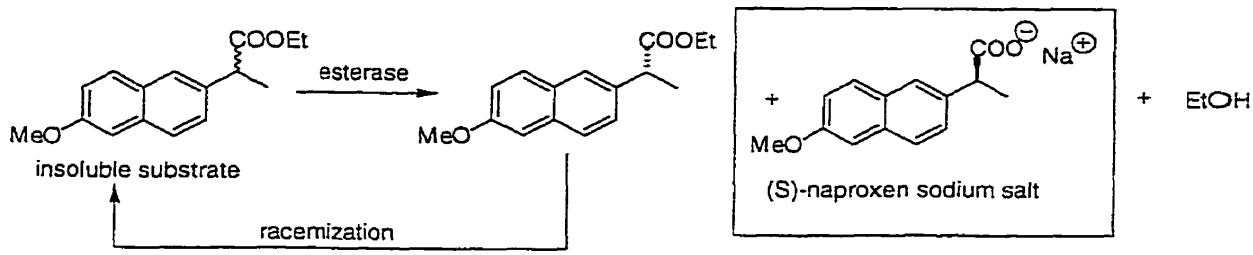


Figure 1.6 Commercial production of (*S*)-Naproxen using an esterase to selectively hydrolyze the (S)-enantiomer. The unreacted (*R*)-Naproxen remains insoluble in solution, is filtered off and chemically racemized for recycling. In 1996, 13,000 kilograms of (*S*)-Naproxen was produced using this method and sold to generic drug firms.

1.7 Identifying active and selective hydrolases

The perception of hydrolases as exotic and delicate catalysts that require a separate set of skills in the laboratory has considerably diminished but the difficult task of selecting a suitable hydrolase remains the major deterrent to their widespread application. One of the hallmarks of hydrolases as biocatalysts is their promiscuity to accept a wide range of compounds that are not their natural substrates. This promiscuity is often afforded at the expense of selectivity, so hydrolases do not always display excellent enantioselectivity.

Several "workhorse" hydrolases such as *Candida rugosa* lipase, *Pseudomonas cepacia* lipase, and pig liver esterase frequently appear in the literature as chiral catalysts but usually a researcher conducts considerable reaction optimization to obtain suitable enantioselectivities with these hydrolases. Currently, hundreds of hydrolases and strains of microorganisms that secrete hydrolases are commercially available. For example, Fluka Chemical company currently sells over 150 hydrolases. A researcher increases the chance of identifying enantioselective hydrolases by having a large library of hydrolases available for testing and, importantly, has efficient methods to select the best hydrolases from this library for a given transformation.

There are several approaches that have been developed to help a researcher "screen" a collection of hydrolases for enantioselectivity towards a target substrate. Broadly speaking, a researcher can:

- Use empirical rules and active site models to predict the enantiopreference and crudely estimate the enantioselectivity of hydrolases towards a target substrate.

- Determine the enantioselectivities of hydrolases towards a target substrate by accurately determining their enantiomeric ratios, E, using one of several techniques.

- Estimate the enantioselectivities of hydrolases towards a target substrate rather than accurately measuring them.

All three techniques are complimentary to each other. However, a researcher must inevitably measure the enantiomeric ratio, E, of the hydrolase-catalyzed resolution to confirm the hydrolase's enantioselectivity. The following three sections explore these approaches in detail.[33]

1.8 Predicting the enantiopreference of hydrolases

To simplify the selection of hydrolases as stereoselective synthetic reagents and eliminate unsuitable hydrolases, researchers have developed empirical rules and active site models to predict the enantioselectivity of hydrolases towards racemic substrates. Empirical rules can predict the enantiopreference (which enantiomer is preferred by the hydrolase) of a hydrolase based upon the relative size and type of substituents of the substrate at its stereocentre. These rules are meant to serve as general guidelines for researchers when choosing hydrolases for synthetic applications. Active site models crudely map the topography and hydrophobicity of the active site of hydrolases and help predict the enantiopreference and enantioselectivity of a hydrolase towards the substrate. These predictive models and rules are particularly useful for defining the active site of hydrolases if X-ray crystal structures of hydrolases are not available.

Empirical rules for predicting the enantiopreference of hydrolases

The first example of a size rule to predict selectivity was Prelog's rule for predicting the enantioselectivity of the reduction of ketones by the yeast *Culvularia falcata* based upon the relative size of the two substituents adjacent to the carbonyl, Figure 1.7.[34] This rule also suggested that increasing the difference in size of the substituents adjacent to the carbonyl could improve enantioselectivity.[35]

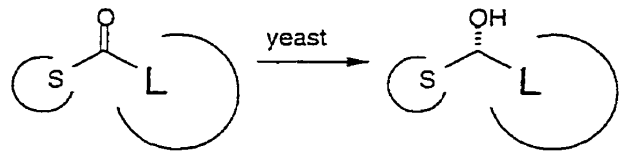


Figure 1.7 Prelog's rule for predicting the enantiopreference for the reduction of carbonyl compounds by yeast. L = large, bulky substituent such as phenyl, s = small substituent such as methyl.

Similar size rules have been developed for the kinetic resolutions of chiral alcohols, carboxylic acids, and amines and the most common examples are briefly outlined below.

Empirical rules for secondary alcohols and amines

Kazlauskas and coworkers proposed a size rule in 1990 to predict with high accuracy which enantiomer of secondary alcohols is preferred by three common hydrolytic enzymes, cholesterol esterase, lipase from *Pseudomonas cepacia*, and lipase from *Candida rugosa*.[36] To develop the rule, they summarized the observed enantiopreferences from the resolution of cyclic and acyclic substrates reported in the literature. This rule suggested that lipases and esterases discriminate between enantiomers of secondary alcohols based upon the size of the substituents at the active site. Since then, this rule has been extended to several other hydrolases towards secondary alcohols, Figure 1.8a.[21]

Several researchers carried out experiments to confirm the relationship between size of substituents of secondary alcohols and enantioselectivity. They found that when the size of one of the substituents of a secondary alcohol with two similarly-sized

substituents is increased, for example through hydroboration of a double bond, enantioselectivity increased.[36, 37]

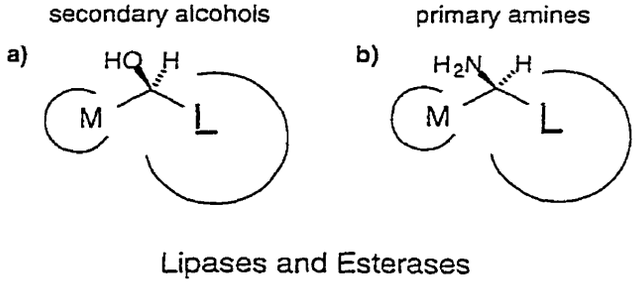


Figure 1.8 Empirical rules that predict which enantiomer of a secondary alcohol and primary amine react faster with lipases and esterases. The rules are valid for all lipases and esterases whose substrate specificity has been currently mapped: twelve lipases and esterases for secondary alcohols, and three lipases for primary amines.[38]

Researchers similarly found that lipases and esterases follow the same empirical size rule towards primary amines of the type NH_2CHRR', Figure 1.8b.[39] This discovery was not surprising because researchers have elucidated the molecular basis for enantiodiscrimination of secondary alcohols in lipases and esterases. Using x-ray crystallography, researchers discovered a common three-dimensional arrangement of the catalytic machinery necessary for catalysis in lipases and esterases. The active site of lipases and esterases contains both a large hydrophobic binding site open to the reaction solvent and a smaller, restricted binding pocket.[40] The larger pocket binds the large substituent of primary amines and secondary alcohols while the restricted binding pocket can only bind the smaller substituent. The structural elements necessary for catalysis (the catalytic triad and oxyanion hole described in Section 1.4) and the overall protein folding pattern sets the enantioselectivity of secondary alcohols and amines in the active site. Importantly, these structural elements have been conserved throughout evolution in lipases and esterases. Therefore, despite very different structural and phylogenetic origins and disparate sequence homology, all lipases and esterases studied by x-ray crystal structures contain these structural elements.[41] This common three-dimensional

scaffolding is commonly referred to as the α/β-hydrolase fold, because it is mostly composed of a parallel β-sheet as the core of the protein with α-helices surrounding this core.[42]

Empirical size rules for secondary alcohols and amines were also proposed for the serine protease, subtilisin, and they were found to be mirror images to the empirical rules proposed for lipases and esterases, Figure 1.9.[39b, 43] Subtilisin's catalytic machinery is an approximate mirror image of the α/β-hydrolase fold found in lipases and esterases so it is not surprising that their enantiopreference is also a mirror image to that for lipases and esterases.[42, 44]

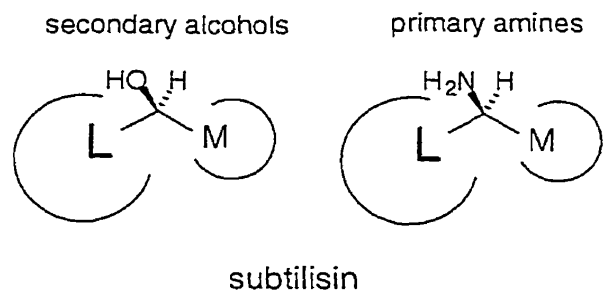


Figure 1.9 Empirical rule that predicts the enantiopreference of subtilisin towards secondary alcohols and primary amines. Note that these rules are the opposite of the rules for lipases and esterases.

Empirical rules for primary alcohols

Developing an empirical rule for the selectivity of lipases and esterases toward primary alcohols has been more difficult than for secondary alcohols. Most lipases and esterases exhibit low enantioselectivity towards primary alcohols in part because they must not only distinguish between the difference in size of substituents at the stereocentre but also different conformations of the substrate along the flexible C(1)-C(2) bond of primary alcohols.

Only two lipases, porcine pancreatic lipase (PPL) and *Pseudomonas cepacia* lipase (PCL), have shown moderate to excellent enantioselectivity towards some primary alcohol substrates and researchers have tried to develop empirical size models to predict their enantiopreference.[45, 46] Surprisingly, an empirical rule proposed for PCL by

Kazlauskas towards primary alcohols was opposite to that for secondary alcohols and was only reliable for substrates without oxygen at the stereocentre.[45a] This finding suggests that factors other than size of the substituents at the stereocentre can contribute to the enantiodiscrimination of primary alcohols by PCL, Figure 1.10. Computer modeling of transition state analogs of substrates bound to PCL suggest that primary alcohols bind in different regions of the lipase than secondary alcohols, accounting for the reversal of enantiopreference towards secondary alcohols.[47] Furthermore, this modeling suggested that H-bonding also sets the enantioselectivity in PCL towards substrates with oxygen at the stereocentre. Such a finding cannot be predicted with empirical size models.

Overall, empirical rules are much less reliable and general for predicting the enantiopreference towards primary alcohols than those for secondary alcohols and amines.

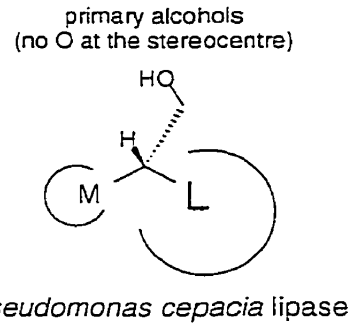


Figure 1.10 Empirical rule that summarizes the enantiopreference of *Pseudomonas cepacia* lipase (PCL) towards chiral primary alcohols. This rule is reliable when an oxygen atom is not directly attached to the stereocentre. Note that PCL follows opposite size rules toward primary and secondary alcohols.

Empirical rules for carboxylic acids

Although there are fewer examples of resolutions of carboxylic acids than secondary alcohols in the literature, hydrolases catalyze their enantioselective hydrolysis.[48] A particularly important class of chiral carboxylic acids is 2-arylpropionic acids, which are non-steroidal anti-inflammatory drugs, for example Naproxen, shown in Figure 1.6. Purified lipase from *Candida rugosa* exhibits excellent enantioselectivity

towards 2-arylpropionic acids.[49] An empirical rule was developed by the Kazlauskas group to predict which enantiomer of chiral carboxylic acids reacts faster with *Candida rugosa* lipase (CRL), Figure 1.11.[50] This rule is also based upon the size of the substituents of the stereocentre but is only reliable for CRL that has been partially purified. The rule is only reliable 68% of the time with crude CRL (random guessing is 50% reliable because there are only two choices), but the rule is completely reliable with the purified form of the lipase.

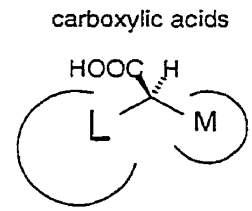


Figure 1.11 Empirical size rule for the enantiopreference of *Candida rugosa* lipase (CRL) toward carboxylic acids with the stereocentre at the α-position. This rule is reliable for purified CRL only. Note that this size rule is opposite to the size rule for secondary alcohols with CRL.

It is difficult to develop an empirical rule for an entire class of hydrolases such as lipases and esterases toward chiral carboxylic acids because the acid portion of an ester binds in regions of the hydrolase that are not conserved structural elements. Subsequently, when chirality is in the acid portion of an ester, binding can vary widely between similar hydrolases. An empirical rule could not be deduced that accurately predicts the preferred enantiomer of carboxylic acids by *Pseudomonas cepacia* lipase although researchers have suggested that stereoelectronic effects can set the enantiopreference.[51] Furthermore, contaminating hydrolases present in commercial preparations of the hydrolase can effect the observed enantioselectivity towards chiral carboxylic acids since they are likely to have varying degrees of selectivity towards the substrate.

Aspergillus niger lipase (ANL) is one of the most common hydrolases reported in the literature for the production of chiral molecules, especially secondary alcohols and carboxylic acids. These applications have been discovered largely through trial and error and empirical rules had not been developed to aid in the logical application of this lipase as a chiral catalyst. We therefore used substrate mapping to identify the structural features necessary for excellent stereoselectivity with ANL, Figure 1.12. Similar to other lipases, ANL obeyed the empirical size rule for secondary alcohols. An empirical rule based on the size of the substituents at the stereocentre could not predict the fast-reacting enantiomer of chiral carboxylic acids. Surprisingly, we found that the presence of a positively-charged substituent at the stereocentre sets the enantiopreference towards carboxylic acids despite the size of the other substituent. The results of this work are presented fully in Chapter 2 of this thesis.

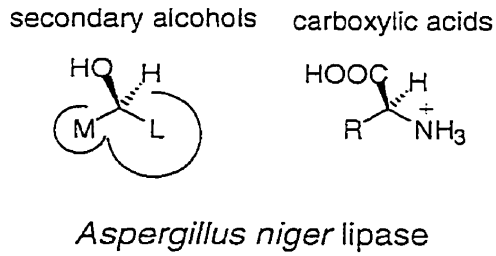


Figure 1.12 Empirical rules for the enantiopreference of lipase from *Aspergillus niger*.

Empirical rules for more limited classes of substrates

More specialized rules have been proposed to account for the enantioselectivity of hydrolases towards narrower classes of substrates. They enable a researcher to make more detailed predictions but they are very limited in their scope and are only accurate for closely related structures.

For example, Itoh and coworkers proposed a model for the resolution of secondary alcohols of 2-(methylthio)-3-acetoxy esters by *Aspergillus niger* lipase (ANL), Fig 1.13.[52] They found that the diastereomers in the *anti* conformation were preferentially hydrolyzed over their corresponding *syn* diastereomers. Also, substrates bearing aromatic

and unsaturated groups at the C-3 position showed higher selectivity than those with aliphatic groups at the C-3 position. The optimal substituents at C-2 position were medium sized, and a sulfur group at this position helped set the stereoselectivity of ANL. Overall, this model is only useful for predicting the stereopreference of compounds that bear an aromatic substituent at the 3-position and a methyl or methylthio substituent at the 2-position.

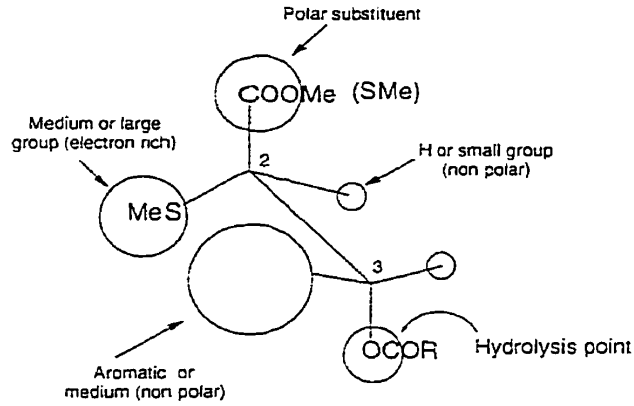


Figure 1.13 Structure and stereochemical requirements for fit in the active site of *Aspergillus niger* lipase proposed by Itoh and coworkers to account for the observed diastereoselectivity towards secondary alcohols of 2-(methylthio)-3-acetoxy esters. The preferred stereoisomer is shown.

A similar substrate model was proposed by Tamm and coworkers to predict the enantiopreference towards *meso* and prochiral diesters by pig liver esterase (PLE), Figure 1.14.[53]

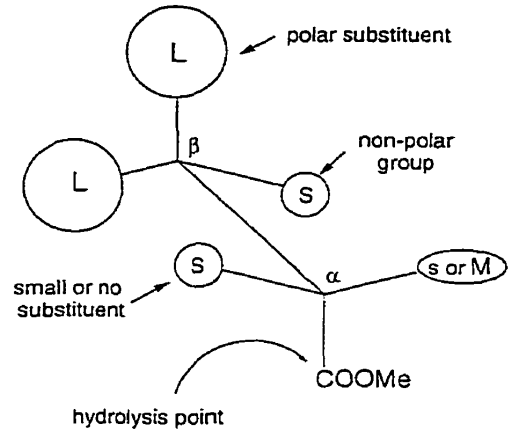


Figure 1.14 Structural model showing the preferred enantiomer of *meso* and prochiral diesters by pig liver esterase. This model summarizes the structural and stereochemical requirements for an ester to be a good substrate for PLE. The substituents are assigned according to their sizes small (s), medium (M) and large (L).

Active site models to predict the enantiopreference of hydrolases

Conversely, researchers also use active site models to more accurately map the topography and hydrophobicity of a hydrolase's active site to define the structure of the hydrolase's ideal substrate. Typically, these cubic-space models are composed of smaller regions or pockets, which control substrate binding. To develop these models, usually a researcher carries out substrate mapping, whereby a series of substrates are tested to explore the size and hydrophobicity/hydrophilicity of functional groups that the active site can accommodate. These models can be used to predict the selectivity of new substrates by considering favorable interactions between the substituents of the substrate and the different binding regions.

A well-known example is the active site model for pig liver esterase (PLE) by Jones and coworkers, Figure 1.15.[54] Unlike the substrate model for PLE above, this model can determine the stereoselectivity of a wide range of methyl ester substrates including novel substrates not previously resolved.[55] To determine the best fit and stereospecificity of a substrate, the ester group to be hydrolyzed is placed within the serine sphere and the remaining substrate is fitted into the H and P pockets following a set of well-defined rules.

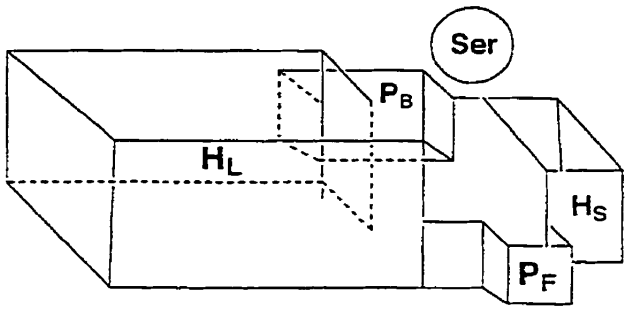


Figure 1.15 The active-site model for pig liver esterase proposed by Jones and coworkers to predict the stereospecificity for racemic acids. The most important binding regions that set the specificity are the large and small hydrophobic areas, marked H_L and H_S. Two pockets have a more polar character, marked P_B (polar back) and P_F (polar front). The sphere marked "Ser" indicates the serine residue involved in catalysis.

Similarly, active site models have also been proposed for another common lipase, *Pseudomonas cepacia* lipase (PCL), Figure 1.16.[56] In both models, differently shaped hydrophobic regions define the binding and orientation of a substrate in the active site of PCL.

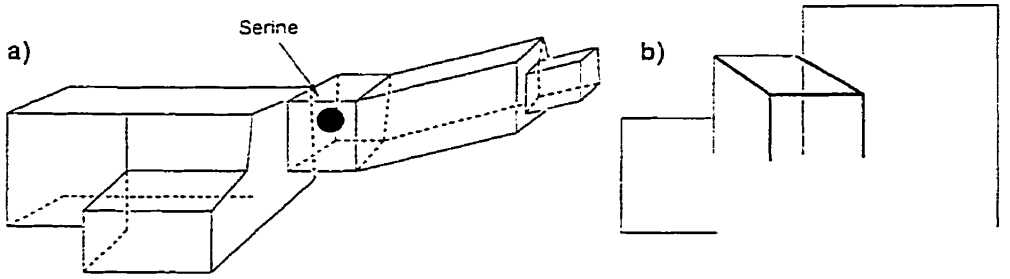


Figure 1.16 a) A three-dimensional active site model of PCL by Theil and coworkers which proposes the size and shape of the hydrophobic binding pockets which control stereoselectivity. **b)** Two-dimensional active site model of PCL proposed by Burgess and Naemura for the acylation of alcohols. The canopy region is the site that recognizes the hydroxyl function of the substrates.

Overall, using empirical rules and active site models can eliminate unsuitable hydrolases towards a target substrate and help predict the outcome of a kinetic resolution. However, there are limitations to their use. Empirical rules do not necessarily display 100% accuracy for predicting the preferred enantiomer (although most are greater than

90%), so some substrates are exceptions to the rule and the absolute stereochemistry of the reaction products must be confirmed experimentally. Empirical rules cannot account for factors, other than size, that contribute to enantioselectivity For example, Hult, Norin and coworkers observed very different enantioselectivities towards three secondary alcohols that are similar in size but very different in electronic effects with *Candida antarctica* lipase B (CALB), Figure 1.17.[57, 58]

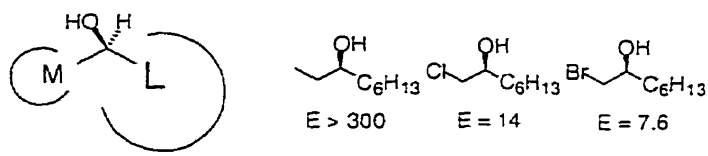


Figure 1.17 Enantioselectivity of CALB-catalyzed acylation of similar sized secondary alcohols. The enantioselectivity towards 3-nonanol is exquisite, E > 300; upon replacement of a CH_3 group with a similarly sized Br, the enantioselectivity drops to 7.6, while replacement with Cl shows an E of 14. All substrates obey the size rule for secondary alcohols shown on the left.

In short, reliable data concerning the rate and enantioselectivity of a reaction can only be given by experimentally measuring the enantiomeric ratio, E of a hydrolase towards the target substrate.

1.9 Measuring the enantioselectivity of hydrolases

There are several methods to quantitatively measure the enantiomeric ratio, E. This determination is the slowest step in the selection process of suitable hydrolases for a given transformation. Each method has its own advantages and disadvantages and they are reviewed below. In particular, their use as methods to rapidly but accurately determine the enantioselectivities of large numbers of hydrolases is evaluated.[59]

Note that the enantioselectivity of commercial hydrolases can be the apparent enantioselectivity since some commercial hydrolases have contaminating hydrolases

present. Although kineticists stress the importance of absolute purity of each enzyme, a synthetic chemist is more interested in the outcome of the kinetic resolution to produce pure stereoisomers than the absolute values of kinetic constants.

Determining E by determining kinetic constants.

From equation 1.16, it is obvious that a researcher can determine the enantioselectivity of a hydrolase towards a substrate by determining the k_{cat} and K_M values for each enantiomer under the desired synthetic conditions.[60] Typically, a researcher measures the initial rates of hydrolysis of the pure enantiomers of a substrate as a function of their concentration under a fixed set of reaction conditions and plots the data using a linear form of Equation 1.11 for analysis. There are several different linear plots available for statistical analysis. The most common are the Lineweaver-Burk plot (1/v against 1/[S])[61], the Eadie-Hofstee plot (v against v/[S])[62], and the direct linear plot (v against [S]), Figure 1.18.[63]

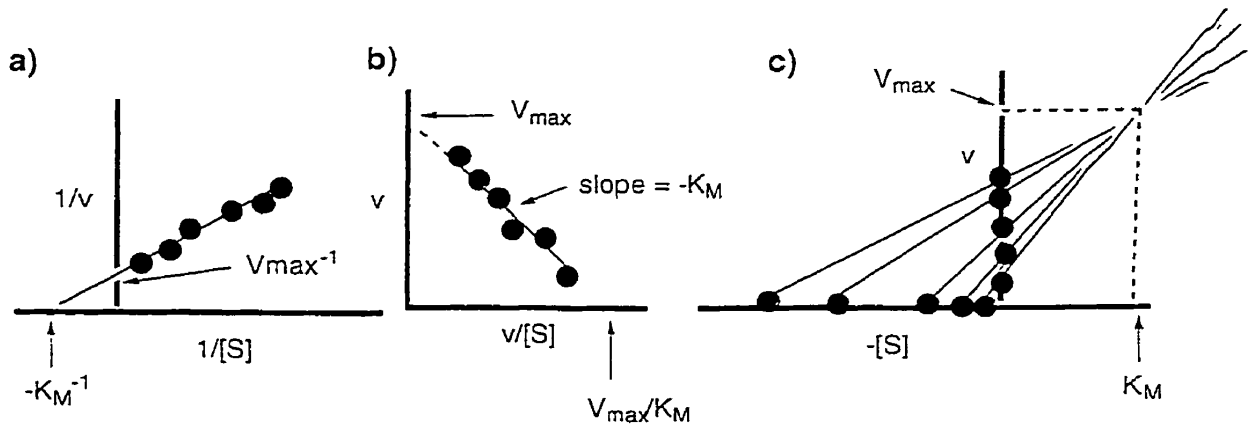


Figure 1.18 Linear plots for determination of kinetic constants from initial rate measurements: **a)** Lineweaver-Burk plot **b)** Eadie-Hofstee plot **c)** direct linear plot. Typically, each plot requires eight to twelve initial rate measurements for accuracy.

Although valuable kinetic information is determined during these experiments, this method is time-consuming and laborious because it requires multiple substrate measurements below and above the K_M value (usually from $K_M/2$ to $10K_M$). Often, it is difficult to accurately determine kinetic constants of non-natural substrates with a

hydrolase because their K_M values are larger than the substrate concentrations that are experimentally possible due of to solubility limits. Also, this procedure requires significant data manipulation and the calculated values are prone to error depending upon the statistical method that is used for analysis.[63]

Overall, determining the enantiomeric ratio, E, by measuring kinetic constants is too slow and labour intensive for screening a library of hydrolases towards a synthetic target for high enantioselectivity.

Determining E by measuring enantiomeric purity

Most chemists prefer to use the integrated form of equation 1.15 developed by Sih and coworkers, commonly referred to as the endpoint method.[24] This formulation is based upon earlier work by Sharpless and coworkers to measure the enantiomeric ratio of non-enzymatic systems.[64] In equation 1.15, $v_S = -d[S]/dt$ and $v_R = -d[R]/dt$. Integration yields the enantiomeric ratio, E, where $[S_o]$ and $[R_o]$ are the initial concentrations of both enantiomers and $[S]$ and $[R]$ are the remaining concentrations after a given reaction time.

$$E = \frac{\ln([S]/[S_o])}{\ln([R]/[R_o])} = \frac{\left(\frac{k_{cat}}{K_M}\right)_S}{\left(\frac{k_{cat}}{K_M}\right)_R} \qquad \text{Eq. (1.18)}$$

During a kinetic resolution, a chemist stops the reaction and measures the concentrations of the (*S*) and (*R*)-enantiomer, expressed in terms of the conversion of reaction, c, and the enantiomeric excesses of the substrate (ee_s) or product (ee_p). This measurement is simpler than measuring the kinetic constants.

First we define the conversion, c, as:

$$c = 1 - \frac{S + R}{S_o + R_o} \qquad \text{Eq (1.19)}$$

and we define the enantiomeric excesses as the relative concentration of the two enantiomers after a certain conversion as:

$$ee_S = \frac{R-S}{R+S} \qquad \text{Eq. (1.20)}$$

$$ee_p = \frac{P-Q}{P+Q} \qquad \text{Eq. (1.21)}$$

where P is the product of the (*S*)-enantiomer and Q is the product of the (*R*)-enantiomer. Substitution of equations 1.19 and 1.20 into 1.18 yields an equation which relates the enantiomeric excess of the remaining substrate and percent conversion to the enantiomeric ratio, E:

$$E = \frac{\ln\left[(1-c)(1-ee_S)\right]}{\ln\left[(1-c)(1+ee_S)\right]} \qquad \text{Eq. (1.22)}$$

Similarly, the enantiomeric ratio can be expressed as a function of the enantiomeric purity of the product and percent conversion by substitution of equations 1.19 and 1.21 into equation 1.18.

$$E = \frac{\ln\left[1-c(1+ee_P)\right]}{\ln\left[1-c(1-ee_P)\right]} \qquad \text{Eq. (1.23)}$$

The value of E is a constant throughout a hydrolase-catalyzed resolution of a racemate but the values of ee_s and ee_p change throughout a kinetic resolution as a function of percent conversion. This relationship is plotted using equations 1.22 and 1.23, Figure 1.19. Overall, these plots indicate the enantiomeric excesses that can be achieved using a hydrolase with a given E towards a substrate. Also, if no hydrolases are available that hydrolyze the desired enantiomer with high enantiomeric excess, a researcher can alternately focus on obtaining the desired enantiomer in high enantiomeric excess as remaining starting material since high ee_S values can be obtained for moderate E values while ee_p values cannot be achieved.

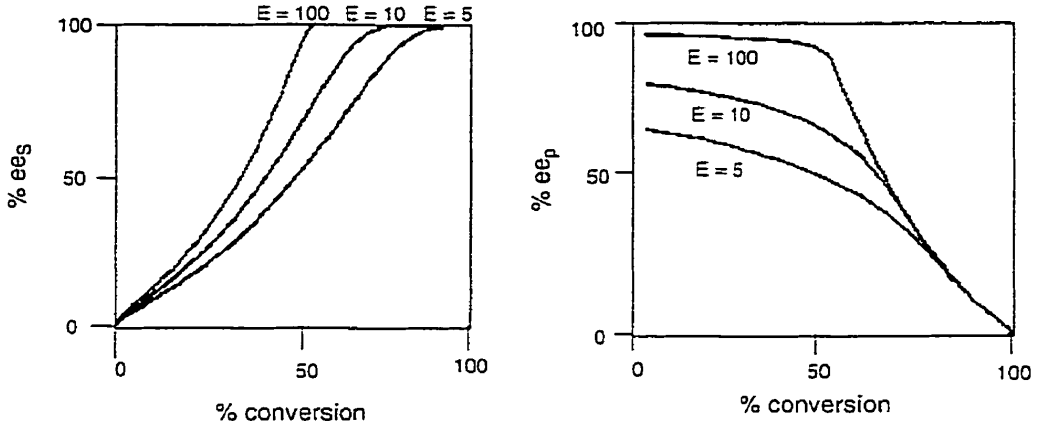


Figure 1.19 Plots of percent enantiomeric excess as a function of percent conversion for various enantiomeric ratios.

The measurement of percent conversion for a given reaction is prone to experimental errors. Alternately, percent conversion can be calculated from both enantiomeric excesses:[65]

$$c = \frac{ee_S}{ee_S + ee_P} \qquad \text{Eq. (1.24)}$$

Thus, percent conversion can be eliminated from equation 1.23 by substituting equation 1.24, yielding an equation for E that uses enantiomeric purities only to determine E:[66]

$$E = \frac{\ln\left[\left(1 - ee_s\right) \Big/ \left(1 + \frac{ee_s}{ee_p}\right)\right]}{\ln\left[\left(1 + ee_s\right) \Big/ \left(1 + \frac{ee_s}{ee_p}\right)\right]} \qquad \text{Eq (1.25)}$$

The endpoint method is the current standard used to measure the enantiomeric ratio of enzyme-catalyzed kinetic resolutions but it is a relatively slow method. Typically,

to determine an E value, a small-scale reaction with the racemic substrate (usually 50 mg or more) and candidate hydrolase (several units depending upon its activity towards the substrate) is set up, allowed to proceed to around 50% conversion, then worked up to separate reaction products from the hydrolase. Suitable analytical conditions must be found to measure the enantiomeric excesses of the remaining starting material and products with chiral stationary phases for HPLC and GC, or chiral shift reagents. Finding suitable conditions can be time-consuming and difficult despite continuing improvements in the development of chiral stationary phases. For example, in Chapter 2, we use four different chiral stationary phases for HPLC, two different chiral stationary phases for GC and a europium chiral shift reagent to measure the enantiomeric purities of the reaction products from *Aspergillus niger* catalyzed resolutions. In very difficult cases, a suitable method to separate enantiomers cannot be found, so researchers depend upon the optical rotation of the reaction products to calculate enantioselectivity.

Ader and Scheider published an excellent example of screening for suitable hydrolases by the endpoint method.[67] They identified a very active and enantioselective hydrolase with E >100 (lipase from *Pseudomonas* species) toward a precursor of β-adrenergic blockers by measuring the enantiomeric ratio of fifteen common hydrolases towards the racemic substrate, Figure 1.20. They simultaneously measured the enantiomeric purities of the remaining starting material and products using an HPLC chiral stationary phase. More often than not, the reaction products of a kinetic resolution must be separated from the remaining starting material since they require different analytical conditions for optimal separation.

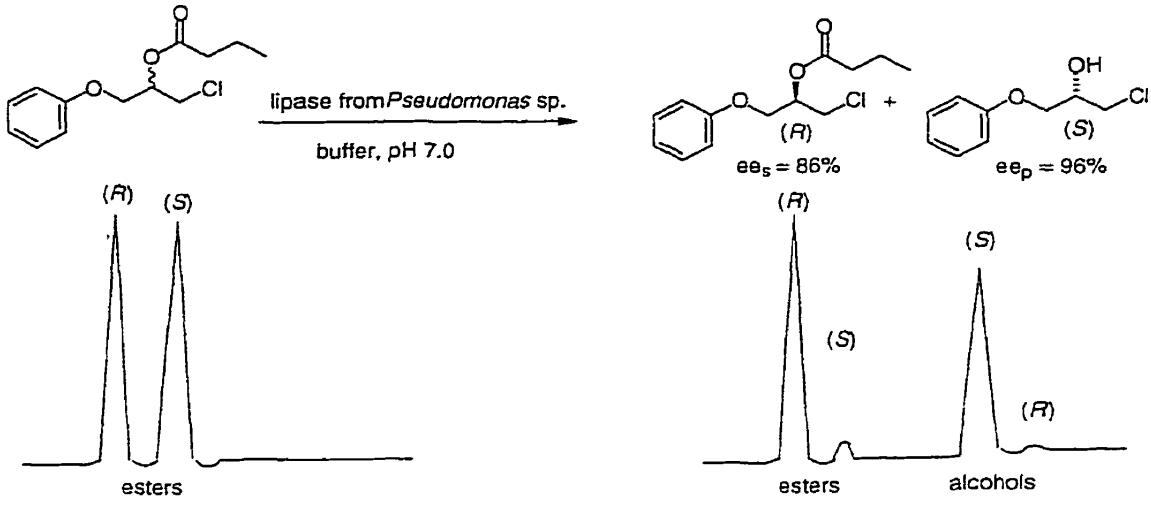


Figure 1.20 Screening a small library of commercial hydrolases toward the butyryl ester of a secondary alcohol precursor of a β-adrenergic blocker identified lipase from *Pseudomonas* sp. with E >100. The schematic shows the HPLC analysis of enantiomeric excess of the remaining starting material and products.

Typically, a researcher screens twenty hydrolases or less towards a target substrate due to time constraints but more or less hydrolases can be tested. In an extreme example, researchers at Tanabe Pharmaceutical (Japan) determined the enantiomeric ratio of 730 commercial hydrolases and cultures of microorganisms towards the racemic trans-3-(4-methoxyphenyl)glycidic acid methyl ester, the key intermediate in the synthesis of diltiazem, shown in Figure 1.5.[68]

Overall, the endpoint method is the standard and accepted procedure for determining the enantioselectivity of hydrolase-catalyzed reactions but it is time-consuming because it requires measuring enantiomeric excesses. Large libraries of hydrolases cannot be screened towards a target substrate without great cost of substrate, enzymes and time. Also, this method is not practical when only small amounts of substrate or hydrolase are available, such as recombinant proteins and substrates from combinatorial libraries.

Determining E by initial rates

Recognizing the need for a method that accurately measures the enantiomeric ratio without measuring enantiomeric excess, Jongejan and coworkers have developed an alternative to the endpoint method.[69] This method uses the initial rates of mixtures of enantiomers to determine E. By varying the ratio of the enantiomers but fixing the total substrate concentration for a series of initial rate measurements, the following equations, which are derivations of equation 1.15, are used to determine E:

$$\frac{1}{(v_x - v_R)} = \frac{v_S/v_R}{E \cdot (v_S - v_R)} \cdot \frac{1}{x} + \frac{E - v_S/v_R}{E \cdot (v_S - v_R)} \qquad \text{Eq. (1.26)}$$

$$\frac{1}{(v_S - v_x)} = \frac{E \cdot v_R/v_S}{(v_S - v_R)} \cdot \frac{1}{(1-x)} + \frac{1 - E \cdot v_R/v_S}{(v_S - v_R)} \qquad \text{Eq. (1.27)}$$

where v_S and v_R represent the initial rate of hydrolysis of the pure (*S*) and (*R*)-enantiomers while v_X is the initial rate of hydrolysis for a substrate mixture with a molar fraction x of the (*S*)-enantiomer. At least three measurements must be carried out, including measurements where $x = 0$ [no (*S*)-enantiomer] and $x = 1$ [no (*R*)-enantiomer]. In practice, eight or more measurements are usually carried out. The data is transformed into linear representations of Eq. 1.26 and Eq. 1.27 and E is evaluated from the slopes of the line once v_S and v_R are determined, Figure 1.21. Several other linear forms of these equations can also be used to determine E.

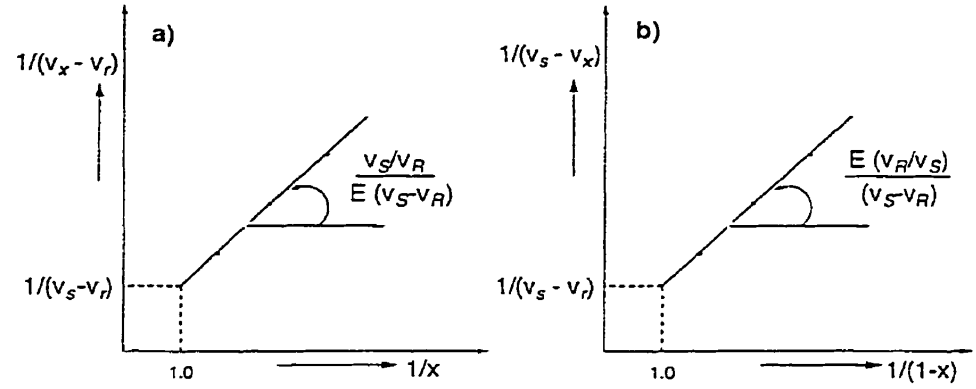


Figure 1.21 Determination of E from initial rates measurements of mixtures with varying enantiomeric excesses. Graph **a** allows for determination of $1/(v_S - v_R)$ at the intersection with $1/x = 1$. The values of v_S and v_R are known so E can be determined from the slopes of the line. Similarly, graph **b** can be used to determine E from eq. 1.27.

This method is particularly useful when only small amounts of substrate and hydrolase are available, or when suitable analytical methods to determine the relative amounts of enantiomers are unavailable. This method also increases the accuracy of the enantiomeric ratio since it does not rely upon a single measurement for calculation as with the endpoint method. The major disadvantage to this method is the time required for each E determination because it requires multiple measurements and graphical analysis to determine E. Overall, this method can be faster than the endpoint method to determine the enantiomeric ratio but is not significantly fast enough to allow for rapid screening of large numbers of hydrolases towards a target substrate.

Measuring enantioselectivity using reaction progress curves

Rather than using just the initial, linear portion of a reaction curves to calculate enantioselectivity as above, researchers have also used the complete reaction progress curve of enzyme-catalyzed kinetic resolutions to calculate the enantiomeric ratio. The relationship of conversion versus time for a kinetic resolution is easily recorded using a pH stat or using analytical methods to measure the loss of substrate or increase in product over time.

If a hydrolase displays perfect enantioselectivity towards a substrate, the kinetic resolution of a racemate stops at 50% when all of the preferred enantiomer is exhausted. A less selective hydrolase continues to hydrolyze substrate after 50% conversion. Figure 1.22. shows that increasing E values typically lead to sharper bending of the progress curves at 50%. Several approaches have been developed to quantitatively determine the enantiomeric ratio from these progress curves. An outline of these methods follows.

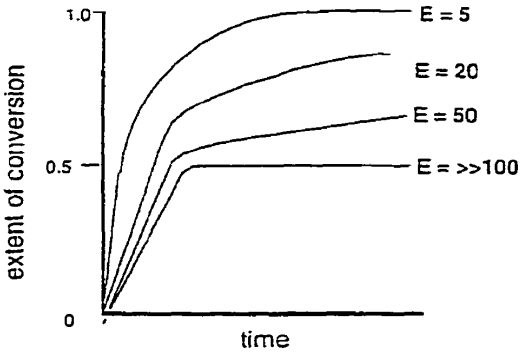


Figure 1.22 Effect of the enantiomeric ratio on the typical shape of a reaction progress curve of a hydrolase-catalyzed kinetic resolution.

Fourneron and coworkers proposed a method to evaluate enantioselectivity by comparing the experimental progress curve of a kinetic resolution to theoretical progress curves.[70] By definition, the enantioselectivity of the reaction equals the rate of hydrolysis of one enantiomer versus the other enantiomer, v_R/v_S. At a given conversion, the overall rate of product formation (v_{total}) equals the sum of the partial rates of both enantiomers, v_R and v_S, and can be expressed by the simple equation:

$$v_{total} = k_R[R] + k_S[S] \qquad \text{Eq. (1.28)}$$

At the initial stages of hydrolysis, the concentrations of the enantiomers (S_o and R_o) can still be considered equal, so the observed rate of reaction can be expressed as:

$$v_{initial} = k_R[R_o] + \frac{k_R}{E}[S_o] \qquad \text{Eq. (1.29)}$$

Alternatively, the reaction can also be expressed in terms of the two reaction products formed from the *S* and *R* enantiomers, *P* and *Q*, respectively:

$$[P+Q] = [R_o]\left(1-e^{k_R t}\right)+[S_o]\left(1-e^{\frac{k_R}{E}t}\right) \qquad \text{Eq. (1.30)}$$

The initial rate ($v_{initial}$) is determined by first taking the tangent at the origin of the experimental progress curve. An arbitrary value of E is chosen to solve Eq. 1.29 for k_R. This value is then substituted into eq. 1.30 and using arbitrary values of time, theoretical curves are generated which have the same tangent at the origin as the experimental curve. This procedure is repeated to generate a series of theoretical curves for different E values. The theoretical curves are then compared to the experimental plot and the theoretical curve that most closely matches the experimental curves determines the value of E, Figure 1.23.

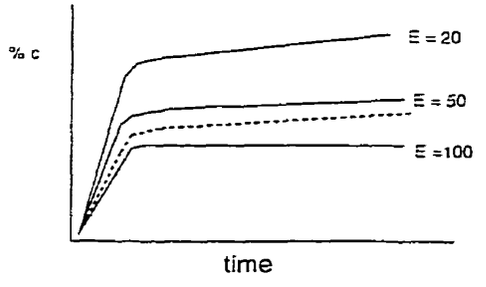


Figure 1.23 Theoretical curves (shown in solid lines) generated by the method of Fourneron and coworkers to determine the enantioselectivity of a hydrolase-catalyzed kinetic resolution (experimental data shown in broken line). E is evaluated to be >50 due to its similarity in shape to the theoretical curve for E = 50.

Lu and coworkers developed a related method.[71] Rather than comparing experimental curve to theoretical curves, two points are chosen on the experimental progress curve and the substrate concentrations are determined (S_1 and S_2). These concentrations then are used to solve Eq. 1.31 for the value of x, derived from equation 1.30, which also describes the change in substrate concentration at two different times of the kinetic resolution:

$$x^2 - 2S_1 x + \left(2S_1^2 - S_2\right) = 0 \qquad where \qquad \ln x = -kt_1 \qquad \text{Eq. (1.31)}$$

This equation can also be expressed in terms of the enantiomeric ratio, E:

$$\frac{1}{E} = \frac{\ln(2S_1 - x_1)}{\ln x_1} \qquad \text{Eq. (1.32)}$$

The value chosen for x from Eq. 1.31 is substituted into Eq. 1.32 and the equation is solved for E.

Lastly, Rakels and coworkers developed a method to determine the kinetic constants, k_{cat} and K_M, from progress curves. This method is more accurate than the previous two progress curve methods because it can account for deviations from Michaelis-Menten kinetics during a kinetic resolution. Progress curves of either the pure enantiomers[72] or a racemic substrate[73] can be used to determine the k_{cat} and K_M values for both enantiomers but greater accuracy is obtained with pure enantiomers than the racemate. Yun and Suelter proposed an earlier and less accurate version of this method.[74]

To use this method, numerical integration is applied to model rate equations that take into account any variations from Michaelis-Menten kinetics, in combination with nonlinear iterative fitting procedures. This procedure simulates progress curves of the reaction. The simulated curves are fitted to the experimental progress curve by adjusting the parameter values, in this case the kinetic constants. The ratio of the kinetic constants, k_{cat} and K_M, for each enantiomer are used to calculate the enantiomeric ratio, Eq. 1.16. For example, Rakels's method was used to determine the enantioselectivity of carboxylesterase NP towards α-substituted propionate esters using the following kinetic equation:

$$-\frac{d([R]+[S])}{dt} = \frac{\frac{k_{cat}^{\ R}}{K_M^{\ R}} \cdot [E] \cdot [R] + \frac{k_{cat}^{\ S}}{K_M^{\ S}} \cdot [E] \cdot [S]}{1 + \frac{[R]}{K_M^{\ R}} + \frac{[S]}{K_M^{\ S}}} + k_h \cdot ([R]+[S]) \qquad \text{Eq. (1.33)}$$

where [E] is the total enzyme concentration and k_h is the first-order autohydrolysis constant to account for any chemical hydrolysis.

Although progress curve analysis alleviates the need to measure enantiomeric excesses, it is not sufficiently fast to screen large numbers of hydrolases towards a synthetic target. A significant amount of data manipulation is required for each E value determination and, depending on the method chosen, these values can be less accurate than the endpoint method. Historically, enzymologists have shown great reluctance to assess kinetic parameters from progress curves because of errors due to changing activity as the reaction progresses despite a large amount of work to increase their accuracy.[75] Currently, progress curves are not widely used to screen for enantioselective hydrolases.

Determining E using computer modeling

When the three-dimensional structure of a hydrolase is available, it is possible to use computer modeling to calculate its enantioselectivity towards a target substrate. Tetrahedral intermediates of the acylation step for each enantiomer are used as transition state analogs and the difference in their potential energies ($\Delta\Delta V^{\ddagger}$) is calculated by force fields. This difference can be used to approximate the difference in activation free energy for the two enantiomers ($\Delta\Delta G^{\ddagger}$) when the entropic portion of Gibbs free energy ($T\Delta\Delta S$) is small, Equation 1.34.

$$\Delta\Delta G^{\ddagger}_{R,S} = \Delta\Delta H - T\Delta\Delta S \cong -RT\ln E \qquad \text{Eq. (1.34)}$$

Several different strategies have been proposed to calculate both large and subtle difference in free energies of the two tetrahedral intermediates. Steric restrictions largely guide the binding of the substrate in the active site but small differences in energy calculations create large differences in the calculated E value. The hydrogen bonds that are essential to stabilize the charge of the tetrahedral intermediate must be included in the calculation. Some strategies largely treat the hydrolase as a fixed set of coordinates and allow for movement of the substrate and amino acid residues close to the substrate only during the simulations.[76] Others account for movement of the hydrolase since some

substrates will induce a fit in the enzyme active site, causing a change in hydrolase geometry.[77] The entropic as well as enthalpic contributions can be included in the calculation of enantioselectivity[77] since entropic contributions have been shown to contribute to the enantiomeric ratio.[78]

The value for E is dependent upon the calculated energy values and, at present, it is difficult to match calculated values to experimentally determined values for E. Clearly, this method is not suitable for screening large numbers of hydrolases towards a target substrate but is more useful to predict and rationalize the enantiopreference towards a target substrate.[79] We employ computer modeling in Chapter 6 to rationalize the observed diastereoselectivity of α-chymotrypsin towards *cis* and *trans* diastereomers of 2-benzyloxymethy-4-carboxylic acid-1,3-dioxolane methyl ester.

Determining E under non-Michaelis-Menten conditions

Finally, it should be noted that although not all hydrolases obey true Michaelis-Menten kinetics, generally a researcher generally chooses the simplest kinetic equations based on knowledge of the mechanism. Because of the large numbers and different types of hydrolases in this thesis, we use equations to calculate the enantiomeric ratio, E, that obey Michaelis-Menten kinetics and have been applied to other hydrolase-catalyze resolutions reported in this literature.

Many of the methods used to calculate E for irreversible, hydrolysis reactions have been extended for use with reactions that do not obey Michaelis-Menten kinetics, *e.g.* reversible esterification reactions. Sih and coworkers extended their equations (Eq. 1.22 and 1.23) to calculate the enantioselectivity of reversible reactions by taking into account rate constants for the forward and reverse directions.[80] Similarly, Anthonsen and Jongejan developed integrated equations to measure the E value of reversible reactions.[81] Straathof and coworkers have carried out extensive research to determine the enantioselectivities of hydrolase-catalyzed reactions that deviate from Michaelis-Menten conditions. For example, they developed equations to measure the enantiomeric ratios of kinetic resolutions with insoluble solid substrates by taking into account mass transfer of the two enantiomers as well as the enantiomeric purities.[82] They also developed methods

to analyze reaction progress curves of reactions that deviate from Michaelis-Menten kinetics[83] We do not explore these reactions in this thesis.

1.10 Estimating the enantioselectivity of hydrolases

Because it is time-consuming and labour-intensive to accurately measure the enantioselectivities of a library of hydrolases towards a synthetic target, researchers sometimes estimate their enantioselectivities. Methods to estimate enantioselectivity are generally faster and more convenient to carry out for large numbers of hydrolases than with the endpoint method. However, the convenience and speed of these methods are usually afforded at the expense of accuracy of the value for E. Common methods to estimate the enantioselectivities of hydrolases towards a target are outlined below.

Eliminating inactive hydrolases

When a researcher screens for enantioselective hydrolases towards a target molecule, not only do suitable hydrolases have to exhibit excellent enantioselectivity towards the target substrate but also must show very good hydrolytic activity to be suitable candidates. Selective but very slow hydrolases are not synthetically useful for the preparative scale production of pure stereoisomers. Thus, a researcher will often first screen for hydrolytic activity towards a target substrate using a variety of techniques including: titration of the liberated acid with base manually or using a pH stat; following the hydrolysis of fluorogenic or chromogenic substrates; thin layer chromatography (TLC) to observe disappearance of starting material or appearance of products; quantification of appearance/disappearance of reaction products using HPLC, GC or NMR. Activity screens focus the researcher on active hydrolases and remove inactive or poorly active hydrolases from consideration for more accurate screening methods.

Using separately measured initial rates to estimate enantioselectivity

The most common method in the literature to rapidly estimate the enantioselectivity of hydrolase-catalyzed kinetic resolutions is to separately measure the hydrolase-catalyzed initial rates of hydrolysis towards the two pure enantiomers of a racemate. The ratio of the rates is used as a qualitative estimation of E.

The rate of hydrolysis of one enantiomer versus the other is the enantiomeric ratio, Equation 1.16. However, this equation assumes both enantiomers are present in solution and compete with each other for the enzyme's active site. Separately measured initial rates of hydrolysis ignore some or all of the effects of K_M on the initial rates. The ratio of separately-measured initial rates is therefore only accurate as a measure of E when both enantiomers have identical K_M values or when substrate concentrations are well below the K_M values (pseudo-first order conditions). Despite these shortcomings, this method continues to be applied to rapidly identify enantioselective hydrolases towards a target, largely due to speed and convenience.

It is convenient to use chromogenic substrates in these assays since their rates of hydrolysis are easily monitored spectrophotometrically. For example, Reetz and coworkers separately measured the rates of hydrolysis of a large library of mutant proteins of *Pseudomonas aeruginosa* lipase towards (*R*) and (*S*)-4-nitrophenyl-2-methyldecanoate, Figure 1.24.[84] Mutant lipases that showed large differences in rates towards both enantiomers were selected as active and enantioselective hydrolases for further rounds of mutation.

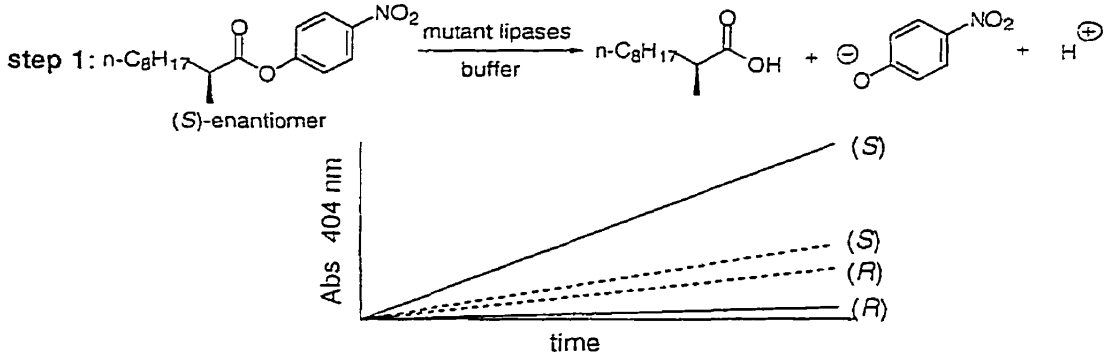


Figure 1.24 In the first step to estimate E, the initial rates of hydrolysis of the (*S*)-substrate is determined spectrophotometrically for each hydrolase. The second step uses the (*R*)-substrate, and the ratio of initial rates from both steps estimates the enantioselectivity. One lipase (——) shows a high estimated enantioselectivity because there is a large difference in initial rates of the two pure enantiomers while another lipase (- - - -) shows similar rates of hydrolysis towards both enantiomers, suggesting poor enantioselectivity.

Many target substrate cannot be easily derivatized into chromogenic substrates, so the initial rates of hydrolysis must be determined by analytical methods such as HPLC or

GC to observe the rate of change of appearance of product or disappearance of starting material. To simplify and generalize this method to estimate E to any substrate, not just chromogenic ones, we developed a method to spectrophotometrically determine the hydrolase-catalyzed rates of hydrolysis of an ester using pH-indicators. With a well-chosen reaction buffer and pH indicator, the rate of change of absorbance of the pH indicator can be directly related to the rate of enzyme-catalyzed hydrolysis. We used this method to rapidly screen a library of 70 commercial hydrolases for activity and enantioselectivity towards an important chiral synthon, a butyryl ester of solketal (2,2-dimethyl-1,3-dioxolane-4-methanol). The complete results of this work are presented in Chapter 4 of this thesis.

Using progress curves to estimate enantioselectivity

Reaction progress curves can be used as an index of enantioselectivity as mentioned in Section 1.9 of this chapter. For example, Griengl and coworkers used reaction progress curves to rapidly estimate the enantioselectivity of *Candida rugosa* lipase (CRL) towards secondary alcohols of norbornane-type compounds, Figure 1.25.[85] Measuring E by endpoint method was used to confirm CRL as enantioselective towards the substrate.

In general, this method can be a fast way of identify a hydrolase with exceptional enantioselectivity towards a target substrate (E >100) but is less useful when enantioselectivity is modest to poor. Since the intrinsic shape of a progress curve depends not only on enantioselectivity but also the reaction conditions such as cosolvents, pH, temperature, and enzyme stability throughout the course of the reaction, it can be difficult to estimate enantioselectivity by this method.

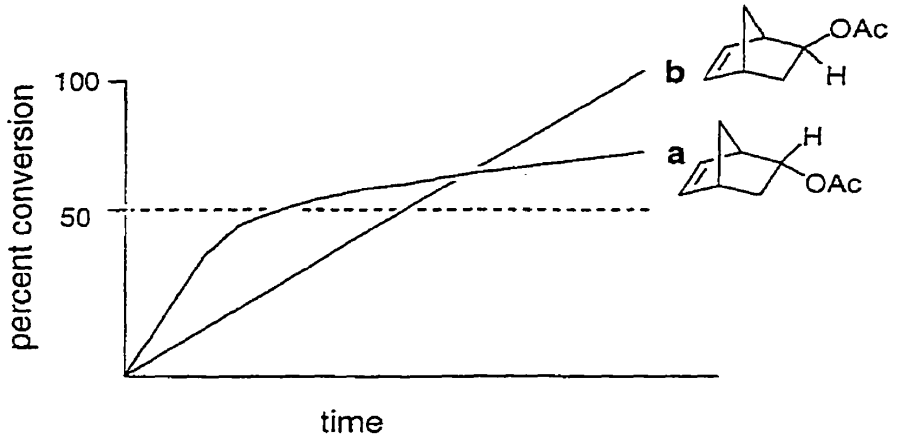


Figure 1.25 The progress curves of the resolution of two norbornane-type alcohols by *Candida rugosa* lipase (CRL) suggests that CRL is enantioselective towards *endo* substrate **a** but is non-selective towards *exo* substrate **b**.

Using pure enantiomers dissolved in agar plates to estimate enantioselectivity

If pure enantiomers of a target substrate are available, separate agar-type plates can be prepared with the pure enantiomers present as suspensions. The substrates must be soluble in the agar solution for this method to work. Aliquots of different hydrolase solutions are dropped onto the plates and, if substrate hydrolysis occurs, clearing zones or halos are formed.[86] Some researchers add pH indicators to help visualize hydrolytic activity.[87] A hydrolase that shows a clearing zone for one enantiomer only is identified as enantioselective, Figure 1.26.

It is difficult to accurately quantify the size of the clearing zone to hydrolytic activity, so this method only identifies hydrolases with exquisite enantioselectivity. This method is not commonly used to estimate enantioselectivity of commercial hydrolases towards a target substrate but is sometimes applied to screening libraries of recombinant proteins produced through cloning techniques for activity and selectivity.[88]

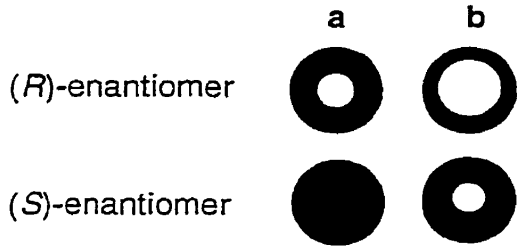


Figure 1.26 Screening for enantioselectivity using pure enantiomers dissolved in agar plates. Hydrolase **a** is identified as enantioselective because hydrolysis is detected towards one enantiomer only while hydrolase **b** is active towards both enantiomers and is classed as nonselective.

1.11 Development of a new method to rapidly identify enantioselective hydrolases

Currently, the best method to select enantioselective hydrolases is Sih's endpoint method, which requires measuring enantiomeric purities. It can be slow and labor intensive when used to screen a large library of hydrolases. To shorten screening time, researchers can either limit the number of hydrolases tested, thereby missing potentially selective catalysts, or they can estimate the enantiomeric ratio, which may result in identifying unselective hydrolases. The development of a rapid but accurate method to measure enantioselectivity without measuring enantiomeric purity or exhaustive data manipulation is critical to the continued application of hydrolases as synthetic reagents for the production of pure stereoisomers.

In this thesis, I focus upon the development of a method to rapidly but accurately measure the enantiomeric ratios of a library of hydrolases towards a target substrate without measuring enantiomeric purities. The criteria were threefold in the development of a new method to measure enantioselectivity. First, the method must accurately measure the enantiomeric ratio, E, and not just estimate it. Second, the method must be suitable for rapidly screening a large library (hundreds to thousands) of hydrolases towards a target substrate. Third, the method must be sensitive enough for screening libraries of small (microgram) quantities of mutant hydrolases. Researchers have developed directed evolution methods to engineer enzymes for increased activity, stability and organic solvent tolerance by random mutagenesis and recombination, coupled with screening steps to select improved variants.[89] By accumulating beneficial environments over

multiple generations, large increases in activity and stability have been achieved. Using directed evolution to enhance a hydrolase's enantioselectivity towards a target substrate requires a sensitive and accurate screen to rapidly but accurately measure E. Developing a sensitive screen to perform directed evolution of a hydrolase for increased enantioselectivity was our ultimate goal.[84, 90]

Chapters 3 to 5 detail the stepwise development of a novel spectrophotometric method to accurately determine the enantiomeric ratio of hydrolase-catalyzed resolutions of a target substrate based upon the measurement of initial rates. We separately measure the initial rates of hydrolysis of each pure enantiomer, but both pure enantiomers compete with an achiral reference compound. The specificity constants (k_{cat}/K_M) of both pure enantiomers are obtained relative to a reference compound. The ratio of the two relative specificity constants yields, by definition, the enantiomeric ratio, E. We call this method "quick E" due to its speed and ease.

To illustrate the usefulness of this technique, we use the quick E method to screen a library of ninety commercial hydrolases for high selectivity towards a mixture of diastereomers of a key chiral intermediate in the synthesis of nucleoside analogs. The quick E method rapidly identified two hydrolases with high selectivity. These results are detailed in Chapter 6.

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Chapter 2

Lipase from the fungus *Aspergillus niger* (ANL) is one of the oldest commercial microbial sources of lipase and has been widely applied as an enantioselective catalyst for the production of chiral building blocks in synthesis. It is an extracellular, 32 kDa hydrolase that is commercially available in a crude form at a very modest price from several suppliers. Most of us are familiar with *Aspergillus niger* fungus as a black growth on shower curtains and bathtubs.

Throughout the past decade, researchers have developed empirical rules to help predict the enantiopreference of hydrolases such as *Candida rugosa* lipase and *Pseudomonas cepacia* lipase towards chiral alcohols and carboxylic acids based upon the size of the substituents at their stereocentres. These rules summarize the observed enantiopreference of substrates reported in the literature. Surprisingly, empirical rules were not developed for ANL.

In this chapter, we present empirical rules for the enantiopreference of ANL towards secondary alcohols and carboxylic acids. These rules aid in the application of ANL as an enantioselective catalyst by identifying the structural elements of a substrate that help set the enantioselectivity of ANL.

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Empirical Rules for the Enantiopreference of Lipase from *Aspergillus niger* toward Secondary Alcohols and Carboxylic Acids, especially α-Amino Acids.

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Abstract: Lipase from *Aspergillus niger* (ANL, Amano lipase AP) catalyzes enantioselective hydrolysis and acylation reactions. To aid in the design of new applications of this lipase, we propose two empirical rules that predict which enantiomer reacts faster. For secondary alcohols, a rule proposed previously for other lipases also works for ANL, but with lower reliability (77%, 37 of 48 examples). For carboxylic acids, we examined both crude and partially-purified ANL because commercial ANL contains contaminating hydrolases. Partial purification removed a contaminating amidase and increased the enantioselectivity of ANL toward many α-amino acids, including cyclic amino acids. Unlike other lipases, ANL readily accepts positively-charged substrates and shows the highest enantioselectivity toward α-amino acids. Although a rule based on the sizes of the substituents could not predict the fast-reacting enantiomer, a rule limited to α-amino acids did predict the fast-reacting enantiomer. We estimate that the charged α-amino group contributes a factor of 40-100 ($\Delta\Delta G^{\ddagger}$ = 2.2-2.7 kcal/mol) to the enantioselectivity of ANL towards carboxylic acids.

Introduction

Lipase-catalyzed reactions are one of the best methods to produce enantiomerically pure compounds.[1] Lipase from the fungus *Aspergillus niger* (ANL) is a useful enantioselective catalyst, especially for the resolution of secondary alcohols and carboxylic acids. These reactions were discovered by trial and error. To identify other applications of this lipase, it would be useful to have generalizations or rules that identify

which structures in a substrate are required for high enantioselectivity. The only previous generalization for ANL was limited to secondary alcohols of 2-(methylthio)-3-acetoxy esters.[2]

The simplest rules for hydrolase-catalyzed reactions do not attempt to predict the degree of enantioselectivity, but only which enantiomer reacts faster. For example, a rule based on the size of the substituents at the stereocentre predicts which enantiomer of a secondary alcohol reacts faster,[3] Figure 2.1.

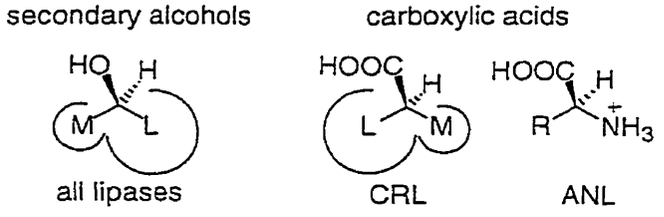

Figure 2.1. Empirical rules that predict the fast-reacting enantiomer in lipase-catalyzed reactions. For secondary alcohols, all lipases, including ANL, follow a rule that compares the size of the substituents at the stereocentre. For carboxylic acids, a rule based upon the size of the substituents at the stereocentre works for CRL, but not for ANL. A rule for ANL includes only α-amino acids (including cyclic amino acids) and suggests that a charged α-amino group is essential for high enantioselectivity. ANL always hydrolyzes the natural L-enantiomer. M represents a medium sized substituent, e.g., CH_3, L represents a large substituent, e.g., Ph, R represents any alkyl or aryl group.

This rule suggests that the lipases distinguish between enantiomers of secondary alcohols primarily based on the relative sizes of the substituents. For the synthetic chemist, this means that lipases resolve secondary alcohols with different-sized substituents better than alcohols with similarly sized substituents. In this paper, we will show that ANL also follows the rule in Figure 2.1, but with lower reliability than for other lipases.

Predicting the fast-reacting enantiomer of a carboxylic acid has been more difficult for lipases. For purified lipase from *Candida rugosa*, a rule based on the size of the substituents was reliable,[4] but we show in this paper that this rule is not reliable for ANL. For ANL, the most efficiently resolved carboxylic acids are α-amino acids and the fast-

reacting enantiomer has the L-configuration, Figure 2.1. This model suggests that charge, not size, of the substituents at the stereocentre sets the enantioselectivity of ANL for carboxylic acids.

Results

Literature survey of the enantiopreference of ANL toward secondary alcohols

To test if the secondary alcohol rule also applies to lipase from *Aspergillus niger*, we summarized all reported ANL-catalyzed reactions of secondary alcohols, Table 2.1. Figure 2.2 shows the structures of the fast-reacting enantiomers. The examples include both hydrolyses of esters and acylations of alcohols, but for consistency the structures in Figure 2.2 are all alcohols. We excluded patents and examples where the enantioselectivity was < 2. For eight examples, the rule is equivocal because the size of both substituents is similar according to CPK models. We marked these examples 'sim. size' in Table 2.1 and excluded them from the tally below. Note, however, that two of these examples showed good enantioselectivity, E > 40.

For the remaining 48 substrates, the rule in Figure 2.1 predicts the absolute configuration of the favored enantiomer in 37 cases, but fails in 11 cases, 77% accuracy. (Guessing alone gives 50% accuracy because there are only two choices.) Six of the 48 substrates showed enantioselectivities > 50 and the rule predicted the favored enantiomer for all six of these. Of the eleven exceptions to the rule, eight showed low enantioselectivities, $E \leq 10$, but three showed moderate enantioselectivity (E = 20-41). Five of the six bicyclic alcohols (compounds **23-27**) did not obey the size rule. Thus, the size rule usually predicts the favored enantiomer, but the reliability is less than for other lipases where the reliability is often > 90%.[3,5] This lower reliability may be an inherent characteristic of ANL or it may be due to contaminating hydrolases, see below.

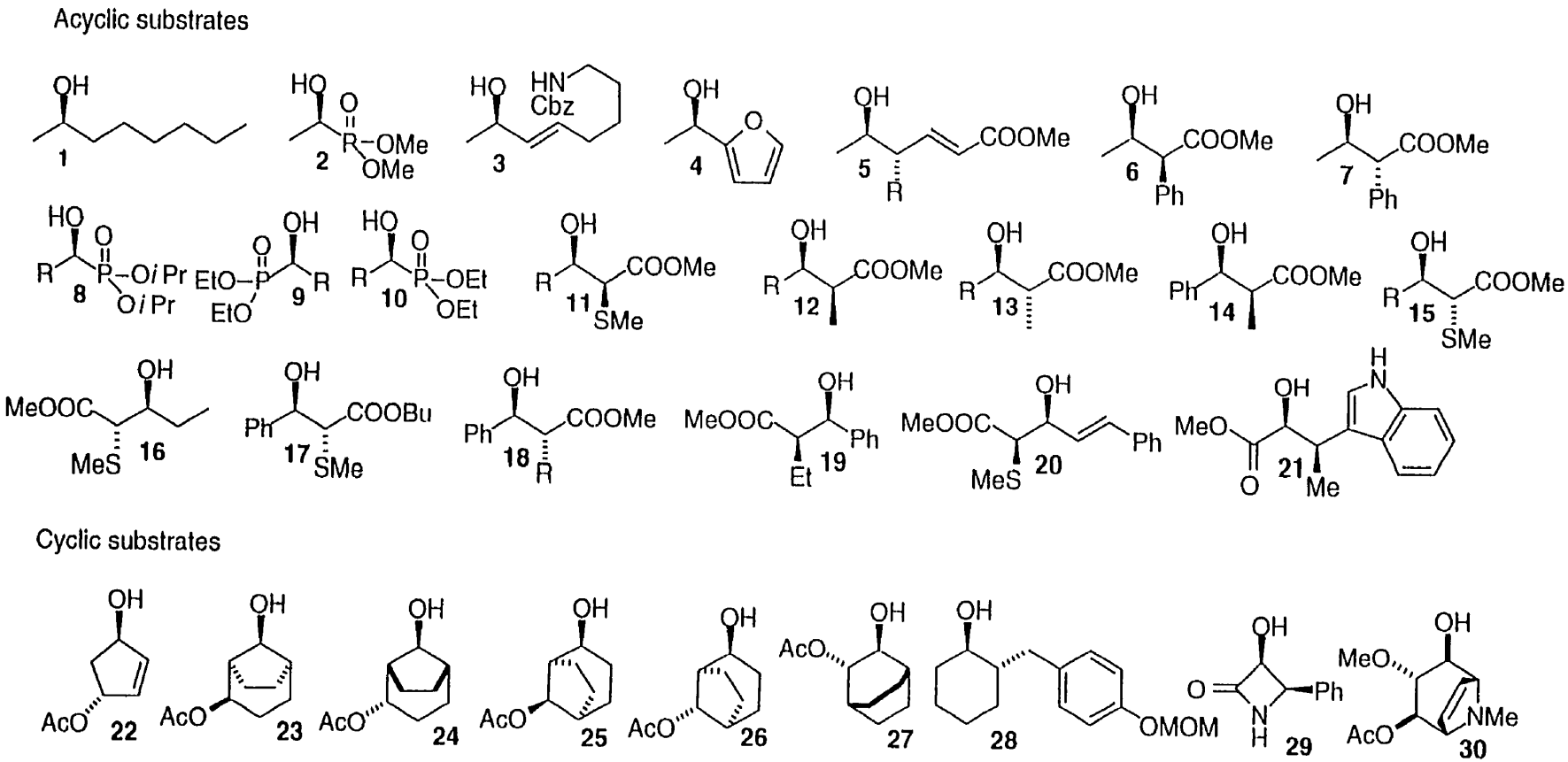

Figure 2.2. Examples from the literature of secondary alcohols resolved or desymmetrized by ANL. Structures show the fast reacting alcohol enantiomer. Most examples follow the rule in Figure 2.1. Details in Table 2.1.

Table 2.1. Literature Survey of the Enantioselectivity of ANL toward Secondary Alcohols.[a]

Struct.	R	E[b]	Follows rule?[c]	Ref	Struct.	R	E[b]	Follows rule?[c]	Ref
1		5	yes	*d*	**12**	CMe=CHEt	12	sim. size	*n*
2		2	yes	*e*	"	CMe=CHMe	10	sim. size	*n*
3		7	yes	*f*	"	CMe=CH_2	18	sim. size	*n*
4		6	yes	*g*	"	CH=CHPh	46	sim. size	*n*
5	(4'-OMe-C_6H_4)-O	8	yes	*h*	**13**	2-Furanyl	20	yes	*n*
"	3',4'-diOMe-C_6H_3	21	yes	*h*	"	2-Thienyl	46	yes	*n*
"	2',4',6'-triOMe-C_6H_2	25[i]	yes	*h*	"	CMe=CHMe	43	sim. size	*n*
6		74	yes	*j*	**14**		15	yes	*j*
7		11	yes	*j*	**15**	Ph	30	yes	*j*
8	Me	31	yes	*e*	"	Me	3	yes	*j*
"	Et	13	yes	*k*	"	Bu	5	yes	*j*
"	n-Propyl	22	yes	*k*	"	CH=CHPh	13	sim. size	*j*
"	n-Pentyl	35, 40[i],150[m]	yes	*k*	**16**		5	no	*j*
"	n-Heptyl	17[i]	yes	*k*	**17**		10	yes	*j*
"	Ph	10[i], 138	yes	*e*	**18**	Me	82	yes	*j*
"	Bn	4.8-6[i]	yes	*l*	"	Et	20	yes	*j*
"	4'-OMe-C_6H_4	17, 11[i]	sim. size	*l*	**19**		3	no	*j*
9	Bn	41	no	*l*	**20**		4	sim. size	*j*
"	*i*-Pr	3	no	*k*	**21**		4	yes	*o*
"	*t*-Butyl	2[i]	no	*k*	**22**		3	no	*p*
10	*n*-Pentyl	9	yes	*k*	**23**		~5	no	*q*
"	*n*-Heptyl	8[i]	yes	*k*	**24**		~10	no	*q*
"	*n*-Nonyl	7[i]	yes	*k*	**25**		~7	no	*q*
11	Me	2	yes	*j*	**26**		~30	no	*q*
"	Et	3	yes	*j*	**27**		20	no	*q*
"	Bu	2	yes	*j*	**28**		2	yes	*r*
12	2-Furanyl	52	yes	*n*	**29**		27	yes	*s*
"	2-Thienyl	8	yes	*n*	**30**		>50	yes	*t*

[a]Hydrolysis of the acetate ester using lipase AP-6 from Amano Enzyme Co. unless noted otherwise. Figure 2.2 shows the absolute configurations of the favored enantiomer. [b]Enantiomeric ratio, E, measures the preference of the enzyme for one enantiomer over the other: Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J.*J. Am. Chem. Soc.* **1982**, *104*, 7294-7299. [c]The rule in Fig. 2.1 is ambiguous

when both substituents are similar in size; these examples are marked 'sim. size'. [d]ANL from Röhm; esterification with dodecanoic acid: Gerlach, D.; Missel, C.; Schreier, P. *Z. Lebensm.-Unters. Forsch.* **1988**, *186*, 315-18. [e]Li, Y.-F.; Hammerschmidt, F. *Tetrahedron: Asymmetry* **1993**, *4*, 109-120. [f]Lipase AP; esterification with vinyl acetate: Hirai, Y.; Nagatsu, M. *Chem. Lett.* **1994**, 21-22. [g]Lipase A; esterification with vinyl acetate in carbon tetrachloride: Kaminska, J.; Gornicka, I.; Sikora, M.; Gora, J. *Tetrahedron: Asymmetry* **1996**, *7*, 907-910. [h]Akita, H.; Umezawa, I.; Takano, M.; Ohyama, C.; Matsukura, H.; Oishi, T. *Chem. Pharm. Bull.* **1993**, *41*, 55-63. [i]Hydrolysis of the chloroacetate ester. [j]Itoh, T.; Kuroda, K.; Tomosada, M.; Takagi, Y. *J. Org. Chem.* **1991**, *56*, 797-804. [k]Drescher, M.; Hammerschmidt, F.; Kahlig, H. *Synthesis* **1995**, *10*, 1267-1272. [l]Drescher, M.; Li, Y.-F.; Hammerschmidt, F. *Tetrahedron* **1995**, *51*, 4933-4946. [m]Reaction carried out at 40 °C. [n]Akita, H.; Matsukura, H.; Oishi, T. *Tetrahedron Lett.* **1986**, *27*, 5241-5244. [o]Lipase A-6 immobilized on Celite: Akita, H.; Enoki, Y.; Yamada, H.; Oishi, T. *Chem. Pharm. Bull.* **1989**, *37*, 2876-2878. [p]Miura, S.; Kurozumi, S.; Toru, T.; Tanaka, T.; Kobayashi, M.; Matsubara, S.; Ishimoto, S. *Tetrahedron* **1976**, *32*, 1893-1898. [q]Lipase A: Naemura, K.; Takahashi, N.; Tanaka, S.; Ida, H. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2337-2343. Estimated E values based on the reported enantiomeric purities. [r]Lipase from Fluka Chemical Co.: Zaravucka, M.; Zuzana, Z.; Rejzek, M., Streinz, L.; Wimmer, Z.; Mackova, M.; Demnerova, K. *Enz. Microb. Tech.* **1995**, *17*, 866-869. E = 6 for esterification with vinyl acetate in benzene. [s]Lipase APF: Patel, R. N.; Banerjee, A.; Ko, R. Y.; Howell, J. M.; Li, W.-S.; Comezoglu, F. T.; Partyka, R. A.; Szarka, L. *Biotechnol. Appl. Biochem.* **1994**, *20*, 23-33. [t]Hoenke, C.; Kluwer, P.; Hugger, U.; Krieger, R.; Prinzbach, H. *Tetrahedron Lett.* **1993**, *34*, 4761-4764.

A literature survey of enantioselectivity of ANL toward carboxylic acids

Table 2.2 summarizes all reported ANL-catalyzed hydrolyses of esters of carboxylic acids in the literature and Figure 2.3 shows the structures of the faster-reacting enantiomers.[6] ANL favors the naturally occurring L-enantiomer of pipecolic acid, **31**, homophenylalanine, **33** (R = CH_2CH_2Ph), and the twelve *N*-Cbz-protected amino acids, **38**. ANL showed no enantioselectivity toward *N*-acetyl pipecolic acid, **39**. For the non-amino acid substrates, ANL showed moderate enantioselectivity toward 2-phenoxypropionic acid, **40**, and low enantioselectivity toward acids **41** and **42**. From such a limited range of substrates (7 structures in total), it is difficult to deduce which classes of carboxylic acids ANL resolves efficiently. For this reason, we resolved a wider range of carboxylic acids with both crude and partially-purified ANL, Table 2.3.

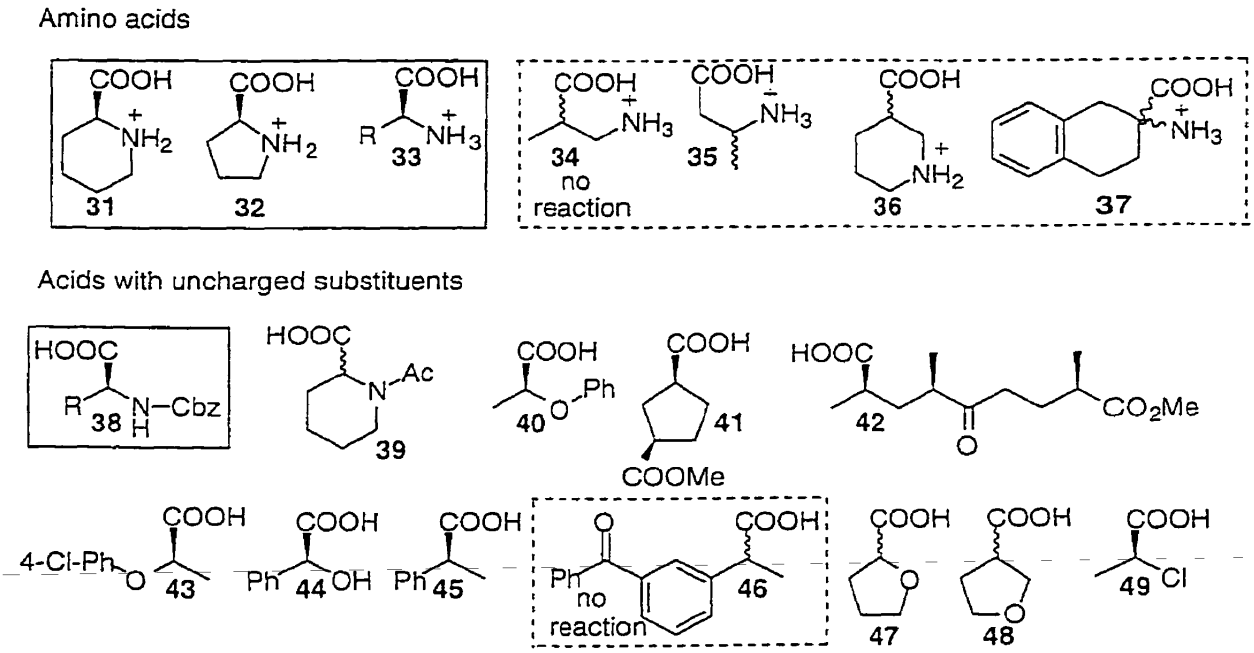

Figure 2.3. Carboxylic acids tested in enantioselective reactions with ANL. Enantioselectivities are moderate or better (E >10) only for the examples in the solid-line boxes. Examples in the dotted-line boxes did not react with ANL. Structures show the fast reacting enantiomer where the absolute configuration is known. Table 2.2 provides details for the examples from the literature and Table 2.3 lists experimental results for examples reported in this paper.

Table 2.2. Literature Survey of the Enantioselectivity of ANL toward Carboxylic Acids.[a]

Structure	R[b]	E[c]	Follows size rule[d]?	Reference
31		20, >100	sim. size	*e*
33	CH_2CH_2Ph	13	yes	*f*
38	Me [Ala]	28, 14	no	*g, h*
"	Et	95	no	*g*
"	*i*-Pr [Val]	28, >100	no	*g, h*
"	*n*-Pr	18	no	*g*
"	*n*-Bu	18	no	*g*
"	*n*-Pentyl	87	sim. size	*g*
"	CH_2=$CHCH_2$	21	no	*g*
"	*n*-Hexyl	49	sim. size	*g*
"	(4-Thiazolyl)methyl	56	no	*g*
"	Bn [Phe]	50, 22	no	*g, h*
"	$CH_3SCH_2CH_2$ [Met]	86	no	*h*
"	$(CH_3)_2CHCH_2$ [Leu]	15	no	*h*
39		1	NA	*e*
40		19	no	*i*
41		3	yes	*j*
42		6	no	*k*

[a]Hydrolysis of the methyl ester using lipase AP-6 from Amano Enzyme Co. unless noted otherwise. Figure 2.3 shows the absolute configuration of the favored enantiomer. [b]When the structure is a common amino acid, its three letter abbreviation is given in brackets. [c]Enantiomeric ratio, E, measures the preference of the enzyme for one enantiomer over the other: Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299. [d]The rule in Fig. 2.1 is ambiguous when both substituents are similar in size; these examples are marked 'sim. size'; 'na' indicates not applicable because E = 1. [e]Crude ANL showed an E of 20, but partially-purified ANL showed an E of > 100: Ng-Youn-Chen, M. C.; Serreqi, A. N.; Huang, Q.; Kazlauskas, R. J. *J. Org. Chem.* **1994**, *59*, 2075-2081. [f]Hydrolysis of the ethyl ester: Houng, J.-Y.; Hsieh, C.-L.; Chen, S.-T. *Biotechnol. Techn.* **1996**, *10*, 353-358. [g]Hydrolysis of the 2-chloroethyl ester: Miyazawa, T.; Takitani, T.; Ueji, S.; Yamada, T.; Kuwata, S. *J. Chem. Soc., Chem. Commun.* **1988**, 1214-1215. [h]Chiou, A.- J.; Wu, S.-H.; Wang, K.- T. *Biotechnol. Lett.* **1992**, *14*, 461-464. [i]Transesterification of the vinyl ester with methanol in heptane: Miyazawa, T.; Kurita, S.; Ueji, S.; Yamada, T.; Kawata, S. *Biotechnol. Lett.* **1992**, *14*, 941-946. [j]Chenevert, R.; Lavoie, M.; Courchesne, G.; Martin, R. *Chem. Lett.* **1994**, 93-96. [k]Ngooi, T. K.; Guo, Z. W.; Sih, C. J. *Biocatalysis* **1990**, *3*, 119-128.

Enantioselectivity of ANL toward carboxylic acids does not depend on the size of the substituents

We tested the methyl esters of nine α-amino acids, **33**, the octyl ester of **32**, three β-amino acids, **34-36**, and one α-disubstituted amino acid, **37**. In addition, we tested esters of an aryloxypropionic acid, **43**, mandelic acid, **44**, two arylpropionic acids, **45** and **46**, two furoic acids, **47** and **48** and 2-chloropropionic acid, **49**. For pipecolic acid, **31**, we previously showed that the enantioselectivity of ANL increased significantly (from E ~ 20 to E >100) upon partial purification of the lipase to remove a contaminating hydrolase.[7] For this reason, we measured the enantioselectivity of both crude and partially-purified ANL.

ANL resolved only the α-amino acids with an enantioselectivity >10. For proline, phenylalanine, and phenylglycine, the enantioselectivity of crude ANL was moderate to good (E = 20-70) and increased to > 90 upon purification. For alanine the enantioselectivity also increased from 3 to 41 upon purification. The specific activity of ANL towards these substrates increased up to two-fold upon purification (0.21-0.49 U/mg for crude, 0.55-0.96 U/mg for partially-purified). On the other hand, the specific activity of ANL towards *N*-Cbz-phenylalanine, **38** (where R = Bn), decreased by a factor of nine upon purification and the enantioselectivity decreased from 20 to 13. These decreases suggests that a contaminating hydrolase in crude ANL contributes to the hydrolysis of *N*-Cbz-phenylalanine-methyl ester. For the non-charged substrates, **43**, **45**, **47-49**, the specific activity increased as much as twenty fold (0.051-1.4 U/mg for crude, 0.072-3.0 U/mg for partially-purified), but the enantioselectivity remained low. Thus, purification increased the specific activity toward many substrates, but increased the enantioselectivity only toward substrates with a charged α-amino group.

The specific activity of crude ANL toward α-amino acids with larger side chains - tryptophan, tyrosine, and *O*-methyl tyrosine - were 5-50 times lower than for the those with smaller side chains. The enantioselectivity was low to moderate (E = 5-25). Little changed upon purification. The specific activity increased two-fold towards tyrosine and *O*-methyl tyrosine, but decreased two-fold towards tryptophan, but the enantioselectivies

remained approximately the same (E = 2.5-21). Three sterically hindered, unnatural α-amino acids, *tert*-leucine (**33**, R = *t*-Bu), 2,6-dimethyltyrosine (**33**, R = 4'-OH-2',6'-Me-C_6H_2) and the α-disubstituted amino acid, **37**, did not react. The slow hydrolysis and lower enantioselectivity towards large substrates suggest that there is a limit to the size of the side chain that ANL can accommodate.

ANL did not catalyze the hydrolysis of any of the three β-amino acids, **34-36**, even though these are not sterically hindered. Both crude and partially-purified ANL showed low enantioselectivity toward the aryloxypropionic acid, **43**, mandelic acid, **44**, the two arylpropionic acids, **45** and **46**, and the two furoic acids, **47** and **48**, and chloropropanoic acid, **49**.

A rule comparing the size of the substituents at the stereocentre predicted the enantiopreference of purified lipase from *Candida rugosa* towards carboxylic acids,[4] but this rule does not work for ANL, Figure 2.1. For the α-amino acids, **33**, the larger group, R, lies to the left, but for the *N*-Cbz-α-amino acids, **38**, the larger group, *N*-Cbz, lies to the right. Yet ANL favors the L-enantiomer for both. For substrates **41** and **43**, the larger group lies to the left, but for **42** the larger group lies to the right. We also compared the overall accuracy of the size rule. Among the 40 carboxylic acids tested in either this work or literature reports, eight did not react. We excluded another six because both substituents had similar sizes (**31**, **32**, **33** (R = CH_3 [Ala]), **38** (R = *n*-pentyl, *n*-hexyl) and **47**, **48**). We also excluded **39**, **44**, **45** from the tally because the enantioselectivity was < 2. For the remaining 23 substrates, only ten obeyed the size rule corresponding to 44% accuracy. This accuracy is similar to guessing alone.

Table 2.3. Enantioselectivity of ANL in the Hydrolysis of Carboxylic Acid Esters.[a]

		Crude ANL[b]				Partially-Purified ANL[b]				
Struct	**R[c]**	**Rate (U/mg)[d]**	**ee_s (%)**	**ee_p (%)**	**E[e]**	**Rate (U/mg)[d]**	**ee_s (%)**	**ee_p (%)**	**E[e]**	**Follows size rule?**
32		0.49	nd	86	20[f,g]	0.62	90	94	100[g]	sim. size
33	Me [Ala]	0.40	59	26	3	0.55	99	78	41	sim. size
"	$CH_3SCH_2CH_2$ [Met]	0.58	78	85	30	0.74	97	99	>100	yes
"	Ph	0.45	92	91	70[h]	0.91	93	93	94[h]	yes
"	Bn [Phe]	0.21	93	86	45	0.96	>99	91	>100	yes
"	Indoyl-CH_2[Trp]	0.057	42	58	5.6	0.026	38	29	2.5	yes
"	4'-OH-C_6H_4-CH_2 [Tyr]	0.014	97	33	7	0.028	53	61	7	yes
"	4'-OMe-C_6H_4-CH_2	0.12	73	84	25	0.21	86	76	20	yes
"	4'-OH-2',6'-Me_2-C_6H_2-CH_2	<0.00001	-	-	-	<0.00001	-	-	-	-
"	*t*-Bu	<0.00001	-	-	-	<0.006	-	-	-	-
34		<0.004	-	-	-	<0.0001	-	-	-	-
35		<0.0001	-	-	-	<0.0009	-	-	-	-
36		<0.001[i]	-	-	-	<0.001[i]	-	-	-	-
37		<0.005	-	-	-	<0.004	-	-	-	-
38	Bn [Phe]	1.4	28	88	20	0.16	9	85	13	no
"	Ph	<0.0001	-	-	-	<0.0001	-	-	-	-
43		0.088	64	43	5	0.15	1	54	3	yes
44		1.00	0	0	1	0.82	0.2	8.1	1.2	na
45		0.051	4	nd	1.2[i,k]	0.072	nd	12	1.3[f,j]	na
46		<0.001	-	-	-	<0.00004	-	-	-	-
47		0.047	10	nd	1.6[g,k]	0.25	15	nd	6[g,k]	sim. size
48		0.045	0	nd	1[g,k]	0.82	4	nd	1.8[g,k]	sim. size
49		1.39	42	51	4.5	3.00	50	66	8.0	yes

[a]Hydrolysis of the methyl ester unless noted otherwise. Figure 2.3 shows the absolute configuration of the favored enantiomer. 'nd' = not determined; '-' = could not be measured because no reaction was detected; 'na' = not applicable because the enantioselectivity is below 2 or because the absolute configuration is unknown. [b]Crude ANL is lipase AP-6 from Amano as received and partially-purified

ANL is lipase AP-6 precipitated with 25-55% saturation of ammonium sulfate. [c]When the structure is a common amino acid, its three letter abbreviation is given in brackets. [d]Initial rate of hydrolysis in units/mg protein. Unit = μmol of ester hydrolyzed per minute. Crude ANL contained 2.8 wt % protein by the Bio-Rad protein assay . [e]E represents the enantiomeric ratio which measures the preference of the enzyme for one enantiomer over the other: Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299. Unless otherwise noted, E was calculated from ee_s and ee_p. [f]E was calculated from ee_p and percent conversion. [g]Hydrolysis of the octyl ester. The methyl ester of **32** was unstable at room temperature; the methyl esters of **47** and **48** were very volatile. [h]Phenylglycine methyl ester partially racemized after 24 hours at pH 5. To determine E accurately, we used short reaction times (typically 1 h) and measured ee_p and ee_s immediately after we stopped the reaction. Others also noted the racemization of phenylglycine, for example, Shiraiwa, T.; Sakata, S.; Fujishima, K; Kurokawa, H. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 191-195. [i]Hydrolysis of the ethyl ester.[j]Hydrolysis of the chloroethyl ester. [k]E was calculated from ee_s and percent conversion.

High enantioselectivity requires a protonated amino group ($-NH_3^+$)

Additional evidence that size of the substituents at the stereocentre is not important comes from good to excellent enantioselectivity toward several carboxylic acids that contain similarly sized substituents. Purified ANL resolved three α-amino acids, pipecolic acid, proline and alanine, with an enantioselectivity of 41 to >100 even though the two substituents differ only in the charge of the substituents at the stereocentre. This difference corresponds to a $\Delta\Delta G^{\ddagger}$ of 2.2-2.7 kcal/mol.

The enantioselectivity of purified ANL toward 2-phenylglycine (**33** where R = phenyl, E = 93) was much higher than for analogs without a protonated amino group. The enantioselectivity toward mandelic acid, **44**, and 2-phenylpropionic acid, **45**, was only 1.2-1.3. Similarly, the enantioselectivity of purified ANL toward alanine (**33** where R = methyl, E = 41) was much higher than toward 2-chloropropionic acid, **49** (E = 8).

To test whether high enantioselectivity requires a protonated amino group, we measured the enantioselectivity of ANL as a function of pH, Table 2.4. Both enantioselectivity and specific activity were highest at pH 5 toward phenylalanine-methyl ester. At higher pH both the rate and the enantioselectivity decreased by factors of 5.3-7.6 and 4-66, respectively. These decreases are consistent with the $-NH_3^+$ form of phenylalanine-methyl ester as the most reactive and best resolved form of the substrate. At pH 4 the enantioselectivity also dropped to 6, but we attribute this drop to partial denaturation of ANL. After incubation at pH 4 for 1 h, the lipase activity measured at pH 7.5 with PNPA as the substrate dropped by 50% for crude lipase and 25 % for partially-purified ANL. Incubation at pH 5-9 showed no drop in activity by PNPA assay.

To confirm that ANL accepts other positively-charged substrates, we tested *S*-acetylthiocholine iodide, a thioester with an $-NMe_3^+$ group in the thiol portion. Indeed, crude ANL catalyzed the efficient hydrolysis of this substrate with a rate of 0.19 U/mg protein. This rate was one hundred times faster than with lipase from *Candida rugosa* and ten thousand times faster than with lipase from *Pseudomonas cepacia*. However, ANL was ten thousand-fold slower at hydrolyzing this substrate than acetylcholine esterase.[8]

Table 2.4. Influence of pH on the Enantioselectivity of Partially-Purified ANL toward Phenylalanine-Methyl Ester[a].

pH	rate U/mg[b]	% substrate protonated[c]	ee_s[d]	ee_p[d]	E[e]
4.02	0.047	>99	0.068	0.68	6±1
5.02	0.91	99	0.68	0.95	80±20
6.00	0.16	87	0.53	0.65	8±1
7.07	0.17	47	0.81	0.63	18±1
7.97	0.12	10	0.11	0.70	1.2±0.04
8.90	0.13	1	0.88	0.45	6±2[f]

[a]Reaction conditions: 15 units of lipase (by PNPA assay), room temperature, 25 mM substrate in 10 ml sodium acetate buffer (10 mM) for pH 4-6 and Tris-HCl buffer (10 mM) for pH 6-9. E values at pH 6 were similar in both buffers. Reactions were monitored by pH stat. [b]Unit = μmol of ester hydrolyzed per minute. [c]The fraction of protonated Phe-methyl ester at the given pH was calculated using a pK_a = 7.00: Jencks, W.P.; Regenstein, J. *CRC Handbook of Biochemistry. Selected Data for Molecular Biology* CRC: Boca Raton, FL, **1986**, pp J187-J226. [d]Determined by HPLC with a Crownpak CR(+) column. The preferred enantiomer was L as established by the order of elution on the CR(+) column and compared to authentic samples; the D-enantiomer elutes first for both acid and methyl ester. [e]E was calculated from ee_s and ee_p as defined by Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299. Error limits are estimates. [f]Phenylalanine-methyl ester hydrolyzed spontaneously at pH 8.9 at a rate of 9.2×10^{-4} min^{-1} (6.6% hydrolysis during the 1 h reaction).The ee values were corrected for this hydrolysis. The uncorrected values are ee_s = 0.72 and ee_p = 0.40. Spontaneous hydrolysis was not detected at other pH values.

ANL is not an amidase

High enantioselectivity toward α-amino acids suggests that partially-purified ANL may be an amidase, not a lipase. However, we confirmed that partially-purified ANL is not an amidase by testing its ability to catalyze hydrolysis of D,L-phenylalaninamide, Table 2.5. Partially-purified ANL showed no detectable hydrolysis of the amide. In contrast, crude ANL catalyzed the slow hydrolysis of D,L-phenylalaninamide favoring the natural L-enantiomer with excellent enantioselectivity, suggesting that crude ANL contains a contaminating amidase. The activity of the crude lipase towards

phenylalaninamide was 2-6 times faster at pH 4-5 than at pH 7.5 and 9. Researchers have previously isolated proteases with optimal activities at acidic pH from *Aspergillus niger*.[9]

Purified ANL is indeed a lipase because it catalyzed hydrolysis of insoluble substrates one hundred times more efficiently than crude ANL: olive oil (0.29-0.62 U/mg protein for partially-purified vs. 0.005 U/mg protein for crude) and ethyl butyrate (0.44-2.42 for partially-purified vs. 0.02 U/mg protein for crude).

Table 2.5. Hydrolysis of D,L-Phenylalaninamide by *Aspergillus niger* Lipase.[a]

	crude ANL				PP ANL
pH	**activity[b] (U/mg)**	**ee_s**	**ee_p**	**E[c]**	**activity[b] (U/mg)**
4.1	0.016	29	>99	>100	<0.0002
5.0	0.036	48	>99	>100	<0.0008
7.5	0.0068	63	>99	>100	<0.0003
8.9	0.0058	59	>99	>100	<0.0002

[a]Reaction conditions: 15 units of lipase, room temperature, 25 mM substrate in 10 ml sodium acetate buffer (100 mM) for pH 4.1 and pH 5 and sodium phosphate buffer (100 mM) for pH 7.5 and 8.9. Reactions were monitored by TLC (1:1; $CHCl_3$:MeOH; 1% NH_4OH) and terminated when product and starting material spots appeared approximately equal in intensity. [b]Unit/mg = μmol of amide hydrolyzed per minute per mg of protein. [c]Enantiomeric excesses and degree of conversion were determined by HPLC (Crownpak CR(+) column). E was calculated from ee_s and ee_p as defined by Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299. The preferred enantiomer was L according to the order of elution on the CR(+) column compared to authentic samples; the D-enantiomer elutes first for both acid and amide.

Discussion

Because all lipases follow the secondary alcohol rule in Figure 2.1, it is not surprising that ANL also follows the rule. However, the reliability of the rule for ANL is significantly lower than for other lipases: 77% for ANL, but > 90% for most other lipases. Although the lower reliability may be an inherent characteristic of ANL (no sequence or structure is known for this lipase), it is most likely due to the contaminating

amidase. This amidase may favor the opposite enantiomer of secondary alcohols and thus account for the exceptions to the rule. A recent survey showed that lipases and the protease subtilisin favor the opposite enantiomers of secondary alcohols,[10] thus it is reasonable to suggest that the amidase in crude ANL may also favor the opposite enantiomer.

Many groups have identified contaminating hydrolases in crude ANL.[7,11,12] For example, Hofelman *et al.* identified esterases, proteases, amylases as well as a lipase. The main commercial use of ANL is in cheese-making to accelerate the ripening of flavors.[13] Both lipases and proteases contribute to this ripening, so there is no need for purer enzymes. For synthetic applications, however, a purer form of ANL would be useful.

Our survey showed ANL, unlike other lipases, readily accepts positively-charged substrates. Carrea *et al.* found that ANL was the best lipase for deprotection of cephalosporin derivatives containing a carboxylate near the reactive site.[12] This result suggests that ANL may also readily accept negatively-charged substrates.

A protonated α-amino substituent was the most important feature for high enantioselectivity of ANL toward carboxylic acids. ANL resolves small to medium sized α-amino acids with very good enantioselectivity, but larger amino acids react slowly with lower enantioselectivity. The L-enantiomer was always favored as shown in Figure 2.1. Although many proteases resolve amino acids esters,[14] ANL is the only lipase that shows high enantioselectivity toward a range of amino acid esters. Although crude lipase from *Humicola lanuginosa* resolved α-amino acids and α, α-disubstituted α-amino acids, Liu *et al.* found that the true catalyst was an impurity. Because this impurity did not catalyze hydrolysis of *o*-nitrophenyl butyrate or olive oil, it was probably a protease.[15] Similarly, Houng *et al.* reported enantioselective hydrolysis of amino acid esters catalyzed by crude PPL, but again the true catalyst may be a contaminating protease.[16]

For carboxylic acids, a rule based on the size of the substituents did not work for either crude or purified ANL. This result suggests that electronic effects, specifically charge, control the enantioselectivity of ANL. We estimate that interactions of the lipase with the $-NH_3^+$ or $=NH_2^+$ group contribute a factor of 40 to 100, corresponding to a 2.2-

2.7 kcal/mol difference in $\Delta\Delta G^{\ddagger}$. This estimate comes from the enantioselectivity of ANL toward substrates whose substituents at the stereocentre differ only in charge. Similarly, Rotticci *et al.* found that electronic effects influence the enantioselectivity of lipase B from *Candida antarctica* (CAL-B).[17] Replacing a bromo substituent by methyl changed the enantioselectivity of CAL-B by a factor of >30 (>2 kcal/mol in $\Delta\Delta G^{\ddagger}$) and the enantioselectivity either decreased or increased depending of the location of the substituent.

Another unique feature of ANL is that it catalyzes the enantioselective hydrolysis of cyclic amino acids - proline and pipecolic acid. This ability may be useful for synthesis of peptides containing cyclic amino acids. Although some proteases can accept proline, they also catalyze the hydrolysis of peptide bonds.[18]

Experimental Section

General

Lipase from *Aspergillus niger* (AP-6) and *Pseudomonas cepacia* (LPL-80) were purchased from Amano International Enzyme Co. (Troy, VI). Lipase from *Candida rugosa* (type VII) and acetyl cholinesterase (isolated from electric eel) were purchased from Sigma Chemical Co. (Oakville, Ontario). Chemicals were purchased from Sigma-Aldrich Chemical Co. (Oakville, ON) and used without further purification unless stated otherwise. D,L-β-Aminoisobutyric acid, **34**, and D,L-3-aminobutyric acid, **35**, were purchased from Fluka Chemical Co. (Oakville, ON). D,L-Dimethyl tyrosine and D,L-2-aminotetralin-2-carboxylic acid, **37**, were provided by Dr. P. Schiller, IRCM, Montréal. (±)-Ketoprofen chloroethyl ester, **46** and (±)-methyl-2-(4-chlorophenoxy)propanoate, **43**, were provided by Dr. Ian Colton.

Ultrafiltration was performed with an Amicon ultrafiltration kit (Oakville, ON) under N_2 using a YM-10 filter. Melting points were taken on an Electrothermal melting point apparatus and were corrected. Protein concentrations were measured using a dye-binding assay from Bio-Rad (Mississauga, ON) with bovine serum albumin as the standard. Purified ANL was desalted with Bio-Gel P6 (Bio-Rad), a size exclusion gel.

Enzyme assays were carried out on a Hewlett Packard 8452A diode array spectrophotometer equipped with a Nelson RTE-100 water bath temperature control unit. The rate of lipase-catalyzed ester hydrolysis was measured on a Radiometer RTS 822 pH stat. HPLC chiral stationary phase columns were purchased from Daicel Chemical Industries Ltd. (Fort Lee, NJ). NMR spectra were recorded on either a Varian Gemini 200 MHz, Jeol 270 MHz or Unity 500 MHz NMR spectrometer.

Enzyme activity

Hydrolase activity was measured using *p*-nitrophenyl acetate (PNPA) as a substrate. An aliquot (5 μL) of enzyme solution was added to phosphate buffer (1.00 mL, pH 7.5, 10 mM), allowed to equilibrate to 25 °C, followed by addition an aliquot (5 μL) of PNPA (50 mM solution in 1:1 acetonitrile/ 10 mM phosphate buffer, pH 7.5). The initial rate of formation of *p*-nitrophenolate was monitored at 25 °C, 404 nm for 30 seconds. Activity was calculated using the Beer-Lambert law, with a cell length of 1 cm, and extinction coefficient of 11,600 $M^{-1}cm^{-1}$ which accounts for the incomplete ionization of *p*-nitrophenolate at pH 7.5. Crude ANL showed an activity of 48 U/g solid (1.7 U/mg protein) with this assay. U = μmol of ester hydrolyzed per minute.

Activity of the partially-purified lipase towards olive oil (0.6-35 mM) and ethyl butyrate (9-250 mM) were measured at pH 5 (sodium acetate buffer, 10 mM) using a pH stat which maintained the pH at 5 by automatic titration with 0.1 N NaOH. Insoluble substrate remained during the assay to ensure interfacial activation.

Partial purification of ANL by precipitation with ammonium sulphate

The procedure was carried out according to Ng-Youn-Chen *et al.*[7] All steps during enzyme purification were carried out at 4 °C. Crude lipase from *Aspergillus niger* (Amano AP-6, 32 g, 1500 U by PNPA assay) was stirred in Tris-HCl buffer (250 mL, 25 mmol, pH 7.5) for 3 h, then centrifuged (10,000 rpm, 20 min) to remove insoluble material. Solid ammonium sulphate (36 g, 25% saturation) was added to the stirring supernatant in small portions every 15 min over 4 h. The solution stirred overnight. The resulting suspension was centrifuged (10,000 rpm, 20 min), the very small pellet was

discarded, and additional ammonium sulphate (51 g, 55% saturation) was added in small portions over four hours. The solution was stirred overnight then centrifuged (3,000 rpm, 45 min). The supernatant was discarded and a brown pellet containing the lipase was dissolved in sodium acetate buffer (25 mL, 10 mM, pH 5). This solution was desalted with a size exclusion gel (BioGel-P6) using 10 mM phosphate buffer, 10 mM NaCl, pH 7 as eluent at a linear flow rate of 12.7 cm/h. Fractions were collected and assayed using the PNPA assay. Those fractions with lipase activity were pooled yielding 70 mL of solution containing 189 units (13% yield). The solution was concentrated by ultrafiltration with an Amicon YM-10 membrane yielding 13 mL (14.5 U/mL, 4.25 mg protein/mL) of lipase solution. Partially-purified ANL solution showed a specific activity of 3.41 U/mg protein accounting for a two-fold increase in specific activity over the crude preparation, and an enantioselectivity > 100 towards phenylalanine-methyl ester HCl. The dark brown solution retained full activity when stored at 4 °C with 0.02 wt/vol % NaN_3 solution as preservative for at least 3 months. With other lots of crude ANL, the best partial-purification used 25-48% saturation with ammonium sulphate.

Partial purification of ANL by anion-exchange chromatography

Several lots of crude ANL gave < 10% yield when purified by ammonium sulphate precipitation. These lots were partially-purified by first precipitating ANL at 60% saturation with ammonium sulphate, and second, anion exchange chromatography. The 60% pellet (285 units, 19% yield) was dissolved in sodium acetate buffer (23 mL, 10 mM, pH 5) and desalted with a Bio-Gel P-6 column as described above. The fractions were assayed using the PNPA assay and those with lipase activity were pooled, yielding 27 mL of solution containing 236 units. The phosphate buffer was exchanged for Tris-HCl buffer (25 mM, pH 7.48) by ultrafiltration using an Amicon YM-10 membrane, for a final volume of 5 mL (4.00 U/mL, 2.18 U/mg protein).

The solution (2.5 mL) was then injected onto a MonoQ anion exchange column (Mono Q HR 5/5 column, Pharmacia, Baie d'Urfé, QC) equilibrated with Tris-HCl buffer (25 mM, pH 7.48) using a Pharmacia FPLC system. The column was eluted at a flow rate of 0.5 mL/min with the same buffer for 10 mL, followed by a linear gradient of 0-0.5 M NaCl over 70 mL, then eluted with 0.5 M NaCl for 10 mL. Fractions (2 mL) were

collected and assayed by PNPA. Fractions 13-15 contained hydrolase activity. The procedure was repeated and fractions 13-15 from both runs were pooled, yielding 12 mL of solution containing 77 units (27% yield overall from the 60% pellet). ANL partially-purified by this technique showed a specific activity of 7.74 U/mg protein accounting for a 4.5-fold increase in specific activity over the crude preparation and enantioselectivity > 100 towards phenylalanine-methyl ester HCl. The dark brown solution retained full activity when stored at 4 °C with 0.02 wt/vol % NaN_3 solution as preservative for 1 month.

Synthesis of esters of carboxylic acids

Esters of all carboxylic acids were previously prepared in literature. ^{1}H-NMR and ^{13}C-NMR data are provided because not all literature references included complete NMR data. Amino-acid esters were purified by recrystallization from methanol/diethyl ether; all other esters were purified by flash chromatography.

D,L-2-Phenylglycine-methyl ester-HCl was prepared by Fischer esterification.[19] 98% (white solid); mp = 129.1-130.1 °C; R_f = 0.51 (95:5 chloroform/methanol); ^{1}H-NMR (CD_3OD, 200 MHz) δ 3.82 (s, 1H), 4.88 (br s, 3H), 5.30 (s, 3H), 7.49 (m, 5H); ^{13}C-NMR (CD_3OD, 200 MHz) δ 54.9, 58.5, 129.3, 130.6, 131.3, 133.3, 169.6.

D,L-Proline-octyl ester-HCl, methyl ester of **32**, was prepared by Fischer esterification.[19] 74% (clear oil); R_f= 0.81 (50:50 chloroform/methanol); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.87 (t, 3H), 1.45 (m, 10H), 2.11 (m, 2H), 2.39 (m, 2H), 3.43 (m, 2H), 4.10 (m, 1H).

2,6-Dimethyl-DL-tyrosine-methyl ester-HCl was prepared according to Pitzele *et al.*:[20] 70% (light brown solid); mp = 139.4-140.5 °C; R_f = 0.88 (3:2 ethyl acetate/methanol, 1% NH_4OH); ^{1}H-NMR (D_2O, 270 MHz) δ 2.18 (s, 6H), 3.06 (m, 2H), 3.67 (s, 3H), 4.16 (t, 1H, J = 8.2 Hz), 6.58 (s, 2H); ^{13}C-NMR (CD_3OD, 200 MHz) δ 21.6, 21.8, 32.5, 54.4, 54.6, 116.6, 123.3, 139.5, 157.1, 170.6.

O-Me-DL-Tyrosine methyl ester-HCl was prepared according to Moersch *et al.*:[21] 85% (white solid); mp = 167-168 °C; R_f = 0.78 (3:2 ethyl acetate/methanol, 1% NH_4OH); ^{1}H-NMR (d_6-DMSO, 200 MHz) δ 3.10 (m, 2H), 3.68 (s, 3H), 3.75 (s, 3H), 4.21 (t, 1H, J = 6.8 Hz), 6.90 (d, 2H, J = 8.6 Hz), 7.17 (d, 2H, J = 8.6 Hz); ^{13}C-NMR (CD_3OD, 200 MHz) δ 37.7, 54.2, 54.5, 56.2, 56.6, 115.7, 127.0, 131.5, 160.5, 170.0.

D,L-tert-Leucine-methyl ester-HCl was prepared according to Brenner *et al.*:[22] 91% (white solid); mp = 228.4-234.7 °C (sublimes without melting); R_f = 0.80 (3:2 ethyl acetate/methanol, 1% NH_4OH); ^{1}H-NMR (D_2O, 500 MHz) δ 1.07 (s, 9H), 3.29 (s, 3H), 3.72 (s, 1H). ^{13}C-NMR (CD_3OD, 500 MHz) δ 28.1, 34.8, 63.7, 182.9.

(±)-Methyl β-aminoisobutyrate-HCl, methyl ester of **34**, was prepared according to Brenner *et al*:[22] 99% (white solid); mp = 109.9-110.6 °C; R_f = 0.76 (1:1 chloroform/methanol); ^{1}H-NMR (D_2O, 200 MHz) δ 1.16 (d, 3H, J = 7.68 Hz), 2.85-2.93 (m, 1H), 3.01-3.27 (m, 2H), 3.68 (s, 3H); ^{13}C-NMR (D_2O, 200 MHz) δ 17.9, 40.8, 44.8, 56.3, 178.0.

(±)-Methyl 3-aminobutyrate-HCl, methyl ester of **35**, was prepared according to Brenner *et al.*:[22] 96% (yellow oil); R_f = 0.79 (1:1 methanol/chloroform); ^{1}H-NMR (D_2O, 200 MHz) δ 1.29 (d, 3H, J = 6.9 Hz), 2.69 (d, 2H, J = 6.1 Hz), 3.63-3.74 (m, 1H), 3.69 (s, 3H); ^{13}C-NMR (D_2O, 200 MHz) δ 21.6, 41.5, 48.0, 56.1, 180.1.

(±)-Methyl 2-aminotetralin-2-carboxylate, methyl ester of **37**, was prepared by the method of Obrecht *et al.*:[23] 48% (white solid); mp = 131-132 °C; R_f = 0.68 (3:1 hexanes/ethyl acetate); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 1.92 (m, 1H), 2.16 (m, 1H), 2.76

(d, 2H, J = 16 hz), 2.84 (m, 1H), 2.99 (m, 1H), 3.30 (d, 1H, J = 16 hz), 7.06-7.13 (m, 4H). ^{13}C-NMR (CD_3OD, 200 MHz) δ 14.2, 25.3, 31.7, 39.7, 56.6, 61.2, 126.1, 128.8, 129.7, 132.8, 134.5, 176.2.

The *N*-Cbz derivatives **38** (R = Bn, Ph) were prepared from their respective methyl esters following a procedure by Bodansky:[24]

D,L-N-Cbz-Phenylalanine-methyl ester: 62% (yellow oil); R_f = 0.82 (95:5 chloroform /methanol); ^{1}H-NMR ($CDCl_3$, 270 MHz) δ 3.10 (m, 2H), 3.71 (s, 3H), 4.65 (m, 1H), 5.08 (s, 2H), 5.21 (br d, 2H, J = 7.9 Hz), 7.09-7.33 (m, 10H); ^{13}C-NMR ($CDCl_3$, 200 MHz) δ 39.6, 53.5, 55.9, 127.4, 128.3, 128.4, 128.8, 128.9, 129.5, 135.8, 136.4, 155.5, 171.7.

D,L-N-Cbz-Phenyglycine-methyl ester: 54% (white solid); mp = 79.8-82.1 °C; R_f = 0.37 (3:1 hexanes/ethyl acetate); ^{1}H-NMR ($CDCl_3$, 270 MHz) δ3.72 (s, 3H), 5.09 (s, 2H), 5.84 (d, 1H, J = 7.2 Hz), 5.85 (br d, 2H, J = 5.67 Hz), 7.17-7.35 (m, 10H); ^{13}C-NMR ($CDCl_3$, 200 MHz) δ 53.9, 59.0, 68.1, 127.4, 128.3, 128.4, 128.6, 128.8, 128.8, 129.1, 129.2, 136.3, 136.8, 155.2, 171.0.

We prepared the octyl esters of **47** and **48** and the 2-chloroethyl ester of **45** using a modified DCC coupling employing a water soluble coupling reagent (*N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide):[25]

(±)-2-Chloroethyl 2-phenylpropanoate, chloroethyl ester of **45**: 74% (yellow oil); R_f = 0.60 (3:1 hexanes/ethyl acetate); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 1.54 (d, 3H, J = 7.4 Hz), 3.64 (t, 2H, J = 6 Hz), 3.77 (q, 1H, J = 7 Hz), 4.3 (m, 2H), 7.2-7.4 (m, 5H); ^{13}C-NMR (CD_3OD, 200 MHz) δ 20.0, 42.7, 46.6, 65.2, 127.5, 127.8, 128.9, 140.2, 173.9.

(±)-Octyl tetrahydro-2-furoate, octyl ester of **47**: 69% (clear oil); R_f = 0.59 (3:1 hexanes/ethyl acetate); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.86 (br t, 3H, J = 1.13), 1.25 (br s, 12H), 1.62 (m, 2H), 1.94 (m, 3H), 2.28 (m, 1H), 4.08 (m, 2H), 4.12 (t, 2H, J = 6.8 Hz),

4.39 (m, 1H); ^{13}C-NMR (CD_3OD, 200 MHz) δ 15.7, 24.1, 26.7, 27.3, 30.0, 31.6, 33.1, 66.0, 70.3, 173.2.

(±)-Octyl tetrahydro-3-furoate, octyl ester of **48**: 72% (clear oil); R_f = 0.69 (3:1 hexanes/ethyl acetate); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.86 (br t, 3H), 1.27 (m, 12H), 1.60 (m, 2H), 2.1 (m, 2H), 3.07 (q, 1H, J = 8 Hz), 3.85-4.04 (m, 4H), 4.11 (t, 2H, J = 6.6 Hz); ^{13}C-NMR ($CDCl_3$, 500 MHz) δ 13.9, 22.4, 25.0, 25.6, 28.4, 28.9, 30.0, 31.5, 64.8, 69.1, 72.4, 103.0, 161.9.

(±)-Methyl-2-chloropropanoate, methyl ester of **49**. (±)-2-Chloropropionyl chloride was added dropwise to a stirring solution of methanol in an ice bath, under an atmosphere of N_2. The reaction was allowed to warm to room temperature and was stirred for 1 h. The reaction was then neutralized with saturated sodium bicarbonate, extracted with diethyl ether (3 x 15 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*: 89% (yellow oil); R_f = 0.51 (2:1 hexanes/ethyl acetate); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 1.69 (d, 3H, J = 6.9 Hz), 3.79 (s, 3H), 4.41 (q, 1H, J = 6.9 Hz); ^{13}C-NMR ($CDCl_3$, 200 MHz) δ 23.0, 53.5, 54.1, 170.3.

General procedure for ANL-catalyzed hydrolyses of amino acid esters, esters of 32-37

Aspergillus niger lipase (25 units) was added to sodium acetate buffer (8 mL, 10 mM, pH 5) and stirred for 1 h to ensure complete dissolution. D,L-Amino acid esters (100 mg) dissolved in sodium acetate buffer (1 mL, 10 mM, pH 5) were added and the rate of hydrolysis was monitored by pH stat which maintained the pH at 5 by automatic titration with 0.1 N NaOH. Reactions were terminated at approximately 40% conversion, as noted on the pH stat, by removing the enzyme by ultrafiltration with a YM-10 Amicon membrane. The aqueous extract containing both the starting ester and product acid was concentrated *in vacuo*. The enantiomeric purities of the acid and ester were measured as described below and the enantiomeric ratio, E, was calculated according to Sih.[26] When the enantiomeric excess of both starting ester and product acid could not be determined in

a single HPLC run, the starting ester and acid were separated by adjusting the solution to pH 8 with saturated $NaHCO_3$ and extracting the ester with ethyl acetate (3 x 15 mL). Ethanol was added dropwise during the workup to break up emulsions when necessary. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated *in vacuo*, producing the ester. The aqueous phase was concentrated *in vacuo*, yielding the product acid. There was no detectable chemical hydrolysis of the amino acid esters at pH 5.

*General procedure for ANL-catalyzed hydrolyses of carboxylic acid esters, esters of **38, 39, 43-49***

The procedure was similar to above, with the following changes. (±)-Carboxylic acid esters (100 mg) were dissolved in 2 mL diethyl ether and added to the stirring enzyme solutions. The stirring reactions were sealed with parafilm to prevent appreciable evaporation of the ether layer during the reactions. Reactions were terminated at a convenient conversion by adjusting the solution to pH 1 using 1N HCl and the product acids and starting esters were extracted with diethyl ether (3 x 20 mL). The starting ester and acid were separated by adjusting the solution to pH 8 with saturated $NaHCO_3$ and extracting the ester with ethyl acetate (3 x 15 mL). The aqueous layer was adjusted to pH 2 and the acid extracted with ethyl acetate (3 x 15 mL). Ethanol was added dropwise during workup to break up the emulsion and inactivate the enzyme. Both extracts were dried with Na_2SO_4, filtered, and concentrated *in vacuo*. The enantiomeric excesses of the acids and esters were measured as described below and the enantiomeric ratio, E, was calculated according to Sih.[26] There was no detectable chemical hydrolysis of the above substrates at pH 5.

Determination of enantiomeric purity by HPLC using a chiral stationary phase

High performance liquid chromatography (HPLC) was performed on a Spectra-Physics liquid chromatograph, model 8800 equipped with a Spectra FOCUS forward optical scanning detector, SP8880 autosampler and Spectra Physics software. The analytical chiral columns used for enantiomeric resolution were a Chiralpak AD amylose

derivatized column, a Chiralcel OD column, a Chiralpak WH column and a Crownpak CR(+) crown ether derivatized column (Daicel Chemical Industries Ltd., Fort Lee, NJ). Enantiomeric excesses were measured as described below and the enantiomeric ratio, E, was calculated. The area of the peaks was measured either by electronic integration or cut and weigh when electronic integration gave unequal areas for the racemate.

Enantiomers of the proline, **32**, were analyzed with a Chiralpak WH column using 0.25 mM $CuSO_4$ as eluting buffer with the detector set at 254 nm: 50 °C, 1.5 mL/min, k_L' = 2.23; k_D' = 3.90; α= 1.75; R_s = 1.77. Proline was dissolved in eluting buffer (1 mg/mL), and neutralized to pH 7 with dilute NaOH prior to injection. Proline esters were hydrolyzed with aqueous NaOH for analysis. The absolute configuration was established by comparison with an authentic sample of L-proline.

Enantiomers of α- and β-amino acids, **33-35**, their methyl esters, and phenylalaninamide were separated with a Crownpak CR(+) modified crown ether column, using aq. $HClO_4$ solution as eluent, with the detector set at 200 nm. Concentrations of 0.1 mg compound/mL mobile phase were injected for analysis except for alanine, 1.0 mg/mL. Absolute configurations were inferred from the order of elution on a CR(+) column for which the D-enantiomer always elutes first and by the reported orders of elution in literature for the compounds below[27]. CAUTION: Aqueous solutions containing perchloric acid should not be evaporated and can explode if heated.

Alanine: 0 °C, aq. $HClO_4$ pH 1.5, 0.4 mL/min, k_D' = 0.75; k_L' = 2.30; α = 3.07; R_s = 6.00

Alanine-methyl ester: 25 °C, aq. $HClO_4$ pH 2, 0.3 mL/min, k_D' = 0.38; k_L' = 0.70; α =1.84; R_s = 1.8.

Methionine: 25 °C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D' = 0.82; k_L' = 1.6; α =1.95; R_s = 2.12.

Methionine-methyl ester: 25 °C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D' = 1.79; k_L' = 4.04; α = 2.26; R_s = 3.00.

Phenylalanine: 25 °C, aq. $HClO_4$ pH 2, 0.8 mL/min, $k_D' = 3.93$; $k_L' = 5.20$; $\alpha = 1.32$; $R_s = 3.86$.

Phenylalanine-methyl ester: 25 °C, aq. $HClO_4$ pH 2, 0.8 mL/min, $k_D' = 8.29$; $k_L' = 10.09$; $\alpha = 1.22$; $R_s = 2.76$; and at 5 °C, aq. $HClO_4$ pH 1.5, 0.8 mL/min, $k_D' = 11.7$; $k_L' = 20.9$; $\alpha = 1.78$; $R_s = 3.87$.

Phenylalaninamide: 5 °C, aq. $HClO_4$ pH 1.5, 0.8 mL/min, $k_D' = 7.66$; $k_L' = 14.8$; $\alpha = 1.93$; $R_s = 5.05$.

2-*Phenylglycine:* 40 °C, aq. $HClO_4$ pH 2, 1.0 mL/min, $k_D' = 1.37$; $k_L' = 4.46$; $\alpha = 3.25$; $R_s = 10.1$.

2-Phenylglycine-methyl ester: 40 °C, aq. $HClO_4$ pH 2, 1.0 mL/min, $k_D' = 3.89$; $k_L' = 10.18$; $\alpha = 2.62$; $R_s = 10.0$.

Tyrosine: 25 °C, aq. $HClO_4$ pH 2, 0.8 mL/min, $k_D' = 2.11$; $k_L' = 2.84$; $\alpha = 1.34$; $R_s = 1.09$.

Tyrosine-methyl ester: 25 °C, aq. $HClO_4$ pH 2, 0.8 mL/min, $k_D' = 4.27$; $k_L' = 6.24$; $\alpha = 1.46$; $R_s = 1.62$.

4-O-Me-Tyrosine: 25 °C, 1.2 mL/min, aq. $HClO_4$ pH 2 $k_D' = 6.62$; $k_L' = 8.73$; $\alpha = 1.31$; $R_s = 2.80$.

4-OMe-Tyrosine-methyl ester: 25 °C, aq. $HClO_4$ pH 2, 1.2 mL/min, $k_D' = 14.72$; $k_L' = 19.88$; $\alpha = 1.35$; $R_s = 1.89$.

2,6-Dimethyltyrosine: 25 °C, aq. $HClO_4$ pH 2, 0.8 mL/min, $k_D' = 10.19$; $k_L' = 11.29$; $\alpha = 1.11$; $R_s = 0.84$.

2,6-Dimethyltyrosine-methyl ester: 25 °C, aq. $HClO_4$ pH 2, 0.8 mL/min, $k_D' = 10.89$; $k_L' = 12.11$; $\alpha = 1.11$; $R_s = 1.08$.

Tryptophan: 25 °C, aq. $HClO_4$ pH 2, 1.2 mL/min, $k_D' = 11.61$; $k_L' = 14.32$; $\alpha = 1.23$; $R_s = 1.20$.

Tryptophan-methyl ester: 25 °C, aq. $HClO_4$ pH 2, 1.2 mL/min, $k_D' = 24.61$; $k_L' = 32.47$; $\alpha = 1.32$; $R_s = 1.40$.

Enantiomers of β-amino acids, **34** and **35**, and their methyl esters were separated with a Crownpak CR(+) column.

β-Aminoisobutyic acid, **34**: 0 °C, aq. $HClO_4$ pH 1, 0.4 mL/min, $k_D' = 3.48$; $k_L' = 4.15$; $\alpha = 1.19$; $R_s = 1.04$.

Methyl β-aminoisobutyrate: 0 °C, aq. $HClO_4$ pH 1.5, 0.4 mL/min, $k_D' = 3.03$; $k_L' = 4.00$; $\alpha = 1.32$; $R_s = 1.63$.

3-Aminobutyric acid, **35**: 0 °C, aq. $HClO_4$ pH 1.5, 0.4 mL/min, $k_D' = 2.05$, $k_L' = 2.64$, $\alpha = 1.29$; $R_s = 1.29$.

Methyl 3-aminobutyrate: 0 °C, aq. $HClO_4$ pH 1.5, 0.4 mL/min, $k_D' = 4.19$; $k_L' = 5.17$; $\alpha = 1.23$; $R_s = 1.73$.

Enantiomers of **43**, 2-(4-chlorophenoxy)propionic acid, were separated using a Chiralpak AD column as described previously by Colton *et al.*:[28] 254 nm, 25 °C, 95/5/1 hexanes/isopropanol/triflouroacetic acid, 0.5 mL/min, $k_S' = 2.13$; $k_R' = 2.80$; $\alpha = 1.31$; $R_s = 3.78$. The absolute configuration was established by comparison with the reported order of elution by the column manufacturer. The ester of **43** was hydrolyzed to **43** with 1.5 equivalents of aqueous NaOH prior to analysis.

Enantiomers of **38** (R = Bn, Ph) and **45** were separated using a Chiralcel OD column. The ester of **45** was hydrolyzed to **45** with 1.5 equivalents of aqueous NaOH prior to analysis.

2-Phenylpropionic acid, **45**: 254 nm, 25 °C, 98/2/1 hexanes/isopropanol/formic acid, 0.5 mL/min, $k_R' = 3.35$; $k_S' = 4.05$; $\alpha = 1.21$; $R_s = 2.13$.

N-Cbz-Phenylalanine: 254 nm, 25 °C, 79/20/1 hexanes/ isopropanol/triflouroacetic acid, 0.5 mL/min, $k_D' = 1.84$; $k_L' = 2.13$; $\alpha = 1.16$; $R_s = 1.52$.

N-Cbz-Phenylalanine-methyl ester: 254 nm, 25 °C, 80/20 hexanes/ isopropanol, 0.5 mL/min, $k_L' = 4.74$; $k_D' = 5.34$; $\alpha = 1.13$; $R_s = 1.47$.

N-Cbz-Phenylglycine-methyl ester: 254 nm, 25 °C, 80/20 hexanes/ isopropanol, 0.5 mL/min, , $k_S' = 2.87$; $k_R' = 3.37$; $\alpha = 1.17$; $R_s = 2.00$.

The absolute configurations were established by comparison with the reported order of elution by the column manufacturer.

Determination of enantiomeric purity by ^{1}H-NMR

The racemic esters of **47** and **48** were dissolved in $CDCl_3$ and the ^{1}H-NMR spectra were obtained using a 200 MHz spectrometer. Solid tris[(3-heptafluoropropylhydroxymethylene)-(+)-camphorato] europium(III), $Eu(hfc)_3$, was added portion-wise until separate signals for the $COOCH_2$ methylene triplet of the racemic octyl esters were obtained. The triplets of **47** were separated by 2.4 Hz and those of **48** were separated by 3.7 Hz. The number of equivalents of shift reagent necessary to obtain the separations was then added to the esters for which the enantiomeric purity was to be determined. The absolute configurations were not determined.

Determination of enantiomeric purity by gas chromatography using a chiral stationary phase

Gas chromatography was performed on a Varian 589-Series II gas chromatograph equipped with chiral stationary phases.

Enantiomers of methyl mandelate (methyl ester of **44**) were separated on a Chiraldex G-TA30 column (Astec Inc., Whippany, NJ) which contained 2,6-di-*O*-pentyl-3-*O*-trifluoroacetyl derivative of γ-cyclodextrin as the stationary phase: 110 °C, split ratio 115:1, $k_S' = 17.8$; $k_R' = 18.4$; $\alpha = 1.03$; $R_s = 0.91$. Product acid was converted to the ester derivative with ethereal CH_2N_2 for analysis. The absolute configuration was established by comparison with the reported order of elution by the column manufacturer.

Enantiomers of the methyl ester of **49** were separated using a Chirasil-DEX CB

column (Chrompack Inc., Raritan, NJ) which contained a polydimethylsiloxane derivative of β-cyclodextrin as the stationary phase: 60 °C, $k_S' = 3.8$; $k_R' = 4.47$; $\alpha = 1.17$; $R_s = 5.00$. Product acid **49** was converted to the methyl ester for analysis by dissolution in methanol containing a catalytic amount of sulfuric acid. The absolute configuration was established by comparing the order of elution with an authentic sample of (*S*)-methyl-2-chloropropanoate.

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Chapter 3

"Studying the photograph of a racehorse cannot tell you how fast it can run"

Professor Jeremy Knowles
Harvard University

In the previous chapter, we explored the use of empirical substrate models to aid in the selection of enantioselective lipases towards a target substrate. Empirical rules can predict the enantiopreference and crudely estimate enantioselectivity, but a researcher cannot avoid determining the enantiomeric ratio, E, to confirm a hydrolase's enantioselectivity towards a substrate.

Currently, the most popular method to measure E is the endpoint method. Small-scale reactions are run for each candidate hydrolase and the substrate, the reactions are stopped at a convenient percent conversion and the enantiomeric purities of the products and/or starting materials are measured using appropriate analytical conditions. Typically, it takes several hours to test a single hydrolase, so researchers rarely screen large numbers of hydrolases.

In this chapter, we describe a novel, spectrophotometric method we call "quick E" to accurately measure the enantiomeric ratio of hydrolase-catalyzed reactions using initial rate measurements. This method avoids the time-consuming step of measuring enantiomeric purities and most importantly, agrees with the slower endpoint method for both low and high enantioselectivities and is fast enough to screen large libraries of hydrolases towards a target substrate.

Note that this chapter was written as a communication for publication. Comprehensive details on the development of this method and experimental are presented in Chapter 5. It is necessary to present the quick E method at this point in the thesis to logically show the stepwise developments of this method documented in future chapters.

Quick E. A Fast Spectrophotometric Method to Measure the Enantioselectivity of Hydrolases.

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Abstract: The slow step in finding and optimizing enantioselective biocatalysts is measuring their enantioselectivity. The current endpoint method for measuring enantioselectivity typically requires several hours to run a reaction, work it up, and measure the enantiomeric purity of the product and/or remaining starting material. We report a fast spectrophotometric method that measures enantioselectivity of hydrolases in less than one minute. The initial rates of hydrolysis of pure enantiomers of a chromogenic substrate, such as 4-nitrophenyl 2-phenylpropanoate, 1, are easily measured by monitoring the release of 4-nitrophenoxide at 404 nm. However, the ratio of separately measured initial rates does *not* give the correct enantioselectivity because it ignores the competitive binding of the two enantiomers. To reintroduce competition, we added a chromogenic reference compound, resorufin tetradecanoate, to each reaction. In the first reaction, one enantiomer competes with the reference compound, while in the second reaction the other enantiomer competes with the same reference compound. The ratio of the two relative rates gives the enantioselectivity. The quick E method agrees with the slower endpoint method for both low and high enantioselectivities and is fast enough to screen biocatalyst libraries.

Communication

Hydrolase-catalyzed resolutions of racemates are often the best route to enantiomerically-pure compounds.[1] These reactions are often more selective and cheaper than chemical methods. To find an enantioselective hydrolase for a target compound, researchers first screen commercial enzymes and cultures of microorganisms and then optimize the reaction conditions. Both screening and optimization require measuring the

enantioselectivity of the reaction. The enantioselectivity of an enzyme is the ratio of the specificity constants, k_{cat}/K_M, for the enantiomers, eq 3.1.[2, 3]

$$\textit{Enantiomeric ratio} = E = \frac{(k_{cat}/K_M)_{fast\ enantiomer}}{(k_{cat}/K_M)_{slow\ enantiomer}} \qquad \text{Eq. (3.1)}$$

Currently, the best method to measure E is the endpoint method developed by C. J. Sih's group,[3] but screening hundreds of commercial enzymes or cultures of microorganisms by this method is difficult. To measure E, researchers run a test resolution, work up the reaction and measure two of the following: enantiomeric purity of the starting material (ee_s), enantiomeric purity of the product (ee_p), or conversion (c). Equation 3.2 gives the enantiomeric ratio.

$$E = \frac{\ln[(1-c)(1-ee_S)]}{\ln[(1-c)(1+ee_S)]} = \frac{\ln[(1-c)(1+ee_P)]}{\ln[(1-c)(1-ee_P)]} = \frac{\ln\left[(1-ee_S)\Big/\left(1+\frac{ee_S}{ee_P}\right)\right]}{\ln\left[(1+ee_S)\Big/\left(1+\frac{ee_S}{ee_P}\right)\right]} \qquad \text{Eq. (3.2)}$$

The easiest cases - a fast reaction, minimal workup and rapid chromatographic determination of enantiomeric purity - require an hour. More difficult cases involve slow reactions, difficult workups, or separation and derivatization to measure enantiomeric purities and can take more than a day. A typical example, measuring the enantioselectivity of a hydrolase towards **1**, required 4.5 hours. We first carried out the kinetic resolution to 40% conversion (2 h), then separated remaining 1 from the product acid (30 min), hydrolyzed unreacted 1 to the acid with aqueous NaOH (1 h including workup) and finally measured the enantiomeric purity of both samples by HPLC on a chiral stationary phase (1 h).

Recognizing this difficulty, researchers have reported alternative methods to measure E by measuring initial rates of samples with varying ratios of enantiomers[4] or by analyzing reaction progression curves.[5] Unfortunately, they are not significantly faster and can be less accurate than the endpoint method. In this paper, we report a method to measure E in one minute from relative initial rates of hydrolysis of pure enantiomers and

a reference compound. Researchers previously used mixtures of substrates to measure enzyme selectivity.[6] We extend these techniques to enantioselectivity and to rapid spectrophotometric measurements.

Hydrolyses of pure enantiomers of 4-nitrophenyl 2-phenylpropanoate, (*S*)-**1** and (*R*)-**1**, and 4-nitrophenyl 2-(4-isobutylphenyl)propanoate (ibuprofen 4-nitrophenyl ester), (*S*)-**2** and (*R*)-**2**, liberates the yellow *p*-nitrophenoxide ion.[7] The increase in absorbance at 404 nm revealed the initial rates of hydrolysis of each enantiomer, but the ratio of these rates did not give the enantiomeric ratio, Table 3.1. The ratio of rates over- or underestimated E by as much as 70 percent because it ignored competitive binding of the two enantiomers to the enzyme. In other cases, researchers found that differences in K_M for the enantiomers contributed a factor of three to four to the enantioselectivity.[8]

To reintroduce competition, we added resorufin tetradecanoate as a reference compound.[9] We monitored the initial rates of hydrolysis of (*S*)-**1** at 404 nm and the reference compound at 572 nm in the same solution, Figure 3.1.

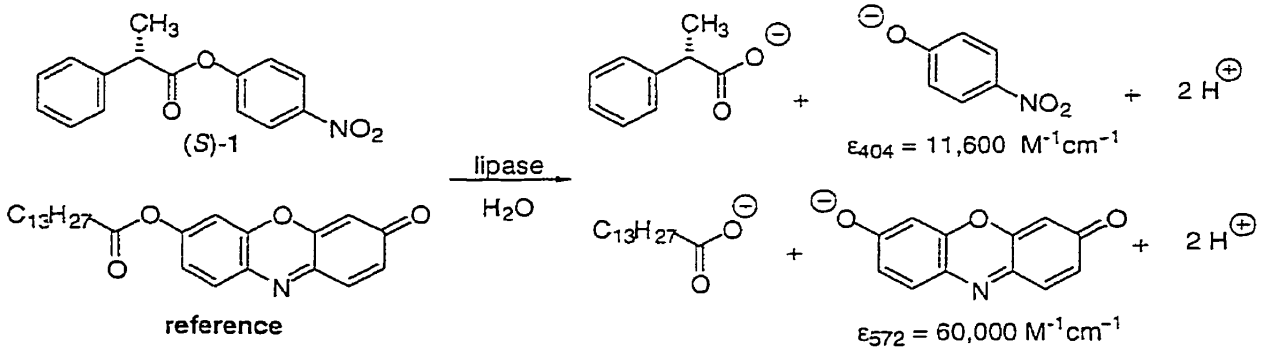

Figure 3.1. The first step of the quick E measure of enantioselectivity of lipases toward 4-nitrophenyl 2-phenylpropanoate, **1**. Lipase-catalyzed hydrolysis of (*S*)-**1** and the reference compound, resorufin tetradecanoate yields yellow and pink chromophores, respectively. The solution turns deep orange if both substrates are hydrolyzed; pink if only the reference compound is hydrolyzed. The second step of the quick E is the same, except that it uses the (*R*)-enantiomer of the chiral ester. Equations 3.3 and 3.4 yield the enantioselectivity.

Table 3.1. Enantiomeric ratios of hydrolases toward (±)-**1** and (±)-**2** measured using the endpoint method[a] and the ratio of separately-measured initial rates of hydrolysis of the enantiomers.

Substrate	Lipase[b]	ee_s[c] (%)	ee_p[c] (%)	c[d] (%)	E[e] endpoint	Initial rate[f] (*S*)	Initial rate[f] (*R*)	Rate ratio S/R[g]
1	PCL	nd	67	60	29 ± 3	15.32	0.771	20 ± 1
1	CRL	17	50	25	3.5 ± 0.2	2.81	2.38	1 ± 0.2
1	IPA-CRL	43	98	30	>100	3.56	0.092	40 ± 2
1	PPL	nd	2	4	1.1 ± 0.1	1.11	0.757	1.4 ± 0.1
1	CAL-A	20	23	37	1.9 ± 0.1	1.40	0.355	4 ± 0.2
2	PCL	28	43.2	39	3.3 ± 0.1 (R)	0.535	0.579	1.1 ± 0.1 (R)
2	CRL	5.3	14.5	27	1.4 ± 0.1	0.330	0.274	1.2±0.1
2	IPA-CRL	40.1	>99	29	>100	0.702	0.0128	55±5

[a]Reaction conditions: room temperature, 10 mM Tris buffer, pH 8, 100 mg crude lipase or 0.7 mg protein for IPA-CRL, 1 mmol of substrate dissolved in 1 mL of acetonitrile. [b]For spectrophotometric measurements, the lipases were dissolved in Tris buffer (25 mM, pH 7.5) and centrifuged to remove insoluble material. PCL (lipase from *Pseudomonas cepacia,* Amano lipase PS, 29.3 mg solid/mL), CRL (lipase from *Candida rugosa,* Sigma, 36.9 mg solid/mL; 0.5 mg protein/mL by the Bio-Rad protein assay), IPA-CRL (CRL treated with isopropanol as in reference 12a; 0.7 mg/mL protein by the Bio-Rad protein assay), PPL (porcine pancreatic lipase, Sigma, 42.3 mg solid/mL), CAL-A (lipase A from *Candida antarctica,* Boehringer Mannheim, 18.2 mg solid/mL). [c]ee_s = enantiomeric excess of remaining substrate, ee_p = enantiomeric excess of product. Determined by HPLC as described in reference 7. [d]Extent of conversion. [e]Enantiomeric ratio calculated using equation 3.2. Error limits for E were estimated assuming an error of ±1% for enantiomeric purity.

All hydrolyses favored the (*S*)-enantiomer except the PCL-catalyzed hydrolysis of **3** which favored the (*R*)-enantiomer. *f*Substrates were emulsified in aqueous solution according to Vorderwülbecke, T.; Kieslich, K.; Erdmann, H. *Enzyme Microb. Technol.* **1992**, *14*, 631-639. An acetonitrile solution of **1** (7.8 mM, 0.5 mL) or **2** (9.04 mM, 0.5 mL) was added dropwise to Tris buffer (9 mL, 50 mM, pH 8.0) containing 0.45-0.90 w/v% Triton X-100 (Pierce Surfact-Amps) and vortexed until clear. This emulsion remained clear for at least 3 h. To measure initial rates of hydrolysis, lipase solution (100 μL) was added to the substrate emulsion (900 μL) at 25 ^{0}C and the increase in absorbance at 404 nm was monitored for 15 s. No spontaneous chemical hydrolysis was detected. Values are in absorbance units per second x 10^{3}. *g*Average and standard deviation for three experiments.

After taking into account the initial concentrations of both substrates, the ratio of these rates yielded the selectivity of the hydrolase for (*S*)-**1** over the reference compound, eq 3.3.

$$\frac{(S)-1}{\textbf{reference}}\ selectivity = \frac{(k_{cat}/K_M)_{(S)-1}}{(k_{cat}/K_M)_{\textbf{reference}}} = \frac{v_{(S)-1}}{v_{\textbf{reference}}} \times \frac{[\textbf{reference}]}{[(S)-1]} \qquad \text{Eq. (3.3)}$$

A second experiment using (*R*)-**1** and the reference compound yielded the selectivity of (*R*)-**1** over the reference compound. The ratio of these two selectivities yields the enantiomeric ratio, eq 3.4.

$$E = \frac{(S)-1}{(R)-1}\ selectivity = \frac{\frac{(S)-1}{\textbf{reference}}\ selectivity}{\frac{(R)-1}{\textbf{reference}}\ selectivity} \qquad \text{Eq. (3.4)}$$

The quick E method agreed with the endpoint method for both **1** and **2** using five different lipases, Table 3.2. We measured low (E = 1.4), average (E = 27) and excellent (E = 210) enantiomeric ratios correctly by this technique. Each hydrolysis experiment requires 30 seconds; thus, the measurement time for E was only one minute plus a small amount of time to fill each cuvette.

The ability to measure high enantioselectivities with quick E is important because this is the goal of most screening studies. Using the endpoint method, measurement errors in conversion or enantiomeric purity limit make it difficult to measure enantiomeric ratios >100. Using quick E, however, we were able to measure an E of 210±20 for the IPA-CRL catalyzed hydrolysis of **1**, and set a lower limit of >140 for **2**, Table 3.2. Quick E measures these high values by measuring the rate of hydrolysis of the slow enantiomer. Our inability to measure a higher enantiomeric ratio for **2** was due to the unusually low reactivity of **2**. IPA-CRL favored the reference compound by a factor of 23 over the fast enantiomer of **2** and by >3000 over the slow enantiomer. Using a slower-reacting reference compound, it may be possible to measure higher enantiomeric ratios. Another limit to measuring high enantiomeric ratios with quick E is the enantiomeric purity of the starting slow enantiomer. Reaction of the contaminating fast enantiomer will give a

measured E that is lower than the true E. If the enantiomeric purity of the slow enantiomer is >99.9% ee, then quick E should be able to measure enantioselectivities up to 1000.

There are three advantages to the quick E method. First, it is many times faster than a typical endpoint measurement, yet has equivalent or better accuracy. Accuracy may be particularly important for screening in directed evolution where the improvements of each generation are small.[10] Note that quick E is based on the same equations as the endpoint methods, so inaccuracies of the endpoint method also apply to quick E.[11] Second, it requires much smaller amounts of hydrolase because the entire reaction occurs in the spectrophotometer. This advantage may be especially useful for screening biocatalyst libraries. Third, the quick E method can measure high enantioselectivities more easily than the endpoint method and high enantioselectivity can be visualized, Figure 3.2.

There are also several disadvantages to this screen. First, it requires pure enantiomers, albeit in small amounts (~20 μg per measurement in a 200 μL cuvette). Semi-preparative HPLC with chiral stationary phases may yield enough pure enantiomer for screening. Second, the current version of quick E measures the enantioselectivity only for chromogenic substrates. (Note that for **1** and **2** the enantiomeric ratios for the 4-nitrophenyl esters were the same as those for the corresponding methyl or ethyl esters.[12,8a] Future versions of quick E will extend the range of substrates. Third, spectrophotometric measurements require clear solutions. Dissolving hydrophobic substrates in aqueous solution, even with the help of surfactants, is sometimes difficult.

Acknowledgements

Financial support of this work by NSERC (Canada) gratefully acknowledged. Acknowledgement is made to the donors of The Petroleum Research Fund, administered by the ACS, for partial support of this research. We thank Ms. Cecile Vaugelaude, from INSA Rouen, France for the synthesis of (±)-**1** and Mr. Michael Trani of the NRC Biotechnology Research Institute, Montréal for the gift of (*R*)- and (*S*)-**2**.

Table 3.2. Enantiomeric ratios of hydrolases toward (±)-**1** and (±)-**2** using the quick E method.

Substrate	Lipase[a]	(*S*)-enantiomer + reference[b]		(*R*)-enantiomer + reference[b]		Quick E[c]
		404 nm	572 nm	404 nm	572 nm	
1	PCL	13.2	9.79	0.379	7.50	29 ± 3
1	CRL	1.02	9.48	0.28	9.02	3.5 ± 0.3
1	IPA-CRL	1.89	7.58	0.004	3.68	210 ± 20
1	PPL	0.199	0.186	0.125	0.164	1.4 ± 0.2
1	CAL-A	2.15	1.53	0.824	1.47	2.3 ± 0.2
2	PCL	0.3341	6.78	0.5750	4.709	2.5 ± 0.3 (*R*)
2	CRL[d]	3.408	49.3	1.342	52.18	3 ± 2
2	IPA-CRL	2.154	8.78	<0.01	5.767	>140

[a]See Table 3.1. [b]Substrate solutions as for Table 3.1, but an acetonitrile solution of resorufin tetradecanoate (0.5 mL, 1.6 mM) was also added. The liberated 4-nitrophenoxide and resourufin were measured in two separate measurements at 404 nm and 572 nm, respectively, at 25 ^{0}C for 15 s. No spontaneous chemical hydrolysis was detected. Values are in absorbance per second x 10^3 [c]Enantiomeric ratio calculated using eq 3.4. Average and standard deviation for three measurements. [d]CRL (lipase from *Candida rugosa*, Sigma, 214 mg solid/mL; 1.9 mg protein/ml by the Bio-Rad protein assay).

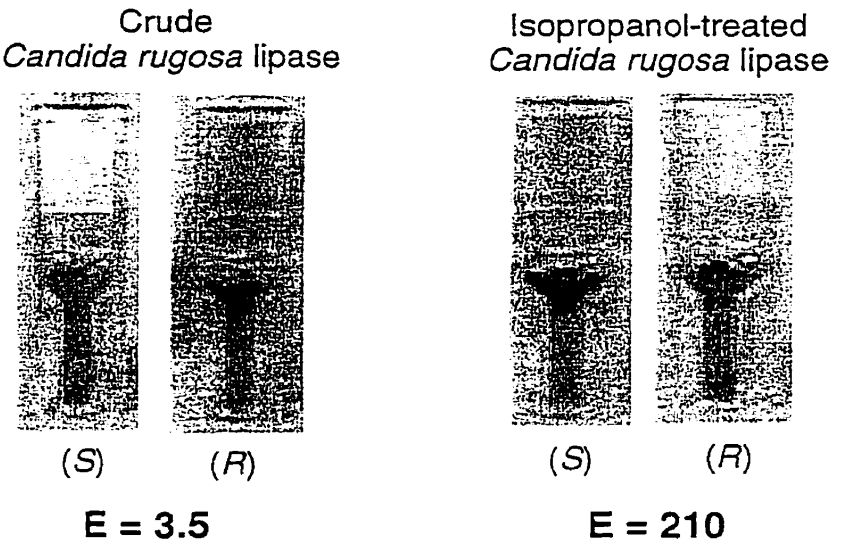

Figure 3.2 High enantioselectivity can be visually detected in the quick E measurement of lipases toward 4-nitrophenyl 2-phenylpropanoate, **1**. Lipase-catalyzed hydrolysis of 4-nitrophenyl esters of the pure enantiomers and the reference compound yield pink and yellow chromophores. The reaction solution turns orange if both substrates are hydrolyzed, pink if only the reference compound is hydrolyzed. With crude CRL, both steps of the quick E measurement result in orange solutions, indicating low enantioselectivity. With isopropanol-treated CRL, the (*R*) selectivity step produces a pink solution, indicating that only the reference compound is hydrolyzed in this step and the lipase has high enantiopreference towards the (*S*)-enantiomer.

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All enantiomeric purities were measured by HPLC using a Chiracel OD-H column (Daicel) at 25 °C, eluted at 1 ml/min. 2-phenylpropanoic acid: (98/2/1 hexanes/isopropanol/trifluoroacetic acid, k_R' = 3.35; k_s' = 4.05; α = 1.2; R_s = 2.13); 2-(4-isobutylphenyl)propanoic acid: (100/1/0.1 hexanes/isopropanol/ trifluoroacetic acid, k_R' = 3.31; k_s' = 4.27; α = 1.29; R_s = 2.43); **2**: (100/1/0.1 hexanes/isopropanol/trifluoroacetic acid, k_R' = 2.41; k_s' =2.93; α = 1.21; R_s = 1.66). For analysis, samples of **1** were hydrolyzed to the acid in aqueous NaOH.

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Chapter 4

"...whereupon pouring into a small phial, full of impregnated water, a very little spirit of vinegar, I found that, according to my expectation, the ceruleous colour immediately vanished, but was deceived in the expectation that I had, that the golden colour would do so too; for, which way soever I turned the phial, either to or from the light, I found the liquor to appear always of a yellowish colour and no other. Upon this I imagined that the acid salts of the vinegar having been able to deprive the liquor of its ceruleous colour, a sulphurous salt being of a contrary nature, would be able to mortify the saline particles of vinegar. And destroy their effects; and accordingly having placed my self betwixt the window, and the phial, and into the same liquor dropt a few drops of oil of tartar per deliquium (as chymistas call it) I observed with pleasure, that immediately upon the diffusion of this liquor, the impregnated water was restored to its former ceruleous colour..."

Robert Boyle
Experimental History of Colours, **1664**

The word indicator is derived from the Latin *indicare*, "to disclose" and, in general terms, is a substance which indicates the state of the system with respect to a selected component. The first use of an indicator for pH analysis was reported by W. Lewis in 1767[1] although Robert Boyle is generally regarded as the first to recognize the remarkable powers of plant extracts to change colours upon treatment with acids and bases.[2] In the 20th century, researchers began to apply pH indicators to monitor the progress of enzyme-catalyzed reactions that produce or consume protons. In this chapter, we extend the application of pH indicators to "disclose" the enantioselectivity of hydrolytic enzymes toward chiral substrates.

In Chapter 3, we presented a spectrophotometric method to rapidly determine the enantioselectivity of hydrolytic enzymes towards chromogenic esters. We used

chromogenic esters to first demonstrate quick E because their relative rates of hydrolysis are easily determined spectrophotometrically. We wished to make the quick E assay more general and extend quick E to synthetically useful, non-chromogenic esters. To do so, we needed a spectrophotometric method to detect the rates of hydrolysis of esters. In this chapter, we detail the development of a quantitative, colourimetric assay to measure enzyme-catalyzed rates of hydrolysis of esters using a pH indicator. Hydrolysis of an ester releases a proton(s). We measure the rate of proton release using a pH indicator and use a modified form of the Beer-Lambert law to relate the absorbance kinetics of the indicator to the true rates of hydrolysis. This pH indicator assay sets the stage to carry out quick E measurements of non-chromogenic esters in Chapters 5 and 6.

We can also use this pH indicator assay to rapidly estimate enantioselectivity. Using pure enantiomers of a chiral ester, the ratio of separately measured initial rates of enzyme-catalyzed hydrolysis estimates the enantioselectivity. Although this ratio is not the true enantioselectivity, it can rapidly identify active and enantioselective hydrolases from a large library towards a target substrate. We used the primary alcohol, solketal butyrate, as a test substrate because it is an important chiral building block and previous screenings with hydrolases by other researchers showed only limited success.

The pH indicator assay is currently under patent review, in collaboration with Thermogen, Inc. (Chicago, IL).

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Quantitative screening of hydrolase libraries using pH indicators: Identifying active and enantioselective hydrolases.

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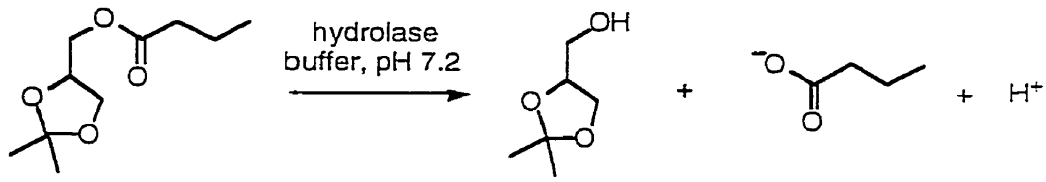
Abstract: The slowest step in finding a selective hydrolase for synthesis is often the screening step. Researchers must run small test reactions and measure the amounts of stereoisomers formed by HPLC, GC, or NMR. We have developed a colourimetric method to speed up this screening. We quantitatively detect ester hydrolysis using a pH indicator, 4-nitrophenol. We estimate the selectivity by measuring the initial rates of hydrolysis for pure stereoisomers separately. To demonstrate the utility of this method, we screened seventy-two commercial enzymes towards racemic solketal butyrate, an important chiral building block. First, we eliminated the twenty hydrolases that did not catalyze hydrolysis of either enantiomer. Next, we measured initial rates of hydrolysis of the pure enantiomers of solketal butyrate. For horse liver esterase, these initial rates differed by a factor of twelve. Subsequent GC experiments confirmed an enantiomeric ratio of fifteen for this hydrolase. Although this enantioselectivity is moderate, it is the highest enantioselectivity reported for a hydrolysis of solketal esters.

Introduction

Chemists often exploit the high stereoselectivity and regioselectivity of hydrolytic enzymes to solve synthetic problems.[1] For example, researchers use selective reactions catalyzed by lipases to prepare enantiomerically pure pharmaceutical intermediates and to selectively deprotect sensitive synthetic intermediates. One limitation to the wider use of

hydrolases is the difficulty in finding the best hydrolase for a given reaction. Although several empirical rules[2] can help one select likely candidates from the fifty to one hundred hydrolases commercially available, most researchers also use screening. To screen for selective hydrolases today, researchers run a small reaction for each hydrolase, workup the reaction, and determine the ratio of stereoisomers by HPLC, GC or NMR.[3] At best, one determination takes four hours. To save time researchers rarely screen all commercial hydrolases and likely miss good hydrolases.

To speed up this screening, we have developed a quantitative, colorimetric assay for hydrolysis of esters using pH indicators. Hydrolysis of an ester at neutral pH, for example, solketal butyrate (butyryl ester of 2,2-dimethyl-1,3-dioxolane-4-methanol) below, releases a proton. We measure the rate of proton release using a pH indicator.



Researchers have used pH indicators to monitor the progress of enzyme-catalyzed reactions that release or consume protons since the 1940's.[4, 5] For example, researchers have monitored reactions catalyzed by amino acid decarboxylase,[6] carbonic anhydrase,[7] cholinesterase,[8] hexokinase[9, 10] and proteases.[11] Many researchers either used a pH indicator assay qualitatively or calibrated the color change with additional experiments.

However, by choosing the reaction conditions carefully, one can ensure that the color change is proportional to the number of protons. In particular, both the buffer and the indicator must have the same affinity for protons (pK_a's within 0.1 unit of each other) so that the relative amount of buffer protonated and indicator protonated stays constant as the pH shifts during the reaction. Researchers defined the proportionality between the rate of indicator absorbance change and reaction rate as the buffer factor, Q.[6, 7, 12] When the pK_a's of the indicator and buffer are the same, Q is given by Eq. 4.1 where C represents the total molar concentration (sum of acid and base forms) of buffer (B) or

indicator (In), $\Delta\varepsilon$ represents the difference in extinction coefficient between the protonated and deprotonated forms of the indicator and l represents the path length.

$$Q = \frac{C_B}{C_{In}} \cdot \frac{1}{\Delta\varepsilon \cdot l} \qquad \text{Eq. (4.1)}$$

The true reaction rate is given by Eq. 4.2, where dA/dt is the rate of indicator absorbance change. The highest sensitivity (largest dA/dt) occurs when Q is small. Thus, lowering the buffer concentration or increasing the indicator concentration increases the sensitivity of the assay.

$$rate\ (\mu mol/min) = \frac{dA}{dt} \times Q \times reaction\ volume \times 10^6 \qquad \text{Eq. (4.2)}$$

We used this assay to screen for enantioselective hydrolases in 96-well microplates, Figure 4.1. Using pure enantiomers, we separately measured the initial rates of hydrolysis of each enantiomer. Hydrolases that showed large differences in the initial rates of hydrolysis of the two enantiomers were further analyzed by traditional methods to determine enantioselectivities.[3] Note that the ratio of separately measured initial rates of hydrolysis of the enantiomers is NOT the true enantioselectivity, so this screening provides only an estimated enantioselectivity.[13] To minimize the amount of pure enantiomers needed, we first screened using racemic substrate to eliminate hydrolases that did not catalyze hydrolysis of the racemic substrate. Whittaker *et al.* measured the esterase activity of proteases in 96-well microplates using a similar assay.[11] However, their assay required additional calibration experiments because it did not use an indicator-buffer pair with the same pK_a values.

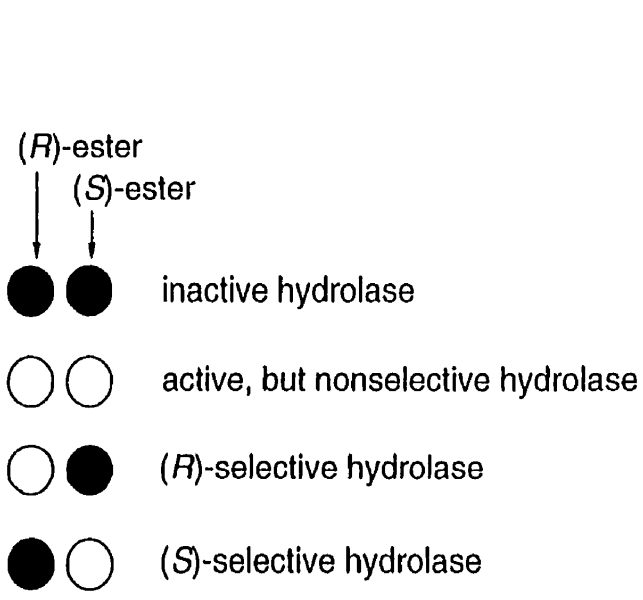
To demonstrate this method, we screened a library of seventy-two commercial hydrolases (lipases, esterases and proteases) towards solketal butyrate, an important

chiral building block in the synthesis of pharmaceuticals and biologically-active compounds.[14] Many researchers have searched, without success, for a highly enantioselective hydrolase that could resolve this substrate.[15] For hydrolysis in water, the highest enantiomeric ratio was 9 for a proteinase from *Aspergillus oryzae*,[16] while for acylation of solketal in organic solvent, the highest enantiomeric ratio was 20-25 for a lipase from *Pseudomonas* species (lipase AK).[15] Our screening, which is easily completed in one afternoon, has identified a new hydrolase, horse liver esterase, with an enantiomeric ratio of 15 for the hydrolysis of solketal butyrate. This is the highest enantioselectivity yet reported for hydrolysis of a solketal ester.

Results

Optimizing sensitivity of the assay

Since most hydrolases have maximal activity near neutral pH, we developed the assay for pH 7.2. As a pH indicator, we chose 4-nitrophenol. The similarity of its pK_a (7.15[17]) to the pH of the reaction mixture ensures that changes in pH give a large and linear color change.[6] The large difference in the extinction coefficients of the protonated and deprotonated forms (200 vs. 18,000 M^{-1} cm^{-1} at 404 nm) gives good sensitivity.[18] Finally, nitrophenols bind less to proteins than some polyaromatic indicators.[19] The concentration of the pH indicator should be as high as possible to maximize sensitivity, see Eq. 4.1 above. In our assay, the high initial absorbance of 4-nitrophenoxide/4-nitrophenol limited the concentration to 0.45 mM.[20] This concentration gave a starting absorbance of ~1.2 where the accuracy is still not compromised by low light levels.



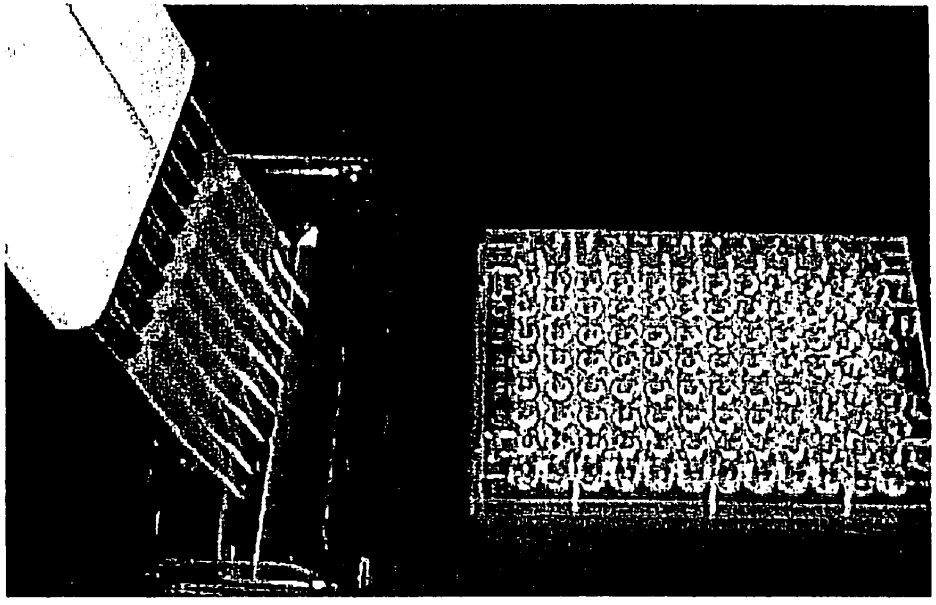


Figure 4.1. Schematic of the colourimetric screen for enantioselectivity. The circles represent wells in a microplate containing either the (*R*)- or the (*S*)- enantiomer. Hydrolysis of an ester releases acid that decolorizes the pH indicator. Dark circles represent no reaction, while the white circles represent wells in which hydrolysis occurred. This diagram exaggerates the color change; in practice the color change is not visible to the eye. The hydrolase is rejected if neither enantiomer reacts or if both enantiomers react at similar rates. If one enantiomer reacts significantly faster than the other, then the hydrolase is tested further. Note that measuring the rates of hydrolysis of pure enantiomers separately gives only an estimated enantioselectivity, not the true enantioselectivity (see reference 13). The right photo shows a typical screening step for estimated E.

As a buffer, we chose BES (*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) because its pK_a (7.15[21]) is identical to that of 4-nitrophenol. This identity ensures that changes in proton concentration during the reaction gives linear changes in absorbance.[22] We empirically determined an optimum buffer concentration of approximately 5 mM, see below. This value is a compromise between low buffer concentrations to maximize sensitivity, see Eq. 4.1 above, and high buffer concentrations to ensure accurate measurements and small pH changes throughout the assay (<0.05 pH units for 10% hydrolysis at our conditions). The small pH changes are important because kinetic constants can change with changing pH.

Suitable substrate concentrations ranged from 0.5 to 2 mM, typically 1 mM. At substrate concentration below 0.5 mM, the absorbance changes are too small to be detected accurately. For example, hydrolysis of 5% of a 0.25 mM substrate concentration at our standard conditions (pH 7.2, 0.45 mM 4-nitrophenol, 5 mM BES), changes the absorbance by only 0.005 absorbance units. Solubility in water sets the upper limit of substrate concentration because spectrophotometric measurements require clear solutions. Typical organic substrates dissolve poorly in water, so we added organic cosolvent - 7 vol % acetonitrile. For very insoluble substrates, we previously prepared clear emulsions using detergents.[23]

Quantitative validation of the assay

To confirm that color changes accurately measured the release of protons, we experimentally determined the factor Q and compared it to the theoretical Q, calculated using Eq. 4.1. First, we mimicked the proton release upon hydrolysis of the substrate by addition of HCl, Figure 4.2. The absorbance decreased linearly due to protonation of the 4-nitrophenoxide. The reciprocal of the slopes corresponds to the buffer factor, Q, calculated using Eq. 4.1. As expected, the decreases were all linear and the slopes increased with decreasing buffer concentration. However, below 2 mM buffer, the experimentally measured slopes disagreed with the theoretical slopes by more than 10%.

We chose 5 mM as the buffer concentration for our assay as a compromise between accuracy and sensitivity.

Small changes in reaction conditions did not compromise the sensitivity or accuracy of this assay. The measured value of Q did not change by more than the experimental error (~5%) upon addition of 7% of acetonitrile or dimethyl sulfoxide. The measured value of Q also did not change due to unknown buffer salts in the commercial hydrolases or due to added $CaCl_2$ (2 mM) in the stock solutions of proteases.

As a test reaction, we monitored the horse liver esterase catalyzed hydrolysis of racemic solketal butyrate. The decrease of the indicator absorbance, Figure 4.3a, was linear and corresponded to a specific activity of 1.85 μmol/min/mg protein.[24] Control experiments with no substrate or with no esterase showed no change in absorbance over one hour. When we scaled up the reaction 100-fold and monitored the reaction with a pH stat, we measured a higher specific activity, 4.99 μmol/min/mg protein. We attribute the difference to activation from the rapid mechanical stirring in the pH stat experiment.

Reaction rates increased linearly with the amount of enzyme added for three typical hydrolases, one from each class of hydrolases in our library, indicating that the enzyme-catalyzed reaction rates determined with this assay are proportional to the total enzyme concentration, Figure 4.3b.

Screening for hydrolases enantioselective towards solketal butyrate

To demonstrate the utility of our method, we screened a library of seventy-two commercial hydrolases towards (±)-solketal butyrate. All hydrolases were dissolved in the assay buffer, 5 mM BES buffer at pH 7.2. Since some solid hydrolase preparations contain buffer salts and extenders, the pH of each solution was checked and readjusted to pH 7.2 when necessary. First, we screened with racemic solketal butyrate to eliminate hydrolases that did not catalyze hydrolysis of either enantiomer. This screen eliminated the twenty hydrolases listed in note *a* of Table 4.1.

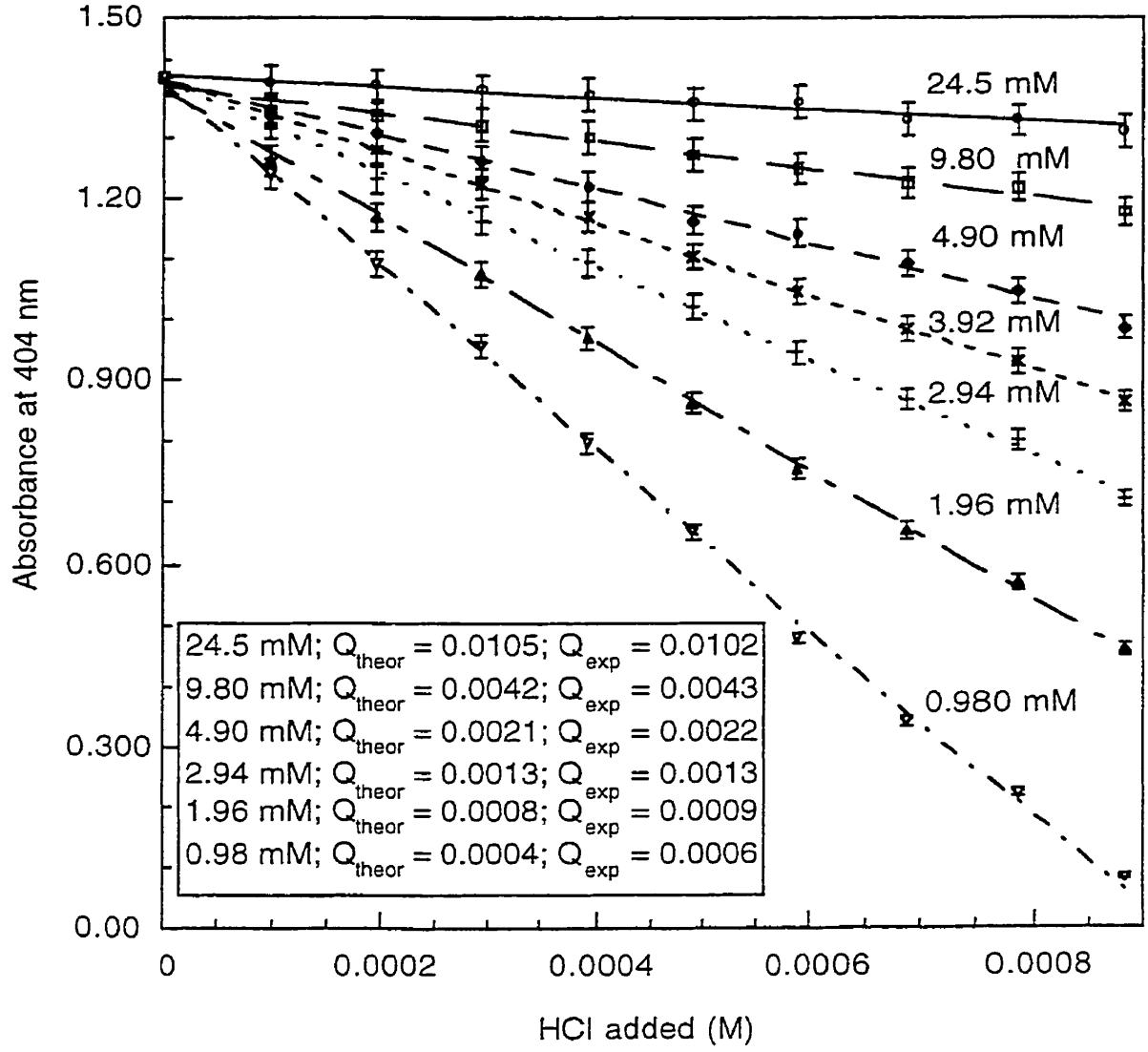


Figure 4.2 Sensitivity of the assay solution to added acid. The yellow color of the assay solution decreased linearly (regression factor = 0.97 for 24.5 mM; >0.99 for all other concentrations) in each solution. For a given amount of acid, solutions with lower concentrations of buffer showed larger color changes. For buffer concentration above 2 mM, the experimentally measured slopes were within 5% of the theoretical slopes calculated using Eq. 4.1 (where $\Delta\varepsilon$ = 17,800 M-1 cm-1, l = 0.292 cm, C_{In} = 0.447 mM). For buffer concentrations below 2 mM, the experimental and theoretical slopes disagreed by >10%. We chose a buffer concentration of 5 mM to maximize sensitivity without compromising accuracy. Each point is an average of four measurements, with variation <3% between each measurement. All lines are normalized to a starting absorbance of 1.4.

Next, we estimated the enantioselectivity of the remaining fifty-two hydrolases toward solketal butyrate by separately measuring the initial rates of hydrolysis of the pure enantiomers, Table 4.1. We used the ratio of these rates as an estimated enantioselectivity.[25] Note that the ratio of these rates is NOT the true enantioselectivity, or enantiomeric ratio E, because we measured the rates of hydrolysis of the enantiomers separately. Nine hydrolases showed estimated enantioselectivity ≥4. The seven lipases and proteases favored the (*R*)-ester, while the two esterases favored the (*S*)-ester. The highest estimated enantioselectivities were found with horse liver esterase (HLE, estimated enantioselectivity = 12), *Rhizopus oryzae* lipase (ROL, estimated enantioselectivity = 11) and protease from *Bacillus subtilis, variation Biotecus A.* (BSP, estimated enantioselectivity = 7). Previous workers identified *Aspergillus oryzae* protease (AOP) as an enantioselective hydrolase.[16] This hydrolase was also among the nine enantioselective hydrolases (estimated enantioselectivity = 5). The identification of HLE, ROL and BSP as enantioselective hydrolases towards solketal butyrate are new results from this screening.

Although we used only the first 3 - 4 minutes of data in the calculations, we monitored the reactions for one hour to ensure that we did not miss slow hydrolases or hydrolases that show a lag time. All substrate/hydrolase solutions were prepared and measured in quadruplicate to ensure accuracy. The total screening time for seventy-two hydrolases in quadruplicate was 180 minutes plus several minutes between each plate to fill the 96-well plates. This time could be easily reduced to less than an hour with a shorter screening time. Complete screening of the library towards a racemate and its enantiomers is easily completed in an afternoon.

We also changed reaction conditions in the assay in an attempt to increase the estimated enantioselectivity toward solketal butyrate. For example, the activity of hydrolases, especially lipases, often increases in the presence of an interface. We reasoned that this interfacial activation may also change the enantioselectivity. We screened the hydrolase library with Triton X-100 (a non-ionic detergent) added to create micelles. The

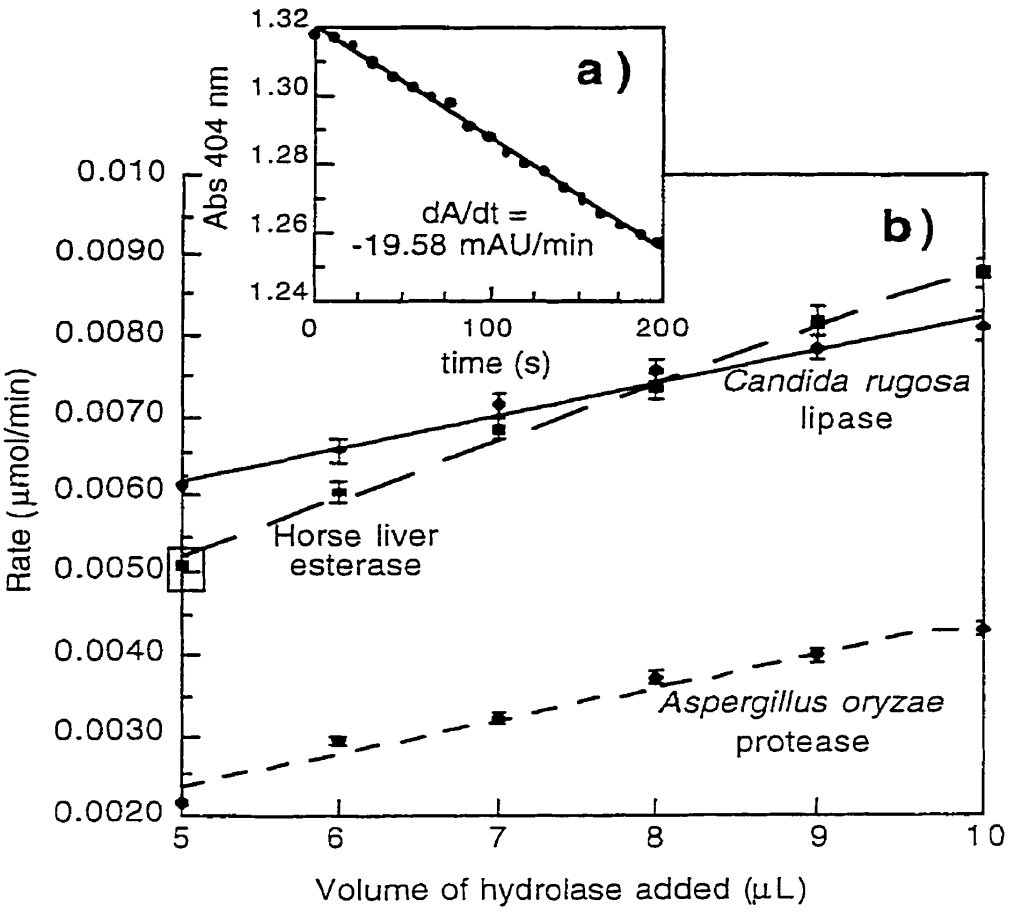


Figure 4.3. a) Initial rate measurement for boxed point showing the measured absorbance change in the horse liver esterase-catalyzed hydrolysis of (±)-solketal butyrate with 5 µL hydrolase solution. Using Eq. 4.2, the calculated rate equals 0.0050 µmol/min; specific activity equals 1.85 µmol/min/mg protein. Measured points fit a straight line with regression factor of 0.997. **b)** Increased rates of hydrolysis of 1 mM (±)-solketal butyrate with increased amount of hydrolase. Rates are calculated over 200 seconds using Eq. 4.2 where $\Delta\varepsilon$ = 17,300 M^{-1} cm^{-1}, l = 0.365 cm (final volume was 125 µL), C_B = 4.70 mM, C_{In} = 0.365 mM, 5.9% acetonitrile. Measured points fit a straight line with regression factor of 0.97 for each hydrolase. Each point is an average of four initial rate measurements, which differed by <2%.

Table 4.1. Activity of commercial hydrolases towards (±)-solketal butyrate and its enantiomers.

Source of active hydrolase[a]	Wt.[b]	Prot.[c]	Sup-plier	Activity (±) [d]	Activity (*R*)[d]	Activity (*S*)[d]	Estimated E[e]
Lipases							
Aspergillus niger	30	0.61	*f*	0.029	1.72	2.01	1.17 (*S*)
Aspergillus oryzae	7.1	4.5	*g*	0.039	0.157	0.0317	4.95 (*R*)
Candida antarctica lipase A	34	4.9	*h*	0.035	0.066	0.036	1.83 (*R*)
Candida antarctica lipase B	29	3.9	*i*	1.15	0.667	0.556	1.20 (*R*)
Candida lipolytica	35	0.23	*f*	0.114	0.381	0.340	1.12 (*R*)
Candida rugosa	31	0.35	*j*	2.76	4.25	1.85	2.30 (*R*)
Candida rugosa (cylindracea)	37	0.71	*k*	2.03	3.06	1.77	1.73 (*R*)
Humicola sp.	13	4.0	*h*	0.027	0.372	0.147	2.53 (*R*)
Penicillin camembertii	86	0.92	*f*	0.060	0.189	0.498	2.63 (*S*)
Penicillin roquefortii	57	0.74	*f*	0.151	0.844	1.23	1.46 (*S*)
Pseudomonas cepacia	31	3.1	*f*	0.038	0.085	0.021	4.05 (*R*)
Pseudomonas fluorescens	5.2	0.73	*g*	0.091	0.572	0.565	1.01 (*R*)
Rhizopus javanicus	44	2.7	*f*	0.063	0.286	0.066	4.33 (*R*)
Rhizopus oryzae	53	4.1	*f*	0.040	0.124	0.011	11.3 (*R*)
Thermus aquaticus	1.1	0.29	*g*	0.148	0.665	0.606	1.10 (*R*)
Esterases							
Acetylcholine esterase	0.26	0.18	*j*	0.745	7.29	5.42	1.35 (*R*)
Bacillus sp.	1.1	0.58	*g*	0.112	0.532	0.180	2.96 (*R*)
Bacillus stearothermophilus	1.1	0.57	*h*	0.066	2.77	1.65	1.68 (*R*)
Bacillus thermoglycosidasius	0.82	0.76	*g*	0.065	1.22	0.470	2.59 (*R*)
Bovine cholesterol esterase	9.8	0.99	*i*	0.402	1.43	1.73	1.21 (*S*)
Candida lipolytica	3.5	1.4	*g*	0.056	0.244	0.306	1.25 (*S*)
Cutinase	2.1	1.1	*m*	2.38	10.0	4.40	2.28 (*R*)
E001	0.40	0.14	*n*	2.08	11.4	7.65	1.49 (*R*)
E002	0.38	0.16	*n*	1.27	1.39	0.721	1.93 (*R*)
E003	1.0	0.23	*n*	2.91	4.81	3.64	1.32 (*R*)
E004	1.0	0.29	*n*	2.21	3.24	3.40	1.05 (*S*)
E005	1.0	0.27	*n*	1.88	1.38	1.10	1.25 (*R*)
E006	0.64	0.13	*n*	3.59	12.3	8.80	1.40 (*R*)
E007	2.1	0.97	*n*	1.26	1.22	1.35	1.11 (*S*)
E009	1.0	0.37	*n*	2.65	4.66	4.01	1.16 (*R*)
E010	1.0	0.26	*n*	1.56	4.19	3.01	1.39 (*R*)
E011	0.80	0.22	*n*	3.44	4.21	7.30	1.73 (*S*)
E013	1.0	0.24	*n*	0.869	0.860	0.843	1.02 (*R*)
E014	1.0	0.31	*n*	0.136	0.875	0.498	1.76 (*R*)
E016	1.0	0.26	*n*	1.22	1.76	0.960	1.83 (*R*)
E017b	1.0	0.33	*n*	1.12	0.694	0.620	1.12 (*R*)
E018	2.0	0.76	*n*	0.135	0.279	0.548	1.96 (*S*)
E019	0.60	0.20	*n*	3.70	6.45	7.79	1.21 (*S*)
E020	0.44	0.16	*n*	4.97	8.35	9.21	1.10 (*S*)
Pig liver esterase	-	0.09	*j*	71.9	3.03	14.9	4.91 (*S*)
Pig liver esterase	0.36	0.49	*g*	5.79	2.26	6.17	2.73 (*S*)
Horse liver esterase	1.7	0.59	*g*	0.975	0.168	2.05	12.2 (*S*)

Table 4.1 continued.

Source of active hydrolase[a]	Wt.[b]	Prot. *c*	Sup-plier	Activity (±) [d]	Activity (*R*)[d]	Activity (*S*)[d]	Estimated E[e]
Proteases							
Saccharomyces cerevisiae	1.2	0.26	*g*	0.085	0.219	0.074	2.96 (*R*)
Aspergillus oryzae	29	7.0	*j*	0.097	0.157	0.032	4.91 (*R*)
Aspergillus satoi	32	0.40	*j*	0.748	3.17	3.13	1.01 (*R*)
Bacillus lichenformis	5.2	2.1	*g*	0.335	0.261	0.083	3.14 (*R*)
Bac. subtilis var. Biotecus A	4.2	1.7	*g*	0.351	0.221	0.0303	7.29 (*R*)
Subtilisin Carlsberg	9.7	4.4	*j*	0.083	0.213	0.082	2.60 (*R*)
Streptomyces griseus	20.1	7.2	*o*	0.012	0.032	0.0135	2.37 (*R*)
Thermolysin, Type X	2.4	0.15	*j*	0.095	0.255	0.738	2.89 (*S*)
Proteinase, bacterial	4.7	2.1	*g*	0.186	0.154	0.028	5.50 (*R*)
Proteinase K	0.42	0.07	*g*	2.14	3.08	2.29	1.34 (*R*)

[a]The following hydrolases showed no detectable activity (<0.01 μmol/min/mg protein) towards racemic solketal butyrate: Lipases: Amano lipases from *Rhizopus stolonifer*, *Mucor javanicus* and *Mucor miehei*, wheat germ lipase (Sigma), porcine pancreatic lipase (Biocatalysts), lipase from *Rhizopus niveus* (Boehringer Mannheim); Esterases: ThermoGen esterases E008, E012 and E015, Fluka esterases from *Thermoanaerobium brockii* and *Mucor miehei*; Proteases: α-chymotrypsin (Sigma), pepsin from hog stomach (Fluka), subtilisin from *Bacillus lichenformis* (Fluka), thrombin from human plasma (Fluka), trypsin (Worthington, Freehold, NJ), Sigma proteases from *Bacillus polymyxa*, bovine pancreas type 1, papaya, *Streptomyces caespitosus*. [b]Amount (mg) of solid enzyme per ml of buffer in the stock solutions. [c]Protein concentration of stock solutions in mg protein/mL determined by the Bio-Rad assay using BSA as a standard. [d]Observed rate of hydrolysis in μmol/min/mg protein. Rates calculated using Eq. 4.2 were divided by the protein content in the well. The values are an average of four measurements, which typically varied by less than 2%. [e]Ratio of the separately measured initial rates for the enantiomers. This ratio is NOT the true enantioselectivity, but is a useful estimate of the enantioselectivity. The absolute configuration of the faster reacting ester is in parentheses. Note that hydrolysis of the (*R*)-ester yields the (*S*)-alcohol due to a change in the priority of the substituents. [f]Amano Enzyme USA Co., Ltd. (Troy, VA). [g]Fluka Chemie (Oakville, ON). [h]Boerhinger-Mannheim (Mannheim, Germany). [i]Novo-Nordisk (Baagsverd, DK). [j]Sigma-Aldrich (Oakville, ON). [k]Biocatalysts Ltd. (Pontypridd, Mid Glam, Wales, UK). [l]Genzyme (Cambridge, MA). [m]Unilever Research labs (Vlardingen, the Netherlands). [n]ThermoGen, Inc. (Chicago, IL). [o]Calbiochem/Behrig Diagnostics (La Jolla, CA).

reaction rates increased for eight hydrolases (three lipases and five esterases), decreased for thirty-two hydrolases, and stayed constant for twelve hydrolases. Unfortunately, the estimated enantioselectivities remained unchanged or decreased slightly for the best hydrolases: AOP (decrease from 4.9 to 4.2), ROL (decrease from 11.3 to 9.6), HLE (decrease from 12.2 to 7.6), BSP (decrease from 7.3 to 5.4). With non-selective hydrolases, the estimated enantioselectivities showed small increases or decreases. For example, subtilisin Carlsberg increased from 2.6 to 2.8, esterase from *Bacillus stearothermophilus* increased from 1.7 to 2.4 and *Aspergillus oryzae* lipase decreased from 4.95 to 1.8. Overall, the selectivities towards solketal butyrate did not significantly change upon addition of Triton X-100.

To confirm these screening results, we measured the enantioselectivity of three selective hydrolases and three poorly-selective hydrolases using the conventional endpoint method,[3] Table 4.2. Under conditions similar to those in the screening solutions (1 mM substrate, 7% acetonitrile as cosolvent), the true enantioselectivity and the estimated enantioselectivity agreed to within a factor of 2.3. Since 1 mM solketal butyrate is too dilute for practical preparative reactions, we also measured the enantioselectivity of these hydrolases at 50 mM solketal butyrate where the reaction mixture contained insoluble droplets of substrate. The enantioselectivity under these conditions also agreed with the enantioselectivity estimate from screening to within a factor of 2.6.

The most enantioselective hydrolase was HLE, E = 14.8 at 1 mM, E = 9.7 at 50 mM substrate concentration, respectively. At 50 mM substrate without acetonitrile, the enantioselectivity of HLE declined slightly again to E = 8.7 (c = 17.1% after 2.5 h). These values agree well with the estimated enantioselectivities of 12.1 (without Triton X-100) and 7.6 (with Triton X-100). Although Partali *et al.* reported an enantiomeric ratio of 9 for AOP[16], we measured an estimated enantioselectivity of 4.9 and a true enantioselectivity of 4.8 under our conditions. Although the estimated enantioselectivity for ROL was also high (11.3), the true enantioselectivity was lower, E = 4.8 - 4.9. Hydrolases with low estimated enantioselectivities (CRL, Esterase E013, cutinase) also showed low true

enantioselectivities. Thus, hydrolases identified as enantioselective indeed were enantioselective and hydrolases identified as nonselective were not enantioselective.

Our screening procedure quickly identified HLE as a new hydrolase for the resolution of solketal butyrate with modest enantioselectivity. It is the most selective hydrolase reported in literature to date for the hydrolysis of an ester of solketal.

Discussion

The most important part of the assay design was to ensure that it accurately measured the rates of hydrolysis. The first requirement is that the buffer and indicator have pK_a's within 0.1 units. A difference in pK_a's of 0.3 units causes an 8% error when the pH changes by 0.1 unit.[12] In a typical assay, the pH changed by 0.05 units (10% hydrolysis of the substrate), thus, differences in pK_a's can lead to non-linear and inaccurate rates. If different pK_a's cannot be avoided, one can still get accurate results by using calibration experiments[10] or a more complex equation.[10] The linear relationship between the amount of acid added and the color change, as shown in Figure 4.2, confirms that the pK_a's lie within the acceptable range in our experiment. Further, the agreement of the theoretical and experimental slopes to within 5% establishes that the assay is quantitative.

The assay tolerates small changes in reaction conditions, such as the addition of 7% acetonitrile. Indeed, the pK_a of 4-nitrophenol changes only slightly from 7.15 to 7.17 upon addition of 10% ethanol.[26] This result suggests that cosolvent concentrations below 10% do not compromise the accuracy of the assay. Also, small amounts of salts present in the hydrolase solutions (buffer salts in commercial hydrolase preparations, 2 mM $CaCl_2$ in the protease solutions) did not affect the accuracy.

This assay is approximately seven times less sensitive than one using hydrolysis of 4-nitrophenyl esters. For example, if the rate of hydrolysis towards a non-chromogenic ester and a 4-nitrophenyl ester were identical, then our assay would require seven times

Table 4.2. True enantioselectivities of hydrolases towards solketal butyrate measured by the endpoint method.

Hydrolase	S[a] (mM)	Time[b] (h)	ee_s[c] (%)	ee_p[c] (%)	C[d] (%)	True E[e]	Estimated E[f]
Rhizopus oryzae lipase	1	16.5	95.4	23.6	80.2	5.0 ± 0.1 (R)	11.3 (*R*)
Rhizopus oryzae lipase	50	1.25	37.8	51.2	42.5	4.4 ± 0.1 (R)	
Horse liver esterase	1	4.0	40.3	81.8	33.0	14.8 ± 0.7 (S)	12.2 (*S*)
Horse liver esterase	50	2.5	22.1	77.3	22.2	9.7 ± 0.1 (S)	
Cutinase	1	2.0	92.8	27.4	77.2	5.0 ± 0.02(R)	2.28 (*R*)
Cutinase	50	0.42	70.3	41.8	62.7	4.8 ± 0.04 (R)	
Aspergillus oryzae protease	1	4.0	10.5	65.8	13.8	5.4 ± 0.1(R)	4.91 (*R*)
Aspergillus oryzae protease	50	14	11.1	62.0	15.2	4.8 ± 0.1 (R)	
Candida rugosa lipase	1	2.5	86.3	14.4	85.7	3.0 ± 0.1 (R)	1.73 (*R*)
Candida rugosa lipase	50	0.1	15.7	40.5	27.8	2.7 ± 0.02 (R)	
Esterase E013	1	9.5	0	0	20	1.0	1.02
Esterase E013	50	2.0	0	nrg	nr	nr	

[a]Substrate concentration in the reaction mixture. [b]Reaction time. [c]Measured enantiomeric purity of the starting material (ee_s) or product (ee_p). [d]Degree of conversion calculated by $ee_s/(ee_s+ee_p)$. [e]The true enantioselectivity was calculated from ee_s and ee_p according to Ref. 3. The absolute configuration of the fast-reacting ester is in parentheses. The error limits were estimated from enantioselectivities measured from three separate GC injections. [f]Values from Table 4.1. [g]nr = no reaction detected by GC.

more hydrolase to observe the same change in absorbance. The assay with 4-nitrophenyl esters releases one molecule of 4-nitrophenol (53% of these will be deprotonated at pH 7.2), while our assay protonates one 4-nitrophenoxide for every twelve protons released.

There are several advantages with this screening method. First, it is hundreds of times faster than conventional screening. The 96-well format allows the analysis of large numbers of samples simultaneously. However, speed is not gained at the expense of accuracy; variation between quadruplicate measurements for a reaction is typically <2%. Moreover, our method is quantitative unlike screening for hydrolytic activity by TLC. Second, since the entire reactions and analyses take place in the microplate wells, workup and analysis by GC, HPLC or NMR is avoided. Third, it requires hundreds to thousands of times less substrate (typically 20 μg/well) and hydrolase (we used between 0.6-35 μg protein/well). For this reason, it may be useful in the screening of mutant hydrolases in directed evolution experiments. Fourth, this assay measures the hydrolysis of any ester, not just chromogenic esters. The most important rule of screening is 'You get what you screen for', so the ability to screen the target compound, not an analog of the target compound, is an important advantage. For speed, we screened in microplates, but one could also adjust the concentrations to use cuvettes and a conventional UV-vis spectrophotometers.

There are few disadvantages with our screening method. First, it requires pure enantiomers, albeit in small amounts. We screened the hydrolase library in quadruplicate with only six milligrams of each enantiomer of solketal butyrate. Second, it provides only an estimated enantioselectivity. This method ignores some or all of the differences in K_M of the enantiomers. Third, it requires clear solutions. To obtain clear solutions with water-insoluble substrates, experimentation is sometimes required to find the best cosolvent or emulsion conditions.

In this paper, we assayed for hydrolase activity at pH 7.2, but other buffer indicator pairs may be suitable for screening at other pH's. For example, at pH 6 chlorophenol red (pK_a 6.0) and MES (2-[*N*-morpholino]ethanesulfonic acid, pK_a 6.1)

may be suitable; at pH 8 phenol red (pK_a 8.0) and EPPS (*N*-[2-hydroxylethyl]piperazine-*N*'-[3-propanesulfonic acid], pK_a 8.0) may be suitable; at pH 9 thymol blue (pK_a 9.2) and CHES (2-[*N*-cyclohexylamino]ethane-sulfonic acid, pK_a 9.3) may be suitable.

We are currently adapting this acid-base indicator assay to measure true enantioselectivity by extending our Quick E method for measuring true enantioselectivity.[23]

Experimental Section

General. Chemicals were purchased from Sigma Chemical Co. (Oakville, ON) and were used without further purification unless stated. Triton X-100 was purchased from ESA Inc. (Chelmesford, MA). Standardized acid was purchased from A & C American Chemicals Ltd. (Montréal, QC). Enzyme suppliers are noted in the footnotes of Table 4.1. All microplate assays were performed on a Spectramax 340 microplate reader with SOFTmax PRO version 1.2.0 software (Molecular Devices, Sunnyvale, CA). Polystyrene 96-well flat-bottomed microplates (maximum volume 360 µl/well, Corning Costar, Acton, MA) were filled using Eppendorff 8-channel pipettes (5-100 µl, 50-1,200 µl) and solution basins for multichannel pipettes (Fisher Scientific, Nepean, ON). The initial rate of the small-scale horse liver esterase-catalyzed ester hydrolysis reaction was measured with a Radiometer RTS 822 pH stat.

(±)-Solketal butyrate. Butyric anhydride (2.87 mL, 17.5 mmol, 1.5 equiv.), 4-dimethylaminopyridine (0.071 g, 0.58 mmol, 0.05 equiv.) and anhydrous sodium carbonate (1.86 g, 17.6 mmol, 1.5 equiv.) were added to a solution of (±)-solketal (1.54 g, 11.7 mmol, 1.0 equiv.) in ethyl acetate (40 mL) and stirred overnight. The reaction mixture was washed several times with water, then with brine, and the organic extract was dried with magnesium sulfate. Flash chromatography (3:1 hexanes:ethyl acetate) afforded the pure butyryl ester as a yellow oil in 91% yield. R_f= 0.56 (3:1 hexanes:ethyl acetate), ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 0.98 (t, ^3J (H,H) = 7.4 Hz, 3H; CH_3), 1.37 (s, 3H; CH_3),

1.43 (s, 3H; CH_3), 1.67 (sextet, ^3J (H,H) = 7.4 Hz, 2H; CH_2), 2.33 (t, ^3J (H,H) = 7.3 Hz, 2H; CH_2), 3.7 (m, 1H; 1 H of CH_2), 4.05-4.16 (m, 3H; 1 H of CH_2, CH_2), 4.27 - 4.32 (m, 1H; CH). ^{13}C-NMR (200 MHz, $CDCl_3$): δ = 15.3 (CH_3), 19.9 (CH_2), 26.9 (CH_3), 28.1 (CH_3), 37.3 (CH_2), 65.5 (CH_2), 67.3 (CH_2), 74.6 (CH), 110.3 (C), 173.1 (C=O); MS (CI) m/z: 203 (MH^+, 9), 187 (55), 145 (100); HRMS under EI showed no molecular ion.

(S)-Solketal butyrate and (R)-solketal butyrate. Samples were prepared from enantiomerically pure solketal as above for the racemate. The enantiomeric purities of the butyrates measured by GC (see below) were 99.2% and 99.8%, respectively. No contaminating butyric acid or solketal were detected by GC or ^{1}H-NMR.

Hydrolase library. The hydrolases were dissolved in BES buffer (5.0 mM, pH 7.2 containing 0.02% NaN_3 as preservative) at the concentrations listed in Table 4.1 (0.5-40 mg solid/mL solution). $CaCl_2$ (2 mM) was added to the protease solutions since some proteases require calcium ions to maintain their structure. For hydrolase samples with low protein content, we used saturated solutions (up to 40 mg solid/mL) and for hydrolase samples with high protein content, we chose lower concentrations (typically, 1 mg solid/mL). Each solution was centrifuged to remove insoluble material (5 min, 2,000 rpm) and titrated to a final pH of 7.2. The protein concentrations were determined using a dye-binding assay from Bio-Rad (Mississauga, ON) with bovine serum albumin (BSA) as the standard. Solutions were stored in a 96-well assay block 'mother plate' equipped with aluminum sealing tape (2 mL maximum volume in each well, Corning Costar, Acton, MA) at -20 °C. This 'mother plate' speeds up repeated screens using the same hydrolases and is a convenient way to store large libraries of hydrolases. Hydrolytic activity of the libraries is maintained over several months.

Screening of commercial hydrolases with pH indicators. The assay solutions were prepared by mixing solketal butyrate (420 µL of a 30.0 mM solution in acetonitrile),

acetonitrile (470 μL), 4-nitrophenol (6,000 μL of a 0.9115 mM solution in 5.0 mM BES, pH 7.2) and BES buffer (5,110 μL of a 5.0 mM solution, pH 7.2). Hydrolase solutions (5 μL/well) were transferred from the mother plate to a 96-well microtiter plate using an 8-channel pipette. Assay solution (100 μL/well) was quickly added to each well using a 1,200 μL 8-channel pipette. The final concentrations in each well were 1.0 mM substrate, 4.65 mM BES, 0.434 mM 4-nitrophenol, 7.1% acetonitrile. The plate was quickly placed in the microplate reader, shaken for 10 s to ensure complete mixing and the decrease in absorbance at 404 nm was monitored at 25 °C as often as permitted by the microplate software, typically every 11 seconds. The starting absorbance was typically 1.2. Data were collected for one hour to ensure we detected slow reactions and reactions with a lag time. Each hydrolysis was carried out in quadruplicate and was averaged. The first 10 s of data were sometimes erratic, possibly due to dissipation of bubbles created during shaking. For this reason, we typically excluded the first 10 s of data from the calculation of the initial rate. Activities were calculated from slopes in the linear portion of the curve usually over the first two hundred seconds. The initial rates were calculated from the average dA/dt, using Eq. 4.2 where $\Delta\varepsilon$ = 17,300 M^{-1} cm^{-1} (experimentally determined for our conditions) and l = 0.306 cm. To calculate specific activity (μmol/min/mg protein), we took into account the total amount of protein in each well.

Screening of commercial hydrolases with pH indicators under interfacial activation conditions. The procedure was the same as above except that the BES buffer (5 mM, pH 7.2) contained Triton-X 100 (8.45 mM). Final concentration of Triton X-100 in the wells was 2.8 mM.

Small-scale reactions with 1 mM (±)-solketal butyrate. These small-scale reactions mimic the conditions in the microplate during pH indicator activity screening except that no indicator is present. Hydrolase solutions (50 μL) were added to solutions of (±)-solketal

butyrate (3.50 mL of a 14.4 mM solution in acetonitrile) and BES buffer (46.45 mL of a 5.0 mM solution, pH 7.2) for a final reaction volume of 50 mL (1.0 mM substrate, 4.65 mM BES, 7% acetonitrile). After stirring at room temperature for a time estimated from the pH indicator screening, the mixture was extracted with diethyl ether (3 x 20 mL). These extracts, which contained both the ester substrate and the alcohol product, were combined, washed with water and dried with magnesium sulfate, filtered and evaporated to dryness.

Small-scale reactions with 50 mM (±)-solketal butyrate. Hydrolase solutions (250 µL for CRL, ROL, HLE, AOP, E013; 50 µL for cutinase) were added to solutions of (±)-solketal butyrate (352 µl of a 0.715 M solution in acetonitrile) and BES buffer (4,398 µl of a 5.0 mM solution, pH 7.2) for a final reaction volume of 5.0 mL (50 mM substrate, 4.65 mM BES, 7% acetonitrile). Reactions were worked up as above.

Determination of enantiomeric purity by GC. Gas chromatography analysis was performed on a Varian 589-Series II Gas Chromatograph equipped with a Chirasil-DEX CB chiral stationary phase (25 m x 0.25 mm x 0.25 µm Chrompack, Raritan, NJ). For analysis, solketal was converted to the acetate by dissolving the mixture of solketal and solketal butyrate in ethyl acetate (5 mL) containing acetic anhydride, 4-pyrrolidinopyridine and anhydrous potassium carbonate. The solution was stirred for one hour at room temperature, then filtered, was washed with brine, then water, dried with magnesium sulfate and evaporated to dryness. Both the starting material, solketal butyrate, and the acetate of the product were simultaneously separated with baseline resolution by using a temperature gradient (100 °C to 130 °C, 2 °C/min). Solketal butyrate: k'_1 = 8.11 (*S*), α = 1.04; solketal acetate: k'_1 = 4.21 (*S*), α = 1.10. The ee-values reported in the tables are the mean of three injections. We did not observe any racemization of the substrate during derivatization.

Acknowledgements

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24. We assume that one proton is released for each ester group hydrolyzed at pH 7.2 due to the high pK_a of solketal. Other esters (for example, 4-nitrophenyl esters) may release more than one proton per ester group hydrolyzed and should be accounted for in Eq. 4.1.

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Chapter 5

The ultimate goal of the research in this thesis was the development of a quantitative method to rapidly screen for enantioselective hydrolases towards a target substrate that does not require the measurement of enantiomeric purity. Chapter 3 introduced the quick E method using chromogenic esters. In this chapter, we extend quick E to measure the enantioselectivity of non-chromogenic esters using the pH indicator screen developed in Chapter 4.

Several problems were encountered during the development of quick E and they are detailed in this chapter. Moreover, the background and theory of the quick E method are fully explored in this chapter.

Experimental details that were not included in Chapter 3 are described in full.

The Development of Spectrophotometric Methods to Rapidly Measure Enantioselectivity.

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Abstract: Spectrophotometric assays are the most frequently used method to monitor the course of an enzyme-catalyzed reaction due to their convenience and speed. We apply the speed and convenience of these assays to measure the enantioselectivity of hydrolase-catalyzed kinetic resolutions of chiral alcohols and carboxylic acids. The established method to measure enantioselectivity is called the endpoint method where a researcher runs a small-scale reaction for each hydrolase with the target substrate, then measures the enantiomeric purity of the reaction products. This method is time-consuming so researchers rarely test large numbers of hydrolases for enantioselectivity towards a chiral substrate. We report the stepwise development of a rapid spectrophotometric method to measure the enantioselectivity of hydrolases using initial rate measurements. The initial rate of hydrolysis of a pure enantiomer of a target substrate, relative to a reference compound, is measured yielding the selectivity ratio, $[(k_{cat}/K_M)_R/(k_{cat}/K_M)_{reference}]$. A second initial rate measurement gives the selectivity ratio for the other enantiomer $[(k_{cat}/K_M)_S/(k_{cat}/K_M)_{reference}]$ and the ratio of the selectivity ratios yields the enantiomeric ratio, E. We call this method "quick E" because of its speed. We first develop this method using chromogenic esters then generalize this method to any ester with the use of pH indicators to quantitatively detect protons released during ester hydrolysis. Quick E measurements for both chromogenic and non-chromogenic esters agree well with E values determined by the endpoint method. Using quick E, we can determine the enantioselectivities of approximately 100 hydrolases per day.

Introduction

Hydrolase-catalyzed resolutions of racemates are useful for the production of enantiomerically-pure compounds. They are performed under mild reaction conditions, can show higher selectivity than classical synthetic methods, and allow for the preparation of a wide variety of enantiomerically-pure molecules such as primary and secondary alcohols, and carboxylic acids.[1] However, the difficult task of selecting a suitable hydrolase from hundreds of commercially available hydrolases and microorganisms remains a major deterrent to their wider use. Researchers have developed empirical rules and models to aid in the selection but these models cannot accurately predict the degree of selectivity. Researchers must therefore carry out screening to identify suitable hydrolases.

When researchers screen for enantioselectivity, they are looking for hydrolases with good hydrolytic activity and very high enantioselectivity toward a racemic substrate. In rare ideal cases, the enantioselectivity is so good that the reaction stops at 50% conversion when all of the preferred enantiomer is consumed, leaving both product and residual starting material with high enantiomeric excess. In practice, most hydrolases do not exhibit this degree of selectivity towards a substrate, so the degree of selectivity, called the enantiomeric ratio or E, must be determined.

In a hydrolase-catalyzed reaction, the observed initial rate of a hydrolysis of a substrate (v) is expressed by the following steady-state equation where [E] is the concentration of free enzyme and [S] is the initial concentration of ester.[2]

$$v = -\frac{d[S]}{dt} = \left(\frac{k_{cat}}{K_M}\right) \cdot [S] \cdot [E] \qquad \text{Eq. (5.1)}$$

When two substrates are present in solution, they both compete for the enzyme's active site. From eq. 5.1, it follows that the ratio of rates of hydrolysis equals the ratio of their k_{cat}/K_M values (the specificity constants) after taking into account the concentration of both substrates. If the two substrates are a pair of enantiomers, the ratio of their specificity constants equals the enantiomeric ratio, E, Equation 5.2.[3]

$$Enantiomeric\ ratio = E = \frac{(k_{cat}/K_M)_{fast\ enantiomer}}{(k_{cat}/K_M)_{slow\ enantiomer}} \qquad \text{Eq. (5.2)}$$

To calculate E by measuring the kinetic constants, k_{cat} and K_M, a researcher measures the initial rates of hydrolysis of each enantiomer as a function of its substrate concentration and transforms the data into a linear form for analysis.[4] Although this method reveals useful information on the kinetics of each enantiomer as well as the overall enantioselectivity, it is unsuitable for screening large numbers of hydrolases for enantioselectivity because it is time-consuming - it requires multiple measurements for each pure enantiomer and significant data analysis.

Most researchers prefer to use the integrated forms of Equation 5.2 developed by Sih and coworkers called the endpoint method,[5] which relate the degree of conversion of the reaction to the enantiomeric purity of the remaining substrate (ee_s) or resulting product (ee_p), Equation 5.3.

$$E = \frac{\ln[(1-c)(1-ee_S)]}{\ln[(1-c)(1+ee_S)]} = \frac{\ln[(1-c)(1+ee_P)]}{\ln[(1-c)(1-ee_P)]} \qquad \text{Eq. (5.3)}$$

To determine E by the endpoint method, a researcher runs a small-scale reaction with a candidate hydrolase and racemic substrate, stops the reaction before completion, works up the reaction usually to separate product from starting material, then determines the enantiomeric purities of the reaction products. Finding analytical conditions to separate enantiomers using chiral stationary phases or NMR chiral shift reagents can be difficult and time-consuming and is often the slow step in screening. In general, a single endpoint measurement takes several hours to perform so researchers rarely screen all commercially available hydrolases. Rather, they tend to focus on hydrolases that have been widely reported in the literature. Less common but enantioselective hydrolases are likely overlooked when a researcher limits the number of hydrolases tested.

To avoid the slow step of measuring enantiomeric purities, researchers have proposed alternate methods to determine enantioselectivity based upon the measurement

of rates of hydrolysis. Jongejan and coworkers developed a method to calculate E by analyzing initial rates of reaction with varying ratios of enantiomers but fixed total substrate concentration.[6] Although this method can be accurate, it is not significantly faster than the endpoint method because it requires multiple initial rate measurements as well as curve fitting to evaluate E. Alternately, researchers have developed methods to analyze reaction progression curves of hydrolase-catalyzed reactions to determine enantioselectivity.[7] These methods can be less accurate than the endpoint method and also require substantial data manipulation to determine E.

In this paper, we present the stepwise development of a novel spectrophotometric method to accurately measure the enantioselectivity of hydrolases based upon the measurement of initial rates. Rather than measure the relative rates of hydrolysis of a racemic solution to determine E (this would require measuring enantiomeric purity to evaluate the relative rate of hydrolysis of each enantiomer), we separately measure the rates of hydrolysis of each enantiomer relative to a reference compound. We obtain a specificity ratio (k_{cat}/K_M) for each pure enantiomer relative to the reference compound and the ratio of the relative specificity constants for each pure enantiomer yields the enantioselectivity. $(k_{cat}/K_M)_R/(k_{cat}/K_M)_S$. We call this method "quick E" due to its speed and ease.

First, we validate the quick E method using chromogenic esters as substrates. Next, we extend quick E to non-chromogenic esters with the use of pH indicators to visualize ester hydrolysis. To validate the quick E method, we compare the enantioselectivity of hydrolases towards substrates using the quick E method and Sih's endpoint method. Quick E measurements for both chromogenic and non-chromogenic esters agree well with enantioselectivities determined by the more traditional endpoint method.

Results

Screening enantiomers of chromogenic esters separately to estimate enantioselectivity

To properly evaluate enantioselectivity based upon initial rates of hydrolysis, the rates of hydrolysis of both enantiomers must be measured simultaneously to reflect their relative binding in the enzyme's active site. However, the relative rate of hydrolysis of

both enantiomers cannot be simultaneously determined without measuring enantiomeric purities, so researchers often estimate enantioselectivity by measuring the initial rates of hydrolysis of the two enantiomers in separate hydrolysis experiments, Equation 5.4.[8]

$$Estimated\ E \approx \frac{initial\ rate_{fast\ enantiomer}}{initial\ rate_{slow\ enantiomer}} \qquad \text{Eq. (5.4)}$$

We separately measured the initial rates of hydrolysis of 4-nitrophenyl esters of pure enantiomers to quickly estimate the enantioselectivity of several lipases towards three common chiral carboxylic acids, 2-phenylpropanoic acid, **1**, 2-(4-isobutylphenyl)propanoic acid (commercially available as Ibuprofen), **2**, and phenylalanine, **3**, Figure 5.1. The rates of hydrolysis of 4-nitrophenyl esters were conveniently determined spectrophotometrically by monitoring the rate of release of 4-nitrophenoxide anion at 404 nm, Figure 5.2. The ratio of separately measured initial rates of hydrolysis estimated the enantioselectivity.

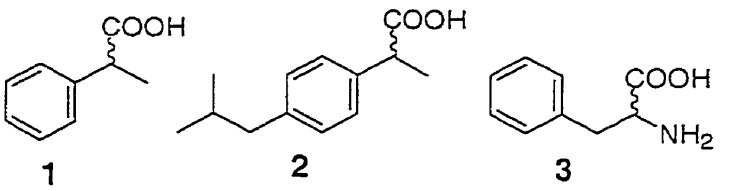

Figure 5.1 Structures of chiral carboxylic acids for testing.

We compared these estimated enantioselectivities to the "true" enantio selectivities determined by the endpoint method, Table 5.1.[9] In all cases, the enantioselectivity was over or underestimated by 1.2 to 3-fold by separately measuring the initial rates of hydrolysis of the pure enantiomers. The highest estimated enantioselectivity measured was 55 for isopropanol treated *Candida rugosa* lipase towards **2**, but its true enantioselectivity was > 100 by the endpoint method. 4-Nitrophenyl esters of

phenylalanine, **3**, were extremely unstable at pH 8 ($t_{1/2}$ = 0.51 h) so we corrected the observed rates for chemical hydrolysis during spectrophotometric measurements. Moreover, we could not determine the enantioselectivity of **3** by the endpoint method because it completely hydrolyzed during workup.

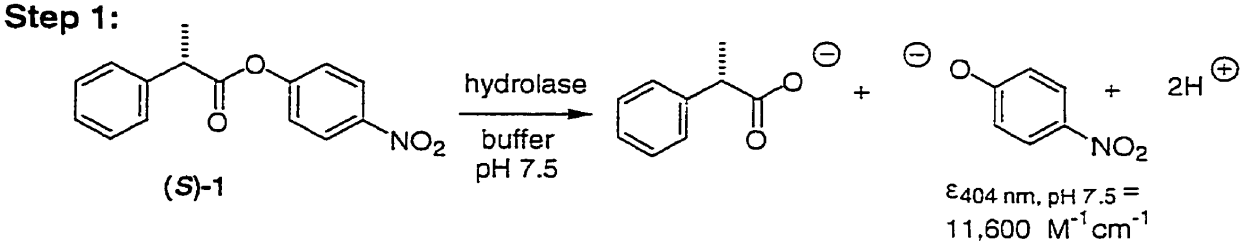

Figure 5.2 Initial rates of hydrolysis of 4-nitrophenol esters are easily measured spectrophotometrically by measuring the linear increase in absorbance at 404 nm over time. The initial rate of hydrolysis of (*S*)-**1** is determined in the first step to estimate enantioselectivity. The second step uses (*R*)-**1** and the ratio of the two rates estimates the enantioselectivity. Note that the extinction coefficient accounts for the partial ionization of 4-nitrophenol at pH 7.5; in practice, 4-nitrophenol (pK_a 7.15) is not fully ionized at pH 7.5 as shown.

Estimated E values do not yield the true enantioselectivities because differences in K_M between the two enantiomers are ignored in separately measured initial rate measurements. When initial rates are measured at saturating substrate concentrations, where [S] > K_M, all of the enzyme's active sites are occupied and the rate of hydrolysis is independent of the substrate concentration. The observed initial rate depends on k_{cat}. However, when the initial rates are measured at concentrations below saturating substrate concentrations, where [S] << K_M, most of the enzyme's active sites are unoccupied and the rate of hydrolysis is considerably slower. The observed initial rate depends upon k_{cat}, K_M and [S]. Thus, the ratio of separately-measured initial rates of pure enantiomers only yields the correct value of E when the K_M value for the (*R*) and (*S*) enantiomers are identical or when the substrate concentration during initial rate measurements is well below the K_M values of both enantiomers.

Table 5.1 Enantiomeric ratios of hydrolases towards 4-nitrophenyl-esters **1**–**3**, using the endpoint method[a] and estimated E.

Sub-strate	Lipase[b]	Wt[c] (prot.)[d]	Sup-plier	ee_s[e]	ee_p[e]	Time[f]	c[g]	E[h] endpoint	Initial Rate (*S*)[i]	Initial Rate (*R*)[i]	Estimated E[j]
1	*Candida antarctica*, type A	18.2 (2.62)	*k*	20	23	6.1	37	1.9 ± 0.1	0.0203	0.00515	4.0 ± 0.2 (S)
1	*Candida rugosa*	36.9 (0.406)	*l*	17	50	20.3	25	3.5 ± 0.2	0.263	0.223	1.2 ± 0.2 (S)
1	IPA-*Candida rugosa*	Na (0.70)	*m*	43	98	4.5	30	>100	0.193	0.00500	40 ± 2 (S)
1	*Pseudomonas cepacia*	29.3 (2.93)	*k*	nd	67	2.4	60	29 ± 3	0.198	0.0100	20 ± 1 (S)
1	Porcine pancreas	42.3 (7.15)	*l*	nd	2.0	22.0	4.0	1.1 ± 0.1	0.00590	0.00403	1.4 ± 0.1 (S)
2	*Candida rugosa*	18.2 (2.62)	*l*	5.3	14.5	4.25	27	1.4 ± 0.1	0.00478	0.00397	1.2 ± 0.1 (S)
2	IPA-*Candida rugosa*	Na (0.70)	*m*	40.1	>99	21.0	29	>100	0.0382	0.000695	55 ± 5 (S)
2	*Pseudomonas cepacia*	29.3 (2.93)	*k*	28	43.2	16.5	39	3.3 ± 0.1 (*R*)	0.00694	0.0075	1.1 ± 0.1 (*R*)
3	*Aspergillus niger*	49.3 (1.38)	*k*	0	0	0.3	100[n]	nd	0.357[n]	0.266[n]	1.34 ± 1 (S)

[a]Reaction conditions: room temperature, 9 mL 10 mM Tris buffer, pH 8.0, 100 mg solid lipase powder or 0.7 mg protein of IPA-CRL, 100 mg substrate dissolved in 1 mL acetonitrile except for **3**: 8 mL 10 mM sodium acetate buffer, pH 5, 280 mg solid lipase, 100 mg substrate dissolved in 1 mL acetonitrile. [b]For spectrophotometric measurements, the lipases were dissolved in Tris buffer (25 mM, pH 7.5) and centrifuged to remove insoluble material. The following lipases showed no activity (<1 x 10^{-5} µmol/min/mL) towards either enantiomer of 4-nitrophenol-2-phenylpropanoate over 60 seconds: *Aspergillus niger* AP-6 (Amano, 45.0 mg/mL), *Candida lypolytica* (Amano, 39.0 mg/mL), *Chromobacterium viscosum* (Sigma-Aldrich, 6.5 mg/mL), *Mucor javanicus* (Amano, 40.2 mg/mL), *Penicillin camemberti* (Amano, 37.0 mg/mL), *Penicillin Roqueforti* (Amano, 33.0 mg/mL), *Rhizopus javanicus* (Amano, 33.1 mg/mL), *Rhizopus oryzae* (Amano, 30.9 mg/mL), *Rhizopus stolonifer* (Amano, 32.0 mg/mL), wheat germ (Sigma-Aldrich, 16.1 mg/mL). [c]Amount (mg) of

solid enzyme per mL of buffer in the stock solutions used for initial rate measurements. "na" = not available [d]Protein concentration of stock solutions in mg/mL determined by the Bio-Rad assay using BSA as the standard. [e]Measured enantiomeric purity of the remaining starting material (ee_s) or product acid (ee_p). [f]Reaction time in hours. [g]Percent conversion. [h]Enantiomeric ratio calculated from ee_s and ee_p as defined by Chen, C. S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem,. Soc.* **1982**, *104*, 7294-7299. All lipases preferred the (*S*)-enantiomer except in one example. Error limits for E were estimated assuming a 1% error in enantiomeric purity. [i]Reaction conditions during spectrophotometric assay: 0.37 mM substrate, 0.38% Triton X-100, 45.2 mM Tris Buffer, 4.76% acetonitrile except for reaction with ANL: 0.25 mM substrate, 0.5% acetonitrile, 10 mM HEPES buffer, pH 8.0. Rates of hydrolysis are reported in µmol/min/mg protein and are calculated using the Beer-Lambert Law with $\varepsilon = 15{,}800\ M^{-1}cm^{-1}$, $l = 1$ cm. [j]Error limits are the average of 3 separate experiments. [k]Amano Enzyme USA Co., Ltd. (Troy, VA).[l]Sigma-Aldrich (Oakville, ON). [m]CRL partially-purified with isopropanol as outlined in the experimental section. [n](±)-Phenylalanine 4-nitrophenyl ester TFA salt hydrolyzed spontaneously at pH 5 at a rate of $3.78 \times 10^{-4}\ s^{-1}$ ($T_{1/2} = 0.51$ h). It was impossible to run the reaction and determine enantiomeric purities without complete hydrolysis of the substrate. [o]Rate of hydrolysis has been corrected for background chemical hydrolysis.

Overall, separately measured initial rates of pure enantiomers can be a fast estimate of enantioselectivity but give inaccurate E values because some or all of the contributions from K_M are ignored.

Using pseudoracemates to determine the enantioselectivity of chromogenic esters.

To accurately determine E using separately measured initial rate measurements, the rates must reflect the competition between the enantiomers for the hydrolase's active site. To introduce competition without introducing the other enantiomer, we added a "pseudoenantiomer" to compete with each pure chromogenic ester. The pseudoracemates are an equimolar pair of esters where each enantiomer of the chiral acid is linked to a structurally similar but different achiral alcohol. For example, the pseudoenantiomer of 4-nitrophenyl-(*S*)-2-phenylpropanoate, (*S*)-**1**, is a non-chromogenic phenyl ester, phenyl-(R)-2-phenylpropanoate, (*R*)-**4**. Both enantiomers compete for the enzyme's active site but we only detect the hydrolysis of the 4-nitrophenyl ester spectrophotometrically. However, the observed rate of hydrolysis of the chromogenic ester reflects the competition between both pseudoenantiomers, Figure 5.3.

In the first step of the measurement of E using pseudoenantiomers, the ratio of rates of hydrolysis of (*S*)-**1**/(*R*)-**4** is measured. In the second step, the reverse pair of pseudoenantiomers is used where the (*R*)-enantiomer releases the chromophore. The ratio of initial rates of hydrolysis of the two chromogenic enantiomers relative to their non-chromogenic pseudoenantiomers yields a value for E, Equation 5.5.

$$E_{pseudoracemate} = \frac{initial\ \ rate_{fast\ \ pseudoracemate}}{initial\ \ rate_{slow\ \ pseudoracemate}} \qquad \text{Eq. (5.5)}$$

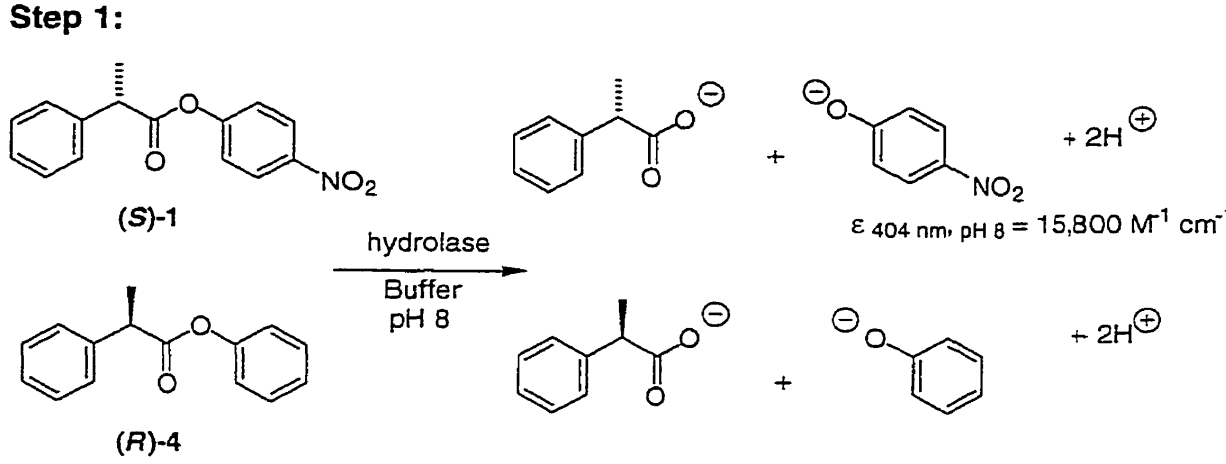

Figure 5.3 Step one of the pseudoracemate screen for enantioselectivity towards esters of 2-phenylpropanoic acid. The pseudoracemate is an equimolar mixture of (*S*)-1 and (*R*)-**4**. A yellow color appears if the hydrolase catalyzes the hydrolysis of the (*S*)-ester. The second step of the pseudoracemate screen (not shown) uses the reverse pair of substrates [4-nitrophenyl-(*R*)-2-phenylpropanoate and phenyl-(*S*)-2-phenylpropanoate]. An enantioselective hydrolase shows a yellow color in only one of the two steps. The ratio the rates of hydrolysis at 404 nm for step 1 and step 2 gives a value for E. Note that the extinction coefficient accounts for the partial ionization of 4-nitrophenol at pH 8; in practice, 4-nitrophenol (pKa = 7.15) is not fully ionized at pH 8 as shown.

To test this method, we measured the enantioselectivity of isopropanol-treated *Candida rugosa* lipase (IPA-CRL) towards 4-nitrophenyl-2-phenylpropanoate **1** using phenyl esters as pseudoenantiomers, **4**. We reported in Table 5.1 that the enantioselectivity of IPA-CRL towards 4-nitrophenyl-2-phenylpropanoate measured by the endpoint method is >100. Using pseudoracemates, we found that the enantioselectivity was underestimated by 2-fold, with E = 55, Table 5.2. Moreover, this value was only slightly closer to the true enantioselectivity than the estimated E in Table 5.1. When we measured the enantioselectivity of isopropanol-treated *Candida rugosa* lipase toward phenyl-(±)-2-phenylpropanoate, **4**, by the endpoint method, we were surprised to find that the enantioselectivity of IPA-CRL towards the phenyl ester of 2-phenylpropionic acid was 10-fold lower than for the 4-nitrophenyl ester analogue, E = 9.5 (ee_s = 42.3%, ee_p = 72.6% at 40% conversion). Enantioselectivity is sensitive to small structural changes.

Table 5.2 Enantioselectivities of isopropanol-treated *Candida rugosa* lipase towards 4-nitrophenol-(±)-2-phenylpropanoate, **1**, determined by the pseudoracemate method.[a]

Pseudoenantiomer pair[b]	**(*S*)[c] pair**	**(*R*)[c] pair**	**E[d] Pseudoenantiomers**
4 (phenyl esters)	1.98	0.0361	55 ± 5
5 (2-chloroethyl esters)	1.15	0.0299	38 ± 2

[a]Concentrations during hydrolysis: 0.43 mM of each pseudoenantiomer, 1.7% DMSO, 0.0028 mg protein for (*S*) pair, 0.055 mg protein for (*R*) pair. [b]Enantiomerically-pure pseudoenantiomers are used in equimolar amounts to the pure enantiomers of 4-nitrophenyl-2-phenylpropanoate during screening. [c]Rate of hydrolysis of the 4-nitrophenyl ester of the pseudoenantiomer pair reported in μmol/min/mg protein using $\varepsilon_{404\ nm,\ pH\ 8} = 15{,}800\ M^{-1}cm^{-1}$ and $l = 1$ cm. The uncertainty in the data was determined to be 5.7% by calculating the standard deviation of single absorbance measurements. [d]Ratio of the fast rate over the slow rate, Equation 5.5. Error represents the standard deviation of three measurements.

We next used 2-chloroethyl esters of 2-phenylpropionic acid **5**, as pseudoenantiomers of 4-nitrophenyl-2-phenylpropanoate **1**. Although these esters appear structurally very different, their enantioselectivity by isopropanol-treated *Candida rugosa* lipase was reported to be >100.[10] Still, the E value by the pseudoracemate method was considerably lower (E = 38) than the E value by the endpoint method.

For an ester to be a true pseudoenantiomer, it must only differ in structure but not in kinetic constants (k_{cat} and K_M). Identical enantioselectivities toward the racemates of both esters do not ensure that two esters are suitable as pseudoenantiomers since there are a limitless number of combinations of kinetic constants that yield the same E value. To use pseudoenantiomers to accurately measure enantioselectivity, the kinetic constants of a chromogenic ester and its corresponding non-chromogenic ester must be identical. Finding a suitable match for each measurement is impractical since the kinetic constants would have to be determined for each hydrolase. Overall, the pseudoenantiomer method is slower to set up than the traditional endpoint method to evaluate enantioselectivity.

Measuring enantioselectivity of chromogenic esters via quick E

To introduce competition into the separately measured initial rate measurements of pure enantiomers, we added an achiral chromogenic ester, resorufin tetradecanoate **6**, to both initial rate measurements of the pure enantiomers. Unlike pseudoenantiomers, the reference compound introduces the same competition with both pure enantiomers, Figure 5.4. We call this method "quick E" because of its speed and ease.

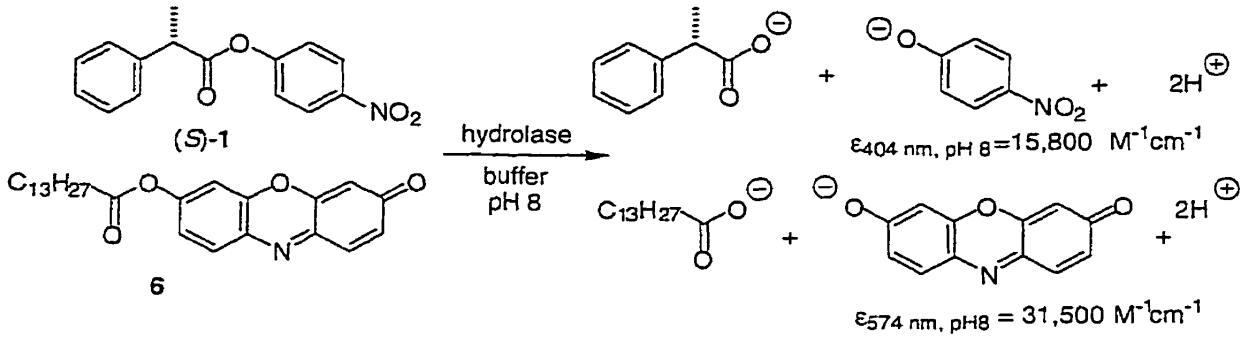

Figure 5.4 First step in the quick E measurement of enantioselectivity of a hydrolase toward (±)-4-nitrophenyl-2-phenylpropanoate, **1**. Hydrolase-catalyzed hydrolysis of (*S*)-**1** and resorufin tetradecanoate **6** releases yellow and pink chromophores, respectively. The solution turns a deep orange colour if both the substrates are hydrolyzed, pink if only the reference compound is hydrolyzed. The second step of the quick E is the same, except that it uses the (*R*)-enantiomer of the chiral ester. Equation 5.6 yields the selectivity ratio for each step. The extinction coefficients of both chromophores account for their partial ionization at pH 8; in practice, neither are fully ionized at pH 8.0 as shown above.

When two substrates compete for an enzyme's active site, the ratio of the specificity constants (k_{cat}/K_M) can be determined simultaneously by measuring the initial rates of hydrolysis of the pair, after taking into account their initial concentrations. The ratio of rates of hydrolysis of the 4-nitrophenyl-(S)-2-phenylpropanoate, **1**, vs. the reference compound, **6**, after taking into account the initial concentrations, yields the selectivity ratio of the hydrolase for the substrate versus the reference compound for the first step of quick E, Equation 5.6. The choice of reference compound is unimportant (see discussion section for practical considerations) as long as the same reference compound for used in both steps in quick E.

$$\frac{(S)-1}{6}\ selectivity\ ratio = \frac{(k_{cat}/K_M)_{(S)-1}}{(k_{cat}/K_M)_6} = \frac{initial\ rate_{(S)-1}}{initial\ rate_6} \times \frac{[6]}{[(S)-1]} \qquad \text{Eq. (5.6)}$$

In a second step, the experiment is repeated with the (*R*)-enantiomer and the reference compound **6**, and the (*R*)-**1**/**6** ratio is determined. The specificity constants

(k_{cat}/K_M) of each pure enantiomer relative to the same reference compound are determined in two initial rate measurements and these specificity constants are directly comparable to each other. The ratio of these two selectivity experiments yields, by definition, the enantioselectivity, Equation 5.7.

$$\frac{\frac{(S)-1}{6}\ selectivity\ ratio}{\frac{(R)-1}{6}\ selectivity\ ratio} = \frac{\frac{initial\ rate_{(S)-1}}{initial\ rate_{6}} \times \frac{[6]}{[(S)-1]}}{\frac{initial\ rate_{(R)-1}}{initial\ rate_{6}} \times \frac{[6]}{[(R)-1]}} = \frac{(k_{cat}/K_M)_{(S)-1}}{(k_{cat}/K_M)_{(R)-1}} = quick\ E$$

Eq. (5.7)

When both selectivity experiments use the same substrate concentrations, Eq. 5.7 can be simplified to the ratio of initial rates from each selectivity experiment, Eq. 5.8

$$quick\ E = \frac{initial\ rate_{(S)-1}}{initial\ rate_{6}} \times \frac{initial\ rate_{6}}{initial\ rate_{(R)-1}} \qquad \text{Eq. (5.8)}$$

Using the quick E method, we measured E values for **1** - **3** and compared these values to their endpoint values reported in Table 5.1. The Quick E method agreed with the endpoint method for **1** and **2**, Table 5.3. We measured low, average and excellent enantioselectivities accurately by this technique. We could not measure the quick E of phenylalanine, **3**, because of its instability in aqueous solution.

Extending quick E to chiral alcohols

Another important class of chiral building blocks are chiral alcohols and many hydrolase-catalyzed resolutions of secondary and, to a lesser extent, primary alcohols, have been reported in the literature.[11]

Table 5.3 Enantioselectivities of hydrolases towards 4-nitrophenyl-(±)-2-propanoate **1** and 4-nitrophenyl-(±)-2-(4-isobutylphenyl)propanoate **2** determined by the quick E method.

Substrate	Lipase[a]	(*S*)-enantiomer+ ref[b]		(*R*)-enantiomer+ ref[b]		Quick E[c]	Endpoint E[d]
		404 nm	574 nm	404 nm	574 nm		
1	*Candida antarctica*, type A	0.0309	0.0111	0.0119	0.0107	2.3 ± 0.2	1.9 ± 0.1
1	*Candida rugosa*	0.0948	0.445	0.0260	0.423	3.5 ± 0.3	3.5 ± 0.2
1	IPA-*Candida rugosa*	0.102	0.206	0.000215	0.100	230 ± 20	>100
1	*Pseudomonas cepacia*	0.170	0.0636	0.00488	0.0487	27 ± 3	29 ± 3
1	Porcine pancreas	0.00105	0.000495	0.000659	0.000437	1.4 ± 0.2	1.1 ± 0.1
2	*Candida rugosa*[e]	0.0677	0.494	0.0266	0.523	2.7 ± 2	1.2 ± 0.1
2	IPA-*Candida rugosa*	0.116	0.239	< 0.0005	0.157	> 140	>100
2	*Pseudomonas cepacia*	0.00430	0.0441	0.00740	0.0306	2.5 ± 0.3 (*R*)	3.3 ± 0.1 (*R*)

[a]See Table 5.1 for details. [b]Final concentration in the cuvette during assay: 0.071 mM resorufin tetradecanoate, 0.351 mM (*R*)- or (*S*)-ester, 9% acetonitrile, 0.73% Triton X-100, 43 mM Tris buffer, pH 8. The liberated 4-nitrophenxide and resorufin were measured at 404 nm and 574 nm, respectively, at 25 °C for 15 seconds. No measurable spontaneous hydrolysis was detected. Rates are calculated in µmol/min/mg protein using l = 1 cm, $\varepsilon_{574\ nm}$ = 31,500 M^{-1} cm^{-1} (experimentally determined for 9% acetonitrile, pH 8.1) and $\varepsilon_{404\ nm}$ (experimentally determined for 9% acetonitrile, pH 8.1) = 15,900 M^{-1} cm^{-1}. [c]Enantiomeric ratio calculated using Equation 5.8. Error is the standard deviation for 3 measurements. [d]Values from Table 5.1. [e]CRL solution (214 mg/mL; 1.9 mg protein/mL).

A requirement of quick E is the ability to monitor the rate of hydrolysis of a pure enantiomer and a reference compound simultaneously. However, it is difficult to find a chromophore in the acid portion of the molecule to derivative an alcohol into a chromogenic ester. Researchers have previously used thioester analogues of alcohols to spectrophotometrically detect lipase-catalyzed rates of hydrolysis.[12] Hydrolysis of a thioacetate releases a thiol which, in its reactive thiolate anion form, can cleave the disulfide bond of Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid); DTNB] to release a yellow chromophore at 412 nm, Figure 5.5.[13]

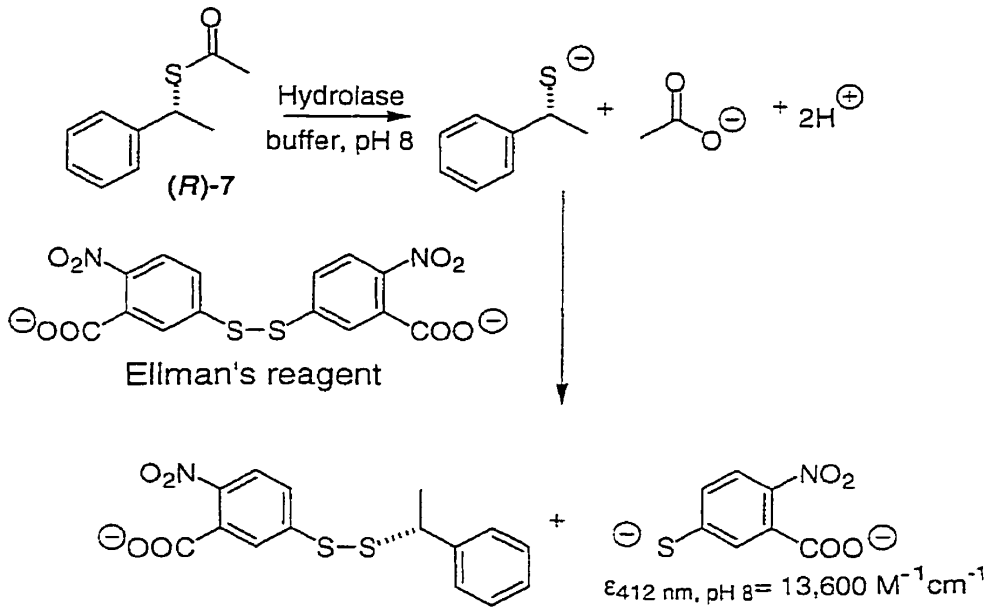

Figure 5.5 Hydrolysis of (*R*)-1-phenethyl thioacetate, 7, releases a thiolate anion which cleaves Ellman's reagent to produce a yellow chromophore (Ellman's anion) which can be detected spectrophotometrically. The degree of ionization of the thiol at pH 8 is dependent upon its pK_a.

Researchers have previously resolved thioacetates with hydrolases and observed enantioselectivities similar to their alcohol counterparts.[14] We therefore prepared (±)-1-phenethyl thioacetate, 7, as a structural analog of (±)-1-phenethyl acetate. We measured the enantioselectivity of subtilisin Carlsberg towards 7, E = 9.7 by the endpoint method (ee_s = 75%, ee_p = 63%, 22 h). This value was higher than the value previously reported

for 1-phenethyl acetate, E = 3.7.[15] In both kinetic resolutions, the (*R*)-enantiomer was the preferred enantiomer by subtilisin. This enantiopreference agrees with empirical rules developed for subtilisin Carlsberg.[16]

We next prepared the pure enantiomers of **7** and measured the quick E value with subtilisin Carlsberg, Table 5.4. We used resorufin acetate as a reference compound (2.27 μmol/min/mg protein) because resorufin tetradecanoate was not a substrate for subtilisin Carlsberg.[17]

The measured rates of subtilisin-catalyzed hydrolysis of the pure enantiomers of **7** detected with Ellman's reagent were only two-fold above the background rate of formation of Ellman's anion using our assay conditions. After correcting the rates of hydrolysis for background formation of Ellman's anion, the quick E value was calculated to be 1.4, 7-fold lower than the E value measured by the endpoint method, Table 5.4. To ensure that we could detect hydrolysis of a thioacetate with Ellman's reagent under our assay conditions, we tested the hydrolysis of S-acetylthiocholine iodide by acetylcholinesterase under identical assay conditions and observed large, linear changes in absorbance at 412 nm (55,000 μmol/min/mg protein).

There are several practical problems associated with using thiol analogues for quick E measurements of alcohols. Since the rate of hydrolysis of the thioacetate is not directly measured, errors in the absorbance kinetics of 5'-thio-2'-nitrobenzoate formation (Ellman's anion) lead to errors in the quick E values. Ellman's reagent can be cleaved by other nucleophiles present in solution, so accurately detecting low hydrolase activity towards the thioacetate can be difficult. Importantly, the reactive form of the product thiol is the thiolate anion, RS^-, so the pK_a of the released thiol is critical. The pK_a of 1-phenylethyl thiol and many other organic building blocks are above pH 9[18], so less than 10% of the released thiol is in its reactive form towards Ellman's reagent at pH 8. Fornasier and Tonellato report the pK_a of thiocholine is 7.85, so more than 50% of acetylthiocholine is in its nucleophilic form at pH 8, which partially accounts for the high activity we observed in the test assay.[19] Whitesides and coworkers found that the maximum rates of thiol-disulphide interchange between thiols and Ellman's reagent are observed for thiols having pK_a values close to the pH of the reaction solutions.[20] It is not

Table 5.4 Enantioselectivity of subtilisin Carlsberg[a] protease towards (±)-1-phenethyl thioacetate, 7, using the Quick E method in the presence of Ellman's reagent (DTNB).

Sub-strate	(*R*)-enantiomer+ ref[b]		(*S*)-enantiomer+ ref[b]		Quick E[c]	Endpoint E[d]
	412 nm	574 nm	412 nm	574 nm		
7	0.00364	1.41	0.00341	1.86	1.4 ± 0.5	9.7 ± 0.4

[a]3.14 mg solid/mL solution; 1.39 mg protein/mL solution by the Bio-Rad assay. [b]Final concentration in the cuvette during assay: 0.433 mM thioacetate, 0.545 mM DTNB, 0.078 mM resorufin acetate, 0.7% Triton X-100, 44 mM Tris buffer, 12% acetonitrile. The simultaneously liberated Ellman's anion and resorufin anion were measured at 412 nm and 574 nm, respectively, at 25 °C for 30 seconds. Rates are calculated in μmol/min/mg protein using l = 1 cm, $\varepsilon_{574\ nm}$ = 31,500 M^{-1} cm^{-1} (9% acetonitrile, pH 8.1) and $\varepsilon_{404\ nm}$ (9% acetonitrile, pH 8.1) = 13,600 M^{-1} cm^{-1} and are corrected for spontaneous hydrolysis of Ellman's reagent under our conditions during the assay [c]Enantiomeric ratio calculated using Equation 5.3. Error is the standard deviation for 3 measurements. [d]Conditions during endpoint reaction: room temperature, pH 8, 50 mM Tris buffer, 50 mg substrate dissolved in 1 mL acetonitrile, 3.14 mg subtilisin Carlsberg. Error bars are an estimate of error from integration that we estimated to be <2%.

practical to assay hydrolases under very basic conditions where the pH of the solution is close to the pK_a value of the released thiol (>9). Finally, it is unpleasant to synthesize and work with the thiol analogues of pure alcohols.

We, therefore, chose to focus on the development of a more versatile and simple method to extend quick E to non-chromogenic esters.

Extending quick E to non-chromogenic esters

For quick E to be a versatile technique, it was necessary to generalize the method for any ester substrate, not just chromogenic esters. To accomplish this goal, we developed a quantitative, spectrophotometric assay to measure the hydrolase-catalyzed rates of hydrolysis of esters using pH indicators.[21] Hydrolysis of an ester bond releases an alcohol and acid moiety and also a proton(s). With a suitable pH-indicator, the released proton(s) produces a change in absorbance of the pH indicator. If the conditions of the assay are chosen carefully, the rate of change of absorbance of the pH-indicator is directly proportional to the rate of enzyme-catalyzed ester hydrolysis, Equation 5.9, where $\Delta\varepsilon_{404nm}$ is the difference in extinction coefficients of the fully protonated and deprotonated form of the pH indicator. Importantly, the reaction buffer and pH indicator must have identical pK_a values to ensure a linear relationship between the absorbance kinetics and the enzyme kinetics. This sensitive pH indicator assay enables us to accurately measure the rates of hydrolysis of any ester substrate.

$$initial\ \ rate(\mu mol/\min) = \frac{dA_{404\,nm}}{dt} \times \frac{[Buffer]}{[indicator]} \times \frac{1}{\Delta\varepsilon_{404\,nm} \cdot l} \times rxn\ volume \times 10^6 \quad \text{Eq. (5.9)}$$

To test this method, we used 4-nitrophenol as a pH indicator to measure the rates of hydrolysis of an ester of the primary alcohol, solketal butyrate, **8**, at neutral pH in the presence of the reference compound, resorufin acetate, **6**. The first step of the extended quick E measurement is shown in Figure 5.6.

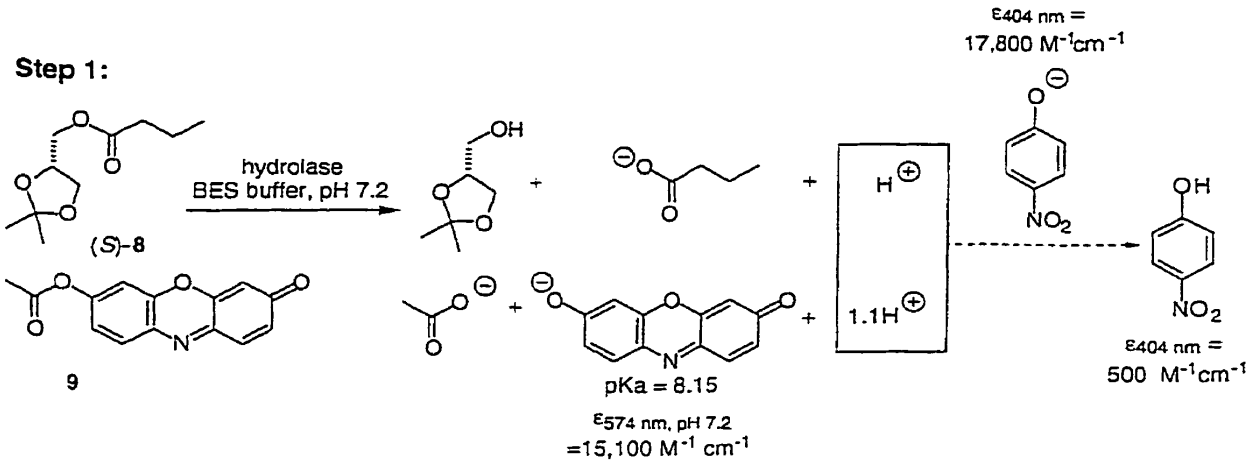

Figure 5.6 The first step of the quick E measurement of a non-chromogenic ester, solketal butyrate **8**, using 4-nitrophenol as the pH indicator. Hydrolase-catalyzed hydrolysis of (*S*)-**8** and resorufin acetate, **9**, releases protons and the pink chromophore, resorufin. We calculate the rate of hydrolysis of resorufin acetate by the change in absorbance at 574 nm. To calculate rate of hydrolysis of (*S*)-**8**, we subtract the rate of protons released during hydrolysis of the reference compound from the total rate of protons detected with 4-nitrophenol. The ratio of relative rates, (*S*)-**8**/**9** is the selectivity ratio for the first step in quick E. The second step is the same but uses the (*R*)-enantiomer and resorufin acetate. The ratio of selectivity ratios from both steps yields quick E.

The rate of hydrolysis of the reference compound is calculated using the extinction coefficient of resorufin for the screening conditions, Equation 5.10. We measured ε = 15,100 $M^{-1}cm^{-1}$ for pH 7.2, 7% acetonitrile.

$$Rate_{reference}(\mu mol/\min) = \frac{dA_{574nm}}{dt} \times \frac{1}{\varepsilon_{574nm,pH7.2} \cdot l} \times rxn\ volume \times 10^6 \qquad \text{Eq.(5.10)}$$

To determine the rate of hydrolysis of the pure enantiomers of solketal butyrate, we first determined the acid dissociation constant of resorufin alcohol, pK_a = 8.15. Only 10% of the resorufin released upon hydrolysis of resorufin acetate is deprotonated at pH 7.2, so 1.1 protons are released for every resorufin acetate molecule hydrolyzed (one proton is from the acetic acid which is fully deprotonated at neutral pH). Since the pH indicator detects all protons, we subtract the rate of protons from hydrolysis of the

reference compound from the total rate of protons detected to obtain the true rate of enzyme-catalyzed hydrolysis of the pure enantiomers of solketal butyrate, Equation 5.11.

$$initial\ \ rate_{(S)-substrate}\ (\mu mol/\min) = \left(\frac{dA_{404\,nm}}{dt} \times \frac{[Buffer]}{[indicator]} \times \frac{1}{\Delta\varepsilon_{404\,nm} \cdot l} \times rxn\ volume \times 10^{6}\right) - 1.1\left(rate_{reference}\right)$$

Eq. (5.11)

Note that for eq. 5.11 to be accurate, it is important to use a strong enough buffer during the assay to ensure that the pH of the solution overall does not change significantly during quick E measurements. The ratio of deprotonated resorufin molecules changes with changing pH. We chose 5 mM BES buffer as a compromise between high buffer concentrations to ensure small changes in pH throughout the assay (0.05 pH units for 10% hydrolysis of the substrates) and low buffer concentrations to maximize the pH indicator sensitivity (<0.055 change in absorbance units for 10% hydrolysis of substrates in a quick E measurement).

We used this extended quick E method to measure the quick E values of several hydrolases towards solketal butyrate, a common chiral building block in organic synthesis, Figure 5.7.[22] We then compared these values to the E values determined by the endpoint method to confirm that quick E measured the enantioselectivities correctly. Enantioselectivities by quick E agree with those by the endpoint method for five out of six hydrolases, Table 5.5. Only with horse liver esterase did we observe a two-fold difference between the endpoint E and the quick E value. We attribute the lower quick E to the added surfactant, Triton X-100, which was not present during the endpoint reaction.

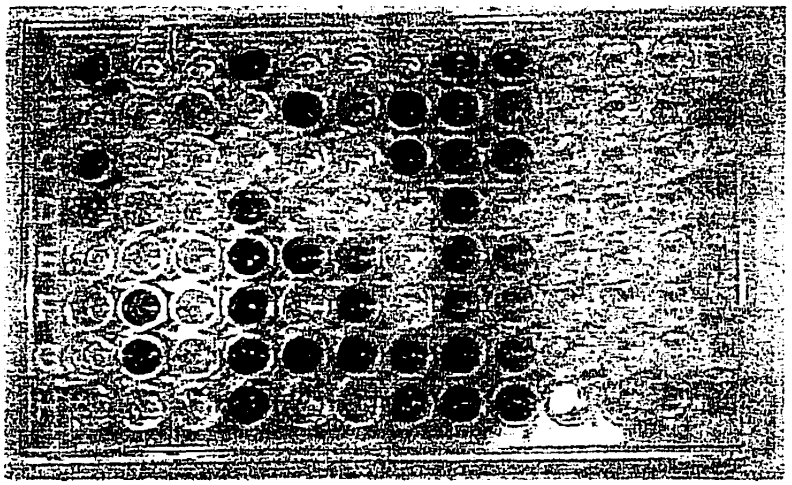

Figure 5.7. Photo of the first step of a typical quick E screen for enantioselective hydrolases towards a target substrate using 4-nitrophenol as the pH indicator and resorufin acetate as a reference compound. A different hydrolase is present in each well. Hydrolase-catalyzed hydrolysis of pure enantiomers and resorufin acetate releases protons and the pink chromophore, resorufin. Wells that are a bright pink color indicate that the reference compound is hydrolyzed quickly by the hydrolase. Yellow wells indicate slow to no hydrolysis of the reference compound. It is not possible to detect the decrease in absorbance of the indicator with our eyes but monitoring the changes in absorbances at 404 nm and 574 nm yield the relative rates of hydrolysis of both substrates. The second step of quick E is the same but uses the opposite enantiomer.

Table 5.5 Enantioselectivities of hydrolases towards (±)-solketal butyrate, **8**, using the quick E method.

Hydrolase[a]	**Time (s)**[b]	**(*S*)-enantiomer+ ref**[c]		**(*R*)-enantiomer+ ref**[c]		**Quick E**[d]	**Endpoint E**[e]
		404 nm	**574 nm**	**404 nm**	**574 nm**		
Aspergillus oryzae protease	10-100	0.00696	0.0998	0.0455	0.103	6.3 ± 1.8 (*R*)	5.4 ± 0.1 (*R*)
Candida rugosa lipase	0-100	0.208	0.855	0.731	0.800	3.7 ± 0.8 (*R*)	3.0 ± 0.1 (*R*)
Cutinase from *Fusarium solani*	0-200	1.07	4.57	4.06	5.82	3.0 ± 1.1 (*R*)	5.0 ± 0.02 (*R*)
Esterase E013	0-100	0.267	4.38	1.04	11.7	1.4 ± 0.3 (*R*)	1.02
Horse liver esterase	0-200	2.55	0.188	0.225	0.137	8.3 ± 2.1(*S*)	14.8 ± 0.7 (*S*)
Rhizopus oryzae lipase[f]	0-100	0.0970	0.922	0.268	0.453	5.6 ± 1.4 (*R*)	5.0 ± 0.1 (*R*)

[a] See Table 4.1. [b]Data collection time. [c]Final concentration in the microplate wells during assay: 0.10 mM resorufin acetate, 1.0 mM (*R*)- or (*S*)- solketal butyrate, 7% acetonitrile, 0.134 mM (0.86%) Triton X-100, 0.434 mM 4-nitrophenol, 4.65 mM BES buffer, pH 7.2. Changes in absorbance at 404 nm and 574 nm were monitored simultaneously. Rates are calculated in µmol/min/mg protein using equations 5.10 and 5.11 after taking into account the amounts of protein in each well as determined by the Bio-Rad assay using BSA as a standard. No spontaneous chemical hydrolysis was detected. [d]Enantiomeric ratio calculated using Equation 5.7. Reported error is the standard deviation for 3 measurements. [e]Endpoint values were determined using the identical conditions as in the microplate wells during screening but no pH indicator or Triton X-100 was added, see Table 4.2. [f]Resorufin *t*-butylacetate **10** was used as a reference compound in the quick E measurement at 0.1 mM concentration in the well during assay.

Discussion

The most important consideration in the development of quick E was to ensure that we accurately measured the enantiomeric ratio, E. To do this, we needed to account for differences in k_{cat} and K_M between both enantiomers since the enantiomeric ratio, E, is a ratio of the specificity constants (k_{cat}/K_M) of both enantiomers, Eq. 5.2.

In general, when more than one substrate competes for the enzyme active site, the ratio of their initial rates of hydrolysis equals their relative specificity constants (k_{cat}/K_M values) after taking into account the initial substrate concentrations, Eq. 5.1. When the two substrates are enantiomers, the ratio equals the enantiomeric ratio, E, and a researcher measures enantiomeric purity of the reaction products to determine the relative rates of hydrolysis. However, a researcher can also determine the k_{cat}/K_M value of an enantiomer relative to a substrate that is not its enantiomer as long as the rates of hydrolysis of both substrates can be measured simultaneously. This measurement is the basis of quick E.

In quick E, we add an achiral, chromogenic reference compound to the separate solutions of pure enantiomers, then measure the initial rates of hydrolysis of the pure enantiomers relative to the reference compound. In a single spectrophotometric measurement, we obtain k_{cat}/K_M of each pure enantiomer relative to the reference compound.[23] The ratio of the two steps yields the enantiomeric ratio, E. Without a competing reference compound, the measured initial rates only reflect k_{cat}/K_M when the substrate concentrations are well below the K_M values. Other groups have used multiple substrate kinetics to determine relative k_{cat}/K_M values toward mixtures of peptide substrates.[24, 25, 26] We apply this technique to the rapid determination of enantioselectivity of hydrolytic enzymes.

Competitive substrate inhibition does not affect the enantioselectivity of an enzyme so the presence of a reference compound, which acts as a competitive inhibitor to the pure enantiomers, does not alter the enantioselectivity of a hydrolase towards a chiral substrate.[6] In quick E, the rate of hydrolysis of each pure enantiomer is diminished to an extent consistent with the competition between the pure enantiomer and the reference compound for the enzyme's active site. This is true regardless of the substrate concentrations relative to each other as well as relative to their individual K_M values, so

competition experiments between pure enantiomers and a reference compound can be performed under conditions where the substrate concentrations are well below their K_M values.[27]

A major requirement of the quick E method is the ability to accurately measure initial rates of hydrolysis of the pure enantiomers and the reference compound simultaneously. We first developed quick E using chromogenic esters because it is convenient to monitor their rates of hydrolysis spectrophotometrically. We chose an ester of the alcohol, resorufin, as a reference compound because it releases a brilliant pink chromophore with an absorbance maximum at 574 nm. Importantly, this absorbance does not overlap with the absorbance maximum of 4-nitrophenoxide, so the rates of hydrolysis of both the 4-nitrophenyl esters of the pure enantiomers and resorufin acetate are easily determined simultaneously. Resorufin analogues have been used previously to spectrophotometrically monitor the activity of enzymes including O-dealkylases[28], galactosidases[29], cellulases[30], lipases[31], proteases, esterases, and phospholipases.[32]

To measure the rates on non-chromogenic esters, we developed a pH indicator assay to visualize the hydrolysis of any ester.[21] We chose 4-nitrophenol as the pH indicator because the absorbance of common polyaromatic indicators overlapped with the absorbance of resorufin. We needed to know the pK_a of resorufin at our screening conditions to quantitatively account for protons from the hydrolysis of the reference compound, eq. 5.11. Other groups reported that the pK_a value of the phenolic oxygen of resorufin is approximately 6.[33] However, we observed two distinct pK_a values for resorufin at pH 6.0 and 8.1. We believe the former is the pK_a of the proton on the bridging nitrogen of resorufin while the latter is the pK_a of the phenolic oxygen. We expect that the nitrogen of resorufin, a planar molecule with delocalized electronic structure, would display a pK_a comparable to the nitrogen of pyridine which has a pK_a of 5.2. Also, the pK_a value of 6.0 is low for a phenolic alcohol; in comparison, the pK_a of 4-nitrophenol is 7.15. Finally, we determined the extinction coefficient of resorufin at pH 7.2 is 15,100 M^{-1} cm^{-1}. We, and others, observed that the absorbance of resorufin when fully deprotonated is 53,000-69,000 $M^{-1}cm^{-1}$ depending on experimental conditions.[31, 33] If the pK_a of the phenol is 5.8, over 95% of the resorufin is deprotonated at pH 7.2 and the extinction coefficient should be very close to the absorbance maximum.

Quick E measurements also require that initial rates are measured under competitive conditions. Two substrates do not compete if there are very large differences in their kinetic parameters and the larger the difference, the lower the accuracy of the measured selectivity ratio. Schellenberger and coworkers found that relative rate constants cannot be reliably estimated for a mixture of two or more substrates that differ by two or more logarithmic unit in their rate constants, unless substrate concentration can be quantified with extremely high precision.[23] In quick E, when there are very large differences in hydrolytic activity between the reference compound and the enantiomers, a more suitable reference compound must be chosen. For example, we used resorufin *t*-butylacetate **10**, when measuring the quick E of *Rhizopus oryzae* lipase (ROL) towards solketal butyrate **8**, because resorufin acetate, which was suitable with all other hydrolases tested, was too quickly hydrolysed by ROL relative to the pure enantiomers of solketal butyrate.

The selectivity ratios, k_{cat}/K_M, are strictly comparable only to other selectivity ratios relative to the same reference compound. For example, determining enantioselectivity with pairs of pseudoenantiomers gave the incorrect E value because the rates of hydrolysis of the two pure enantiomers of the substrate were relative to different reference compounds. Similarly, Hermetter and coworkers used "enantiomer analogs" of alkyldiacyl glycerol analogs labeled with two different flourophores to measure lipase stereoselectivity. They found that the enantiomeric analogs displayed different kinetics so direct comparison of the two rates could only estimate the stereoselectivity, not accurately measure it.[34]

There are several advantages to the quick E method. Foremost, quick E is significantly faster than the endpoint method because the entire quick E reaction takes place in a microplate well or cuvette. Workup and measuring enantiomeric purities is avoided. Using microtiter plates to simultaneously determine the quick E values of a library of hydrolases also increases the speed of quick E. Quick E measurements require little substrate and enzyme so our method is well suited for measuring the enantioselectivities of small amounts of recombinant hydrolases. Finally, quick E measurements are sensitive to very small changes in enantioselectivity and may therefore

be useful in directed evolution experiments to increase the enantioselectivity of hydrolases where improvements between generations of hydrolases are small.[35]

There are also disadvantages to the quick E method. Clear solutions with fully dissolved substrates are a requirement of spectrophotometric measurements. In particular, precipitation of substrates during rate measurements produce false negative rates which cause large error in the measured rates of hydrolysis using 4-nitrophenol as a pH indicator. The reference compounds and most organic substrates are insoluble in aqueous solutions so finding conditions for clear solutions can be challenging. To dissolve substrates, we added water-miscible organic cosolvents and Triton X-100, a non-ionic detergent, but these additives can alter the enantioselectivity of an enzyme toward the substrate. Finally, pure enantiomers are required for quick E measurements albeit in small quantities – we can typically measure the quick E of 100 hydrolases in quadruplicate towards a target substrate using less than 10 mg of each pure enantiomer.

Note that quick E is prone to the same problems that face the endpoint determination of E. Due to assumptions made in deriving Equation 5.3, both the endpoint method and quick E will give inaccurate enantioselectivities in two common situations – impure biocatalysts and reactions inhibited by products. First, E values calculated using impure biocatalysts are a weighted average of all the enzymes.[5] If these enzymes differ significantly in their affinity for the substrate, then different enzymes will dominate the activity at different substrate concentrations. Second, when product inhibits the reaction the apparent enantioselectivity can change. To include product inhibition in the quantitative analysis, researchers use more complex equations that take into account the mechanism of lipase-catalyzed reaction (ping-pong bi-bi).[36] The deviations are usually small, so researchers consider them only in the final process optimization.

The most important confirmation of the quick E method is that E values match E values determined by the endpoint method. Using both chromogenic esters and non-chromogenic esters, quick E values agreed with enantiomeric ratios determined by the endpoint method. The extension of the quick E method to non-chromogenic esters increased the utility of this method since it is not always possible or convenient to convert a target substrate into a chromogenic ester, such as phenylalanine, **3**. Importantly, substrate analogs do not always have the same enantioselectivity as the target substrate.

Overall, quick E is a simple but quantitative method to determine the enantioselectivity of hydrolytic enzymes and simplifies the selection process for chemists to find enantioselective hydrolases. We next apply this method to identify stereoselective hydrolases useful for the production of pharmacological agents and to develop highly active and selective hydrolases via directed evolution.[8b, 37]

Experimental

General. Chemicals were purchased from Sigma-Aldrich Chemical Co. (Oakville, ON) and were used without further purification unless stated. EDC-HCl (N-ethyl-N'-[3-(dimethylamino) propyl]carbodiimide hydrochloride) and 1-hydroxybenzotriazole (anhydrous) were purchased from Chem-Impex Int. (Wood Dale, IL). TRIS buffer was purchased from ICN Biomedicals, Inc. (Aurora, OH). The pure enantiomers and racemate of 2-(4-isobutylphenyl)propanoic acid were supplied by Mr. Michael Trani of the Biotechnology Research Institute (Montreal, Quebec). Enzyme suppliers are noted in the footnotes of Tables. Spectrophotometric assays in cuvettes were performed on a Hewlett Packard 8452A diode array spectrophotometer equipped with a Neslab RTE-100 water bath temperature control unit. Microplate assays were performed on a Spectramax 340 microplate reader with SOFTmax PRO version 1.2.0 software (Molecular Devices, Sunnyvale, CA). Polystyrene 96-well flat-bottomed microplates (maximum volume 360 µl/well, Corning Costar, Acton, MA) were filled using Eppendorf 8-channel pipettes (5-100 µl; 50-1,200 µl) and solution basins for multi-channel pipettes (Fisher Scientific, Nepean, ON). The initial rates of small-scale enzyme-catalyzed hydrolysis reactions were measured with a Radiometer RTS 822 pH stat. NMR were recorded on a Varian Gemini 200 MHz spectrometer. Melting points were taken on an Electrothermal melting point apparatus and were corrected. Mass spectra were acquired using EI (70 eV) conditions or CI (NH_3 as ionization gas) on a Kratos MS25RFA double focussing mass spectrometer. High performance liquid chromotography (HPLC) was performed on a Spectra Physics liquid chromatograph, model 8800, equipped with a Spectra FOCUS forward optical

scanning detector, SP8800 autosampler and Spectra Physics software. HPLC chiral stationary phases were purchased from Daicel Chemical Industries Ltd. (Fort Lee, NJ).

Synthesis of Substrates

Esters were synthesized using a modified DCC coupling employing a water soluble coupling reagent (EDC-HCl; N-ethyl-N'-[3-(dimethylamino) propyl]carbodiimide hydrochloride) unless stated otherwise.[38] Alcohol (1.1 equiv.), acid and 1-hydroxybenzotriazole (1.1 equiv.) were added to anhydrous dichloromethane in a round bottom flask stirring in an ice bath. The mixtures stirred for 15 minutes, then EDC-HCl (1.1 equiv.) was added. The reactions were allowed to warm to room temperature and stirred for 48 hours, followed by washings with saturated sodium bicarbonate, water, 1 N HCl, then water, in that order. Esters were purified by silica gel chromatography eluted with hexanes/ethyl acetate and recrystallized, when solids, from hexanes/ethyl acetate, unless stated otherwise.

(±)-4-nitrophenyl-2-phenylpropanoate, **1**. 4-nitrophenol was recrystallized from chloroform and (±)-2-phenylpropanoic acid was purified by vacuum distillation prior to coupling. The reaction afforded 2.35 g (10.4 mmol, 78% yield) of the title compound as a yellow solid: R_f = 0.53 (3:1 hexanes:ethyl acetate); mp = 155.8 - 162.5 °C; ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.64 (d, 3H, J = 7.0 Hz), 3.96 (q, 1H, J = 7.2 Hz), 7.18 (d, 2H, J = 9.0 Hz), 7.38 (m, 5H), 8.23 (d, 2H, J = 9.2 Hz); ^{13}C-NMR (200 MHz, $CDCl_3$) δ 19.9, 46.9, 122.6, 125.4, 127.7, 128.0, 129.2, 139.5, 145.4, 155.5, 171.8; MS (EI) m/z: 271 ($M^{\cdot+}$, 5); 133 (59), 105 (100), 103 (4), 79 (4), 77 (4), 63 (1), 51 (1); HRMS (EI): calcd. for $C_{15}H_{13}N_1O_4$: 271.08460; found: 271.08445, 0.6 ppm error.

(R)- and (S)-4-nitrophenyl-2-phenylpropanoate, (*R*) and (*S*)-**1**. Samples were prepared from enantiomerically-pure 2-phenylpropionic acid as above for the racemate. The enantiomeric purities of the esters measured by HPLC (see below) were 99.7% and 99.4%, respectively. Yields for the reactions were 51% and 56%, respectively.

(±)-4-nitrophenyl 2-(4-isobutylphenyl)propanoate, **2**. The reaction afforded 0.70 g (2.14 mmol, 44% yield) of the product as a yellow solid after silica gel chromatography with 100% chloroform as eluent, followed by recrystallization: R_f = 0.57 (chloroform); mp = 57.8 - 60.0 °C; ^{1}H-NMR (200 MHz, $CDCl_3$) δ 0.917 (d, 6H, J = 6.6 Hz), 1.63 (d, 3H, J = 7.2 Hz), 1.91 (m, 1H), 2.48 (d, 2H, J = 7.0 Hz), 3.96 (q, 1H, J = 7.2 Hz), 7.14 – 7.31 (m, 6H), 8.23 (d, 2H, J = 9.2 Hz); ^{13}C-NMR (200 MHz, $CDCl_3$) 19.9, 23.9, 31.6, 46.3, 46.5, 111.4, 122.6, 125.4, 127.4, 129.9, 136.6, 141.3, 172.0; MS (EI) m/z: 327 ($M^{\bullet+}$, 6), 189 (3), 161 (100), 145 (4), 117 (11.2), 105 (3), 91 (5); HRMS (EI): calcd. for $C_{19}H_{21}N_1O_4$: 327.14720; found: 327.14705, 0.5 ppm error.

(R)-and (S)-4-nitrophenyl 2-(4-isobutylphenyl)propanoate, (*R*) and (*S*)-**2**. Samples were prepared from enantiomerically-pure 2-(4-isobutylphenyl)propanoic acid as above for the racemate. The enantiomeric purities of the esters measured by HPLC (see below) were 98.2% and 99.6%, respectively, after hydrolysis to the acid using aqueous NaOH for analysis. Yields for the reactions were 68 and 65%, respectively.

D,L-phenylalanine-4-nitrophenol ester TFA salt, **3**. D,L-phenylalanine was converted to its N-*t*-BOC derivative using di-*tert*butyl-pyrocarbonate following a procedure of Tarbell *et al.*[39] The protected amino acid was then converted to its 4-nitrophenyl ester following the coupling procedure above. The *t*-BOC group was subsequently removed by stirring the solid compound in 5 mL neat TFA for 10 minutes, followed by removal of TFA *in vacuo*. The final product was recrystallized from chloroform affording the title compound as a white, fluffy solid in 45% yield overall from three steps: mp = 177.1 – 178.9 °C (sample darkens at 160 °C); ^{1}H-NMR (200 MHz, CD_3OD) δ 3.41 (d, 2H, J = 7.2 HZ), 4.68 (t, 1H, J = 7.2 Hz), 7.29 (d, 2H, J = 9.4 Hz), 7.32-7.43 (mM, 5H), 8.36 (d, 2H, J = 9.2 Hz); MS (EI) m/z: 286 ($M^{\bullet+}$, 2), 240 (2), 195 (23), 167 (4), 120 (100), 91 (29), 69 (19), 46 (23); HRMS (EI): calcd. for $C_{15}H_{14}N_2O_4$: 286.09536; found: 286.09520, 0.5 ppm error.

D- and L-phenylalanine-4-nitrophenol ester TFA salt, D and L-3. Samples were prepared from enantiomerically-pure *L-* and *D-* N-*t*-BOC-phenylalanine-4-nitrophenol ester (Sigma-Aldrich Co., 99% purity) by removal of the N-*t*-BOC group as for the racemate. The enantiomeric purities of the esters measured by HPLC (see below) were both > 99.5% (see below).

(±)-phenyl-2-phenylpropanoate, **4**. Phenol was recrystallized from dichloromethane prior to coupling. The reaction afforded 1.01 g (4.47 mmol, 67% yield) of the title compound as a clear oil: R_f = 0.86 (3:1 hexanes:ethyl acetate); ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.66 (d, 3H, *J* = 8 Hz), 3.96 (q, 1H, *J* = 8 Hz), 7.04 (d, 2H, *J* = 8 Hz), 7.40 (m, 8H); ^{13}C-NMR (200 MHz, $CDCl_3$) δ 20.1, 46.9, 121.7, 126.0, 127.6, 127.8, 129.0, 129.6, 140.2, 150.8, 172.7; MS (EI) m/z: 226 ($M^{\bullet+}$, 2), 132 (7), 105 (100), 94 (10), 77 (9), 65 (3), 51 (3), 37 (2); HRMS (EI): calcd. for $C_{15}H_{14}O_2$: 226.09970; found: 226.09937, 1.4 ppm error.

(R)- and (S)-phenyl-2-phenylpropanoate, (*R*) and (*S*)-**4**. Samples were prepared from enantiomerically-pure 2-phenylpropanoic acid as above for the racemate. The enantiomeric purities of the esters measured by HPLC (see below) were 98.4% and 98.8%, respectively, after hydrolysis to the acids using aqueous NaOH. The yield for both enantiomers was 51%.

(R)- and (S)-2-chloroethyl-2-phenylpropanoate, (*R*) and (*S*)-**5**. Samples were prepared from enantiomerically-pure 2-phenylpropanoic acid. The enantiomeric purities of the esters measured by HPLC (see below) were 98.7% and 98.9%, respectively, after hydrolysis to the acids using aqueous NaOH. The yield was 27% and 47%, respectively as yellow oils: R_f = 0.60 (3:1 hexanes:ethyl acetate); ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.54 (d, 3H, *J* = 7.4 Hz), 3.64 (t, 2H, *J* = 6 Hz), 3.77 (q, 1H, *J* = 7 Hz), 4.3 (m, 2H), 7.2 – 7.4 (m, 5H); ^{13}C-NMR (200 MHz, CD_3OD) δ 20.0, 42.7, 46.6, 65.2, 127.5, 128.9, 140.2, 173.9; MS (EI) m/z: 212 ($M^{\bullet+}$, 21), 133 (6), 105 (100), 77 (9), 63 (4), 51 (4); HRMS (EI): calcd. for $C_{11}H_{13}O_2Cl$: 212.06041; found: 226.09937, 0.5 ppm error.

(±)-1-phenethyl thioacetate, **7**. The racemate and its pure enantiomers were synthesized following a procedure by Corey and Cimprich.[40] To a stirred solution of triphenylphosphine, freshly recrystallized from ethanol (0.861 g, 3.28 mmol, 2 eq), in 30 mL of anhydrous THF at 0 °C in a 50 mL round bottom flask equipped with a stir bar, was added diisopropylazodicarboxylate (655 µL, 0.663 g, 3.28 mmol, 2 eq) dropwise. The reaction stirred in the ice bath for 30 minutes. To this cloudy, yellow mixture was added dropwise a solution of thiolacetic acid (96% purity, 237 µL, 0.249 g, 3.28 mmol, 2 eq) and (±)-1-phenethyl alcohol (197 µL, 0.2 g, 1.64 mmol, 1 eq) dissolved in 4 mL of THF. The reaction turned a greenish-black colour and, after 1 h at 0 °C, the solution was allowed to warm to room temperature, then stirred for an additional 1 h. The final reaction was a clear yellow solution which appeared as 4 spots by TLC (96:4 hexanes: ethyl acetate). The reaction was concentrated *in vacuo* to 10 mL and washed with a solution of saturated sodium bicarbonate (4 x 15 mL). Hexanes (10 mL) was added, producing a white slurry which was filtered through a pad of celite, then through a 4 cm pad of silica gel. The reaction was dried over $MgSO_4$ and evaporated. The reaction products were resuspended in hexanes (4 mL) and the solution filtered to remove excess triphenylphosphine oxide which precipitated as a white solid. Silica gel chromatography with 1:1 dichloromethane:hexanes as eluent afforded 81.2 mg of the product (0.45 mmol, 28% yield) as a water-white oil: $R_f = 0.35$ (1:1 dichloromethane:hexanes); ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.66 (d, 3H, J = 7.2 Hz), 2.30 (s, 3H), 4.75 (q, 1H, J = 7.2 Hz), 7.3 (m, 5H). ^{13}C-NMR (200 MHz, $CDCl_3$) 23.8, 31.9, 44.3, 127.5, 127.6, 128.9, 142.7, 194.5; MS (EI) m/z: 180 ($M^{\bullet+}$, 13), 138 (5), 105 (100), 77 (11), 59 (4), 51 (6), 44 (19); HRMS (EI): calcd. for $C_{10}H_{12}OS$: 180.06088; found: 180.06100, 0.7 ppm error.

(S) and (R)-1-phenethyl-thioacetate, (*R*) and (*S*)-**7**. Samples were prepared from enantiomerically pure 1-phenethyl alcohol as above for the racemate. The yields were 67% and 63% respectively. Both were water white oils. The enantiomeric purities of the thioacetates measured by HPLC (see below) were 99.5% and 99.3%.

(±)-Solketal butyrate, **8** and (*R*) and (*S*)-**8**. Synthesis and characterization data were previously reported in Chapter 4.[21]

Synthesis of reference compounds

Resorufin acetate, **9**. This compound was prepared using a modified procedure by Kramer and Guilbault.[32] To a slurry of resorufin sodium salt (95% purity, 1.015 g, 4.3 mmol, 1 eq.) in 60 mL anhydrous dichloromethane, was added anhydrous pyridine (0.349 mL, 4.3 mmol, 1 eq). The solution was cooled in an ice bath, then acetyl chloride (0.614 mL, 8.6 mmol, 2 eq) was added dropwise over 10 minutes. The deep purple reaction mixture immediately turned orange. The reaction was warmed to room temperature and stirred overnight. The reaction was next diluted with dichloromethane to 300 mL and filtered through a coarse glass frit to remove unreacted resorufin, and the solvent removed *in vacuuo*. The reddish-orange residue was recrystallized from ethanol to yield 0.48 g (1.89 mmol, 44% yield). of a crimson powder: R_f = 0.20 (2:1 hexanes:ethyl acetate); mp = 217.4 – 220.2 °C (sample darkens at 215.4 °C) [literature = 223-225 °C (uncorrected)[32]]; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 2.37 (s, 3H), 6.34 (d, 1H, J = 1.8 Hz), 6.90-6.84 (dd, 1 H, J = 2.1 Hz, 9.9 Hz), 7.11 –7.16 (dd, 1H, J = 2.2 Hz, 7.3 Hz), 7.16 (s, superimposed, 1H), 7.44 (d, 1H, J = 9.7 Hz), 7.81 (d, 1H, J = 4.4 Hz); ^{13}C-NMR (200 MHz, D_6-DMSO) δ 22.2, 106.3, 110.1, 119.7, 130.8, 130.9, 134.6, 135.0, 143.8, 147.9, 149.2, 152.9, 168.2, 184.9; MS (EI) m/z: 255 ($M^{\bullet+}$, 14); 213 (100); 185 (72); 156 (7); 128 (4); 63 (14); 43 (9). HRMS (EI): calcd. for $C_{14}H_9N_1O_4$: 255.05315; found: 255.05330, 0.6 ppm error.

Resorufin tetradecanoate, **6**. The procedure was similar to the acetate derivative but myristic anhydride was added dropwise over 10 minutes to the solution, stirring in an ice bath. The deep purple reaction mixture immediately turned yellow. Several attempts to recrystallize the crude product were unsuccessful. We, therefore, dissolved the crude reaction in chloroform, added 1 x 3 cm of silica gel to the reaction, evaporated the slurry to dryness, then added the mixture to the top of a prepared silica gel column (5 x 15 cm) and ran a flash column using 2:1 hexanes: ethyl acetate as eluent. The spots containing the

product were combined, evaporated, then recrystallized from ethyl acetate/hexanes to afford 0.578 g (1.30 mmol, 33% yield) of the title compound as a bright orange solid: R_f = 0.46 (2:1, hexanes: ethyl acetate); mp = 113.0 – 114.8 °C; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.88 (t, 3H, J = 6.8 Hz), 1.26 (br. m, 20 H), 1.57 (m, 2H), 2.61 (t, 2H, J = 7.6 Hz), 6.34 (d, 1H, J = 2.0 Hz), 6.84 - 6.91 (dd, 1 H, J = 2.0 Hz, 9.8 Hz), 7.09 - 7.14 (dd, 1H, J = 2.8 Hz, 7.3 Hz), 7.15 (s, superimposed, 1H), 7.44 (d, 1H, J = 9.8 Hz), 7.81 (dd, 1H, J = 0.96 Hz, 8.3 Hz); ^{13}C-NMR (200 MHz, D_6-DMSO) δ 15.7, 24.2, 26.3, 30.5, 30.6, 30.7, 30.8, 30.9, 31.0, 31.1, 31.9, 32.0, 33.4, 106.7, 119.7, 121.8, 127.5, 131.3, 134.0, 135.0, 144.5, 148.2, 149.4, 153.5, 169.2, 185.9; MS (CI) m/z: 424 (MH^+, 27); 213 (100); 185 (18); 156 (3); HRMS under EI showed no molecular ion.

Resorufin t-butylacetate (resorufin 3,3-dimethylbutyrate), **10**. The procedure was the same as above but *t*-butylacetyl chloride (1.1 eq) was added dropwise over 10 minutes. The solution immediately turned a yellow-brown color. After 24 h, additional methylene chloride was added and then reaction was washed with saturated sodium bicarbonate, twice with distilled water, and dried over $MgSO_4$. The resulting orange solid was triturated with ethanol and the slurry filtered to afford 0.294 g (0.945 mmol, 25% yield) of the title compound as a bright orange solid: R_f = 0.33 (2.5:1 hexanes:ethyl acetate); mp = 163.1 – 163.7° C; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 1.16 (s, 9H), 2.49 (s, 2H), 6.34 (d, 1H, J = 1.9 Hz), 6.84-6.90 (dd, 1 H, J = 1.8 Hz, 9.9 Hz), 7.09 –7.14 (dd, 1H, J = 2.8 Hz, 7.3 Hz), 7.15 (s, superimposed, 1H), 7.44 (d, 1H, J = 10.0 Hz), 7.80 (d, 1H, J = 8.3 Hz); ^{13}C-NMR (200 MHz, $CDCl_3$) δ 31.1, 32.7, 49.0, 107.7, 110.2, 119.7, 131.3, 131.4, 135.0, 135.3, 144.4, 148.2, 149.3, 153.5, 169.6, 185.8; MS (EI) m/z: 311 ($M^{\cdot+}$, 8.2); 254 (4.6); 213 (100); 185 (24); 156 (4); 128 (3); 99 (10); 57 (22); HRMS (EI): calcd. for $C_{18}H_{17}N_1O_4$: 311.1158; found: 311.1155, 0.8 ppm error.

Spectrophotometric Assays

Separately measured initial rates of chromogenic esters. 4-nitrophenyl esters of 2-phenylpropanoic acid, **1**, and 2-(4-isobutylphenyl)propanoic acid, **2**, were emulsified in

aqueous solutions according to Vorderwübecke.[31] A solution of (*R*) or (*S*)-4-nitrophenyl-ester (500 μL of a 7.8 mM solution in acteonitrile) was added dropwise to Tris buffer (9000 μL, 50 mM, pH 7.5) containing 0.45 w/v% Triton X-100 (Pierce Surfact-Amps) and vortexed until clear. This emulsion remained clear for at least 3 hours. To measure the initial rates of hydrolysis, lipase solutions from Table 5.1 (100 μL) were added to the substrate emulsion (900 μL) in a cuvette at 25 °C and the linear increase in absorbance at 404 nm was monitored for 15 s. The final concentrations in the cuvettes were 0.369 mM substrate, 0.39% Triton X-100, 45 mM Tris buffer, 4.76% acetonitrile. No spontaneous chemical hydrolysis was detected. Solutions of 4-nitrophenyl esters of phenylalanine, **3**, in acetonitrile (5 μL of a 50 mM solution in acetonitrile) were added to HEPES buffer (1000 μL, 10 mM , pH 7.5). To measure the rates of hydrolysis, 5 μL of ANL solution was added and the change in absorbance monitored as above. The reported rates are corrected for spontaneous chemical hydrolysis ($t_{1/2} = 0.51$ h).

Pseudoracemate screen to determine the enantioselectivity of isopropanol-treated Candida rugosa lipase (IPA-CRL) towards 4-nitrophenyl-2-phenylpropanoate, **1**. Two sets of pseudoenantiomers were used. In the first set which use phenyl esters of 2-phenylpropionic acid **4**, the first assay solution was prepared by adding 4-nitrophenyl-(*S*)-2-phenylpropanoate (***S***)-**1** (5 μL of a 50 mM solution in DMSO) and phenyl-(*R*)-2-phenylpropanaote (***R***)-**4** (5 μL of a 50 mM solution in DMSO) to TES buffer, (559.4 μL of a 10 mM solution, pH 8) and were allowed to equilibrate to 25 °C. An aliquot of IPA-CRL (6.6 μL, 0.42 mg protein/mL) was added to the reaction mixture and the reaction was allowed to stir for various times. The final concentration during measurement were 0.43 mM of each pseudoenantiomer, 1.7% DMSO. The reaction was terminated upon addition of 500 μL of DMSO which fully dissolved the substrates and inactivated the enzyme. The absorbance of 4-nitrophenolate anion was monitored at 25 °C, 404 nm for different reaction times. The initial rate of formation was calculated by plotting the formation of 4-nitrophenolate anion vs. time and using the slope of the line as the rate. Rates of

hydrolysis in μmol/min/mg protein were calculated using an extinction coefficient of 15,800 $M^{-1}cm^{-1}$ and *l* of 1 cm. The ratio of the fast hydrolyzing pseudoracemate to the slow hydrolyzing pseudoracemate was used to calculate E, equation 5.4. The second step of the screen is prepared in a similar fashion using 4-nitrophenyl-(*R*)-2-phenylpropanoate, (R)-**1**, and phenyl-(*S*)-2-phenylpropanaote, (*S*)-**4**, as substrates but only 500 μL of TES buffer was added. We added 10 times more enzyme to these assay solutions to linearly and accurately measure the rate of the slow reacting 4-nitrophenol ester (66 μL IPA-CRL solution, 0.84 mg protein/mL).

The second set of pseudoenantiomers for 4-nitrophenyl-(±)-2-phenylpopanoate, **1**, use the enantiomerically pure esters of 2-chloroethyl-2-phenylpropanoate, **5.** Measurements are carried out identically as above.

Quick E measurements of chromogenic esters, **1-2**. Substrate solutions were the same as for separately measured initial rate measurements, but a solution of resorufin tetradecanaote **6** (0.5 mL of a 1.59 mM solution in acetonitrile) was added slowly to the vortexing solutions of pure enantiomers. It was necessary to use a 2-fold increase in the concentration of Triton X-100 to solubilize resorufin tetradecanoate. The emulsions remained clear for at least 2 hours. To measure the initial rates of hydrolysis, lipase solutions from Table 5.3 (100 μL) was added to the substrate emulsion (900 μL) in a cuvette at 25°C and the linear increase in absorbances at 404 nm and 574 nm were monitored for 15 s. Rates are calculated in μmol/min/mg protein after taking into account the amount of protein in each cuvette. The final concentrations in the cuvettes were 0.351 mM substrate, 0.071 mM resorufin tetradecanoate, 0.73% Triton X-100, 43 mM Tris buffer, 9% acetonitrile. No spontaneous chemical hydrolysis was detected at 404 nm or 574 nm.

Quick E measurements of 1-phenethyl thioacetate,**7**. The procedure was the same as above with the following exceptions. A solution of (*R*) or (*S*)-1-phenethyl thioacetate, 7 (118 μL of a 42.4 mM solution in acetonitrile), and Ellman's reagent (500 μL of a 12.6 mM

solution dissolved in 4:1 acetonitrile:50 mM Tris buffer, pH 8).was added dropwise to Tris buffer (9000 μL, 50 mM, pH 8.0) containing 0.9 w/v% Triton-X100 (Pierce Surfact-Amps) and 382 μL acetonitrile as cosolvent. To this, a solution of resorufin acetate **9** (500 μL of a 1.80 mM solution in acetonitrile) was added slowly to the vortexing solutions of pure enantiomers. To measure the initial rates of hydrolysis, subtilisin Carlsberg (100 μL of a 3.14 mg solid/mL solution in 50 mM Tris buffer, pH 8) was added to the substrate emulsion (1000 μL) in a cuvette at 25 °C and the increase in absorbance at 412 nm and 574 nm were monitored for 200 s. The final concentrations in the cuvettes for measurements were 0.433 mM thioacetate, 0.545 mM Ellman's reagent, 0.078 mM resorufin acetate, 0.7% Triton X-100, 44 mM Tris buffer, 12 % acetonitrile. A significant rate of spontaneous cleavage of DTNB was detected under these conditions (1.93×10^{-2} μmol/min). Rates of hydrolysis by subtilisin Carlsberg were corrected for background formation of 4-nitrothiolate anion. We did not detect an increase in the background rate of cleavage of DTNB in the presence of 0.1 mM resorufin. Buffers should be degassed prior to use to minimize oxidation of sulfhydryl groups during the measurements.

Quick E measurements of solketal butyrate, 8, using pH indicators These measurements were carried out using 96-well microplates and a microplate reader. The assay solutions were prepared by mixing the pH-indicator, 4-nitrophenol (3,000 μL of a 0.9115 mM solution in 5.9 mM BES, pH 7.2), BES buffer (2560 μL of a 5.0 mM solution containing 0.33 mM (2.11%) Triton X-100, pH 7.2), and acetonitrile (28.6 μL), then vortexing the solution. (*R*)-solketal butyrate (R)-**8** (37.4 μL of a 168.6 mM solution in acetonitrile), and resorufin acetate, **9** (374 μL of a 1.685 mM solution in acetonitrile) were added dropwise to the slowly vortexing solution to ensure the formation of micelles and clear solutions. Hydrolase solutions (5 μL/well) were added to the wells and the assay solution (100 μL/well) was added quickly using an 8-channel pipette. The final concentration in each well is 1.0 mM solketal butyrate, 0.1 mM resorufin acetate, 4.65 mM BES buffer, 0.434 mM 4-nitrophenol, 0.134 mM (0.86%) Triton X-100, 7% acetonitrile. The plate was

placed quickly in the microplate reader, shaken for 10 s to ensure complete mixing and the simultaneous decrease in absorbance at 404 nm and increase at 574 nm were monitored at 25 °C as often as permitted by the microplate software, typically every 11 seconds. Data were collected for 15 minutes. Each hydrolysis was carried out in quadruplicate and was averaged. The procedure was repeated for the other enantiomer (*S*)-**8**. Activities were calculated with equations 5.10 and 5.11 using the slopes of the linear, initial portions of the curves where $\Delta\varepsilon_{404\ nm}$ = 17,300 $M^{-1}cm^{-1}$ and $\Delta\varepsilon_{574\ nm}$ = 15,140 $M^{-1}cm^{-1}$ (both experimentally determined for our conditions) and l = 0.306 cm. To calculate the specific activities (μmol/min/mg protein), we took into account the total amount of protein in each well as determined by the Bio-Rad protein Assay.

Isopropanol treatment of Candida rugosa lipase. Lipase was purified by the method of Colton *et. al.*[10] Crude Candida rugosa lipase (20 g. solid, 800 units by PNPA assay) was dissolved in MES buffer (100 mL, 50 mM, pH 6.0, 4 °C) by stirring for 30 minutes. Isopropanol (100 mL, 4 °C) was added dropwise over 30 minutes and allowed to stir for 48 h at 4 °C. The solution turned from clear to cloudy while stirring. A precipitate was removed by centrifuging at 3000 rpm for 30 min at 4 °C. The supernatant was dialyzed against doubly distilled water (3 x 4 L) and concentrated to 20 mL by ultrafiltration under N_2 using an Amicon PM-10 membrane: 692 units with PNPA assay, 16.8 mg protein by the Bio-Rad protein assay using BSA as the standard, 87% yield. The clear yellow enzyme solution was stored at 4 °C with 0.02% wt/vol % NaN_3 as preservative.

General procedure for small-scale enzyme-catalyzed hydrolyses of esters to determine the true E. Lipases (100 mg solid or 0.7 mg protein of IPA-CRL) were added to Tris HCl buffer (9 mL, 10 mM, pH 8) and stirred for 30 minutes to ensure complete dissolution. Substrates (100 mg) were dissolved in 1 mL acetonitrile and added to the stirring solution and the rate of hydrolysis was monitored by pH stat which maintained the pH at 8 by automatic titration with 0.0965 N NaOH. Reactions were terminated by extracting the remaining starting material with diethyl ether (3 x 20 mL). The aqueous phase was then acidified to pH 2 with 1 N HCl and the product acid extracted with diethyl ether (3 x 20

mL). Ethanol was added dropwise during the workup to break up the emulsions and inactivate the enzymes. Both extracts were dried with $MgSO_4$, filtered and concentrated *in vacuo*. For analysis, the remaining starting material was converted to the acid using aqueous NaOH (1.5 equiv.) in ethanol. The enantiomeric excesses were measured by HPLC as described below.

Small-scale enzyme-catalyzed hydrolyses of (±)-sec-phenethyl thioacetate, 7, to determine the true E. The procedure was similar to above with the following changes. Subtilisin Carlsberg (2 mg) was added to Tris buffer (9 mL, 10 mM, pH 8) prior to addition of the substrate. Reactions were terminated by extracting the mixture with ethyl acetate (3 x 20 mL). These extracts, containing both the ester substrate and alcohol product, were dried over $MgSO_4$ the mixture concentrated *in vacuo*. The product alcohol was separated from the remaining acetate by preparative-scale TLC using 3:1 hexanes:ethyl acetate as developing solvent. The enantiomeric excesses were measured by HPLC as described below. Specific activity towards (±)-1-phenthyl thioacetate = 2.85 μmol/min/mg protein.

Enantiomeric excess determination by HPLC.

Enantiomers of **1** and **2** were analyzed using a Daicel OD-H column at 25 °C, 254 nm, 1.0 mL/min. Absolute configurations were confirmed with authentic samples.

2-phenylpropanoic acid: 98:2:1 hexanes:isopropanol:TFA; $k_{R'} = 3.35$; $k_{S'} = 4.05$; $\alpha = 1.2$; $R_s = 2.13$.

4-nitrophenyl-2-(4-isobutylphenyl)propanoate: 100:1:0.1 hexanes:isopropanol:TFA; $k_{R'} = 2.41$; $k_{S'} = 2.93$; $\alpha = 1.21$; $R_s = 1.66$.

2-(4-isobutylphenyl)propanoic acid: 100:1:0.1 hexanes:isopropanol:TFA; $k_{R'} = 3.31$; $k_{S'} = 4.27$; $\alpha = 1.29$; $R_s = 2.43$.

Enantiomers of 1-phenethyl thioacetate, **7**, were analyzed using a Daicel OJ-R column at 25 °C, 254 nm, 98:2:1 hexanes: isopropanol: TFA; 0.8 mL/min. Absolute configurations were confirmed with authentic samples and by comparison to the literature.[40]

1-phenethyl -thioacetate: $k_{S'} = 3.25$; $k_{R'} = 5.45$; $\alpha = 1.67$; $R_s = 2.49$.

1-phenethyl thiol: $k_{S'} = 3.71$; $k_{R'} = 4.26$; $\alpha = 1.15$; $R_s = 1.56$.

Enantiomers of phenylalanine were analyzed using a Crownpak CR(+) column at 25 °C, 200 nm, 0.8 mL/min, with aq. $HClO_4$, pH 2 as the mobile phase.

Phenylalanine: $k_D' = 3.93$; $k_L' = 5.20$; $\alpha = 1.32$; $R_s = 3.86$.

pK_a measurements

pKa of Resorufin Several groups report the pK_a of resorufin to be approximately 6. We believed this value was the pK_a of the ring nitrogen and not the alcohol moiety so we determined the pK_a using two methods to confirm our results. Note that the commercial form of resorufin sodium salt (Sigma-Aldrich) contains approximately 25% impurities so we carried out all pK_a measurements with resorufin (95%, Sigma-Aldrich).

Spectrophotometric titration: A solution of resorufin (659 µL of a 3.15 mM solution in 5 mM BES buffer) wad diluted to 25 mL with 5 mM BES buffer to give a final concentration of 0.083 mM resorufin. This concentration is similar to that in solution at initial rates concentrations in the Quick E measurements. [41] This solution was acidified with 2 M HCl to pH 2.8, an aliquot removed (100 µL) and its absorbance at 574 nm determined with a microplate reader. An aliquot of NaOH (100 µL of a 0.0981 N solution) was added to the resorufin solution and allowed to stir. This procedure was repeated until a final pH of 10.6. The absorbance at 574 nm was plotted vs. the pH, and the pH value where the absorbance was 50% of the maximal value equaled the pK_a of resorufin under these conditions. By the method, a pK_a value of 8.15 was measured. The experiment was repeated using doubly distilled water rather than buffer, and pK_a value was 8.13.

Potentiometric titration: A solution of resorufin was titrated with 9.81 mM NaOH and the change in pH per volume of base added was recorded. The volume of base at the equivalence points was noted and the half volume of the equivalence points gave two pK_a values at 6.0 and 8.13.

pka of 4-nitrophenol. The spectrophotometric titration was carried out as above, for 4-nitrophenol (10 mg dissolved in 10 mL of 5 mM BES buffer). The pK_a was measured to be 7.1. This value agreed with the reported literature value.

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Chapter 6

Since the discovery of AZT as a potent inhibitor of the human immunodeficiency virus (HIV)[1], 2',3'-dideoxynucleoside analogs have figured prominently in the development of effective antiviral agents. However, different stereoisomers of these nucleoside analogs exhibit different anti-viral activities and cytotoxicities, so synthetic routes that control the relative and absolute stereochemistry at the two epimerizable stereocentres of nucleoside analogues are of considerable interest.

BioChem Pharma Inc. (Laval, Quebec), developed the powerful anti-AIDS drug, 3TC, and is a world leader in the research and development of nucleoside analogues as therapeutic agents. 3TC and related dideoxynucleoside analogs are effective inhibitors of HIV replication because they are phosphorylated *in vivo* to their corresponding 5'-triphosphates, which act as both competitive inhibitors of the reverse viral transcriptase and as chain terminators. Several of the chemical synthetic routes to pure stereoisomers of nucleoside analogues are difficult and/or expensive to carry out. We collaborated with BioChem Pharma Inc. to discover alternate routes to pure stereoisomers of nucleoside analogues using hydrolases.

In this chapter, we apply the screening methods described in Chapters 3 through 5 to rapidly identify stereoselective hydrolases for the production of the key diastereomer intermediates of dioxolane nucleoside analogues. To do this, we extend our methods for screening for enantioselectivity to screening for diastereoselectivity.

Finally, this chapter illustrates that enzymes are amazing, versatile chemists. We use a hydrolytic enzyme to produce a key intermediate of a therapeutic agent. In turn, this therapeutic agent can inhibit DNA polymerase, the key synthetic enzyme used for viral replication in the human immunovirus.[2]

This work is currently under patent review in collaboration with BioChem Pharma Inc. and will, thereafter, be submitted for publication.

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Hydrolase-catalyzed separation of *cis* and *trans* diastereomers of 2-benzyloxymethyl-4-(*S*)-carboxylic acid-1,3-dioxolane methyl ester; useful intermediates for the synthesis of dioxolane nucleosides.

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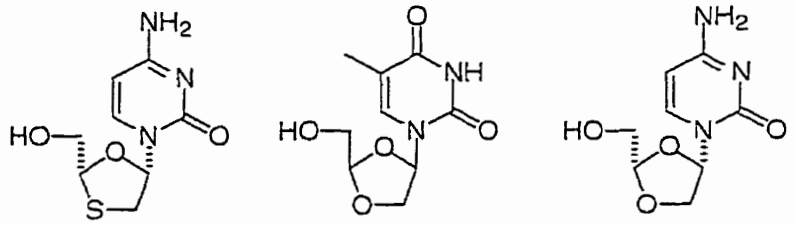
Abstract: Dioxolane nucleosides, in which an oxygen replaces the carbon in the 3' position in the ribose moiety of dideoxy nucleosides, are powerful pharmacological agents, particularly for the treatment of AIDS and hepatitis-B virus. However, their synthesis remains a significant challenge since the relative and absolute stereochemistry of the two stereocentres must be controlled. Several promising routes to pure stereoisomers of a key intermediate dioxolane yield a mixture of configurations at the 2-stereocenter. Separation of these mixtures by silica gel chromatography yields the pure diastereomers but this process is tedious and expensive for large-scale preparation. In this paper, we report that two inexpensive, commercially-available proteases - α-chymotrypsin and bovine pancreatic protease – discriminate between the *cis* and *trans* diastereomers of 2-(*R,S*)-benzyloxymethyl-1,3-dioxolane-4-(*S*)-carboxylic acid methyl ester (diastereoselectivity = 29-35). We discovered these selective hydrolases by screening a library of 91 commercial hydrolases with our previously developed activity and stereoselectivity screens that use pH indicators. A small-scale α-chymotrypsin-catalyzed hydrolysis of a mixture of *cis* and *trans* diastereomer methyl esters yields the desired *cis* dioxolane methyl ester in >98% diastereomeric excess and in good yield. Computer modeling of

transition state analogues of both diastereomers in the active site of α-chymotrypsin rationalizes the observed preference of the *trans* diastereomer on the molecular level.

Introduction

3'-Thia- and oxa-substituted 2', 3'-dideoxynucleoside analogues are an important class of pharmacological agents, for example 3'-thia-dideoxynucleoside 3TC (β-*L*-(-)-2'-deoxy-3'-thiacytidine) **1**, is a potent inhibitor of the human immunodeficiency virus (HIV), Figure 6.1.[1] Similarly, 3'-oxa-dideoxynucleosides possess anti-viral activity as well as anti-tumour activity. (±)-Dioxolane-T, **2**, and its natural (+)-*L*-diastereomer possess anti-HIV and anti-HBV (hepatitis-B virus) activities[2] while β-dioxolane 5-(2-bromovinyl)uracil is active against herpes simplex virus type 1 and 2[3]. β-*L*-(2*S*, 4*S*)-dioxolane cytidine (BCH-4556) **3**, the 3'- oxa derivative of 3TC exhibits potent anti-tumour activity towards human prostate cancer and renal carcinoma, the latter for which there is currently no effective therapeutic agent.[4]



1 3TC

2 (±)-dioxolane-T (*cis* conformations)

3 BCH-4556

Figure 6.1. Examples of dideoxynucleoside analogues in clinical use or under clinical evaluation.

Since stereoisomers of dioxolane nucleosides usually have different biological activities and toxicities, many researchers have explored synthetic routes to pure stereoisomers of dioxolane nucleosides. These routes must control the relative and

absolute stereochemistries at two potentially epimerizable centres of the dioxolane ring.

Researchers have published multi-step routes to pure stereoisomers. Two routes use expensive carbohydrates as starting materials. 1,6-Anhydro-β-*L*-gulose is used for the synthesis of *L*-(2*S*)-dioxolane nucleosides[5] and 1,6-anhydro-*D*-mannose is used for the synthesis of *D*-(2*R*)-dioxolane nucleosides.[2b,6] Glycolic acid and *tert*-butyldiphenylsilyl-protected glyco aldehyde is used to synthesize β-(2S)-dioxolane thymidine.[7] Although these procedures yield pure stereoisomers of dioxolane nucleosides, they are too lengthy and expensive for large-scale synthesis.

Routes starting from less expensive carbohydrates set one stereocentre but not the other, thus yielding mixtures of diastereomers of 2-benzyloxymethyl-4-carboxylic acid-1,3-dioxolanes, the key intermediate in the synthesis of dioxolane nucleosides. Combining protected benzyloxyacetaldehyde with D-mannitol,[8] L-ascorbic acid,[9] (±)-methyl glycerate[2a] or combining benzyloxyacetaldehyde with 2,3-O-isopropylidene-(*S*)-glyceric acid[2a, 10] yield, after a few steps, the diastereomers in Figure 6.2. Separation of the mixtures of diastereomers by silica gel chromatography or fractional crystallization sets the stereochemistry at the 2-position. Next, oxidative decarboxylation to their 4-acetates prepare the molecules for coupling to the base with a Lewis acid catalyst, but destroys the stereochemistry at the 4-position.[11] A second separation of diastereomers resets the stereochemistry at the 4-position.

To replace the expensive separation of diastereomers with silica gel, we explored hydrolase-catalyzed routes to separate diastereomers. Hydrolytic enzymes (hydrolases) are well documented as useful chiral catalysts for the production of enantiomerically-pure alcohols and carboxylic acids. Their commercial availability, relatively low cost, and tolerance for a wide class of substrates make them attractive biocatalysts.[12]

Different diastereomers of 2-benzyloxymethyl-1,3-dioxolane-4-carboxylic acid are common intermediates in the syntheses of mixtures of dioxolane nucleosides, Figure 6.2.

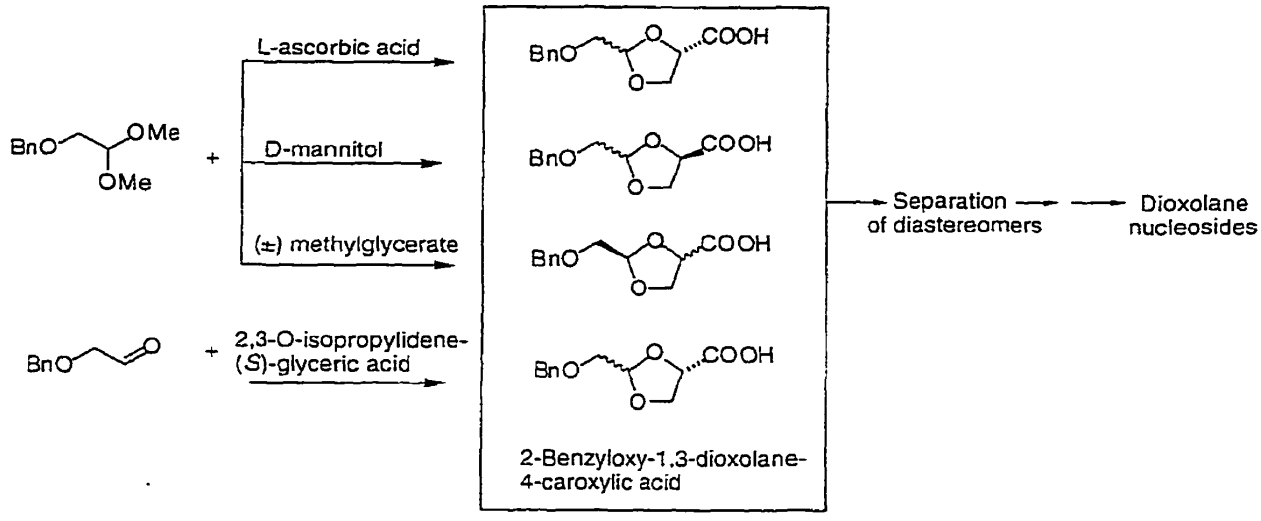
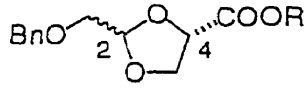


Figure 6.2 Synthetic routes to diastereomerically-pure dioxolanyl nucleosides via mixtures of key intermediate dioxolane acids.

We produce a mixture of (2*S*, 4*S*)- and (2*R*, 4*S*) dioxolanes **3** using a synthetic procedure starting from benzyloxyacetaldehyde and 2,3-O-isopropylidene-(S)-glyceric acid methyl ester.[2a] We therefore focus upon hydrolase-catalyzed routes to the separation of mixtures of *cis*-(2*S*, 4*S*) and *trans*-(2*R*, 4*S*) dioxolanes by selective hydrolysis of their methyl ester derivatives, **4**. Importantly, this separation sets the ultimate stereochemistry of the final dioxolane nucleoside analogue at the 4'-position (equivalent to the 2-position of the dioxolane intermediate).



3: R = H
4: R = Me

Researchers have previously used hydrolases to separate enantiomers of thiaoxolane nucleosides and their precursors.[13] Researchers from the University of Leeds and Glaxo-Wellcome used lipase from *Pseudomonas fluorescens* for the preparation of

homochiral (ee >90%) α-acetoxysulfides and α,β-diacetoxy sulfides via kinetic[14,15] and dynamic[16] resolutions. These optically-pure intermediates can subsequently undergo acid catalyzed cyclization with control of stereochemistry at the thioacetal chiral centre, to yield optically-pure thiaoxolanes, the key intermediate of 3TC. Hoong *et. al.* used pig liver esterase (PLE) to selectively hydrolyse butyryl esters of enantiomeric mixtures of FTC (2',3'-dideoxy-5-flouro-3'-thiacytidine) and BCH-189, the racemic form of 3TC.[17] Storer and coworkers used a 5'-ribonucleotide phosphohydrolase from *Crotalus atrox* to selectively hydrolyse the (+)-monophosphate enantiomer of (±)-BCH-189 with > 99% diastereomeric excess.[18]

Less work has been carried out with the dioxolane nucleosides. To our knowledge, the only reported enzyme-catalyzed route to diastereomerically-pure dioxolane nucleosides or their precursors used adenosine deaminase to selectively deaminate purine dioxolane nucleosides in high diastereomeric excess.[19]

To identify selective hydrolases towards a target substrate, traditionally a researcher carries out several small-scale kinetic resolutions with a small number of commercially-available hydrolases, then determines the selectivity by determining the optical purities of the reaction products.[20] This method is time-consuming because it requires workup of the reactions and measuring optical purities. In general, a researcher does not test all available hydrolases so potentially selective hydrolases are likely missed. To increase our chance of finding a highly selective commercial hydrolase towards dioxolane methyl ester, **4**, we used our previously developed methods to rapidly screen a library of commercial hydrolases for activity and diastereoselectivity towards a 1:1 *cis*: *trans* mixture of **4** in 96-well plates using pH indicators.[21] Our screening has identified two hydrolases, α-chymotrypsin and bovine pancreatic protease, with high diastereoselectivities, D = 29 - 35. We optimized these reactions then carried out a preparative-scale separation of a mixture of the diastereomers to obtain the desired *cis* dioxolane **4** in very high diastereomeric excess (>98%) and in good yield. This work represents our first application of screening for diastereoselectivity.

Results

Estimated diastereoselectivity

We first estimated the diastereoselectivity of ninety-one commercial hydrolases towards the *cis*-(2*S*, 4*S*) and *trans*-(2*R*, 4*S*) dioxolanes, **4**, by measuring the initial rates of hydrolysis of the two pure diastereomers separately. The ratio of the two rates is the estimated diastereoselectivity, Table 6.1. Note that this ratio is NOT the true diastereoselectivity, or diastereomeric ratio, D, because we measured the rates of the pure diastereomers separately.[22] To rapidly and accurately determine the rates of ester hydrolysis, we used a microplate reader and pH indicator (4-nitrophenol) as described previously, Figure 6.3.[21]

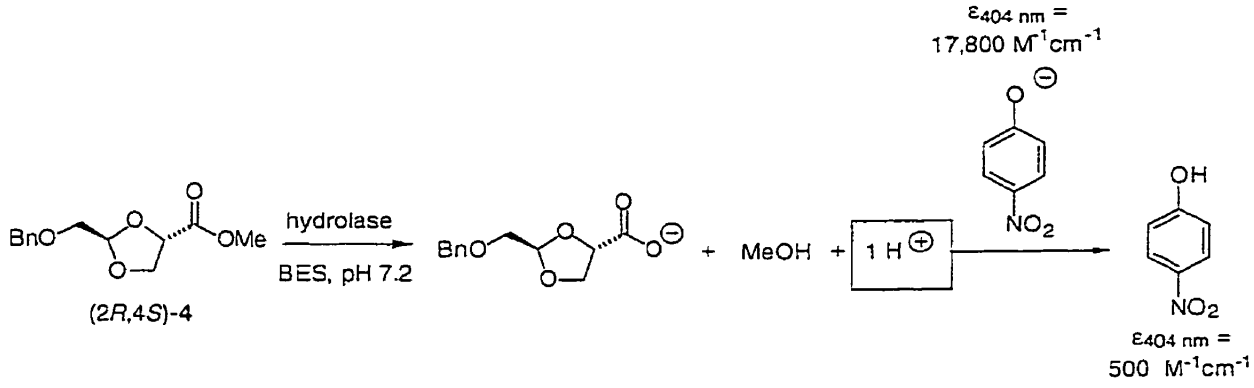


Figure 6.3 First step in the screen for estimated diastereoselectivity. Enzyme-catalyzed hydrolysis of *trans*-(2*R*,4*S*)-dioxolane methyl ester releases a proton. We measure this rate of release with a pH indicator; the rate of color change of the pH indicator is proportional to the enzyme-catalyzed rate of hydrolysis. The second step of the screen is the same but uses the *cis*-(2*S*,4*S*) dioxolane methyl ester. The ratio of rates of hydrolysis estimates the diastereoselectivity.

Fourteen of the ninety-one hydrolases did not catalyze hydrolysis of either diastereomer (activity <0.01 μmol/min/mg protein). Of the remaining seventy-seven active hydrolases, seven showed estimated diastereoselectivities >8, all favoring the *trans* dioxolane. Three of these hydrolases - bovine pancreas protease, α-chymotrypsin and subtilisin from *Bacillus licheniformis* - showed no detectable hydrolysis toward the *cis*

dioxolane during 20 minutes of screening. For these hydrolases we report an estimated diastereoselectivity of >100. Bovine cholesterol esterase showed an estimated diastereoselectivity of 157. Protease from *Streptomyces caespitosus*, Diversa clonezyme ESL-001-02, and horse liver esterase showed modest estimated diastereoselectivities of 18, 13 and 8, respectively. Although twenty-one hydrolases favored the *cis* diastereomer, their estimated diastereoselectivites were low, D < 1.1 – 6.9. All subsequent experiments focus on six of the *trans* selective hydrolases. We omitted the Diversa clonezyme because it was expensive and showed only a moderate estimated diastereoselectivity.

True diastereoselectivity via quick D

To measure the diastereoselectivity of these six hydrolases more accurately, we used our previously published method called "quick E".[23] Quick E is a spectophotmetric assay to rapidly determine the enantioselectivity of hydrolases towards chiral substrates without measuring enantiomeric purities. We add an achiral reference ester, resorufin acetate **5**, to the assay solutions of the pure dioxolane methyl esters. This addition introduces competition between two substrates for the enzyme active site in each step of the assay.

We first measured the rates of hydrolysis of the *trans* dioxolane methyl ester and resorufin acetate simultaneously, Figure 6.4. The ratio of relative rates (in μmol/min) calculated using Equations 6.2 and 6.3 (see experimental section) after accounting for the initial concentration of both substrates, yields the selectivity of the hydrolase toward the *trans* dioxolane, **4**, vs. resorufin acetate, **5**, $(k_{cat}/k_M)_{\text{trans dioxolane}}/(k_{cat}/k_M)_{\text{resorufin acetate}}$. In the second step of the screen, we measured the true selectivity of the hydrolase toward the *cis*-dioxolane, **4**, vs. resorufin acetate, **5**. Since the reference compound is the same in both experiments, the ratio of the two true selectivity ratios calculated using Equation 6.4 (see Experimental section) yields the true diastereoselectivity, $(k_{cat}/K_M)_{\text{trans dioxolane}}/(k_{cat}/K_M)_{\text{cis dioxolane}}$.

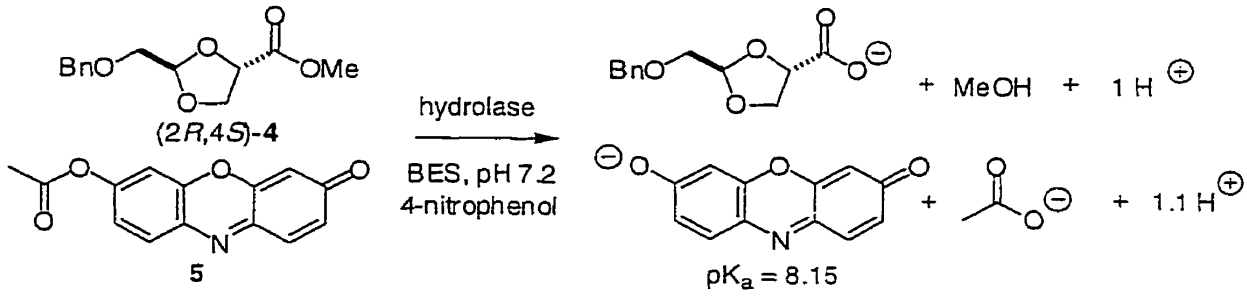


Figure 6.4 First step of the Quick D measurement toward *trans*-(2*R*, 4*S*) dioxolane methyl ester, **4**. Enzyme-catalyzed hydrolysis of the *trans*-(2*R*,4*S*)-dioxolane methyl ester and the reference compound, resorufin acetate **5**, releases protons which produce a decrease in the yellow absorbance of the pH-indicator present in solution, and a brilliant pink chromophore, resorufin anion. Both of the absorbance changes, at 404 nm and 574 nm respectively, can be monitored simultaneously. The simultaneous initial rates of hydrolysis of each compound are calculated in μmol/min using Equations 6.2 and 6.3. The ratio of relative rates yields the selectivity ratio after taking into account the initial concentrations of each substrate. The second step of the Quick D is the same, except that it uses the *cis*-(2*S*, 4*S*)-dioxolane methyl ester. The ratio of selectivity ratios is the diastereoselectivity, Equation 6.4.

Table 6.1. Activity and estimated diastereoselectivity of commercial hydrolases towards *cis*-(2*S*,4*S*) and *trans*-(2*R*,4*S*) dioxolane methyl esters, **4**.

Source of active hydrolase[a]	Wt.[b]	Prot.[c]	Supplier	Activity *trans*[d]	Activity *cis*[d]	Estimated D[e]
Lipases						
Aspergillus niger	30	0.61	*f*	0.188	0.0615	3.06 (*trans*)
Aspergillus oryzae	7.1	4.5	*g*	0.141	0.0613	2.30 (*trans*)
Candida antarctica lipase A	34	4.9	*h*	0.0457	0.0730	1.60 (*cis*)
Candida lipolytica	35	0.23	*f*	0.358	0.256	1.40 (*trans*)
Candida rugosa	31	0.35	*i*	0.358	0.225	1.59 (*trans*)
Candida rugosa (cylindracea)	37	0.71	*j*	0.777	0.327	2.38 (*trans*)
Candida utilis	4.7	0.053	*g*	5.94	1.69	3.51 (*trans*)
Humicola sp.	13	4.0	*h*	0.259	0.342	1.32 (*cis*)
Mucor javanicus	32	1.1	*f*	0.755	0.374	2.02 (*trans*)
Mucor miehei	60	1.3	*f*	0.536	0.729	1.36 (*cis*)
Penicillin camembertii	86	0.92	*f*	1.83	2.09	1.14 (*cis*)
Penicillin roquefortii	57	0.74	*f*	0.839	0.625	1.34 (*trans*)
Pseudomonas cepacia	31	3.1	*f*	0.00644	0.0199	3.09 (*cis*)
Pseudomonas sp., lipoprotein lipase	1.0	0.91	*g*	0.229	0.106	2.16 (*trans*)
Pseudomonas sp type B., lipoprotein lipase	5.2	3.0	*g*	1.15	0.283	4.06 (*trans*)
Rhizopus arrhizus	2.1	1.7	*g*	0.168	0.176	1.05 (*cis*)
Rhizopus javanicus	44	2.7	*f*	0.142	0.0625	2.27 (*trans*)
Rhizopus niveus	57	1.9	*h*	0.408	0.503	1.23 (*cis*)
Rhizopus oryzae	53	4.1	*f*	0.0690	0.0424	1.63 (*trans*)
Rhizopus stolonifer	31	1.2	*f*	0.760	0.682	1.11 (*trans*)
Thermus aquaticus	1.1	0.29	*g*	0.238	0.154	1.55 (*trans*)
Esterases						
Acetylcholine esterase	0.26	0.18	*i*	0.967	0.951	1.02 (*trans*)
Bacillus sp.	1.1	0.58	*g*	0.141	0.125	1.13 (*trans*)
Bacillus stearothermophilus	1.1	0.57	*g*	1.30	1.33	1.02 (*cis*)
Bacillus thermoglycosidasius	0.82	0.76	*g*	0.671	0.334	2.01 (*trans*)
Bovine cholesterol esterase	9.8	0.99	*k*	2.77	0.0176	157 (*trans*)
Candida lipolytica	3.5	1.4	*g*	0.183	0.229	1.25 (*cis*)
Candida rugosa	na	1.3	*l*	0.440	0.393	1.12 (*trans*)
Cutinase from *Fusarium solani pisi*, purified	2.1	1.1	*m*	3.47	1.81	1.92 (*trans*)
Cutinase from *Fusarium solani pisi*, crude	18	2.3	*m*	1.55	0.759	2.04 (*trans*)
E001	0.40	0.14	*n*	17.1	14.6	1.17 (*trans*)
E002	0.38	0.16	*n*	16.3	16.6	1.02 (*cis*)
E003	1.0	0.23	*n*	9.52	5.95	1.60 (*trans*)
E004	1.0	0.29	*n*	6.20	7.15	1.15 (*cis*)
E005	1.0	0.27	*n*	11.9	6.61	1.80 (*trans*)
E006	0.64	0.13	*n*	13.2	5.37	2.46 (*trans*)
E007	2.1	0.97	*n*	1.77	1.49	1.19 (*trans*)
E008	1.0	0.18	*n*	15.8	9.59	1.65 (*trans*)
E009	1.0	0.37	*n*	2.36	1.70	1.39 (*trans*)
E010	1.0	0.26	*n*	8.54	11.4	1.33 (*cis*)
E011	0.80	0.22	*n*	10.3	5.62	1.83 (*trans*)
E013	1.0	0.24	*n*	4.74	3.12	1.52 (*trans*)
E014	1.0	0.31	*n*	6.57	5.78	1.14 (*trans*)
E015	0.52	0.26	*n*	0.386	0.606	1.57 (*cis*)
E016	1.0	0.26	*n*	6.68	12.0	1.80 (*cis*)

Table 6.1 continued.

Source of active hydrolase[a]	Wt.[b]	Prot.[c]	Supplier	Activity *trans*[d]	Activity *cis*[d]	Estimated D[e]
E017b	1.0	0.33	*n*	7.32	4.84	1.51 (*trans*)
E018	2.0	0.76	*n*	1.10	2.05	1.86 (*cis*)
E019	0.60	0.20	*n*	5.52	4.43	1.25 (*trans*)
E020	0.44	0.16	*n*	11.6	10.3	1.13 (*trans*)
ESL-001-01	1.0	0.91	*o*	15.1	18.8	1.24 (*cis*)
ESL-001-02	1.0	0.44	*o*	8.81	0.657	13.4 (*trans*)
ESL-001-03	1.0	0.85	*o*	0.397	0.215	1.85 (*trans*)
ESL-001-04	1.0	1.0	*o*	0.128	0.0327	3.91 (*trans*)
ESL-001-05	1.0	0.70	*o*	1.125	0.359	3.13 (*trans*)
ESL-001-07	1.0	1.0	*o*	0.279	0.125	2.23 (*trans*)
Horse liver esterase	1.7	0.59	*g*	3.86	0.453	8.52 (*trans*)
Pig liver esterase	na	1.8	*i*	127	89.9	1.41 (*trans*)
Pig liver esterase	0.36	0.49	*g*	23.4	11.2	2.09 (*trans*)
Saccharomyces cerevisiae	1.2	0.26	*g*	1.03	0.841	1.22 (*trans*)
Proteases						
Aspergillus oryzae	29	7.0	*i*	0.140	0.127	1.10 (*trans*)
Aspergillus satoi	32	0.40	*i*	3.16	1.64	1.93 (*trans*)
Bacillus lichenformis	5.2	2.1	*g*	1.94	0.295	6.58 (*trans*)
Bacillus polymyxa	31	0.46	*i*	0.0876	0.0866	1.01 (*trans*)
Bac. subtilis var. Biotecus A	4.2	1.7	*g*	1.00	0.258	3.88 (*trans*)
bovine pancreas type 1	5.2	2.2	*i*	0.275	<0.01	>100 (*trans*)
α-chymotrypsin	5.0	3.0	*i*	0.329	<0.01	>100 (*trans*)
Optimase L660	na	29	*p*	0.108	0.113	1.05 (*cis*)
pepsin from hog stomach	24	0.49	*g*	0.0332	0.228	6.87 (*cis*)
Proteinase, bacterial	4.7	2.1	*g*	0.869	0.234	3.71 (*trans*)
Proteinase K	0.42	0.066	*g*	2.64	3.04	1.15 (*cis*)
Streptomyces caespitosus	23	0.26	*i*	0.237	0.0132	18.0 (*trans*)
subtilisin from *Bacillus licheniformis*	4.5	1.3	*g*	1.35	<0.01	>100 (*trans*)
Subtilisin Carlsberg	9.7	4.4	*i*	0.858	0.188	4.56 (*trans*)
Thermolysin, Type X	2.4	0.15	*i*	0.281	0.186	1.51 (*trans*)
thrombin from human plasma	2.0	0.026	*g*	0.473	2.18	4.61 (*cis*)
Acylases						
Aspegillus melleus	26	0.96	*g*	0.0519	0.0609	1.17 (*cis*)
hog kidney	1.1	0.71	*g*	0.781	0.238	3.28 (*trans*)

[a]The following hydrolases showed no detectable activity (<0.01 μmol/min/mg protein) towards either diastereomer: Lipases: *Candida antarctica B* (Novo-Nordisk), lipoprotein lipase from *Chromobacterium viscosum* (Fluka), porcine pancreatic (Biocatalysts), *Pseudomonas fluorescens* (Fluka); wheat germ (Sigma-Aldrich). Esterases: E012 (ThermoGen), ESL-001-06 (Diversa), *Thermoanaerobium brockii* (Fluka). Proteases: papaya (Sigma-Aldrich), Penicllin amidase from *E. coli* (Fluka), Prolidase from *Lactococcus lactis* (Fluka), rennin from *Mucor meihie* (Fluka), *Streptomyces griseus* (Calbiochem/Behrig), trypsin (Worthington). [b]Amount (mg) of solid enzyme per ml of buffer in the stock solutions. "na" = not available; received as a solution. [c]Protein concentration of stock solutions in mg protein/mL determined by the Bio-Rad assay using BSA as a standard. [d]Observed rate of hydrolysis in μmol/min/mg protein. Rates are

calculated in μmol/min using Equation 6.1 then divided by the total amount of protein in each well. The values are an average of four measurements, which typically varied by less than 2%. [e]Ratio of the separately measured initial rates for the diastereomers. This ratio is NOT the true diastereoselectivity, but is a useful estimate. The absolute configuration of the faster reacting ester is in parentheses. [f]Amano Enzyme USA Co., Ltd. (Troy, VA). [g]Fluka Chemie (Oakville, ON). [h]Boerhinger-Mannheim (Mannheim, Germany). [i]Sigma-Aldrich (Oakville, ON). [j]Biocatalysts Ltd. (Pontypridd, Mid Glam, Wales, UK). [k]Genzyme (Cambridge, MA). [l]Altus Biochemicals (Cambridge, Ma.). [m]Unilever Research Labs (Vlardingen, the Netherlands). [n]ThermoGen, Inc. (Chicago, IL). [o]Diversa Corp. (San Diego, CA) [p]Genencor (Palo Alto, CA).

The quick D values for the six hydrolases were significantly lower, sometimes more than twenty times lower, than the estimated diastereoselectivities, Table 6.2. The three hydrolases with estimated diastereoselectivities >100 showed quick D values of only 4.4 – 13. Cholesterol esterase (estimated diastereoselectivity of 157) showed the highest measured quick D value, D = 17. Horse liver esterase (estimated diastereoselectivity of 8.5) showed a true diastereoselectivity of 3.5, while protease from *Streptomyces caespitosus* (estimated diastereoselectivity of 18) showed a quick D diastereoselectivity of 1.6.

Quick D measurements reveal large differences in K_M values between the two pure diastereomers that are not always detected in measurements for estimated D. For example, bovine pancreatic protease showed very large differences in initial rates, estimated D > 100. However, its quick D value was significantly smaller, D = 12.8. Differences in the rates of hydrolysis of the reference compound in quick D reveal large differences in binding between the two pure diastereomers which are ignored in the estimated D assay, Figure 6.5. Resorufin acetate is hydrolyzed 6-fold more slowly in the presence of the *trans* diastereomer than in the presence of the *cis* diastereomer, suggesting that resorufin acetate binds in the active site, disrupting the binding of the *cis* diastereomer. Resorufin competes more fairly with the *trans* dioxolane substrate for the active site, so both exhibit comparable rates of hydrolysis. In the estimated D measurement, we could not detect hydrolysis of the *cis* dioxolane methyl ester, possibly due to tight binding of the substrate, with subsequently low substrate turnover. Differences in binding are difficult to detect when separately measuring initial rates of hydrolysis.

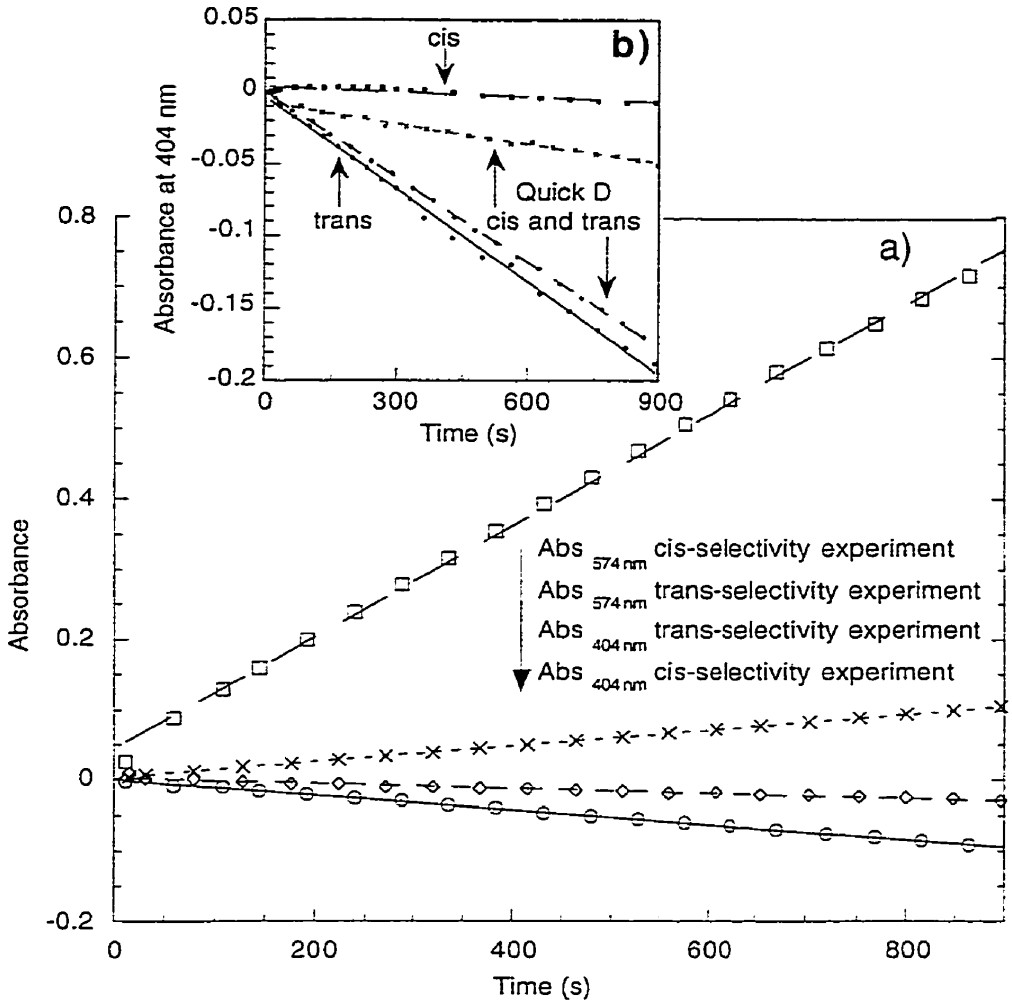


Figure 6.5 a) Initial rate measurements of the quick D measurement with bovine pancreatic protease. The measured absorbance changes at 404 nm (accounting for the protons released from both the pure diastereomer ester and resorufin acetate) and 574 nm (accounting for the released resorufin anion) are measured simultaneously in two separate selectivity steps. All absorbances are normalized to zero. Conditions in the well during assay: 2.0 mM *cis* or *trans* dioxolane methyl ester, 0.1 mM resorufin acetate, 4.65 mM BES, 0.434 mM 4-nitrophenol, 0.86% Triton X-100, 7% acetonitrile. Regression factors for each slope were >0.97. **b)** Relative rates of hydrolysis of the two diastereomers in the quick D measurement and in estimated D measurement, where no reference compound is present. For quick D measurements, the rates of hydrolysis of the pure diastereomer methyl esters were determined by subtracting the contribution of the protons released during resorufin hydrolysis from the total number of protons detected with the pH indicator, Equation 6.3. Conditions in the well during estimated D measurements were similar to quick D but no Triton X-100 was added.

Table 6.2. Diastereomeric ratios of hydrolases towards *cis*-(2*S*, 4*S*)and *trans*-(2*R*, 4*S*) dioxolane methyl esters, **4**, using the Quick D method.

Hydrolase[a]	time (s)[b]	*trans*-diastereomer + ref[c]		*cis*-diastereomer + ref[c]		Quick D[d]	Estimated D[e]
		$rate_{trans}$	$rate_{reference}$	$rate_{cis}$	$rate_{reference}$		
Bovine pancreatic protease	10-900	0.257	0.157	0.128	1.0	12.8 ± 2.3	>100
Cholesterol esterase	10-50	16.6	6.16	0.995	6.24	16.9 ± 4.6	157
α-Chymotrypsin	10-500	0.558	0.651	0.099	0.734	6.4 ± 1.42	>100
Horse liver esterase	10-200	7.69	0.286	2.019	0.265	3.5 ± 0.6	8.5
Streptomyces caespitosus protease	10-900	0.289	0.0826	0.187	0.0854	1.6 ± 0.4	18
Subtilisin from *Bacillus licheniformis*	10-50	4.91	9.43	1.15	9.79	4.4 ± 0.9	>100

[a]See Table 6.1 for details. [b]Data collection time. [c]Final concentration in each well: 2.0 mM dioxolane methyl ester, 0.1 mM resorufin acetate, 0.43 mM pnp, 4.65 mM BES buffer, 7.0% AcCN, 0.134 mM (0.86%) Triton X-100. Changes in absorbance at 404 nm and 574 nm were monitored simultaneously. Rates were calculated in μmol/min using Equations 6.2 and 6.3. Values are in μmol/min x 10^{-3}. No spontaneous chemical hydrolysis was detected. [d]Quick D calculated using Equation 6.4; for a complete derivation, see Janes, L. E.; Kazlauskas, R. J. *J. Org. Chem.* **1997**, *62*, 4560-4561. Average and standard deviation of four measurements. [e]Values from Table 6.1.

True diastereoselectivity via the endpoint method

To confirm that we measured the true diastereoselectivities correctly via "quick D" measurements, we also measured the diastereoselectivities by the traditional endpoint method (small-scale preparative reactions). Starting from a 1:1 mixture of the *cis*-(2*S*,4*S*) and *trans*-(2*R*,4*S*) diastereomers of **4** and using the same reaction conditions as the screening (2 mM substrate, 7% acetonitrile), we allowed the hydrolase to partially hydrolyze the ester. After separation of the product acid from the remaining ester, we measured the diastereomer composition of each by both ^{1}H-NMR spectroscopy and HPLC. Diastereoselectivities from these preparative reactions agree with those measured by the 'Quick D' screen to within the error limits for five of the six hydrolases, Table 6.3. Only with horse liver esterase, did two values differ significantly – 11.4 (preparative reaction) vs. 3.5 (Quick D). We attribute the lower diastereoselectivity in the Quick D experiment to the added surfactant, Triton X-100, which was not present in the preparative reaction. We previously noted a decrease in the enantioselectivity of horse liver esterase upon addition of Triton X-100.[21] These experiments confirm that the Quick D method accurately measures diastereoselectivities.

Optimization of reaction conditions

We also determined the diastereoselectivities at 80 mM substrate concentration which is more typical of a preparative reaction. Insoluble droplets of the substrate were visible at this concentration but no acetonitrile was added. Diastereoselectivities were measured by the endpoint method, Table 6.3. Quick D measurements could not be performed at this substrate concentration because spectrophotometric measurements require clear solutions. Under these new conditions, the diastereoselectivity of three hydrolases did not change (cholesterol esterase, protease from *Streptomyces c.*, subtilisin), that of one hydrolase decreased (horse liver esterase), and that of two hydrolases increased by a factor of 3.6 (α-chymotrypsin and bovine pancreatic protease). To test

Table 6.3. Diastereomeric ratios of hydrolases towards *cis* and *trans* dioxolane methyl esters **4** measured by the endpoint method.

Hydrolase[a]	S[b] (mM)	Time[c] (h)	de_s[d] (%)	de_p[d] (%)	C[e] (%)	Endpoint D[f]	Quick D[g]
Bovine pancreatic protease	2	5.1	53.8	70.6	43.3	9.83 ± 0.3	12.8 ± 2.3
Bovine pancreatic protease	80	3.2	65.9	89.5	42.3	35.7 ± 3.2	
Cholesterol esterase	2	1.4	44.2	78.3	36.1	12.6 ± 0.53	16.9 ± 4.6
Cholesterol esterase	80	2.6	68.5	69.7	49.6	11.3 ± 0.30	
α-Chymotrypsin	2	3.8	42.3	67.4	38.6	7.74 ± 0.21	6.4 ± 1.4
α-Chymotrypsin	80	7.5	75.5	85.5	46.9	28.9 ± 1.7	
Horse liver esterase	2	1.5	61.0	72.16	45.8	11.4 ± 0.35	3.5 ± 0.6
Horse liver esterase	80	8.0	28.7	46.2	38.3	3.56 ± 0.06	
Protease from *Streptomyces caespitosus*	2	17.1	12.9	20.2	39.0	1.70 ± 0.02	1.6 ± 0.4
Protease from *Streptomyces caespitosus*	80	26.2	10.9	20.0	35.3	1.66 ± 0.02	
Subtilisin from *Bacillus licheniformis*	2	1.25	54.0	57.9	48.3	6.33 ± 0.13	4.4 ± 0.9
Subtilisin from *Bacillus licheniformis*	80	4.5	58.8	55.1	51.6	6.1 ± 0.11	

[a]See Table 6.1 for details. [b]Substrate concentration in the reaction mixture. [c]Reaction time. [d]Measured diastereomeric excess of the remaining starting material (de_s) or product acid (de_p). [e]Degree of conversion calculated by $de_s/(de_s+de_p)$. [f]The diastereoselectivity was calculated from de_s and de_p as defined by Chen, C. S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299. The *trans* diastereomer was preferentially hydrolysed in all cases. Error limits on diastereoselectivities were calculated using an estimated 1% error in integration since the diastereomers were separated with baseline resolution. [g]Values from Table 6.2.

whether this increase in diastereoselectivity came from the increase in the substrate concentration (from 2 to 80 mM) or elimination of the acetonitrile, we also measured the diastereoselectivity of α-chymotrypsin at 80 mM substrate with 7% acetonitrile. Some substrate remained undissolved as droplets in the reaction mixture. The diastereoselectivity was an intermediate value, 16, higher that at 2 mM substrate with 7% acetonitrile (D = 7.7), but lower that at 80 mM substrate with no acetonitrile (D = 29). This intermediate value suggests that while 7% acetonitrile lowers the diastereoselectivity, the higher substrate concentration also lowers the diastereoselectivity. Other researchers have observed loss of activity and selectivity of α-chymotrypsin in the presence of organic solvents.[24]

For synthetic use, we tested the separation of 1:2 *trans:cis* mixture of **4** with α-chymotrypsin because our current synthetic route to the dioxolane acids yields a 1:2 mixture of *trans: cis* acids.[10] Using Sih's equation, we calculated that the desired *cis*-(2S, 4S) dioxolane methyl ester will have >98% de above 42% conversion.[25] We carried out the reaction at 80 mM substrate concentration, allowed the α-chymotrypsin to hydrolyse the substrate to 43% conversion, and isolated the remaining *cis*-ester in 83% theoretical yield and >98% de, Figure 6.6.

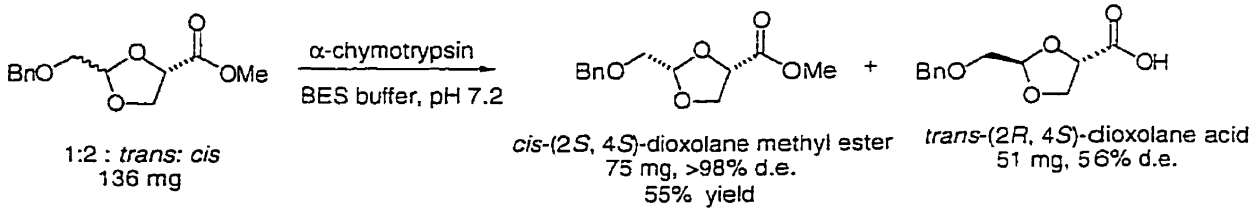


Figure 6.6 Preparative-scale separation of a 1:2 *trans:cis* mixture of dioxolane methyl esters using α-chymotrypsin

Computer modeling

To rationalize the high diastereoselectivity of α-chymotrypsin toward the dioxolanes, **4**, we used computer modeling of enzyme-transition state analog complexes.

Since α-chymotrypsin is a serine protease, the first step in an ester hydrolysis is the attack of the Oγ of the active site serine at the ester carbonyl forming a tetrahedral intermediate.[26] Collapse of this tetrahedral intermediate releases the alcohol. We assume that the transition state involved in formation or collapse of this first tetrahedral intermediate defines the selectivity of chymotrypsin. To mimic this transition state, we used the phosphonates linked to α-chymotrypsin shown in Figure 6.7. Indeed, researchers have shown that phosphonates mimic well the transition state for ester hydrolysis.[27]

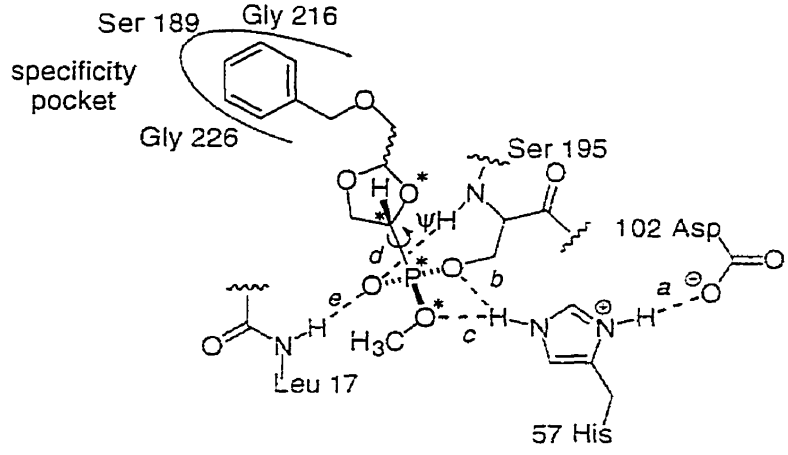


Figure 6.7 Transition state analog for the α–chymotrypsin-catalyzed hydrolysis of dioxolane esters *trans*- and *cis*-**4**. The Oγ of the catalytic serine (Ser 195) attacks the ester carbonyl once it binds in the active site. This attack forms the tetrahedral intermediate. The phosphonate above (black lines) mimics this tetrahedral intermediate. Chymotrypsin residues are drawn in gray. Five hydrogen bonds, marked *a*-*e*, stabilize the charges. To be judged catalytically competent, the minimized structures must contain all five hydrogen bonds. Three conformations for each dioxolane were constructed by varying the torsion angle marked ψ and defined by the atoms marked with asterisks. For each conformation, the phenyl ring was fit into the hydrodphic pocket manually.

To position the phosphonate within the active site, we first linked the phosphorus covalently to the Oγ of serine 195. To position the dioxolane ring, we considered all three possible staggered conformations along the phosphorus-carbon bond.

This gave three conformations for *cis*-**4** (a-c) and for *trans*-**4** (a-c). When *N*-acyl amino acids esters or amides bind to α-chymotrypsin, the *N*-acyl group favors a region called the *am*-site (amide site). This site is polar because it contains a hydrogen bond acceptor, the carbonyl oxygen of Ser 214. For the dioxolanes, the three different staggered conformations alternately place either the oxygen, the methylene, or the hydrogen in this *am*-site. For each conformation, we manually adjusted the orientation of the benzyl moiety so that it fit into α-chymotrypsin's specificity pocket. This specificity pocket accounts for α-chymotrypsin's preference for amides and esters of amino acids with aromatic side chains, e.g. phenylalanine.[28] The binding of hydrophobic groups to this pocket (S_1 pocket) dominates the binding of molecules to the active site, so we assumed that the benzyl group binds in this pocket.

We assumed that a catalytically productive complex required all five hydrogen bonds within the catalytic machinery shown in Figure 6.7. To assign a hydrogen bond, we looked, first, for a donor to acceptor atom distance of less than 3.1 Å. Second, we looked for a nearly linear arrangement of donor atom, hydrogen, and acceptor atom. We considered an angle of 120° or more as nearly linear. We classified any structure lacking two or more of the five hydrogen bonds as non-productive. However, if a structure lacked only one hydrogen bond and it was only slightly outside the limits set above, then we classified it as possibly productive. The two lowest energy structures for the trans isomer (*trans*-**4**c and –**4**b) are possibly catalytically productive structures, Table 6.4. One or two hydrogen bonds in each structure are weak. On the other hand, the lowest energy structures for the cis isomer (*cis*-**4**a and –**4**b) are probably not productive. The lengths of the hydrogen bonds are significantly too long. We interpret these modeling results as follows. The most favorable orientations for the trans isomer are productive complexes, so this isomer reacts. The most favorable orientation for the cis isomer is not productive, so this isomer binds, but does not react. To react it must bind in a much higher energy orientation.

Table 6.4 Minimized structures for the transition state analogs for the α-chymotrypsin-catalyzed hydrolysis of dioxolane esters *trans*-**4** and *cis*-**4**.

Conformation	ψ[a]	Group in *am*-site[b]	H-bond 'c' distance, Å [c] (N–H–O angle)	Steric energy,[d] kcal/mol	Comments
trans-4c	61°	H	3.55 (154°)	1.1	possibly productive, H-bond 'b' is also weak: 3.40 Å, 142°.
trans-4b	-64°	CH_2	3.67 (161°)	2.7	possibly productive
trans-4a	-177°	O	4.25 (164°)	6.8	not productive
cis-4a	-179°	O	3.99 (157°)	0	not productive
cis-4b	-62°	CH_2	3.80 (152°)	2.7	unlikely productive
cis-4c	59°	H	3.22 (143°)	6.7	possibly productive, H-bond 'd' between amide NH of Ser 195 and phosphonyl oxygen is very weak: 3.65 Å, 140°

[a]Dihedral angle along the phosphorus-carbon bond as defined by the asterisks in Figure 6.7. [b]When *N*-acyl amino acid esters or amide bind to the active site of α-chymotrypsin, the *N*-acyl group binds in a polar region containing a hydrogen bond acceptor, the carbonyl oxygen of Ser 214. The part of the dioxolane that binds in this region is indicated. [c]See Figure 6.7 for the definitions of the hydrogen bonds. Unless otherwise noted, hydrogen bonds 'a', 'b', 'd', and 'e' (His 57 to Aps 102, amide NH of Ser 195 to the phosphonyl oxygen, and amide NH of Leu 17 to the phosphonyl oxygen) were present (N–O distance 2.8 – 3.1 Å, N–H–O angle 130° – 170°) in all structures. [d]Energies are relative to the lowest energy of the six structures.

Discussion

Extending two previously developed screening methods for activity and enantioselectivity to measure diastereoselectivity, we rapidly identified two active and selective proteases, α-chymotrypsin from bovine pancreas and bovine pancreatic protease, useful for the separation of diastereomeric mixtures of *cis*-(2*S*, 4*S*) and *trans*-(2*R*, 4*S*) dioxolane methyl esters. We then optimized the α-chymotrypsin catalyzed separation of a 1:2 *trans:cis* mixture of the dioxolane methyl ester **4** to yield the cis-(2*S*, 4*S*) dioxolane methyl ester in high d.e. (>98%) and good yield (55% overall from a 1:2 *trans:cis* mixture). This enzymatic separation simplifies the preparation of a key intermediate in the synthesis of dioxolane nucleosides.

We previously reported that screening for estimated E with separate enantiomers accurately estimated the enantioselectivities of a library of hydrolases towards solketal butyrate.[21] Other researchers have also used the initial rates of hydrolysis of pure enantiomers to estimate enantioselectivity.[29] However, we found the separately measuring the initial rates of hydrolysis of dioxolane methyl esters **4** overestimated the diastereoselectivities of the most selective hydrolases by as much as 20-fold. Only one of six estimated D values agreed with the true diastereoselectivities determined by the endpoint method. The true diastereoselectivity is the ratio of the specificity constants (k_{cat}/K_M) for each diastereomer. When we measure the diastereomers separately in the screen for estimated D, we eliminate competitive binding between the two diastereomers. At saturating substrate concentrations, the initial rate equals the k_{cat}; at partially saturating substrate conditions, the initial rate also depends on the K_M value.[30] Thus, the ratio of initial rates ignored differences in binding between the two dioxolane methyl esters.

Quick D accurately determined diastereoselectivity because competition for the active site is reintroduced in the screen upon addition of a reference compound. When multiple substrates compete for the enzyme active site (in this case, a pure dioxolane of **4**

and resorufin acetate, **5**), the ratio of their initial rates of hydrolysis, after taking into account their initial concentrations, equals their relative k_{cat}/K_M values.[31] Importantly, the rate of turnover of all substrates decreases but the stereoselectivity of an enzyme does not change in the presence of a competitive inhibitor, so addition of resorufin acetate does not change the hydrolase's diastereoselectivity towards the dioxolane methyl esters, **4**.[32] In the presence of the reference compound, relative k_{cat} and K_M values for each substrate are determined and the ratio of the two selectivity experiments yields the diastereoselectivity.

The two most selective hydrolases identified by our screening methods were both proteases isolated from bovine pancreas and may indeed be the same enzymes. Although bovine pancreatic protease (Sigma) is a crude preparation while α-chymotrypsin (Sigma) is a purified protein, both had similar activity toward 4-nitrophenyl acetate and both had essentially the similar protein content, 30 and 32% wt. % protein/wt. % solid, respectively. Both had similar activity in the scale-up reactions with 80 mM substrate (2.4 U/mg protein for α-chymotrypsin, 1.8 U/mg protein for bovine pancreatic protease) and in screening reactions toward 2 mM *trans*-dioxolane methyl ester (0.329 μmol/min/mg protein for α-chymotrypsin; 0.275 μmol/min/mg for bovine pancreatic protease). The initial rates towards the *cis*-dioxolane methyl ester were too slow to measure for both hydrolases. These similar activities and diastereoselectivities suggest that chymotrypsin is present in commercial bovine pancreatic protease and may be responsible for the observed activity and diastereoselectivity. Both hydrolases are also similar in cost (α-chymotrypsin = $0.55/U, bovine pancreatic pancreas = $0.42/U) so either is useful for preparative scale separations.[33]

Other groups have separated diastereomers by selective hydrolysis of their ester derivatives.[34] Konigsberger *et. al.* use *Candida rugosa* lipase (CRL) to separate a mixture of *cis* and *trans* methyl esters of 4-substituted of 4-cyclohexanecarboxylates.[35] Ogasawara and coworkers use *Pseudomonas cepacia* (PCL) lipase to selectively acylate and hydrolyze mixtures of *cis* and *trans* 4-*tert*-butylcylcohexanemethanols and 1,4-

cyclohexanedimethanol.[36, 37] In these cases, both PCL and CRL prefer the *trans* isomer with moderate to excellent diastereoselectivity. Dioxolane methyl esters are diastereomers of 5-membered ring systems which are much more flexible than the six-membered cyclohexane ring systems above so stereoselectivity is more demanding. 1,3-Dioxolane rings are flexible even with bulky alkyl substituents at the 2- and 4-positions.[38]

We chose to separate diastereomers of a key intermediate rather than diastereomers of the final dioxolane nucleosides. The cost of each intermediate increases with each step in the synthesis of the dioxolane nucleosides so separating mixtures of the final compound is costly and wastes material. We have also observed that hydrolases exhibit lower activity towards the final nucleosides than towards their smaller, key intermediates.[39]

In summary, a procedure has been developed for the hydrolase-catalyzed separation of a mixture of *cis* and *trans* 2-benzyloxymethyl-4-carboxylic acid-1,3-dioxolane methyl esters. Our rapid screening techniques identified α-chymotrypsin from bovine pancreas and bovine pancreatic protease as active, diastereoselective catalysts from a library of 91 commercial lipases, esterases, proteases and acylases. This separation is adaptable to the large-scale preparation of 2-(S)-benzyloxymethyl-4-(S)-carboxylic acid-1,3-dioxolane methyl ester, the key intermediate in anti-viral and anti-cancer agents, including BCH-4556.

Experimental

General. Chemicals were purchased from Sigma-Aldrich Co. (Oakville, ON) and were used without further purification unless stated. Enzyme suppliers are noted in the footnotes of Table 6.1. All microplate assays were performed on a Spectramax 340 microplate reader with SOFTmax PRO version 1.2.0 software (Molecular Devices, Sunnyvale, CA). Polystyrene 96-well flat-bottomed microplates (maximum volume 360 µl/well, Corning Costar, Acton, MA) were filled using Eppendorf 8-channel pipettes (5-

100 µl, 50-1,200 µl) and solution basins for multi-channel pipettes (Fisher Scientific, Nepean, ON). The initial rates of the small-scale enzyme-catalyzed hydrolysis reactions were measured with a Radiometer RTS 822 pH stat. NMR were recorded on a Varian Gemini 200 MHz and a Bruker 400 MHz spectrometer. Melting points were taken on an Electrothermal melting point apparatus and were corrected.

Dioxolane methyl esters, **4**. The (2*S*, 4*S*)-*cis* and (2*R*, 4*S*)-*trans* dioxolane substrates were synthesized from benzyloxyacetaldehyde and 2,3-O-isopropylidene-(S)-glyceric acid methyl ester following a procedure by Norbeck et. al.[2a] The *cis* and *trans* acids were separated by silica gel chromatography using 99/0.5/0.5 dichloromethane/methanol/acetic acid as eluent, then converted to methyl esters using dimethoxypropane and catalytic HCl.[40] The diastereomeric excess of the *cis* ester was 98.7%, while the *trans* ester was 99.6% by HPLC. R_f=0.41 (2:1 hexanes:ethyl acetate).

2-(R)-benzyloxymethyl-4-(S)-carboxylic acid-1,3-dioxolane methyl ester (trans diastereomer): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 3.56-3.65 (m, 2H), 3.78 (s, 3H), 3.99 (dd, 1H, 8.2 Hz, 5.4 Hz), 4.31 (dd, 1H, *J* = 8.2 Hz, 11.8 Hz), 4.66 (dd, 1H, *J* = 7.1 Hz, 6.3 Hz), 4.69 (s, 2H), 5.33 (t, 1H, *J* = 4.6 Hz), 7.27-7.35 (m, 5H). ^{13}C-NMR ($CDCl_3$, 400 MHz) δ 51.4, 67.2, 69.2, 72.7, 73.0, 103.3, 125.7, 126.8, 127.4, 136.8, 170.3

2-(S)-benzyloxymethyl-4-(S)-carboxylic acid-1,3-dioxolane methyl ester (cis diastereomer): ^{1}H-NMR ($CDCl_3$, 400 MHz) 3.63-3.72 (m. 2H), 3.74 (s, 3H), 4.10 (dd, 1H, *J* = 8.6 Hz, 7.5 Hz), 4.25 (dd, 1 H, *J* = 8.7 Hz, 3.8 Hz), 4.60 (dd, 1H, *J* = 7.3 Hz, 3.8 Hz), 4.63 (s, 2H), 5.23 (t, 1H, 4.6 Hz), 7.27-7.37 (m, 5H). ^{13}C-NMR ($CDCl_3$, 400 MHz) δ 51.4, 67.5, 69.8, 72.7, 72.8, 103.8, 125.3, 125.8, 127.4, 135.9, 170.0.

Resorufin acetate, **5**. This compound was prepared using a modified procedure by Kramer

and Guilbault.[41] To a slurry of resorufin sodium salt (95% purity, 1.02 g, 4.32 mmol) in 60 mL anhydrous dichloromethane, was added anhydrous pyridine (0.349 mL, 4.32 mmol, 1 eq). The solution was cooled in an ice bath, then acetyl chloride (0.614 mL, 8.63 mmol, 2 eq) was added dropwise over 10 minutes. The deep purple reaction mixture immediately turned orange. The reaction was warmed to room temperature and stirred overnight. The reaction was diluted with dichloromethane to 300 mL and filtered through a coarse glass frit to remove unreacted resorufin, and the solvent was removed *in vacuuo*. The reddish-orange residue was recrystallized from ethanol yielding a crimson powder, 0.48 g (1.89 mmol, 44% yield). R_f = 0.20 (2:1, hexanes/ethyl acetate); mp = 217 – 220 °C (sample darkens at 215 °C) [literature = 223 – 225 °C (uncorrected)[41]]; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 2.37 (s, 3H), 6.34 (d, 1H, J = 1.8 Hz), 6.90-6.84 (dd, 1 H, J = 2.1 Hz, 9.9 Hz), 7.11 –7.16 (dd, 1H, J = 2.2 Hz, 7.3 Hz), 7.16 (s, superimposed, 1H), 7.44 (d, 1H, J = 9.7 Hz), 7.81 (d, 1H, J = 4.4 Hz). This ^{1}H-NMR spectrum matches that reported previously.[42] ^{13}C-NMR (d_6-DMSO, 200 MHz) δ 22.2, 106.3, 110.1, 119.7, 130.8, 130.9, 134.6, 135.0, 143.8, 147.9, 149.2, 152.9, 168.2, 184.9; MS (EI) m/z: 255 ($M^{\bullet+}$, 14); 213 (100); 185 (72); 156 (6.6); 128 (4.2); 63 (14); 43 (9.0). HRMS (EI): calcd. for $C_{14}H_9N_1O_4$: 255.0532; found: 255.0533, 0.6 ppm error.

Hydrolase library. Since most hydrolases have maximal activity near neutral pH 7, we screened all hydrolases at pH 7.2. Previously, we optimized our pH indicator assay for neutral pH.[21] The hydrolases were dissolved in BES buffer (5.0 mM, pH 7.2 containing 0.02% NaN_3 as preservative) at the concentrations listed in Table 6.1 (0.26 - 86 mg solid/mL solution). $CaCl_2$ (2 mM) was added to the protease solutions since some proteases require calcium ions to maintain their structure. For crude samples of hydrolase, we used saturated solutions (up to 86 mg solid/mL) but for purified hydrolases, we chose lower concentrations (typically 1 mg solid/mL). Each solution was centrifuged to remove insoluble material (5 min, 2,000 rpm) and titrated to a final pH of 7.2. The protein

concentrations were determined using a dye-binding assay from Bio-Rad (Mississauga, ON) with bovine serum albumin (BSA) as the standard. Solutions were stored in a 96-well assay block 'mother plate' equipped with aluminum sealing tape (2 mL maximum volume in each well, Corning Costar, Acton, MA) at –20 °C. This 'mother plate' speeds up repeated screens using the same hydrolases and is a convenient way to store large libraries of hydrolases. Hydrolytic activity of the libraries is maintained over several months.

Estimated diastereoselectivity. The assay solutions were prepared by mixing *cis* or *trans* dioxolane methyl ester (504 μL of a 50.0 mM solution in acetonitrile), acetonitrile (386 μL), 4-nitrophenol (6,000 μL of a 0.9115 mM solution in 5.0 mM BES, pH 7.2) and BES buffer (5,110 μL of a 5.0 mM solution, pH 7.2). This solution was vortexed to ensure complete mixing. Hydrolase solutions (5 μL/well) were transferred from the mother plate to a 96-well microtiter plate using an 8-channel pipette. Assay solution (100 μL/well) was quickly added to each well using a 1,200 μL 8-channel pipette. The final concentrations in each well were 2.0 mM substrate, 4.65 mM BES, 0.430 mM 4-nitrophenol, 7.1% acetonitrile. The plate was quickly placed in the microplate reader, shaken for 10 s to ensure complete mixing and the decrease in absorbance at 404 nm was monitored at 25°C as often as permitted by the microplate software, typically every 11 seconds. The starting absorbance was typically 1.2. Data were collected for twenty minutes to ensure we detected slow reactions and reactions with a lag time. Each hydrolysis was carried out in quadruplicate and was averaged. The first 10 s of data were sometimes erratic, possibly due to dissipation of bubbles created during shaking. For this reason, we typically excluded the first 10 s of data from the calculation of the initial rate. Activities were calculated from slopes in the linear portion of the curve usually over the first two hundred seconds. The initial rates were calculated from the average dA/dt, using Equation 6.1 where $\Delta\varepsilon = 17{,}300\ M^{-1}\ cm^{-1}$ (accounting for the fully deprotonated and protonated forms of the pH indicator, experimentally determined under our conditions) and $l = 0.306$ cm.

To calculate specific activity (μmol/min/mg protein), we took into account the total amount of protein in each well.

$$rate = \frac{dA_{404nm}}{dt} \times \frac{[buffer]}{[indicator]} \times \frac{1}{\Delta\varepsilon_{404nm} \cdot l} \times rxn\ volume \times 10^6 \qquad \text{Eq. (6.1)}$$

True diastereoselectivity using Quick D. The assay solutions were prepared by mixing 4-nitrophenol (6,000 μL of a 0.9115 mM solution in 5.0 mM BES, pH 7.2), BES buffer (5,120 μL of a 5.0 mM solution containing 0.33 mM (2.11%) Triton X-100 pH 7.2). and acetonitrile (41.6 μL), then vortexing the solution. Dioxolane methyl ester (90.4 μL of a 279 mM solution in acetonitrile) and resorufin acetate (748 μl of a 1.685 mM solution in acteonitrile, N.B. The solubility limit of resorufin acetate in acetonitrile is approximately 2 mM in acetonitrile) were added dropwise to the slowly vortexing solution to ensure the formation of micelles and clear solutions. Hydrolase solutions (5 μL/well) were transferred from the mother plate to a 96-well microtiter plate using an 8-channel pipette. Assay solution (100 μL/well) was quickly added to each well using a 1,200 μL 8-channel pipette. The final concentrations in each well were 2.0 mM dioxolane methyl ester, 0.1 mM resorufin acetate, 4.65 mM BES, 0.434 mM 4-nitrophenol, 0.134 mM (0.86%) Triton-X100, 7% acetonitrile. The plate was quickly placed in the microplate reader, shaken for 10 s to ensure complete mixing and the simultaneous decrease in absorbance at 404 nm and increase in absorbance at 574 nm were monitored at 25° C as often as permitted by the microplate software, typically every 17 seconds. Data were collected for twenty minutes. Each hydrolysis was carried out in quadruplicate and was averaged. The first 10 s of data were sometimes erratic, possibly due to dissipation of bubbles created during shaking. For this reason, we excluded the first 10 s of data from the calculation of the initial rate. This procedure was repeated for the other enantiomer with the reference

compound. Rates of hydrolysis in μmol/min were calculated using Equations 6.2 and 6.3 from slopes of the linear, initial portion of the curve where $\Delta\varepsilon_{404} = 17{,}300\ M^{-1}\ cm^{-1}$ and $\varepsilon_{574} = 15{,}140\ M^{-1}\ cm^{-1}$ (both experimentally determined for our conditions) and $l = 0.306$ cm.

$$rate_{reference} = \frac{dA_{574nm}}{dt} \times \frac{1}{\varepsilon_{574} \cdot l} \times rxn\ \ volume \times 10^6 \qquad \text{Eq. (6.2)}$$

$$rate_{dioxolane\ ester} = \left[\frac{dA_{404\,nm}}{dt} \times \frac{[buffer]}{[indicator]} \times \frac{1}{\Delta\varepsilon_{404\,nm} \cdot l} \times rxn\ volume \times 10^6 \right] - 1.1\left[rate_{reference} \right] \qquad \text{Eq. (6.3)}$$

For very active hydrolases, we used the slope of 50 seconds but for less active hydrolases, we used up to the first 900 seconds. The quick D value was calculated from the ratio of initial rates from the two screening steps, after taking into account the initial concentrations of the substrates, Equation 6.4.

$$D = \frac{rate_{trans\ dioxolane}}{rate_{reference}} x \frac{[reference]}{[trans\ dioxolane]} \Big/ \frac{rate_{cis\ dioxolane}}{rate_{reference}} x \frac{[reference]}{[cis\ dioxolane]} \qquad \text{Eq. (6.4)}$$

True diastereoselectivity using small-scale reactions. The reaction conditions mimic those in the microplate during estimated diastereoselectivity except that no indicator is present. Hydrolase solutions (500 μL) were added to solutions of (1:1 *cis:trans*)-dioxolane methyl ester (3.5 mL of a 28.77 mM in acetonitrile) and BES buffer (46mL of a 5.0 mM solution, pH 7.2) for a final reaction volume of 50 mL (2.0 mM substrate, 4.65 mM BES, 7% acetonitrile). The final solutions were clear. The rates of hydrolysis were monitored by pH-stat which maintained the pH at 7.2 by automatic titration with 0.0981 N NaOH.

Reactions were usually terminated at 40% conversion, as noted on the pH-stat by extracting the starting material ester with ethyl acetate (3 x 20 mL). The aqueous layer was adjusted to pH 2 with 1 N HCl and the dioxolane acid extracted with ethyl acetate (3 x 20 mL). Ethanol was added dropwise during the workups when necessary to break up emulsions. Both extracts were dried with $MgSO_4$, filtered and concentrated *in vacuo*. The diastereomeric excesses were measured as described below and the diastereomeric ratio, D, was calculated according to Sih.[20] There was no detectable chemical hydrolysis of the substrate at pH 7.2. We did not observe any diastereomer enrichment during extraction of ester and acid into ethyl acetate.

Scale-up reactions with 80 mM substrate. Hydrolase solution (500 µL) was added to a solution of 1:1 cis/trans dioxolane methyl ester (212 mg) and BES buffer (9,800 µL of a 5.0 mM solution, pH 7.2) for a final reaction volume of 10.5 mL (80.0 mM substrate, 4.9 mM BES). The substrate was present as oil droplets. Reactions were carried out and worked up as above.

Determination of diastereomeric purity by NMR. Analysis was performed on a Varian Gemini 200 MHz NMR spectrometer in $CDCl_3$. The *trans* ester shows a triplet at δ 5.33 (^3J = 4.6 Hz) and the *cis* ester shows a triplet up field at δ 5.23 (^3J = 4.6 Hz). The *trans* acid shows a triplet at δ 5.33 (^3J = 3.6 Hz), while the *cis* acid shows a broad singlet up field at δ 5.19. We did not observe any epimerization of the substrate or product acid during workup.

Determination of diastereomeric purity by HPLC. To further confirm the diastereomeric excesses determined by NMR, we also determined diastereomeric excesses using HPLC. Diastereomeric excesses agreed well, typically <1% variation between values determined by NMR and HPLC. Analyses were performed on a Waters Alliance 2690 Separations

module (Solvent and Sample Management), Waters 996 photodiode array detector and Millennium 2010 Chromatography Manager Version 2.15. Diastereomers of the dioxolane acids were analyzed using a YMC ODS-AM 5 μm, 120 A, 4.6 mm ID x 250 mm column (YMC, Wilmington, N.C) and a Waters in-line pre-column filter kit. The mobile phase was composed of H_2O + 0.01% TFA, pH 3.28 (pH was adjusted using 5 N NaOH) and CH_3CN + 0.01% TFA. A gradient was run from 30% CH_3CN to 55% CH_3CN over 50 minutes with a flow rate of 0.5 mL/min. The detector was set at 210 nm: k'_{trans} = 3.21, k'_{cis}= 3.52, α = 1.10, R_s = 1.33. The same HPLC system was used to analyze the diastereomers of the dioxolane methyl esters but we used Millennium 32 Version 3.00 software and a Phenomenex Prodigy ODS 3 μm 100 A 4.6 mm ID x 250 mm column (Phenomenex, Torrence, CA) with a Waters in-line pre-column filter kit. The composition of the mobile phase was isocratic 25% CH_3CN + 0.01% TFA and 75% H_2O + 0.01% TFA, pH 3.28. The flow rate was 1.0 mL/min. and the detector was set at 200 nm: k'_{cis} = 23.6, k'_{trans} = 24.9, α = 1.05, R_s = 2.5.

Enzyme-catalyzed synthesis of cis-(2S, 4S)-dioxolane methyl ester. A 2:1 mixture of *cis:trans* dioxolane methyl ester (136.5 mg, 0.541 mmol) was weighed into a reaction vessel and BES buffer (6,263 μL of a 5 mM solution, pH 7.2) was added. The substrate remained as insoluble droplets. α-Chymotrypsin[43] (500 μL, 0.019 units by PNPA assay) was added to begin the reaction and the rate and degree of hydrolysis was monitored by a pH-stat which maintained the pH at 5 by automatic titration with 0.0981 N NaOH. The reaction was terminated at 43 % conversion for high diastereomeric purity as determined by Sih's equations for recycling, by extracting the remaining starting material ester with ethyl acetate (3 x 20 mL). The aqueous layer was adjusted to pH 2 and the product acid extracted with ethyl acetate (3 x 20 mL). Both extracts were dried with $MgSO_4$, filtered and concentrated *in vacuo*. The diastereomeric excesses were measured by NMR spectroscopy as described above and the diastereomeric ratio, D, was calculated according

to Sih.[20] By this method, we obtained the *cis*-(2*S*, 4*S*)-dioxolane methyl ester (2-(*S*)-benzyloxymethyl)-4-(*S*)-carboxylic acid-1,3-dioxolane methyl ester) as a clear oil in 55% overall yield, with >98 % diastereomeric excess.

Modeling of transition state analogs in α-chymotrypsin. All modeling was done with *Discover*, version 2.9.7 (Biosym/MSI, San Diego, CA) using the AMBER force field. We used a distance dependent dielectric constant of 4.0 and scaled the 1-4 van der Waals interactions by 50%. The distance dependent dielectric constant damps long range electrostatic interactions to compensate for the lack of explicit solvation.[44] Results were displayed using *Insight II* version 95.0 (Biosym/MSI). The starting structure was the x-ray crystal structure of tosylated bovine alpha chymotrypsin[45] obtained from Brookhaven protein data bank (file 2cha). Using the Biopolymer module of *Insight II*®, hydrogen atoms were added to correspond to pH 7.0. Histidines were uncharged, aspartates and glutamates were negatively charged and arginines and lysines were positively charged. The catalytic histidine (His) was protonated. The tosyl group, which covalently linked to Ser195 in the x-ray structure, was replaced by a phosphonate analog shown in Figure 6.7.

Energy minimization proceeded in four stages. First, 200 iterations of steepest descent algorithm, all protein atoms constrained with a force constant of 10 kcal mol^{-1} $Å^{-2}$; second, 200 iterations of conjugate gradients algorithm with the same constraints; and third, 500 iterations of conjugate gradients algorithm with only the backbone constrained by a 10 kcal mol^{-1} $Å^{-2}$ force constant. For the fourth stage, minimization was continued using conjugate gradients algorithm without any constraints until the rms deviation reached less than 0.005 Å mol^{-1}. Crystallographic water molecules were included in all minimizations. Water molecules and the substrate were not constrained through any of the minimization cycles.

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Summary

Hydrolytic enzymes are excellent catalysts for the production of pure stereoisomers but selecting suitable hydrolases from hundreds of commercial hydrolases towards a synthetic target is a major deterrent to their widespread application in organic synthesis. In this thesis, we report the development of methods to speed up the selection process.

For a first approach, researchers often use empirical rules to predict the enantiopreference of a hydrolase towards a substrate. These rules are a summary of the observed enantiopreferences from resolutions previously reported in the literature and crudely identify the structural features of a substrate necessary for good enantioselectivity. Lipase from *Aspergillus niger* (ANL) has been widely applied as a chiral catalyst for the production of stereoisomers but these applications were largely discovered by trial and error. We focussed on the development of empirical rules for ANL towards chiral secondary alcohols and chiral carboxylic acids to help chemists rationally apply ANL to solve synthetic problems. Similar to other lipases, we found that the relative size of the substituents at the stereocentre sets the enantioselectivity of ANL towards secondary alcohols. With carboxylic acids, we found that a positively charged amino group sets the enantioselectivity for ANL. This structural feature is unusual since lipases have evolved in nature to hydrolyze uncharged, lipophilic substrates. Purification of ANL increased its enantioselectivity towards α-amino acids, particularly cyclic amino acids such as proline, by removing a contaminating amidase.

Although empirical models help predict the outcome of a hydrolase-catalyzed kinetic resolution, a researcher must always experimentally determine the enantiomeric ratio, E, which is the degree of selectivity the hydrolase has for one enantiomer over the other. Currently, the endpoint method is the standard approach to evaluate E. A researcher runs a small scale kinetic resolution of a racemic substrate with each hydrolase, then determines the enantiomeric purities of the reaction products to calculate E. This approach is unsuitable for rapidly testing large number of hydrolases because it requires measuring enantiomeric purities.

To avoid this slow process, researchers often estimate the enantioselectivity by measuring the initial rates of hydrolysis of chromogenic esters of both pure enantiomers

in separate hydrolysis experiments. The ratio of initial rates estimates the enantioselectivity. We developed a quantitative, colorimetric assay to measure the rate of hydrolysis of any ester using pH indicators. It is not always possible to derivatize a target substrate into a chromogenic ester. Hydrolysis of an ester releases protons; the rate of proton release can be easily measured using a pH indicator. Moreover, when the buffer and the pH indicator have the same affinity for protons (similar pK_a values) the absorbance kinetics of the indicator are directly proportional to the true enzyme kinetics. We used this method to estimate the enantioselectivities of a library of commercial hydrolases towards a chiral primary alcohol, solketal butyrate, and quickly identified horse liver esterase as the most enantioselective hydrolase reported to date for the hydrolysis of an ester of solketal.

Estimating enantioselectivity is fast but can be inaccurate because some or all of the effects of competition between the enantiomers for the active site, reflected in the K_M values of the pure enantiomers, is ignored with separately measured initial rates. To account for differences in K_M between the two enantiomers, we added a reference compound to the separately measured initial rate measurements of pure enantiomers. The relative rates of hydrolysis of the pure enantiomers versus the reference compound yields a ratio of the specificity constants in each hydrolysis experiment that accounts for contributions from K_M to the observed rates, $(k_{cat}/K_M)_S/(k_{cat}/K_M)_{reference}$. The ratio of the two competition experiments yields the enantiomeric ratio, $(k_{cat}/K_M)_S/(k_{cat}/K_M)_R$. We called this method quick E because it accurately determines the enantiomeric ratio in minutes.

We first validated quick E using chromogenic esters as substrates because their rates of hydrolysis are easily measured spectrophotometrically. Next, to generalize quick E to any ester, we applied our pH indicator assay to measure initial rates of hydrolysis during quick E measurements.

Finally, as proof of the usefulness of the screening methods developed in this thesis, we apply these methods to solve an industrial problem. Dioxolane nucleosides are powerful pharmacological agents used for the treatment of HIV and renal and prostate cancers. However, their synthesis remains a significant challenge since the relative and absolute stereochemistry of the two stereocentres must be controlled. Current routes to

pure stereoisomers yield mixtures of diastereomers that must be separated by silica gel chromatography. To avoid this time-consuming and expensive step, we used our screening techniques to identify two hydrolases, α-chymotrypsin and bovine pancreatic protease, that are useful for the separation of *cis* and *trans* diastereomers of a key intermediate dioxolane ester. Both hydrolases yield the desired *cis* diastereomer in good yield and excellent diastereomeric excess (>98%).

Contributions to Knowledge

1. We developed two empirical rule that can predict which enantiomer of secondary alcohols and chiral carboxylic acids reacts faster with lipase from *Aspergillus niger* (ANL). For secondary alcohols, a rule previously proposed for other lipases and esterases based upon the size of the substituents at the stereocentre, also works for ANL but with lower reliability (77%). For carboxylic acids, the size of the substituents at the stereocentre could not predict the enantiopreference, but a positively-charged amino group at the stereocentre sets the enantiopreference of ANL towards α-amino acids. A rule limited to α-amino acids is completely reliable. These rules can help chemists develop new applications for ANL as a chiral biocatalyst.

2. Partial purification of ANL with ammonium sulphate precipitation or using an FPLC anion exchange column removes contaminating hydrolases from the commercial preparation and increases the enantioselectivity of ANL towards many α-amino acids, including the cyclic α-amino acids, proline and pipecolic acid ($E > 100$).

3. Currently, there is no simple method to rapidly screen libraries of hydrolases for enantioselectivity towards a chiral substrate without measuring enantiomeric purities. We developed a fast spectrophotometric method called "quick E" that accurately measures enantioselectivity of hydrolases in less than one minute. This method does not require enantiomeric purity measurements but rather uses the initial rates of hydrolysis of pure enantiomers relative to a reference compound to determine the relative k_{cat}/K_M values of both pure enantiomers of a racemic pair. The ratio of these rates yields the enantioselectivity. This method is amenable for measuring the enantioselectivities of chromogenic esters (e.g. 4-nitrophenyl esters) and non-chromogenic esters (e.g. methyl esters and acetates) of a substrate.

4. We developed a fast spectrophotometric assay to quantitatively measure the hydrolytic activity of hydrolases using pH indicators. If a buffer and pH indicator are

chosen with the same pK_a, the true rate of enzyme-catalyzed hydrolysis is easily calculated using a modified form of the Beer-Lambert law.

5. Our pH indicator assay can also be used to separately measure the initial rates of hydrolysis of pure enantiomers of a chiral substrate and the ratio of the rates used to estimate the enantioselectivity. Using this method, we identified horse liver esterase as a useful biocatalysts for the kinetic resolution of solketal esters, enantioselectivity = 15. Although modest, this value is the highest enantioselectivity reported to date for the hydrolysis of a solketal ester.

6. Currently, there is no method to accurately measure the enantioselectivity of large libraries (>1000) of small quantities (μg) of mutant hydrolases generated in directed evolution experiments. We, therefore, optimized the sensitivity of the quick E method and the pH indicator assay to screen large libraries of hydrolases and substrates in microtiter plates, the current standard for automated high-throughput screenings. Using microtiter plates greatly increases the speed of measurements and reduces the amount of substrate and enzyme needed per measurement (typically 20 μg of substrate and 1-35 μg enzyme per measurement). For these reasons, these screens will be used to screen mutant hydrolases in directed evolution experiments to increase the enantioselectivity of a hydrolase towards a synthetic target.

7. We identified two hydrolases, α-chymotrypsin and bovine pancreatic protease, useful for the hydrolase-catalyzed separation of *cis* and *trans* diastereomers of 2-benzyloxymethyl-4-(*S*)-carboxylic acid-1,3-dioxolane methyl ester. Dioxolane acids are key intermediates in the synthesis of anti-viral and anti-cancer agents. Current routes to the pure stereoisomers require expensive and tedious separation by silica gel chromatography. Using α-chymotrypsin and bovine pancreatic protease eliminates this slow step in the synthesis of pure stereoisomers.

Future Work

"...if we wish to catch up with Nature, we shall need to use the same methods as she does, and I can foresee a time in which physiological chemistry will not only make greater use of natural enzymes, but will actually resort to creating synthetic ones."

Emil Fischer
Nobel Lecture 1902

Suggested future work with Aspergillus niger lipase

Lipase isolated from the fungus *Aspergillus niger* (ANL) is an unusual lipase. While most lipases do not accept charged substrates, ANL prefers them and readily accepts α-amino-acids as substrates. We demonstrated that ANL has very high activity and enantioselectivity towards two cyclic amino acids, proline and pipecolic acid. This property potentially make ANL an excellent catalyst for lipase-catalyzed syntheses of biologically-active peptides containing proline and pipecolic acid in their sequence, for example the tripeptide OH-Pip-AIB-Pip (AIB = aminoisobutyric acid, Pip = pipecolic acid) which is part of efrapeptin D, a toxin against insects[1]. Although enzyme-catalyzed peptide syntheses have been reported by other groups, enzyme-catalyzed synthesis of peptides that contain proline and pipecolic acid in their sequence has been largely unsuccessful.[2]

Enzyme-catalyzed synthesis of small peptides is a suitable alternative to traditional chemical methods because of mild reaction conditions, no racemization of the amino acids upon coupling to other residues, and minimal use of protecting groups and coupling reagents.

Several groups have used proteases to synthesize peptides[3] because they catalyze peptide bond formation. However, proteases also catalyze the reverse reaction and hydrolyse the growing peptide. Other shortcomings of proteases include low yield and narrow substrate specificity.

Alternately, lipases have been used to catalyze the formation of peptide bonds because, unlike proteases, they cannot hydrolyse the growing peptide. Other groups have used lipases to make peptides[4] but none report the synthesis of peptides with proline or pipecolic acid in their sequence. We believe ANL may catalyze the synthesis of peptides that include cyclic α-amino acids in their sequence since is has excellent enantioselectivity and activity towards proline and pipecolic acid.

Suggested future work with the quick E method

With the recent confluence of chemistry and molecular biology, a new and elegant method has emerged to enhance the properties of enzymes called directed evolution. It has been used to develop enzymes with improved thermostability, substrate specificity and tolerance to organic solvents and it is currently emerging as a technique to enhance the enantioselectivity of hydrolases.[5]

Directed or "in vitro" evolution accelerates the evolution of enzymes using molecular biology techniques such as error prone PCR[6] and gene shuffling[7] to generate large libraries of mutant enzymes. The library is then screened for improvements in the desired property to select the best enzymes from that generation for another round of mutation. This cycle is continued, with the best enzymes from each cycle serving as parents for the next generation. Beneficial mutations evolve over several cycles until the desired catalytic goal is reached.

A critical part of any directed evolution experiment is the selection method used to choose the best enzymes from each round of mutation. The first rule of directed evolution as proposed by Dr. Frances Arnold of the California Institute of Technology is "you get what you screen for" so efficient screening systems are paramount to the success of these experiments. Researchers recently used the ratio of separately measured initial rates of hydrolysis of pure enantiomers to rapidly estimate the enantioselectivity of libraries of mutant lipases produced using error-prone PCR.[8] Estimating enantioselectivity by separately measuring initial rates of pure enantiomers ignores some or all of the contributions of K_M to enantioselectivity so mutant enzymes that distinguish between enantiomers based upon differences in K_M are not selected with this screen.

The ultimate goal when we developed the quick E screen was to measure the enantioselectivities of libraries of mutant hydrolases in directed evolution experiments. Improvements between generations of enzymes in directed evolution are often small but the quick E method is sensitive to small changes in k_{cat} and K_M. Importantly, quick E requires only microgram quantities of hydrolases and substrate to determine E so it is suitable for high-throughput screenings of large numbers of mutant hydrolases. Our next major application of quick E is to screen libraries of mutant lipases for small but stepwise increases in enantioselectivity towards a synthetic target.

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I would greatly appreciate if you could me a copy of your response by FAX at the fax number below as soon as possible as well as a paper copy by mail.

I thank you in advance for your time and co-operation.
Sincerely,
Lana E. Janes

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A. C. Löwendahl, Ph. D.
Research Scientist

Mölndal
November 5, 1998

To whom it may concern,

I have no objection to the paper entitled "Quantitative Screening of Hydrolase Libraries Using pH Indicators: Identifying Active and Enantioselective Hydrolases", of which I am co-author, being included as chapter 4 in the thesis of Lana E. Janes entitled "Screening Methods to Identify Stereoselective Hydrolases."

Dr. A. Christina Löwendahl

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To whom it may concern,

I have no objection to the following published papers, of which I am co-author, being included in the Ph.D. thesis of Lana E. Janes entitled "Screening Methods to Identify Stereoselective Hydrolases for Synthetic Applications":

"Quantitative screening of hydrolase libraries using pH indicators: identifying active and enantioselective hydrolases." *Chem. Eur. J.* **1998**, *4*, 2324-2331.

"Empirical Rules for the enantiopreference of lipase from *Aspergillus niger* toward secondary alcohols and carboxylic acids, especially α-amino acids", *Tetrahedron: Asymmetry* **1997**, *8*, 3719-3733.

"Quick E. A fast spectrophotometric method to measure the enantioselectivity of hydrolases." *J. Org. Chem.* **1997**, *62*, 4560-4561.

Professor Romas J. Kazlauskas

Romas Kazlauskas

Appendix II
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Tetrahedron: Asymmetry, Vol. 8, No. 22, pp. 3719–3733, 1997

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Empirical rules for the enantiopreference of lipase from *Aspergillus niger* toward secondary alcohols and carboxylic acids, especially α-amino acids

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Abstract: Lipase from *Aspergillus niger* (ANL, Amano lipase AP) catalyzes enantioselective hydrolysis and acylation reactions. To aid in the design of new applications of this lipase, we propose two empirical rules that predict which enantiomer reacts faster. For secondary alcohols, a rule proposed previously for other lipases also works for ANL, but with lower reliability (77%, 37 of 48 examples). For carboxylic acids, we examined both crude and partially-purified ANL because commercial ANL contains contaminating hydrolases. Partial purification removed a contaminating amidase and increased the enantioselectivity of ANL toward many α-amino acids, including cyclic amino acids. Unlike other lipases, ANL readily accepts positively-charged substrates and shows the highest enantioselectivity toward α-amino acids. Although a rule based on the sizes of the substituents could not predict the fast-reacting enantiomer, a rule limited to α-amino acids did predict the fast-reacting enantiomer. We estimate that the charged α-amino group contributes a factor of 40–100 ($\Delta\Delta G^{\ddagger}$=2.2–2.7 kcal/mol) to the enantioselectivity of ANL towards carboxylic acids. © 1997 Elsevier Science Ltd

Introduction

Lipase-catalyzed reactions are one of the best methods to produce enantiomerically pure compounds.[1] Lipase from the fungus *Aspergillus niger* (ANL) is a useful enantioselective catalyst, especially for the resolution of secondary alcohols and carboxylic acids. These reactions were discovered by trial and error. To identify other applications of this lipase, it would be useful to have generalizations or rules that identify which structures in a substrate are required for high enantioselectivity. The only previous generalization for ANL was limited to secondary alcohols of 2-(methylthio)-3-acetoxy esters.[2]

The simplest rules for hydrolase-catalyzed reactions do not attempt to predict the degree of enantioselectivity, but only which enantiomer reacts faster. For example, a rule based on the size of the substituents at the stereocenter predicts which enantiomer of a secondary alcohol reacts faster[3] (Figure 1).

This rule suggests that the lipases distinguish between enantiomers of secondary alcohols primarily based on the relative sizes of the substituents. For the synthetic chemist, this means that lipases resolve secondary alcohols with different-sized substituents better than alcohols with similarly sized substituents. In this paper, we will show that ANL also follows the rule in Figure 1, but with lower reliability than for other lipases.

Predicting the fast-reacting enantiomer of a carboxylic acid has been more difficult for lipases. For purified lipase from *Candida rugosa*, a rule based on the size of the substituents was reliable,[4] but we show in this paper that this rule is not reliable for ANL. For ANL, the most efficiently resolved carboxylic acids are α-amino acids and the fast-reacting enantiomer has the L-configuration

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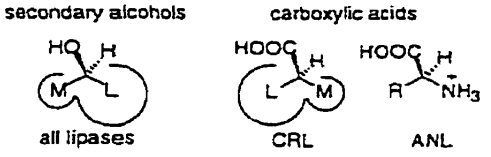

Figure 1. Empirical rules that predict the fast-reacting enantiomer in lipase-catalyzed reactions. For secondary alcohols, all lipases, including ANL, follow a rule that compares the size of the substituents at the stereocenter. For carboxylic acids, a rule based upon the size of the substituents at the stereocenter works for CRL, but not for ANL. A rule for ANL includes only α-amino acids (including cyclic amino acids) and suggests that a charged α-amino group is essential for high enantioselectivity. ANL always hydrolyzes the natural L-enantiomer. M represents a medium sized substituent, e.g., CH_3; L represents a large substituent, e.g., Ph; R represents any alkyl or aryl group.

(Figure 1). This model suggests that charge, not size, of the substituents at the stereocenter sets the enantioselectivity of ANL for carboxylic acids.

Results

Literature survey of the enantiopreference of ANL toward secondary alcohols

To test if the secondary alcohol rule also applies to lipase from *Aspergillus niger*, we summarized all reported ANL-catalyzed reactions of secondary alcohols (Table 1). Figure 2 shows the structures of the fast-reacting enantiomers. The examples include both hydrolyses of esters and acylations of alcohols, but for consistency the structures in Figure 2 are all alcohols. We excluded patents and examples where the enantioselectivity was <2. For eight examples, the rule is equivocal because the size of both substituents is similar according to CPK models. We marked these examples 'sim. size' in Table 1 and excluded them from the tally below. Note, however, that two of these examples showed good enantioselectivity, E>40.

For the remaining 48 substrates, the rule in Figure 1 predicts the absolute configuration of the favored enantiomer in 37 cases, but fails in 11 cases, 77% accuracy. (Guessing alone gives 50% accuracy because there are only two choices.) Six of the 48 substrates showed enantioselectivities >50 and the rule predicted the favored enantiomer for all six of these. Of the 11 exceptions to the rule, eight showed low enantioselectivities, E<10, but three showed moderate enantioselectivity (E=20–41). Five of the six bicyclic alcohols (compounds **23**–**27**) did not obey the size rule. Thus, the size rule usually predicts the favored enantiomer, but the reliability is less than for other lipases where the reliability is often >90%.[3,5] This lower reliability may be an inherent characteristic of ANL or it may be due to contaminating hydrolases, see below.

A literature survey of enantioselectivity of ANL toward carboxylic acids

Table 2 summarizes all reported ANL-catalyzed hydrolyses of esters of carboxylic acids in the literature and Figure 3 shows the structures of the faster-reacting enantiomers.[6] ANL favors the naturally occurring L-enantiomer of pipecolic acid, **31**, homophenylalanine, **33** (R=CH_2CH_2Ph), and the 12 *N*-Cbz-protected amino acids, **38**. ANL showed no enantioselectivity toward *N*-acetyl pipecolic acid, **39**. For the non-amino acid substrates, ANL showed moderate enantioselectivity toward 2-phenoxypropionic acid, **40**, and low enantioselectivity toward acids **41** and **42**. From such a limited range of substrates (seven structures in total), it is difficult to deduce which classes of carboxylic acids ANL resolves efficiently. For this reason, we resolved a wider range of carboxylic acids with both crude and partially-purified ANL (Table 3).

Enantioselectivity of ANL toward carboxylic acids does not depend on the size of the substituents

We tested the methyl esters of nine α-amino acids, **33**, the octyl ester of **32**, three β-amino acids, **34**–**36**, and one α-disubstituted amino acid, **37**. In addition, we tested esters of an aryloxypropionic acid, **43**, mandelic acid, **44**, two arylpropionic acids, **45** and **46**, two furoic acids, **47** and **48** and 2-

Table 1. Literature survey of the enantioselectivity of ANL toward secondary alcohols[a]

Struct.	R	E[b]	Follows rule?[c]	Ref	Struct.	R	E[b]	Follows rule?[c]	Ref
1		5	yes	*d*	12	CMe=CHEt	12	sim. size	*n*
2		2	yes	*e*	"	CMe=CHMe	10	sim. size	*n*
3		7	yes	*f*	"	$CMe=CH_2$	18	sim. size	*n*
4		6	yes	*g*	"	CH=CHPh	46	sim. size	*n*
5	(4'-OMe-C_6H_4)-O	8	yes	*h*	13	2-Furanyl	20	yes	*n*
"	3',4'-*di*OMe-C_6H_3	21	yes	*h*	"	2-Thienyl	46	yes	*n*
"	2',4',6'-*tri*OMe-C_6H_2	25[i]	yes	*h*	"	CMe=CHMe	43	sim. size	*n*
6		74	yes	*j*	14		15	yes	*j*
7		11	yes	*j*	15	Ph	30	yes	*j*
8	Me	31	yes	*e*	"	Me	3	yes	*j*
"	Et	13	yes	*k*	"	Bu	5	yes	*j*
"	*n*-Propyl	22	yes	*k*	"	CH=CHPh	13	sim. size	*j*
"	*n*-Pentyl	35, 40[i], 150[m]	yes	*k*	16		5	no	*j*
"	*n*-Heptyl	17[i]	yes	*k*	17		10	yes	*j*
"	Ph	10[i], 138	yes	*e*	18	Me	82	yes	*j*
"	Bn	4.8-6[i]	yes	*l*	"	Et	20	yes	*j*
"	4'-OMe-C_6H_4	17, 11[i]	sim. size	*l*	19		3	no	*j*
9	Bn	41	no	*l*	20		4	sim. size	*j*
"	*i*-Pr	3	no	*k*	21		4	yes	*o*
"	*t*-Butyl	2[i]	no	*k*	22		3	no	*p*
10	*n*-Pentyl	9	yes	*k*	23		−5	no	*q*
"	*n*-Heptyl	8[i]	yes	*k*	24		−10	no	*q*
"	*n*-Nonyl	7[i]	yes	*k*	25		−7	no	*q*
11	Me	2	yes	*j*	26		−30	no	*q*
"	Et	3	yes	*j*	27		20	no	*q*
"	Bu	2	yes	*j*	28		2	yes	*r*
12	2-Furanyl	52	yes	*n*	29		27	yes	*s*
"	2-Thienyl	8	yes	*n*	30		>50	yes	*t*

[a]Hydrolysis of the acetate ester using lipase AP-6 from Amano Enzyme Co. unless noted otherwise. Figure 2 shows the absolute configurations of the favored enantiomer. [b]Enantiomeric ratio, E, measures the preference of the enzyme for one enantiomer over the other: Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* 1982, *104*, 7294-7299. [c]The rule in Fig. 1 is ambiguous when both substituents are similar in size; these examples are marked 'sim. size'. [d]ANL from Röhm; esterification with dodecanoic acid: Gerlach, D.; Missel, C.; Schreier, P. *Z. Lebensm.-Unters. Forsch.* 1988, *186*, 315-18. [e]Li, Y.-F.; Hammerschmidt, F. *Tetrahedron: Asymmetry* 1993, *4*, 109-120. [f]Lipase AP; esterification with vinyl acetate: Hirai, Y.; Nagatsu, M. *Chem. Lett.* 1994, 21-22. [g]Lipase A; esterification with vinyl acetate in carbon tetrachloride: Kaminska, J.; Gornicka, I.; Sikora, M.; Gora, J. *Tetrahedron: Asymmetry* 1996, *7*, 907-910. [h]Akita, H.; Umezawa, I.; Takano, M.; Ohyama, C.; Matsukura, H.; Oishi, T. *Chem. Pharm. Bull.* 1993, *41*, 55-63. [i]Hydrolysis of the chloroacetate ester. [j]Itoh, T.; Kuroda, K.; Tomosada, M.; Takagi, Y. *J. Org. Chem.* 1991, *56*, 797-804. [k]Drescher, M.; Hammerschmidt, F.; Kahlig, H. *Synthesis* 1995, *10*, 1267-1272. [l]Drescher, M.; Li, Y.-F.; Hammerschmidt, F. *Tetrahedron* 1995, *51*, 4933-4946. [m]Reaction carried out at 40°C. [n]Akita, H.; Matsukura, H.; Oishi, T. *Tetrahedron Lett.* 1986, *27*, 5241-5244. [o]Lipase A-6 immobilized on Celite: Akita, H.; Enoki, Y.; Yamada, H.; Oishi, T. *Chem. Pharm. Bull.* 1989, *37*, 2876-2878. [p]Miura, S.; Kurozumi, S.; Toru, T.; Tanaka, T.; Kobayashi, M.; Matsubara, S.; Ishimoto, S. *Tetrahedron* 1976, *32*, 1893-1898. [q]Lipase A: Naemura, K.; Takahashi, N.; Tanaka, S.; Ida, H. *J. Chem. Soc., Perkin Trans 1*, 1992, 2337-2343. Estimated E values based on the reported enantiomeric purities. [r]Lipase from Fluka Chemical Co.: Zaravucka, M.; Zuzana, Z.; Rejzek, M., Streinz, L.; Wimmer, Z.; Mackova, M.; Demnerova, K. *Enz. Microb. Tech.* 1995, *17*, 866-869. E = 6 for esterification with vinyl acetate in benzene. [s]Lipase APF: Patel, R. N.; Banerjee, A.; Ko, R. Y.; Howell, J. M.; Li, W.-S.; Comezoglu, F. T.; Partyka, R. A.; Szarka, L. *Biotechnol. Appl. Biochem.* 1994, *20*, 23-33. [t]Hoenke, C.; Kluwer, P.; Hugger, U.; Krieger, R.; Prinzbach, H. *Tetrahedron Lett.* 1993, *34*, 4761-4764.

chloropropionic acid, **49**. For pipecolic acid, **31**, we previously showed that the enantioselectivity of ANL increased significantly (from E~20 to E>100) upon partial purification of the lipase to remove a contaminating hydrolase.[7] For this reason, we measured the enantioselectivity of both crude and partially-purified ANL.

ANL resolved only the α-amino acids with an enantioselectivity >10. For proline, phenylalanine, and phenylglycine, the enantioselectivity of crude ANL was moderate to good (E=20–70) and increased to >90 upon purification. For alanine the enantioselectivity also increased from 3 to 41 upon purification. The specific activity of ANL towards these substrates increased up to two-fold upon purification (0.21–0.49 U/mg for crude, 0.55–0.96 U/mg for partially-purified). On the other hand, the specific

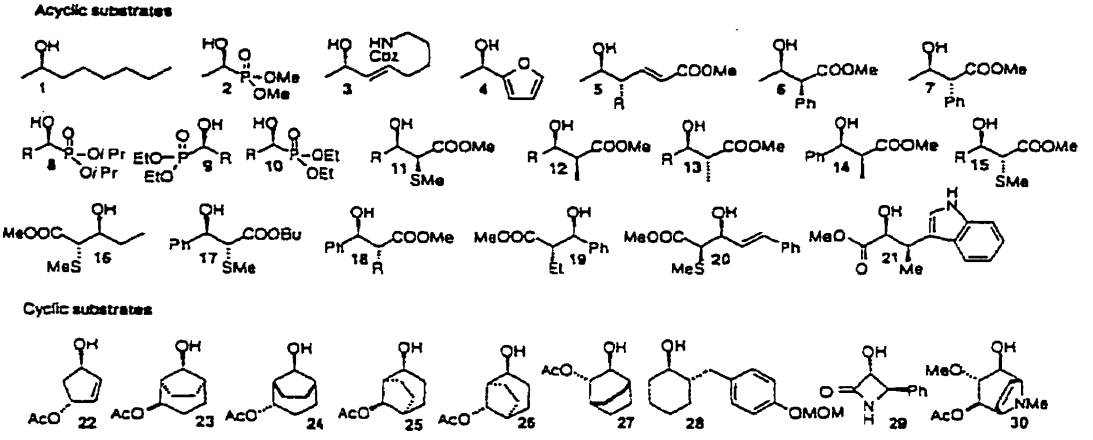

Figure 2. Examples from the literature of secondary alcohols resolved or desymmetrized by ANL. Structures show the fast reacting alcohol enantiomer. Most examples follow the rule in Figure 1. Details in Table 1.

Table 2. Literature survey of the enantioselectivity of ANL toward carboxylic acids[a]

Structure	R[b]	E[c]	Follows size rule[d]?	Reference
31		20, >100	sim. size	*e*
33	CH_2CH_2Ph	13	yes	*f*
38	Me [Ala]	28, 14	no	*g, h*
"	Et	95	no	*g*
"	*i*-Pr [Val]	28, >100	no	*g, h*
"	*n*-Pr	18	no	*g*
"	*n*-Bu	18	no	*g*
"	*n*-Pentyl	87	sim. size	*g*
"	CH_2=$CHCH_2$	21	no	*g*
"	*n*-Hexyl	49	sim. size	*g*
"	(4-Thiazolyl)methyl	56	no	*g*
"	Bn [Phe]	50, 22	no	*g, h*
"	$CH_3SCH_2CH_2$ [Met]	86	no	*h*
"	$(CH_3)_2CHCH_2$ [Leu]	15	no	*h*
39		1	na	*e*
40		19	no	*i*
41		3	yes	*j*
42		6	no	*k*

[a]Hydrolysis of the methyl ester using lipase AP-6 from Amano Enzyme Co. unless noted otherwise. Figure 3 shows the absolute configuration of the favored enantiomer. [b]When the structure is a common amino acid, its three letter abbreviation is given in brackets. [c]Enantiomeric ratio, E, measures the preference of the enzyme for one enantiomer over the other: Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* 1982, *104*, 7294-7299. [d]The rule in Fig. 1 is ambiguous when both substituents are similar in size; these examples are marked 'sim. size'; 'na' indicates not applicable because E = 1. [e]Crude ANL showed an E of 20, but partially-purified ANL showed an E of > 100: Ng-Youn-Chen, M. C.; Serreqi, A. N.; Huang, Q.; Kazlauskas, R. *J. J. Org. Chem.* 1994, *59*, 2075-2081. [f]Hydrolysis of the ethyl ester: Houng, J.-Y.; Hsieh, C.-L.; Chen, S.-T. *Biotechnol. Techn.* 1996, *10*, 353-358. [g]Hydrolysis of the 2-chloroethyl ester: Miyazawa, T.; Takitani, T.; Ueji, S.; Yamada, T.; Kuwata, S. *J. Chem. Soc., Chem. Commun.* 1988, 1214-1215. [h]Chiou, A.-J.; Wu, S.-H.; Wang, K.-T. *Biotechnol. Lett.* 1992, *14*, 461-464. [i]Transesterification of the vinyl ester with methanol in heptane: Miyazawa, T.; Kurita, S.; Ueji, S.; Yamada, T.; Kawata, S. *Biotechnol. Lett.* 1992, *14*, 941-946. [j]Chenevert, R.; Lavoie, M.; Courchesne, G.; Martin, R. *Chem. Lett.* 1994, 93-96. [k]Ngooi, T. K.; Guo, Z. W.; Sih, C. J. *Biocatalysis* 1990, *3*, 119-128.

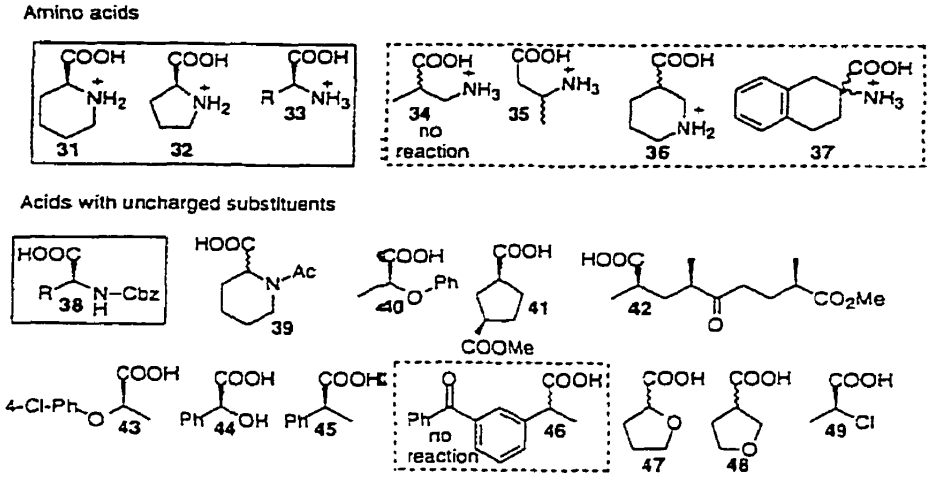

Figure 3. Carboxylic acids tested in enantioselective reactions with ANL. Enantioselectivities are moderate or better (E>10) only for the examples in the solid-line boxes. Examples in the dotted-line boxes did not react with ANL. Structures show the fast reacting enantiomer where the absolute configuration is known. Table 2 provides details for the examples from the literature and Table 3 lists experimental results for examples reported in this paper.

activity of ANL towards *N*-Cbz-phenylalanine, **38** (where R=Bn), decreased by a factor of nine upon purification and the enantioselectivity decreased from 20 to 13. These decreases suggest that a contaminating hydrolase in crude ANL contributes to the hydrolysis of *N*-Cbz-phenylalanine-methyl ester. For the non-charged substrates, **43**, **45**, **47–49**, the specific activity increased as much as 20-fold (0.051–1.4 U/mg for crude, 0.072–3.0 U/mg for partially-purified), but the enantioselectivity remained low. Thus, purification increased the specific activity toward many substrates, but increased the enantioselectivity only toward substrates with a charged α-amino group.

The specific activity of crude ANL toward α-amino acids with larger side chains — tryptophan, tyrosine, and *O*-methyl tyrosine — were 5–50 times lower than for the those with smaller side chains. The enantioselectivity was low to moderate (E=5–25). There was little change upon purification. The specific activity increased two-fold towards tyrosine and *O*-methyl tyrosine, but decreased two-fold towards tryptophan, however the enantioselectivies remained approximately the same (E=2.5–21). Three sterically hindered, unnatural α-amino acids, *tert*-leucine (**33**, R=*t*-Bu), 2,6-dimethyltyrosine (**33**, R=4′-OH-2′,6′-Me-C_6H_2) and the α-disubstituted amino acid, **37**, did not react. The slow hydrolysis and lower enantioselectivity towards large substrates suggest that there is a limit to the size of the side chain that ANL can accommodate.

ANL did not catalyze the hydrolysis of any of the three β-amino acids, **34–36**, even though these are not sterically hindered. Both crude and partially-purified ANL showed low enantioselectivity toward the aryloxypropionic acid, **43**, mandelic acid, **44**, the two arylpropionic acids, **45** and **46**, the two furoic acids, **47** and **48**, and chloropropanoic acid, **49**.

A rule comparing the size of the substituents at the stereocenter predicted the enantiopreference of purified lipase from *Candida rugosa* towards carboxylic acids,[4] but this rule does not work for ANL (Figure 1). For the α-amino acids, **33**, the larger group, R, lies to the left, but for the *N*-Cbz-α-amino acids, **38**, the larger group, *N*-Cbz, lies to the right. Yet ANL favors the L-enantiomer for both. For substrates **41** and **43**, the larger group lies to the left, but for **42** the larger group lies to the right. We also compared the overall accuracy of the size rule. Among the 40 carboxylic acids tested in either this work or literature reports, eight did not react. We excluded another seven because both substituents had similar sizes (**31**, **32**, **33** (R=CH_3 [Ala]), **38** (R=*n*-pentyl, *n*-hexyl) and **47**, **48**). We also excluded

Table 3. Enantioselectivity of ANL in the hydrolysis of carboxylic acid esters[a]

		Crude ANL[b]				Partially-Purified ANL[b]				
Struct	R[c]	Rate (U/mg)[d]	ee_s (%)	ee_p (%)	E[e]	Rate (U/mg)[d]	ee_s (%)	ee_p (%)	E[e]	Follows size rule?
32		0.49	nd	86	20[f,g]	0.62	90	94	100[g]	sim. size
33	Me [Ala]	0.40	59	26	3	0.55	99	78	41	sim. size
"	$CH_3SCH_2CH_2$ [Met]	0.58	78	85	30	0.74	97	99	>100	yes
"	Ph	0.45	92	91	70[h]	0.91	93	93	94[h]	yes
"	Bn [Phe]	0.21	93	86	45	0.96	>99	91	>100	yes
"	Indoyl-CH_2[Trp]	0.057	42	58	5.6	0.026	38	29	2.5	yes
"	4'-OH-C_6H_4-CH_2 [Tyr]	0.014	97	33	7	0.028	53	61	7	yes
"	4'-OMe-C_6H_4-CH_2	0.12	73	84	25	0.21	86	76	20	yes
"	4'-OH-2',6'-Me_2-C_6H_2-CH_2	<0.00001	-	-	-	<0.00001	-	-	-	-
"	*t*-Bu	<0.00001	-	-	-	<0.006	-	-	-	-
34		<0.004	-	-	-	<0.0001	-	-	-	-
35		<0.0001	-	-	-	<0.0009	-	-	-	-
36		<0.001[i]	-	-	-	<0.001[i]	-	-	-	-
37		<0.005	-	-	-	<0.004	-	-	-	-
38	Bn [Phe]	1.4	28	88	20	0.16	9	85	13	no
"	Ph	<0.0001	-	-	-	<0.0001	-	-	-	-
43		0.088	64	43	5	0.15	1	54	3	yes
44		1.00	0	0	1	0.82	0.2	8.1	1.2	na
45		0.051	4	nd	1.2[j,k]	0.072	nd	12	1.3[f,j]	na
46		<0.001	-	-	-	<0.00004	-	-	-	-
47		0.047	10	nd	1.68[g,k]	0.25	15	nd	68[g,k]	sim. size
48		0.045	0	nd	18[g,k]	0.82	4	nd	1.88[g,k]	sim. size
49		1.39	42	51	4.5	3.00	50	66	8.0	yes

[a]Hydrolysis of the methyl ester unless noted otherwise. Figure 3 shows the absolute configuration of the favored enantiomer. 'nd' = not determined; '-' = could not be measured because no reaction was detected; 'na' = not applicable because the enantioselectivity is below 2 or because the absolute configuration is unknown. [b]Crude ANL is lipase AP-6 from Amano as received and partially-purified ANL is lipase AP-6 precipitated with 25-55% saturation of ammonium sulfate. [c]When the structure is a common amino acid, its three letter abbreviation is given in brackets. [d]Initial rate of hydrolysis in units/mg protein. Unit = μmol of ester hydrolyzed per minute. Crude ANL contained 2.8 wt% protein by the Bio-Rad protein assay. [e]E represents the enantiomeric ratio which measures the preference of the enzyme for one enantiomer over the other: Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* 1982, *104*, 7294-7299. Unless otherwise noted, E was calculated from ee_s and ee_p. [f]E was calculated from ee_p and percent conversion. [g]Hydrolysis of the octyl ester. The methyl ester of 32 was unstable at room temperature: the methyl esters of 47 and 48 were very volatile. [h]Phenylglycine methyl ester partially racemized after 24 hours at pH 5. To determine E accurately, we used short reaction times (typically 1 h) and measured ee_p and ee_s immediately after we stopped the reaction. Others also noted the racemization of phenylglycine, for example, Shiraiwa, T.; Sakata, S.; Fujishima, K; Kurokawa, H. *Bull. Chem. Soc. Jpn.* 1991, *64*, 191-195. [i]Hydrolysis of the ethyl ester. [j]Hydrolysis of the chloroethyl ester. [k]E was calculated from ee_s and percent conversion.

39, **44**, **45** from the tally because the enantioselectivity was <2. For the remaining 22 substrates, only 10 obeyed the size rule corresponding to 45% accuracy. This accuracy is similar to guessing alone.

High enantioselectivity requires a protonated amino group ($-NH_3^+$)

Additional evidence that size of the substituents at the stereocenter is not important comes from good to excellent enantioselectivity toward several carboxylic acids that contain similarly sized substituents. Purified ANL resolved three α-amino acids, pipecolic acid, proline and alanine, with an enantioselectivity of 41 to >100 even though the two substituents differ only in the charge of the substituents at the stereocenter. This difference corresponds to a $\Delta\Delta G^{\ddagger}$ of 2.2–2.7 kcal/mol.

The enantioselectivity of purified ANL toward 2-phenylglycine (**33** where R=phenyl, E=94) was much higher than for analogs without a protonated amino group. The enantioselectivity toward mandelic acid, **44**, and 2-phenylpropionic acid, **45**, was only 1.2–1.3. Similarly, the enantioselectivity of purified ANL toward alanine (**33** where R=methyl, E=41) was much higher than toward 2-chloropropionic acid, **49** (E=8).

To test whether high enantioselectivity requires a protonated amino group, we measured the enantioselectivity of ANL as a function of pH (Table 4). Both enantioselectivity and specific activity

Table 4. Influence of pH on the enantioselectivity of partially-purified ANL toward phenylalanine-methyl ester[a]

pH	rate U/mg[b]	% substrate protonated[c]	ee_s[d]	ee_p[d]	E[e]
4.02	0.047	>99	0.068	0.68	6±1
5.02	0.91	99	0.68	0.95	80±20
6.00	0.16	87	0.53	0.65	8±1
7.07	0.17	47	0.81	0.63	18±1
7.97	0.12	10	0.11	0.70	1.2±0.04
8.90	0.13	1	0.88	0.45	6±2[f]

[a]Reaction conditions: 15 units of lipase (by PNPA assay), room temperature, 25 mM substrate in 10 ml sodium acetate buffer (10 mM) for pH 4-6 and Tris-HCl buffer (10 mM) for pH 6-9. E values at pH 6 were similar in both buffers. Reactions were monitored by pH stat. [b]Unit = μmol of ester hydrolysed per minute. [c]The fraction of protonated Phe-methyl ester at the given pH was calculated using a pK_a = 7.00: Iencks, W.P.; Regenstein, J. *CRC Handbook of Biochemistry. Selected Data for Molecular Biology* CRC: Boca Raton, FL, 1986, pp J187-J226. [d]Determined by HPLC with a Crownpak CR(+) column. The preferred enantiomer was L as established by the order of elution on the CR(+) column and compared to authentic samples; the D-enantiomer elutes first for both acid and methyl ester. [e]E was calculated from ee_s and ee_p as defined by Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* 1982, *104*, 7294-7299. Error limits are estimates. [f]Phenylalanine-methyl ester hydrolyzed spontaneously at pH 8.9 at a rate of 9.2 x 10^{-4} min^{-1} (6.6% hydrolysis during the 1 h reaction).The ee values were corrected for this hydrolysis. The uncorrected values are ee_s = 0.72 and ee_p = 0.40. Spontaneous hydrolysis was not detected at other pH values.

were highest at pH 5 toward phenylalanine-methyl ester. At higher pH both the rate and the enantioselectivity decreased by factors of 5.3–7.6 and 4–66, respectively. These decreases are consistent with the $-NH_3^+$ form of phenylalanine-methyl ester as the most reactive and best resolved form of the substrate. At pH 4 the enantioselectivity also dropped to 6, but we attribute this drop to partial denaturation of ANL. After incubation at pH 4 for 1 h, the lipase activity measured at pH 7.5 with PNPA as the substrate dropped by 50% for crude lipase and 25% for partially-purified ANL. Incubation at pH 5–9 showed no drop in activity by PNPA assay.

To confirm that ANL accepts other positively-charged substrates, we tested *S*-acetylthiocholine iodide, a thioester with an $-NMe_3^+$ group in the thiol portion. Indeed, crude ANL catalyzed the efficient hydrolysis of this substrate with a rate of 0.19 U/mg protein. This rate was 100 times faster than with lipase from *Candida rugosa* and 10,000 times faster than with lipase from *Pseudomonas cepacia*. However, ANL was 10,000 times slower at hydrolyzing this substrate than acetylcholine esterase.[8]

ANL is not an amidase

High enantioselectivity toward α-amino acids suggests that partially-purified ANL may be an amidase, not a lipase. However, we confirmed that partially-purified ANL is not an amidase by testing its ability to catalyze hydrolysis of D,L-phenylalaninamide (Table 5). Partially-purified ANL showed no detectable hydrolysis of the amide. In contrast, crude ANL catalyzed the slow hydrolysis of D,L-phenylalaninamide favoring the natural L-enantiomer with excellent enantioselectivity, suggesting that crude ANL contains a contaminating amidase. The activity of the crude lipase towards phenylalaninamide was 2–6 times faster at pH 4–5 than at pH 7.5 and 9. Researchers have previously isolated proteases with optimal activities at acidic pH from *Aspergillus niger*.[9]

Purified ANL is indeed a lipase because it catalyzed hydrolysis of insoluble substrates 100 times more efficiently than crude ANL: olive oil (0.29–0.62 U/mg protein for partially purified vs 0.005 U/mg protein for crude) and ethyl butyrate (0.44–2.42 for partially purified vs 0.02 U/mg protein for crude).

Discussion

Because all lipases follow the secondary alcohol rule in Figure 1, it is not surprising that ANL also follows the rule. However, the reliablity of the rule for ANL is significantly lower than for other lipases: 77% for ANL, but >90% for most other lipases. Although the lower reliablity may be an inherent characteristic of ANL (no sequence or structure is known for this lipase), it is most likely due to the contaminating amidase. This amidase may favor the opposite enantiomer of secondary alcohols

Table 5. Hydrolysis of D,L-phenylalaninamide by *Aspergillus niger* lipase[a]

	crude ANL				PP ANL
pH	activity[b] (U/mg)	ee_s	ee_p	E[c]	activity[b] (U/mg)
4.1	0.016	29	>99	>100	<0.0002
5.0	0.036	48	>99	>100	<0.0008
7.5	0.0068	63	>99	>100	<0.0003
8.9	0.0058	59	>99	>100	<0.0002

[a]Reaction conditions: 15 units of lipase, room temperature, 25 mM substrate in 10 ml sodium acetate buffer (100 mM) for pH 4.1 and pH 5 and sodium phosphate buffer (100 mM) for pH 7.5 and 8.9. Reactions were monitored by TLC (1:1; $CHCl_3$:MeOH; 1% NH_4OH) and terminated when product and starting material spots appeared approximately equal in intensity. [b]Unit/mg = μmol of amide hydrolysed per minute per mg of protein. [c]Enantiomeric excesses and degree of conversion were determined by HPLC (Crownpak CR(+) column). E was calculated from ee_s and ee_p as defined by Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299. The preferred enantiomer was L according to the order of elution on the CR(+) column compared to authentic samples; the D-enantiomer elutes first for both acid and amide.

and thus account for the exceptions to the rule. A recent survey showed that lipases and the protease subtilisin favor the opposite enantiomers of secondary alcohols,[10] thus it is reasonable to suggest that the amidase in crude ANL may also favor the opposite enantiomer.

Many groups have identified contaminating hydrolases in crude ANL.[7,11,12] For example, Hofelman *et al.* identified esterases, proteases and amylases as well as a lipase. The main commercial use of ANL is in cheese-making to accelerate the ripening of flavors.[13] Both lipases and proteases contribute to this ripening, so there is no need for purer enzymes. For synthetic applications, however, a purer form of ANL would be useful.

Our survey showed ANL, unlike other lipases, readily accepts positively-charged substrates. Carrea *et al.* found that ANL was the best lipase for deprotection of cephalosporin derivatives containing a carboxylate near the reactive site.[12] This result suggests that ANL may also readily accept negatively-charged substrates.

A protonated α-amino substituent was the most important feature for high enantioselectivity of ANL toward carboxylic acids. ANL resolves small to medium sized α-amino acids with very good enantioselectivity, but larger amino acids react slowly with lower enantioselectivity. The L-enantiomer was always favored as shown in Figure 1. Many proteases resolve amino acids esters,[14] but ANL is the only lipase that shows high enantioselectivity toward a range of amino acid esters.

Although crude lipase from *Humicola lanuginosa* resolved α-amino acids and α,α-disubstituted α-amino acids, Liu *et al.* found that the true catalyst was an impurity. Because this impurity did not catalyze hydrolysis of *o*-nitrophenyl butyrate or olive oil, it was probably a protease.[15] Similarly, Houng *et al.* reported enantioselective hydrolysis of amino acid esters catalyzed by crude PPL, but again the true catalyst may be a contaminating protease.[16]

For carboxylic acids, a rule based on the size of the substituents did not work for either crude or purified ANL. This result suggests that electronic effects, specifically charge, control the enantioselectivity of ANL. We estimate that interactions of the lipase with the $-NH_3^+$ or $=NH_2^+$ group contribute a factor of 40 to 100, corresponding to a 2.2–2.7 kcal/mol difference in $\Delta\Delta G^{\ddagger}$. This estimate comes from the enantioselectivity of ANL toward substrates whose substituents at the stereocenter differ only in charge. Similarly, Rotticci *et al.* found that electronic effects influence the enantioselectivity of lipase B from *Candida antarctica* (CAL-B).[17] Replacing a bromo substituent by methyl changed the enantioselectivity of CAL-B by a factor of >30 (>2 kcal/mol in $\Delta\Delta G^{\ddagger}$) and the enantioselectivity either decreased or increased depending of the location of the substituent.

Another unique feature of ANL is that it catalyzes the enantioselective hydrolysis of cyclic amino acids — proline and pipecolic acid. This ability may be useful for synthesis of peptides containing cyclic amino acids. Although some proteases can accept proline, they also catalyze the hydrolysis of peptide bonds.[18]

Experimental section

General

Lipase from *Aspergillus niger* (AP-6) and *Pseudomonas cepacia* (LPL-80) were purchased from Amano International Enzyme Co. (Troy, VI). Lipase from *Candida rugosa* (type VII) and acetylcholine esterase (isolated from electric eel) were purchased from Sigma Chemical Co. (Oakville, Ontario). Chemicals were purchased from Sigma–Aldrich Chemical Co. (Oakville, ON) and used without further purification unless stated otherwise. D,L-β-Aminoisobutyric acid, **34**, and D,L-3-aminobutyric acid, **35**, were purchased from Fluka Chemical Co. (Oakville, ON). D,L-dimethyl tyrosine and D,L-2-aminotetralin-2-carboxylic acid, **37**, were provided by Dr P. Schiller, IRCM, Montréal. (±)-Ketoprofen chloroethyl ester, **46** and (±)-methyl-2-(4-chlorophenoxy)propanoate, **43**, were provided by Dr Ian Colton.

Ultrafiltration was performed with an Amicon ultrafiltration kit (Oakville, ON) under N_2 using a YM-10 filter. Melting points were taken on an Electrothermal melting point apparatus and were corrected. Protein concentrations were measured using a dye-binding assay from Bio-Rad (Missisauga, ON) with bovine serum albumin as the standard. Purified ANL was desalted with Bio-Gel P6 (Bio-Rad), a size exclusion gel. Enzyme assays were carried out on a Hewlett Packard 8452A diode array spectrophotometer equipped with a Neslab RTE-100 water bath temperature control unit. The rate of lipase-catalyzed ester hydrolysis was measured on a Radiometer RTS 822 pH stat. HPLC chiral stationary phase columns were purchased from Daicel Chemical Industries Ltd (Fort Lee, NJ). NMR spectra were recorded on either a Varian Gemini 200 MHz, Jeol 270 MHz or Unity 500 MHz NMR spectrometer.

Enzyme activity

Hydrolase activity was measured using *p*-nitrophenyl acetate (PNPA) as a substrate. An aliquot (5 μL) of enzyme solution was added to phosphate buffer (1.00 mL, pH 7.5, 10 mM), allowed to equilibrate to 25°C, followed by addition of an aliquot (5 μL) of PNPA (50 mM solution in 1:1 acetonitrile/ 10 mM phosphate buffer, pH 7.5). The initial rate of formation of *p*-nitrophenolate was monitored at 25°C, 404 nm for 30 seconds. Activity was calculated using the Beer–Lambert law, with a cell length of 1 cm, and extinction coefficient of 11,600 M^{-1} cm^{-1} which accounts for the incomplete ionization of *p*-nitrophenolate at pH 7.5. Crude ANL showed an activity of 48 U/g solid (1.7 U/mg protein) with this assay. U=μmol of ester hydrolysed per minute.

Activity of the partially-purified lipase towards olive oil (0.6–35 mM) and ethyl butyrate (9–250 mM) were measured at pH 5 (sodium acetate buffer, 10 mM) using a pH stat which maintained the pH at 5 by automatic titration with 0.1 N NaOH. Insoluble substrate remained during the assay to ensure interfacial activation.

Partial purification of ANL by precipitation with ammonium sulphate

The procedure was carried out according to Ng-Youn-Chen *et al.*[7] All steps during enzyme purification were carried out at 4°C. Crude lipase from *Aspergillus niger* (Amano AP-6, 32 g, 1500 U by PNPA assay) was stirred in Tris-HCl buffer (250 mL, 25 mmol, pH 7.5) for 3 h, then centrifuged (10,000 rpm, 20 min) to remove insoluble material. Solid ammonium sulphate (36 g, 25% saturation) was added to the stirring supernatant in small portions every 15 min over 4 h. The solution was stirred overnight. The resulting suspension was centrifuged (10,000 rpm, 20 min), the very small pellet was discarded, and additional ammonium sulphate (51 g, 55% saturation) was added in small portions over 4 h. The solution was stirred overnight then centrifuged (3000 rpm, 45 min). The supernatant was discarded and a brown pellet containing the lipase was dissolved in sodium acetate buffer (25 mL, 10 mM, pH 5). This solution was desalted with a size exclusion gel (BioGel-P6) using 10 mM phosphate buffer, 10 mM NaCl, pH 7 as eluent at a linear flow rate of 12.7 cm/h. Fractions were collected and assayed using the PNPA assay. Those fractions with lipase activity were pooled yielding 70 mL of solution containing 189 units (13% yield). The solution was concentrated by ultrafiltration with

an Amicon YM-10 membrane yielding 13 mL (14.5 U/mL, 4.25 mg protein/mL) of lipase solution. Partially-purified ANL solution showed a specific activity of 3.41 U/mg protein accounting for a two-fold increase in specific activity over the crude preparation, and an enantioselectivity of >100 towards phenylalanine-methyl ester HCl. The dark brown solution retained full activity when stored at 4°C with 0.02 wt/vol% NaN_3 solution as preservative for at least 3 months. With other lots of crude ANL, the best partial-purification used 25–48% saturation with ammonium sulphate.

Partial purification of ANL by anion-exchange chromatography

Several lots of crude ANL gave <10% yield when purified by ammonium sulphate precipitation. These lots were partially-purified by first precipitating ANL at 60% saturation with ammonium sulphate, and second, anion exchange chromatography. The 60% pellet (285 units, 19% yield) was dissolved in sodium acetate buffer (23 mL, 10 mM, pH 5) and desalted with a Bio-Gel P-6 column as described above. The fractions were assayed using the PNPA assay and those with lipase activity were pooled, yielding 27 mL of solution containing 236 units. The phosphate buffer was exchanged for Tris-HCl buffer (25 mM, pH 7.48) by ultrafiltration using an Amicon YM-10 membrane, for a final volume of 5 mL (4.00 U/mL, 2.18 U/mg protein).

The solution (2.5 mL) was then injected onto a MonoQ anion exchange column (MonoQ HR 5/5 column, Pharmacia, Baie d'Urfé, QC) equilibrated with Tris-HCl buffer (25 mM, pH 7.48) using a Pharmacia FPLC system. The column was eluted at a flow rate of 0.5 mL/min with the same buffer for 10 mL, followed by a linear gradient of 0–0.5 M NaCl over 70 mL, then eluted with 0.5 M NaCl for 10 mL. Fractions (2 mL) were collected and assayed by PNPA. Fractions 13–15 contained hydrolase activity. The procedure was repeated and fractions 13–15 from both runs were pooled, yielding 12 mL of solution containing 77 units (27% yield overall from the 60% pellet). ANL partially purified by this technique showed a specific activity of 7.74 U/mg protein accounting for a 4.5-fold increase in specific activity over the crude preparation and enantioselectivity of >100 towards phenylalanine-methyl ester HCl. The dark brown solution retained full activity when stored at 4°C with 0.02 wt/vol% NaN_3 solution as preservative for 1 month.

Synthesis of esters of carboxylic acids

Esters of all carboxylic acids were previously prepared in the literature. ^{1}H-NMR and ^{13}C-NMR data are provided because not all literature references included complete NMR data. Amino acid esters were purified by recrystallization from methanol/diethyl ether; all other esters were purified by flash chromatography.

D,L-*2-Phenylglycine-methyl ester-HCl* was prepared by Fischer esterification:[19] 98% (white solid): mp=129.1–130.1°C; R_f=0.51 (chloroform:methanol=95:5); ^{1}H-NMR (CD_3OD, 200 MHz) δ 3.82 (s, 1H), 4.88 (br s, 3H), 5.30 (s, 3H), 7.49 (m, 5H); ^{13}C-NMR (CD_3OD, 200 MHz) δ 54.9, 58.5, 129.3, 130.6, 131.3, 133.3, 169.6.

D,L-*Proline-octyl ester-HCl*, methyl ester of 32, was prepared by Fischer esterification:[19] 74% (clear oil); R_f=0.81 (chloroform:methanol=50:50); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.87 (t, 3H), 1.45 (m, 10H), 2.11 (m, 2H), 2.39 (m, 2H), 3.43 (m, 2H), 4.10 (m, 1H).

2,6-Dimethyl-DL-*tyrosine-methyl ester-HCl* was prepared according to Pitzele *et al.*:[20] 70% (light brown solid); mp=139.4–140.5°C; R_f=0.88 (ethyl acetate:methanol=3:2, 1% NH_4OH); ^{1}H-NMR (D_2O, 270 MHz) δ 2.18 (s, 6H), 3.06 (m, 2H), 3.67 (s, 3H), 4.16 (t, 1H, J=8.2 Hz), 6.58 (s, 2H); ^{13}C-NMR (CD_3OD, 200 MHz) δ 21.6, 21.8, 32.5, 54.4, 54.6, 116.6, 123.3, 139.5, 157.1, 170.6.

O-*Me*-DL-*Tyrosine methyl ester-HCl* was prepared according to Moersch *et al.*:[21] 85% (white solid); mp=167–168°C; R_f=0.78 (ethyl acetate:methanol=3:2, 1% NH_4OH); ^{1}H-NMR (d_6-DMSO, 200 MHz) δ 3.10 (m, 2H), 3.68 (s, 3H), 3.75 (s, 3H), 4.21 (t, 1H, J=6.8 Hz), 6.90 (d, 2H, J=8.6 Hz), 7.17 (d, 2H, J=8.6 Hz); ^{13}C-NMR (CD_3OD, 200 MHz) δ 37.7, 54.2, 54.5, 56.2, 56.6, 115.7, 127.0, 131.5, 160.5, 170.0.

D,L-tert-*Leucine-methyl ester-HCl* was prepared according to Brenner *et al.*:[22] 91% (white solid);

mp=228.4–234.7°C (sublimes without melting); R_f=0.80 (ethyl acetate:methanol=3:2, 1% NH_4OH); ^{1}H-NMR (D_2O, 500 MHz) δ 1.07 (s, 9H), 3.29 (s, 3H), 3.72 (s, 1H). ^{13}C-NMR (CD_3OD, 500 MHz) δ 28.1, 34.8, 63.7, 182.9.

(±)-Methyl β-aminoisobutyrate-HCl, methyl ester of **34**, was prepared according to Brenner *et al.*:[22] 99% (white solid); mp=109.9–110.6°C; R_f=0.76 (chloroform:methanol=1:1); ^{1}H-NMR (D_2O, 200 MHz) δ 1.16 (d, 3H, *J*=7.68 Hz), 2.85–2.93 (m, 1H), 3.01–3.27 (m, 2H), 3.68 (s, 3H); ^{13}C-NMR (D_2O, 200 MHz) δ 17.9, 40.8, 44.8, 56.3, 178.0.

(±)-Methyl 3-aminobutyrate-HCl, methyl ester of **35**, was prepared according to Brenner *et al.*:[22] 96% (yellow oil); R_f=0.79 (methanol:chloroform=1:1); ^{1}H-NMR (D_2O, 200 MHz) δ 1.29 (d, 3H, *J*=6.9 Hz), 2.69 (d, 2H, *J*=6.1 Hz), 3.63–3.74 (m, 1H), 3.69 (s, 3H); ^{13}C-NMR (D_2O, 200 MHz) δ 21.6, 41.5, 48.0, 56.1, 180.1.

(±)-Methyl 2-aminotetralin-2-carboxylate, methyl ester of **37**, was prepared by the method of Obrecht *et al.*:[23] 48% (white solid); mp=131–132°C; R_f=0.68 (hexanes:ethyl acetate=3:1); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 1.92 (m, 1H), 2.16 (m, 1H), 2.76 (d, 2H, *J*=16 hz), 2.84 (m, 1H), 2.99 (m, 1H), 3.30 (d, 1H, *J*=16 hz), 7.06–7.13 (m, 4H). ^{13}C-NMR (CD_3OD, 200 MHz) δ 14.2, 25.3, 31.7, 39.7, 56.6, 61.2, 126.1, 128.8, 129.7, 132.8, 134.5, 176.2.

The *N*-Cbz derivatives **38** (R=Bn, Ph) were prepared from their respective methyl esters following a procedure by Bodansky:[24]

D,L-N-*Cbz-Phenylalanine-methyl ester:* 62% (yellow oil); R_f=0.82 (chloroform:methanol=95:5); ^{1}H-NMR ($CDCl_3$, 270 MHz) δ 3.10 (m, 2H), 3.71 (s, 3H), 4.65 (m, 1H), 5.08 (s, 2H), 5.21 (br d, 2H, *J*=7.9 Hz), 7.09–7.33 (m, 10H); ^{13}C-NMR ($CDCl_3$, 200 MHz) δ 39.6, 53.5, 55.9, 127.4, 128.3, 128.4, 128.8, 128.9, 129.5, 135.8, 136.4, 155.5, 171.7.

D,L-N-*Cbz-Phenyglycine-methyl ester*: 54% (white solid); mp=79.8–82.1°C; R_f=0.37 (hexanes:ethyl acetate=3:1); ^{1}H-NMR ($CDCl_3$, 270 MHz) δ 3.72 (s, 3H), 5.09 (s, 2H), 5.84 (d, 1H, *J*=7.2 Hz), 5.85 (br d, 2H, *J*=5.67 Hz), 7.17–7.35 (m, 10H); ^{13}C-NMR ($CDCl_3$, 200 MHz) δ 53.9, 59.0, 68.1, 127.4, 128.3, 128.4, 128.6, 128.8, 128.8, 129.1, 129.2, 136.3, 136.8, 155.2, 171.0.

We prepared the octyl esters of **47** and **48** and the 2-chloroethyl ester of **45** using a modified DCC coupling employing a water soluble coupling reagent (*N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide):[25]

(±)-2-Chloroethyl 2-phenylpropanoate, chloroethyl ester of **45**: 74% (yellow oil); R_f=0.60 (hexanes:ethyl acetate=3:1); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 1.54 (d, 3H, *J*=7.4 Hz), 3.64 (t, 2H, *J*=6 Hz), 3.77 (q, 1H, *J*=7 Hz), 4.3 (m, 2H), 7.2–7.4 (m, 5H); ^{13}C-NMR (CD_3OD, 200 MHz) δ 20.0, 42.7, 46.6, 65.2, 127.5, 127.8, 128.9, 140.2, 173.9.

(±)-Octyl tetrahydro-2-furoate, octyl ester of **47**: 69% (clear oil); R_f=0.59 (hexanes:ethyl acetate=3:1); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.86 (br t, 3H, *J*=1.13), 1.25 (br s, 12H), 1.62 (m, 2H), 1.94 (m, 3H), 2.28 (m, 1H), 4.08 (m, 2H), 4.12 (t, 2H, *J*=6.8 Hz), 4.39 (m, 1H); ^{13}C-NMR (CD_3OD, 200 MHz) δ 15.7, 24.1, 26.7, 27.3, 30.0, 31.6, 33.1, 66.0, 70.3, 173.2.

(±)-Octyl tetrahydro-3-furoate, octyl ester of **48**: 72% (clear oil); R_f=0.69 (hexanes:ethyl acetate=3:1); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.86 (br t, 3H), 1.27 (m, 12H), 1.60 (m, 2H), 2.1 (m, 2H), 3.07 (q, 1H, *J*=8 Hz), 3.85–4.04 (m, 4H), 4.11 (t, 2H, *J*=6.6 Hz); ^{13}C-NMR ($CDCl_3$, 500 MHz) δ 13.9, 22.4, 25.0, 25.6, 28.4, 28.9, 30.0, 30.6, 31.5, 64.8, 69.1, 72.4, 77.7, 103.0, 161.9.

(±)-Methyl-2-chloropropanoate, methyl ester of **49**. (±)-2-Chloropropionyl chloride was added dropwise to a stirring solution of methanol in an ice bath, under an atmosphere of N_2. The reaction was allowed to warm to room temperature and was stirred for 1 h. The reaction was then neutralized with saturated sodium bicarbonate, extracted with diethyl ether (3×15 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*: 89% (yellow oil); R_f=0.51 (hexanes:ethyl acetate=2:1); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 1.69 (d, 3H, *J*=6.9 Hz), 3.79 (s, 3H), 4.41 (q, 1H, *J*=6.9 Hz); ^{13}C-NMR ($CDCl_3$, 200 MHz) δ 23.0, 53.5, 54.1, 170.3.

General procedure for ANL-catalyzed hydrolyses of amino acid esters, esters of 32–37

Aspergillus niger lipase (25 units) was added to sodium acetate buffer (8 mL, 10 mM, pH 5) and stirred for 1 h to ensure complete dissolution. D,L-Amino acid esters (100 mg) dissolved in sodium acetate buffer (1 mL, 10 mM, pH 5) were added and the rate of hydrolysis was monitored by pH stat which maintained the pH at 5 by automatic titration with 0.1 N NaOH. Reactions were usually terminated at approximately 40% conversion, as noted on the pH stat, by removing the enzyme by ultrafiltration with a YM-10 Amicon membrane. The aqueous extract containing both the starting ester and product acid was concentrated *in vacuo*. The enantiomeric purities of the acid and ester were measured as described below and the enantiomeric ratio, E, was calculated according to Sih.[26] When the enantiomeric excess of both starting ester and product acid could not be determined in a single HPLC run, the starting ester and acid were separated by adjusting the solution to pH 8 with saturated $NaHCO_3$ and extracting the ester with ethyl acetate (3×15 mL). Ethanol was added dropwise during the workup to break up emulsions when necessary. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated *in vacuo*, producing the ester. The aqueous phase was concentrated *in vacuo*, yielding the product acid. There was no detectable chemical hydrolysis of the amino acid esters at pH 5.

General procedure for ANL-catalyzed hydrolyses of carboxylic acid esters, esters of 38, 39, 43–49

The procedure was similar to above, with the following changes. (±)-Carboxylic acid esters (100 mg) were dissolved in 2 mL diethyl ether and added to the stirring enzyme solutions. The stirring reactions were sealed with Parafilm to prevent appreciable evaporation of the ether layer during the reactions. Reactions were terminated at a convenient conversion by adjusting the solution to pH 1 using 1 N HCl and the product acids and starting esters were extracted with diethyl ether (3×20 mL). The starting ester and acid were separated by adjusting the solution to pH 8 with saturated $NaHCO_3$ and extracting the ester with ethyl acetate (3×15 mL). The aqueous layer was adjusted to pH 2 and the acid extracted with ethyl acetate (3×15 mL). Ethanol was added dropwise during workup to break up the emulsion and inactivate the enzyme. Both extracts were dried with Na_2SO_4, filtered, and concentrated *in vacuo*. The enantiomeric excesses of the acids and esters were measured as described below and the enantiomeric ratio, E, was calculated according to Sih.[26] There was no detectable chemical hydrolysis of the above substrates at pH 5.

Determination of enantiomeric purity by HPLC using a chiral stationary phase

High performance liquid chromatography (HPLC) was performed on a Spectra Physics liquid chromatograph, model 8800 equipped with a Spectra FOCUS forward optical scanning detector, SP8880 autosampler and Spectra Physcis software. The analytical chiral columns used for enantiomeric resolution were a Chiralpak AD amylose derivatized column, a Chiralcel OD column, a Chiralpak WH column and a Crownpak CR(+) crown ether derivatized column (Daicel Chemical Industries Ltd, Fort Lee, NJ). Enantiomeric excesses were measured as described below and the enantiomeric ratio, E, was calculated. The area of the peaks was measured either by electronic integration or cut and weigh when electronic integration gave unequal areas for the racemate.

Enantiomers of the proline, **32**, were analysed with a Chiralpak WH column using 0.25 mM $CuSO_4$ as eluting buffer with the detector set at 254 nm: 50°C, 1.5 mL/min, k_L'=2.23; k_D'=3.90; α=1.75; R_s=1.77. Proline was dissolved in eluting buffer (1 mg/mL), and neutralized to pH 7 with dilute NaOH prior to injection. Proline esters were hydrolysed with aqueous NaOH for analysis. The absolute configuration was established by comparison with an authentic sample of L-proline.

Enantiomers of α- and β-amino acids, **33–35**, their methyl esters, and phenylalaninamide were separated with a Crownpak CR(+) modified crown ether column, using aq. $HClO_4$ solution as eluent, with the detector set at 200 nm. Concentrations of 0.1 mg compound/mL mobile phase were injected for analysis except for alanine, 1.0 mg/mL. Absolute configurations were inferred from the order of elution on a CR(+) column for which the D-enantiomer always elutes first and by the reported orders of

elution in literature for the compounds below.[27] CAUTION: Aqueous solutions containing perchloric acid should not be evaporated and can explode if heated.

Alanine: 0°C, aq. $HClO_4$ pH 1.5, 0.4 mL/min, k_D'=0.75; k_L'=2.30; α=3.07; R_s=6.00.

Alanine-methyl ester: 25°C, aq. $HClO_4$ pH 2, 0.3 mL/min, k_D'=0.38; k_L'=0.70; α=1.84; R_s=1.8.

Methionine: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=0.82; k_L'=1.6; α=1.95; R_s=2.12.

Methionine-methyl ester: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=1.79; k_L'=4.04; α=2.26; R_s=3.00.

Phenylalanine: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=3.93; k_L'=5.20; α=1.32; R_s=3.86.

Phenylalanine-methyl ester: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=8.29; k_L'=10.09; α=1.22; R_s=2.76; and at 5°C, aq. $HClO_4$ pH 1.5, 0.8 mL/min, k_D'=11.7; k_L'=20.9; α=1.78; R_s=3.87.

Phenylalaninamide: 5°C, aq. $HClO_4$ pH 1.5, 0.8 mL/min, k_D'=7.66; k_L'=14.8; α=1.93; R_s=5.05.

2-Phenylglycine: 40°C, aq. $HClO_4$ pH 2, 1.0 mL/min, k_D'=1.37; k_L'=4.46; α=3.25; R_s=10.1.

2-Phenylglycine-methyl ester: 40°C, aq. $HClO_4$ pH 2, 1.0 mL/min, k_D'=3.89; k_L'=10.18; α=2.62; R_s=10.0.

Tyrosine: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=2.11; k_L'=2.84; α=1.34; R_s=1.09.

Tyrosine-methyl ester: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=4.27; k_L'=6.24; α=1.46; R_s=1.62.

4-O-Me-Tyrosine: 25°C, 1.2 mL/min, aq. $HClO_4$ pH 2 k_D'=6.62; k_L'=8.73; α=1.31; R_s=2.80.

4-O-Me-Tyrosine-methyl ester: 25°C, aq. $HClO_4$ pH 2, 1.2 mL/min, k_D'=14.72; k_L'=19.88; α=1.35; R_s=1.89.

2,6-dimethyltyrosine: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=10.19; k_L'=11.29; α=1.11; R_s=0.84.

2,6-dimethyltyrosine-methyl ester: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=10.89; k_L'=12.11; α=1.11; R_s=1.08.

Tryptophan: 25°C, aq. $HClO_4$ pH 2, 1.2 mL/min, k_D'=11.61; k_L'=14.32; α=1.23; R_s=1.20.

Tryptophan-methyl ester: 25°C, aq. $HClO_4$ pH 2, 1.2 mL/min, k_D'=24.61; k_L'=32.47; α=1.32; R_s=1.40.

β-Aminoisobutyic acid, **34**: 0°C, aq. $HClO_4$ pH 1, 0.4 mL/min, k_D'=3.48; k_L'=4.15; α=1.19; R_s=1.04.

Methyl β-aminoisobutyrate: 0°C, aq. $HClO_4$ pH 1.5, 0.4 mL/min, k_D'=3.03; k_L'=4.00; α=1.32; R_s=1.63.

3-Aminobutyric acid, **35**: 0°C, aq. $HClO_4$ pH 1.5, 0.4 mL/min, k_D'=2.05, k_L'=2.64, α=1.29; R_s=1.29.

Methyl 3-aminobutyrate: 0°C, aq. $HClO_4$ pH 1.5, 0.4 mL/min, k_D'=4.19; k_L'=5.17; α=1.23; R_s=1.73

Enantiomers of **43**, 2-(4-chlorophenoxy)propionic acid, were separated using a Chiralpak AD column as described previously by Colton *et al.*:[28] 254 nm, 25°C, hexanes:isopropanol:trifluoroacetic acid=95:5:1, 0.5 mL/min, k_S'=2.13; k_R'=2.80; α=1.31; R_s=3.78. The absolute configuration was established by comparison with the reported order of elution by the column manufacturer. The ester of **43** was hydrolyzed to **43** with 1.5 equivalents of aqueous NaOH prior to analysis.

Enantiomers of **38** (R=Bn, Ph) and **45** were separated using a Chiralcel OD column. The ester of **45** was hydrolyzed to **45** with 1.5 equivalents of aqueous NaOH prior to analysis. The absolute configurations were established by comparison with the reported order of elution by the column manufacturer.

2-Phenylpropionic acid, **45**: 254 nm, 25°C, hexanes:isopropanol:formic acid=98:2:1, 0.5 mL/min, k_R'=3.35; k_S'=4.05; α=1.21; R_s=2.13.

N-Cbz-Phenylalanine: 254 nm, 25°C, hexanes:isopropanol:trifluoroacetic acid=79:20:1, 0.5 mL/min, k_D'=1.84; k_L'=2.13; α=1.16; R_s=1.52.

N-Cbz-Phenylalanine-methyl ester: 254 nm, 25°C, hexanes:isopropanol=80:20, 0.5 mL/min, k_L'=4.74; k_D'=5.34; α=1.13; R_s=1.47.

N-Cbz-Phenylglycine-methyl ester: 254 nm, 25°C, hexanes:isopropanol=80:20, 0.5 mL/min, k_S'=2.87; k_R'=3.37; α=1.17; R_s=2.00.

Determination of enantiomeric purity by ^{1}H-NMR

The racemic esters of **47** and **48** were dissolved in $CDCl_3$ and the ^{1}H-NMR spectra were obtained using a 200 MHz spectrometer. Solid tris[(3-heptafluoropropylhydroxymethylene)-(+)-camphorato]

europium(III), $Eu(hfc)_3$, was added portion-wise until separate signals for the $COOCH_2$ methylene triplet of the racemic octyl esters were obtained. The triplets of **47** were separated by 2.4 Hz and those of **48** were separated by 3.7 Hz. The number of equivalents of shift reagent necessary to obtain the separations was then added to the esters for which the enantiomeric purity was to be determined. The absolute configurations were not determined.

Determination of enantiomeric purity by gas chromatography using a chiral stationary phase

Gas chromatography was performed on a Varian 589-Series II gas chromatograph equipped with chiral stationary phases.

Enantiomers of methyl mandelate (methyl ester of **44**) were separated on a Chiraldex G-TA30 column (Astec Inc., Whippany, NJ) which contained 2,6-di-*O*-pentyl-3-*O*-trifluoroacetyl derivative of γ-cyclodextrin as the stationary phase: 110°C, split ratio 115:1, k_S'=17.8; k_R'=18.4; α=1.03; R_s=0.91. Product acid was converted to the ester derivative with ethereal CH_2N_2 for analysis. The absolute configuration was established by comparison with the reported order of elution by the column manufacturer.

Enantiomers of the methyl ester of **49** were separated using a Chirasil-DEX CB column (Chrompack Inc., Raritan, NJ) which contained a polydimethylsiloxane derivative of β-cyclodextrin as the stationary phase: 60°C, k_S'=3.8; k_R'=4.47; α=1.17; R_s=5.00. Product acid **49** was converted to the methyl ester for analysis by dissolution in methanol containing a catalytic amount of sulfuric acid. The absolute configuration was established by comparing the order of elution with an authentic sample of (*S*)-methyl-2-chloropropanoate.

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Quick *E*. A Fast Spectrophotometric Method To Measure the Enantioselectivity of Hydrolases

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Hydrolase-catalyzed resolutions of racemates are often the best route to enantiomerically pure compounds.[1] These reactions are often more selective and cheaper than chemical methods. To find an enantioselective hydrolase for a target compound, researchers first screen commercial enzymes and cultures of microorganisms and then optimize the reaction conditions. Both screening and optimization require measuring the enantioselectivity of the reaction. The enantioselectivity of an enzyme is the ratio of the specificity constants, k_{cat}/K_M, for the enantiomers, eq 1.[2,3]

$$\text{enantiomeric ratio} = E = \frac{(k_{cat}/K_M)_{\text{fast enant}}}{(k_{cat}/K_M)_{\text{slow enant}}} \quad (1)$$

Currently, the best method to measure E is the endpoint method developed by Sih's group,[3] but screening hundreds of commercial enzymes or cultures of microorganisms by this method is time-consuming. To measure E, researchers run a test resolution, work up the reaction, and measure conversion and enantiomeric purity of the starting material or product. A typical example, measuring the enantioselectivity of a hydrolase toward 1, required approximately 4.5 h.

Recognizing this difficulty, researchers have reported alternative methods to measure E by measuring initial rates of samples with varying ratios of enantiomers[4] or by analyzing reaction progression curves.[5] Unfortunately, they are not significantly faster and can be less accurate than the endpoint method. In this paper, we report a method to measure E in 1 min from relative initial rates of hydrolysis of pure enantiomers and a reference compound. Researchers previously used mixtures of substrates to measure enzyme selectivity.[6] We extend these techniques to enantioselectivity and to rapid spectrophotometric measurements.

Hydrolyses of pure enantiomers of 4-nitrophenyl 2-phenylpropanoate, (*S*)-1 and (*R*)-1, and 4-nitrophenyl 2-(4-isobutylphenyl)propanoate (ibuprofen 4-nitrophenyl ester), (*S*)-2 and (*R*)-2, liberates the yellow *p*-nitrophenoxide ion.[7] The increase in absorbance at 404 nm revealed the initial rates of hydrolysis of each enantiomer, but the ratio of these rates did not give the enantiomeric ratio, Table 1. The ratio of rates over- and underestimated E by as much as 70% because it ignored competitive binding of the two enantiomers to the enzyme. In other cases, researchers found that differences in K_M for the enantiomers contributed a factor of 3−4 to the enantioselectivity.[8]

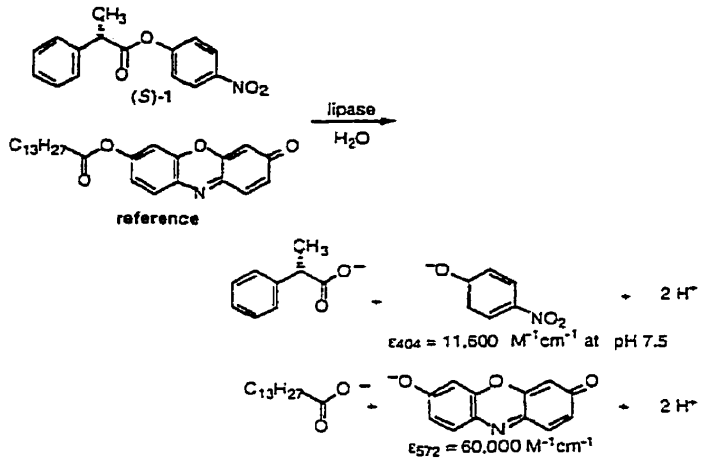

Figure 1. First step of the quick E measure of enantioselectivity of lipases toward 4-nitrophenyl 2-phenylpropanoate, 1. Lipase-catalyzed hydrolysis of (*S*)-1 and the reference compound, resorufin tetradecanoate, yields yellow and pink chromophores, respectively. The solution turns deep orange if both substrates are hydrolyzed, pink if only the reference compound is hydrolyzed. The second step of the quick E is the same, except that it uses the (*R*)-enantiomer of the chiral ester. Equations 2 and 3 yield the enantioselectivity.

To reintroduce competition, we added resorufin tetradecanoate as a reference compound.[9] We monitored the initial rates of hydrolysis of (*S*)-1 at 404 nm and the reference compound at 572 nm in the same solution, Figure 1. After taking into account the initial concentrations of both substrates, the ratio of these rates yielded the selectivity of the hydrolase for (*S*)-1 over the reference compound, eq 2.

$$\frac{(S)\text{-}1}{\text{reference}}\text{selectivity} = \frac{(k_{cat}/K_M)_{(S)\text{-}1}}{(k_{cat}/K_M)_{\text{reference}}} = \frac{v_{(S)\text{-}1}}{v_{\text{reference}}}\,\frac{[\text{reference}]}{[(S)\text{-}1]} \quad (2)$$

A second experiment using (*R*)-1 and the reference compound yielded the selectivity of (*R*)-1 over the refer-

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(7) Enantiomerically pure acid and 4-nitrophenol were coupled using 1 equiv of *N*-ethyl-*N*′-[3-(dimethylamino)propyl] carbodiimide and 1 equiv of 1-hydroxybenzotriazole in anhydrous dichloromethane at 0 °C for 15 min and then stirred at room temperature for 48 h. Esters were purified by column chromatography on silica gel eluted with ethyl acetate and recrystallized from hexanes/ethyl acetate, 44−60% yield. (*R*)-1, 99.7% ee; (*S*)-1, 99.4% ee; (*R*)-3, 98.2% ee; (*S*)-3, 99.6% ee. Starting acids showed the same enantiomeric purity. All enantiomeric purities were measured by HPLC using a Chiracel OD-H column (Daicel) at 25 °C, eluted at 1 mL/min. 2-Phenylpropanoic acid (98/2/1 hexanes/2-propanol/trifluoroacetic acid, $k_R' = 3.35$; $k_S' = 4.05$; $\alpha = 1.2$; $R_s = 2.13$); 2-(4-isobutylphenyl)propanoic acid (100/1/0.1 hexanes/2-propanol/trifluoroacetic acid, $k_R' = 3.31$; $k_S' = 4.27$; $\alpha = 1.29$; $R_s = 2.43$); 3 (100/1/0.1 hexanes/2-propanol/trifluoroacetic acid, $k_R' = 2.41$; $k_S' = 2.93$; $\alpha = 1.21$; $R_s = 1.66$). For analysis, samples of 1 were hydrolyzed to the acid in aqueous NaOH.

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Table 1. Enantiomeric Ratios of Hydrolases toward (±)-1 and (±)-3 Measured Using the Endpoint Method[a] and the Ratio of Separately-Measured Initial Rates of Hydrolysis of the Enantiomers

substrate	lipase[b]	ee_s[c] (%)	ee_p[c] (%)	c[d] (%)	E[e] endpoint	initial rate[f] (S)	initial rate[f] (R)	rate ratio S/R[g]
1	PCL	nd	67	60	29 ± 3	15.32	0.771	20 ± 1
1	CRL	17	50	25	3.5 ± 0.2	2.81	2.38	1 ± 0.2
1	IPA–CRL	43	98	30	>100	3.56	0.092	40 ± 2
1	PPL	nd	2	4	1.1 ± 0.1	1.11	0.757	1.4 ± 0.1
1	CAL–A	20	23	37	1.9 ± 0.1	1.40	0.355	4 ± 0.2
3	PCL	28	43.2	39	3.3 ± 0.1 (*R*)	0.535	0.579	1.1 ± 0.1 (*R*)
3	CRL	5.3	14.5	27	1.4 ± 0.1	0.330	0.274	1.2 ± 0.1
3	IPA–CRL	40.1	>99	29	>100	0.702	0.0128	55 ± 5

[a] Reaction conditions: room temperature, 10 mM Tris buffer, pH 8, 100 mg of crude lipase or 0.7 mg of protein for IPA–CRL, 1 mmol of substrate dissolved in 1 mL of acetonitrile. [b] For spectrophotometric measurements, the lipases were dissolved in Tris buffer (25 mM, pH 7.5) and centrifuged to remove insoluble material. PCL (lipase from *Pseudomonas cepacia*, Amano lipase PS, 29.3 mg solid/mL), CRL (lipase from *Candida rugosa*, Sigma, 36.9 mg of solid/mL; 0.5 mg protein/mL by the Bio-Rad protein assay), IPA–CRL (CRL treated with 2-propanol as in ref 12a; 0.7 mg/mL of protein by the Bio-Rad protein assay), PPL (porcine pancreatic lipase, Sigma, 42.3 mg of solid/mL), CAL-A (lipase A from *Candida antarctica*, Boehringer Mannheim, 18.2 mg of solid/mL). [c] ee_s = enantiomeric excess of remaining substrate, ee_p = enantiomeric excess of product. Determined by HPLC as described in ref 7. [d] Extent of conversion. [e] Enantiomeric ratio calculated using eq 2. Error limits for *E* were estimated assuming an error of ±1% for enantiomeric purity. All hydrolyses favored the (*S*)-enantiomer except the PCL-catalyzed hydrolysis of 3, which favored the (*R*)-enantiomer. [f] Substrates were emulsified in aqueous solution according to: Vorderwulbecke, T.; Kieslich, K.; Erdmann, H. *Enzyme Microb. Technol.* **1992**, *14*, 631–639. An acetonitrile solution of 1 (7.8 mM, 0.5 mL) or 3 (9.04 mM, 0.5 mL) was added dropwise to Tris buffer (9 mL, 50 mM, pH 8.0) containing 0.45–0.90 w/v % Triton X-100 (Pierce Surfact-Amps) and vortexed until clear. This emulsion remained clear for at least 3 h. To measure initial rates of hydrolysis, the lipase solution (100 μL) was added to the substrate emulsion (900 μL) at 25 °C, and the increase in absorbance at 404 nm was monitored for 15 s. No spontaneous chemical hydrolysis was detected. Values are in absorbance per second × 10^3. [g] Average and standard deviation for three experiments.

Table 2. Enantiomeric Ratios of Hydrolases toward (±)-1 and (±)-3 Using the Quick *E* Method

		(*S*)-enantiomer + ref[b]		(*R*)-enantiomer + ref[b]		
substrate	lipase[a]	404 nm	572 nm	404 nm	572 nm	*E*[c] quick *E*
1	PCL	13.2	9.79	0.379	7.50	29 ± 3
1	CRL	1.02	9.48	0.28	9.02	3.5 ± 0.3
1	IPA–CRL	1.89	7.58	0.004	3.68	210 ± 20
1	PPL	0.199	0.186	0.125	0.164	1.4 ± 0.2
1	CAL–A	2.15	1.53	0.824	1.47	2.3 ± 0.2
3	PCL	0.3341	6.78	0.5750	4.709	2.5 ± 0.3 (*R*)
3	CRL[d]	3.408	49.3	1.342	52.18	3 ± 2
3	IPA–CRL	2.154	8.78	<0.01	5.767	>140

[a] See Table 1. [b] Substrate solutions as for Table 1, but an acetonitrile solution of resorufin tetradecanoate (0.5 mL, 1.6 mM) was also added. The liberated 4-nitrophenoxide and resorufin were measured in two separate measurements at 404 and 572 nm, respectively, at 25 °C for 15 s. No spontaneous chemical hydrolysis was detected. Values are in absorbance per second × 10^3. [c] Enantiomeric ratio calculated using eq 3. Average and standard deviation for three measurements. [d] CRL (lipase from *Candida rugosa*, Sigma, 214 mg solid/mL; 1.9 mg protein/mL by the Bio-Rad protein assay).

ence compound. The ratio of these two selectivities yields the enantiomeric ratio, eq 3.

$$E = \frac{(S)\text{-}1}{(R)\text{-}1}\text{selectivity} = \frac{\dfrac{(S)\text{-}1}{\text{reference}}\text{selectivity}}{\dfrac{(R)\text{-}1}{\text{reference}}\text{selectivity}} \quad (3)$$

The quick *E* method agreed with the endpoint method for both **1** and **2** using five different lipases, Table 2. We measured low ($E = 1.4$), average ($E = 27$), and excellent ($E = 210$) enantiomeric ratios correctly by this technique. Each hydrolysis experiment requires 30 s; thus, the measurement time for *E* was only one minute.

There are three advantages to the quick *E* method. First, it is many times faster than a typical endpoint measurement, yet has equivalent or better accuracy. Accuracy may be particularly important for screening in directed evolution where the improvements of each generation are small.[10] Note that quick *E* is based on the same equations as the endpoint methods, so inaccuracies of the endpoint method also apply quick *E*.[11] Second, it requires much smaller amounts of hydrolase because the entire reaction occurs in the spectrophotometer. This advantage may be especially useful for screening biocatalyst libraries. Third, the quick *E* method can measure high enantioselectivities more easily than the endpoint method.

There are also several disadvantages to this screen. First, it requires pure enantiomers, albeit in small amounts (~20 μg per measurement in a 200 μL cuvette). Second, the current version of quick *E* measures the enantioselectivity only for chromogenic substrates. Future versions of quick *E* will extend the range to all esters. Third, spectrophotometric measurements require clear solutions. Dissolving hydrophobic substrates in aqueous solution requires addition of surfactants.

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(11) Due to assumptions made in deriving eq 1, both the endpoint method and quick *E* will give inaccurate enantioselectivities in two common situations—impure biocatalyst and reactions inhibited by product.

Quantitative Screening of Hydrolase Libraries Using pH Indicators: Identifying Active and Enantioselective Hydrolases

Lana E. Janes, A. Christina Löwendahl, and Romas J. Kazlauskas*

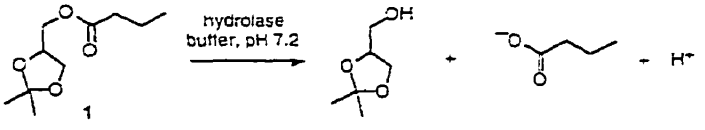
Abstract: The slowest step in finding a selective hydrolase for synthesis is often the screening step. Researchers must run small test reactions and measure the amounts of stereoisomers formed by HPLC, GC, or NMR. We have developed a colorimetric method to speed up this screening. We quantitatively detect ester hydrolysis using a pH indicator, 4-nitrophenol. We estimate the selectivity by measuring the initial rates of hydrolysis for pure stereoisomers separately. To demonstrate the utility of this method, we screened seventy-two commercial enzymes for enantioselective hydrolysis of racemic solketal butyrate, an important chiral building block. First, we eliminated the twenty hydrolases that did not catalyze hydrolysis of either enantiomer. Next, we measured initial rates of hydrolysis of the pure enantiomers of solketal butyrate. For horse-liver esterase, these initial rates differed by a factor of twelve. Subsequent GC experiments confirmed an enantiomeric ratio of fifteen for this hydrolase. Although this enantioselectivity is moderate, it is the highest enantioselectivity reported for a hydrolysis of solketal esters.

Keywords: enantiomeric resolution · enzyme catalysis · lipases · screening · synthetic methods

Introduction

Chemists often exploit the high stereoselectivity and regioselectivity of hydrolytic enzymes to solve synthetic problems.[1] For example, researchers use selective reactions catalyzed by lipases to prepare enantiomerically pure pharmaceutical intermediates and to selectively deprotect sensitive synthetic intermediates. One limitation to the wider use of hydrolases is the difficulty in finding the best hydrolase for a given reaction. Although several empirical rules[2] can assist in the selection of likely candidates from the fifty to one hundred hydrolases commercially available, most researchers also use screening. To screen for selective hydrolases today, researchers run a small reaction for each hydrolase, work up the reaction, and determine the ratio of stereoisomers by HPLC, GC, or NMR.[3] At best, one determination takes four hours. To save time researchers rarely screen all commercial hydrolases and likely miss good hydrolases.

To speed up this screening, we have developed a quantitative, colorimetric assay for hydrolysis of esters using pH indicators. Hydrolysis of an ester at neutral pH, for example solketal butyrate (**1**, butyryl ester of 2,2-dimethyl-1,3-dioxolane-4-methanol), releases a proton (Scheme 1). We measure the rate of proton release using a pH indicator.



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Scheme 1. Hydroyisis of solketal butyrate at neutral pH.

Researchers have used pH indicators to monitor the progress of enzyme-catalyzed reactions that release or consume protons since the 1940's.[4, 5] For example, researchers have monitored reactions catalyzed by amino acid decarboxylase,[6] carbonic anhydrase,[7] cholinesterase,[8] hexokinase,[9, 10] and proteases.[11] Many researchers either used a pH indicator assay qualitatively or calibrated the color change with additional experiments.

However, by choosing the reaction conditions carefully, one can ensure that the color change is proportional to the number of protons. In particular, both the buffer and the indicator must have the same affinity for protons (pK_as within 0.1 unit of each other) so that the relative amount of protonated buffer to protonated indicator stays constant as the pH shifts during the reaction. Researchers defined the proportionality between the rate of indicator absorbance change and reaction rate as the buffer factor, Q.[6, 7, 12] When the pK_as of the indicator and buffer are the same, Q is given by Equation (1), where C represents the total molar concentration (sum of acid

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and base forms) of buffer (B) or indicator (In), $\Delta\varepsilon$ represents the difference in extinction coefficient between the protonated and deprotonated forms of the indicator, and l represents the path length. The true reaction rate is given by Equation (2), where dA/dt is the rate of indicator absorbance change. The highest sensitivity (largest dA/dt) occurs when Q is small. Thus, lowering the buffer concentration or increasing the indicator concentration increases the sensitivity of the assay.

$$Q = \frac{C_B}{C_{In}} \times \frac{1}{\Delta\varepsilon_{404\,nm} l} \quad (1)$$

$$\text{rate } (\mu\text{mol min}^{-1}) = \frac{dA}{dt} \times Q \times \text{reaction volume} \times 10^6 \quad (2)$$

We used this assay to screen for enantioselective hydrolases in 96-well microplates (Figure 1). Using pure enantiomers, we separately measured the initial rates of hydrolysis of each enantiomer. Hydrolases that showed large differences in the initial rates of hydrolysis of the two enantiomers were further analyzed by traditional methods to determine enantioselectivities.[3] Note that the ratio of separately measured initial rates of hydrolysis of the enantiomers is not the true enantioselectivity, so this screening provides only an estimated enantioselectivity.[13] To minimize the amount of pure enantiomers needed, we first screened using racemic substrate to eliminate hydrolases that did not catalyze hydrolysis of the racemic substrate. Whittaker et al. measured the esterase activity of proteases in 96-well microplates using a similar assay.[11] However, their assay required additional calibration experiments because it did not use an indicator–buffer pair with the same pK_a values.

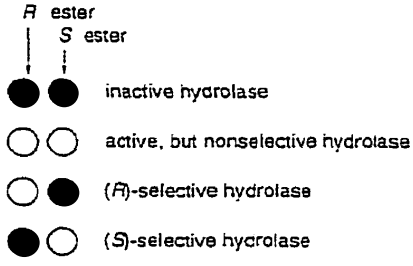

Figure 1. Schematic diagram of the colorimetric screen for enantioselectivity. The circles represent wells in a microplate containing either the *R* or the *S* enantiomer. Hydrolysis of an ester releases acid that decolorizes the pH indicator. Dark circles represent no reaction, while the white circles represent wells in which hydrolysis occurred. This diagram exaggerates the color change; in practice the color change is not visible to the eye. The hydrolase is rejected if neither enantiomer reacts or if both enantiomers react at similar rates. If one enantiomer reacts significantly faster than the other, then the hydrolase is tested further. Note that measuring the rates of hydrolysis of pure enantiomers separately gives only an estimated enantioselectivity, not the true enantioselectivity (see ref. [13]).

To demonstrate this method, we screened a library of seventy-two commercial hydrolases (lipases, esterases, and proteases) for enantioselective hydrolysis of solketal butyrate, an important chiral building block in the synthesis of pharmaceuticals and biologically active compounds.[14] Many researchers have searched, without success, for a highly enantioselective hydrolase that could resolve this substrate.[15] For hydrolysis in water, the highest enantiomeric ratio was 9 for a proteinase from *Aspergillus oryzae*,[16] while for acylation of solketal in organic solvent, the highest enantiomeric ratio was 20–25 for a lipase from *Pseudomonas* species (lipase AK).[15] Our screening, which is easily completed in one afternoon, has identified a new hydrolase, horse-liver esterase, with an enantiomeric ratio of 15 for the hydrolysis of solketal butyrate. This is the highest enantioselectivity yet reported for hydrolysis of a solketal ester.

Results

Optimizing sensitivity of the assay: Since most hydrolases have maximal activity near neutral pH, we developed the assay for pH 7.2. As a pH indicator, we chose 4-nitrophenol. The similarity of its pK_a (7.15[17]) to the pH of the reaction mixture ensures that changes in pH give a large and linear color change.[6] The large difference in the extinction coefficients of the protonated and deprotonated forms (200 versus 18000 $M^{-1}cm^{-1}$ at 404 nm) gives good sensitivity.[18] Finally, nitrophenols bind less strongly to proteins than some polyaromatic indicators.[19] The concentration of the pH indicator should be as high as possible to maximize sensitivity [Eq. (1)]. In our assay, the high initial absorbance of 4-nitrophenoxide/4-nitrophenol limited the concentration to 0.45 mM.[20] This concentration gave a starting absorbance of ≈ 1.2, where the accuracy is still not compromised by low light levels.

As a buffer, we chose BES [*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] because its pK_a (7.15[21]) is identical to that of 4-nitrophenol. This ensures that changes in proton concentration during the reaction give linear changes in absorbance.[22] We empirically determined an optimum buffer concentration of approximately 5 mM (see below). This value is a compromise between low buffer concentrations to maximize sensitivity [Eq. (1)], and high buffer concentrations to ensure accurate measurements and small pH changes throughout the assay (<0.05 pH units for 10% hydrolysis under our conditions). The small pH changes are important because kinetic constants can change with changing pH.

Suitable substrate concentrations ranged from 0.5 to 2 mM, typically 1 mM. At substrate concentration below 0.5 mM, the absorbance changes are too small to be detected accurately under our standard conditions. For example, hydrolysis of 5% of a 0.25 mM substrate concentration under our standard conditions (pH 7.2, 0.45 mM 4-nitrophenol, 5 mM BES), changes the absorbance by only 0.005 absorbance units. Solubility in water sets the upper limit of substrate concentration because spectrophotometric measurements require clear solutions. Typical organic substrates dissolve poorly in water, so we added organic cosolvent—7 vol% acetonitrile. For very insoluble substrates, we used previously prepared clear emulsions with detergents.[23]

Quantitative validation of the assay: To confirm that the color changes accurately measured the release of protons, we experimentally determined the factor Q and compared it with the theoretical Q, calculated using Equation (1). First, we mimicked the proton release upon hydrolysis of the substrate

by addition of HCl, Figure 2. The absorbance decreased linearly owing to protonation of the 4-nitrophenoxide. The reciprocal of the slopes corresponds to the buffer factor, Q,

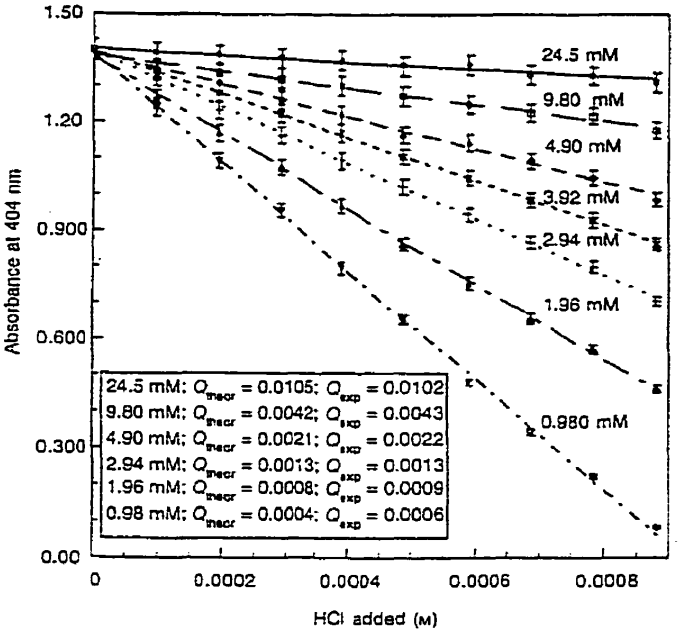

Figure 2. Sensitivity of the assay solution to added acid. The yellow color of the assay solution decreased linearly (regression factor = 0.97 for 24.5 mM; > 0.99 for all other concentrations) in each solution. For a given amount of acid, solutions with lower concentrations of buffer showed larger color changes. For buffer concentration above 2 mM, the experimentally measured slopes were within 5% of the theoretical slopes calculated by means of Equation (1) (where $\Delta\varepsilon = 17800\ M^{-1}cm^{-1}$, $l = 0.292$ cm, $C_{in} = 0.447$ mM). For buffer concentrations below 2 mM, the experimental and theoretical slopes disagreed by > 10%. We chose a buffer concentration of 5 mM to maximize sensitivity without compromising accuracy. Each point is an average of four measurements, with variation < 3% between each measurement. All lines are normalized to a starting absorbance of 1.4.

calculated by means of Equation (1). As expected, the decreases were all linear and the slopes increased with decreasing buffer concentration. However, below 2 mM buffer, the experimentally measured slopes disagreed with the theoretical slopes by more than 10%. We chose 5 mM as the buffer concentration for our assay as a compromise between accuracy and sensitivity.

Small changes in reaction conditions did not compromise the sensitivity or accuracy of this assay. The measured value of Q did not change by more than the experimental error ($\approx$5%) upon addition of 7% of acetonitrile or dimethyl sulfoxide. The measured value of Q also was not changed by unknown buffer salts in the commercial hydrolases or by added $CaCl_2$ (2 mM) in the stock solutions of proteases.

As a test reaction, we monitored the hydrolysis of racemic solketal butyrate catalyzed by horse-liver esterase. The decrease of the indicator absorbance was linear (Figure 3a), and corresponded to a specific activity of 1.85 $\mu mol\,min^{-1}\,mg^{-1}$ protein.[24] Control experiments with no substrate or with no esterase showed no change in absorbance over one hour. When we scaled up the reaction a hundredfold and monitored the reaction with a pHstat, we

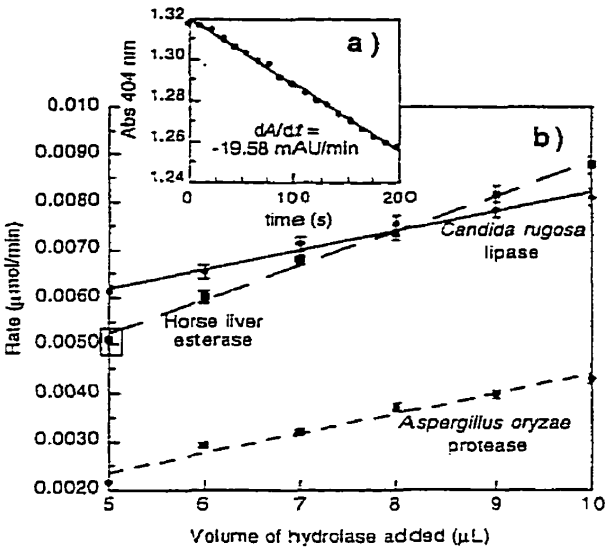

Figure 3. a) Initial rate measurement for boxed point showing the measured absorbance change in the horse-liver esterase-catalyzed hydrolysis of (±)-solketal butyrate with 5 μL hydrolase solution. The calculated rate [Eq. (2)] equals 0.0050 $\mu mol\,min^{-1}$ and the specific activity equals 1.85 $\mu mol\,min^{-1}\,mg^{-1}$ protein. The measured points fit a straight line with regression factor of 0.997. b) Increased rates of hydrolysis of 1 mM (±)-solketal butyrate with increased amount of hydrolase. Rates are calculated over 200 seconds by means of Equation (2), where $\Delta\varepsilon = 17300\ M^{-1}cm^{-1}$, $l = 0.365$ cm (final volume was 125 μL), $C_B = 4.70$ mM, $C_{in} = 0.365$ mM, 5.9% acetonitrile. The measured points fit a straight line with regression factor of 0.97 for each hydrolase. Each point is an average of four initial rate measurements, which differed by < 2%.

measured a higher specific activity, 4.99 $\mu mol\,min^{-1}\,mg^{-1}$ protein. We attribute the difference to activation by the rapid mechanical stirring in the pHstat experiment.

Reaction rates increased linearly with the amount of enzyme added for three typical hydrolases, one from each class of hydrolases in our library, indicating that the enzyme-catalyzed reaction rates determined with this assay are proportional to the total enzyme concentration; see Figure 3b.

Screening for hydrolases enantioselective for solketal butyrate: To demonstrate the utility of our method, we screened a library of seventy-two commercial hydrolases for enantioselective hydrolysis of (±)-solketal butyrate. All hydrolases were dissolved in the assay buffer, 5 mM BES buffer at pH 7.2. Since some solid hydrolase preparations contain buffer salts and extenders, the pH of each solution was checked and readjusted to pH 7.2 when necessary. First, we screened with racemic solketal butyrate to eliminate hydrolases that did not catalyze hydrolysis of either enantiomer. This screen eliminated the twenty hydrolases listed in note [a] of Table 1.

Next, we estimated the enantioselectivity of the remaining fifty-two hydrolases for solketal butyrate by separately measuring the initial rates of hydrolysis of the pure enantiomers, Table 1. We used the ratio of these rates as an estimated enantioselectivity.[25] Note that the ratio of these rates is not the true enantioselectivity, or enantiomeric ratio E, because we measured the rates of hydrolysis of the enantiomers separately. Nine hydrolases showed estimated enantioselectivity ≥ 4. The seven lipases and proteases

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Table 1. Activity of commercial hydrolases towards (±)-solketal butyrate and its enantiomers.

Source of active hydrolase[a]	Wt.[b]	Prot.[c]	Supplier	Activity (±)[d]	Activity (*R*)[d]	Activity (*S*)[d]	Estimated *E*[e]
Lipases							
Aspergillus niger	30	0.61	[f]	0.029	1.72	2.01	1.17 (*S*)
Aspergillus oryzae	**7.1**	**4.5**	[g]	**0.039**	**0.157**	**0.0317**	**4.95 (*R*)**
Candida antarctica lipase A	34	4.9	[h]	0.035	0.066	0.036	1.83 (*R*)
Candida antarctica lipase B	29	3.9	[i]	1.15	0.667	0.556	1.20 (*R*)
Candida lipolytica	35	0.23	[f]	0.114	0.381	0.340	1.12 (*R*)
Candida rugosa	31	0.35	[j]	2.76	4.25	1.85	2.30 (*R*)
Candida rugosa (cylindracea)	37	0.71	[k]	2.03	3.06	1.77	1.73 (*R*)
Humicola sp.	13	4.0	[h]	0.027	0.372	0.147	2.53 (*R*)
Penicillin camembertii	86	0.92	[l]	0.060	0.189	0.498	2.63 (*S*)
Penicillin roquefortii	57	0.74	[f]	0.151	0.844	1.23	1.46 (*S*)
Pseudomonas cepacia	**31**	**3.1**	[f]	**0.038**	**0.085**	**0.021**	**4.05 (*R*)**
Pseudomonas fluorescens	5.2	0.73	[g]	0.091	0.572	0.565	1.01 (*R*)
Rhizopus javanicus	**44**	**2.7**	[f]	**0.063**	**0.286**	**0.066**	**4.33 (*R*)**
Rhizopus oryzae	**53**	**4.1**	[f]	**0.040**	**0.124**	**0.011**	**11.3 (*R*)**
Thermus aquaticus	1.1	0.29	[g]	0.148	0.665	0.606	1.10 (*R*)
Esterases							
Acetylcholine esterase	0.26	0.18	[j]	0.745	7.29	5.42	1.35 (*R*)
Bacillus sp.	1.1	0.58	[g]	0.112	0.532	0.180	2.96 (*R*)
Bacillus stearothermophilus	1.1	0.57	[g]	0.066	2.77	1.65	1.68 (*R*)
Bacillus thermoglycosidasius	0.82	0.76	[g]	0.065	1.22	0.470	2.59 (*R*)
Bovine cholesterol esterase	9.8	0.99	[j]	0.402	1.43	1.73	1.21 (*S*)
Candida lipolytica	3.5	1.4	[g]	0.056	0.244	0.306	1.25 (*S*)
Cutinase	2.1	1.1	[m]	2.38	10.0	4.40	2.28 (*R*)
E001	0.40	0.14	[n]	2.08	11.4	7.65	1.49 (*R*)
E002	0.38	0.16	[n]	1.27	1.39	0.721	1.93 (*R*)
E003	1.0	0.23	[n]	2.91	4.81	3.64	1.32 (*R*)
E004	1.0	0.29	[n]	2.21	3.24	3.40	1.05 (*S*)
E005	1.0	0.27	[n]	1.88	1.38	1.10	1.25 (*R*)
E006	0.64	0.13	[n]	3.59	12.3	8.80	1.40 (*R*)
E007	2.1	0.97	[n]	1.26	1.22	1.35	1.11 (*S*)
E009	1.0	0.37	[n]	2.65	4.66	4.01	1.16 (*R*)
E010	1.0	0.26	[n]	1.56	4.19	3.01	1.39 (*R*)
E011	0.80	0.22	[n]	3.44	4.21	7.30	1.73 (*S*)
E013	1.0	0.24	[n]	0.869	0.860	0.843	1.02 (*R*)
E014	1.0	0.31	[n]	0.136	0.875	0.498	1.76 (*R*)
E016	1.0	0.26	[n]	1.22	1.76	0.960	1.83 (*R*)
E017b	1.0	0.33	[n]	1.12	0.694	0.620	1.12 (*R*)
E018	2.0	0.76	[n]	0.135	0.279	0.548	1.96 (*S*)
E019	0.60	0.20	[n]	3.70	6.45	7.79	1.21 (*S*)
E020	0.44	0.16	[n]	4.97	8.35	9.21	1.10 (*S*)
Pig-liver esterase	–	**0.09**	[j]	**71.9**	**3.03**	**14.9**	**4.91 (*S*)**
Pig-liver esterase	0.36	0.49	[g]	5.79	2.26	6.17	2.73 (*S*)
Horse-liver esterase	**1.7**	**0.59**	[g]	**0.975**	**0.168**	**2.05**	**12.2 (*S*)**
Saccharomyces cerevisiae	1.2	0.26	[g]	0.085	0.219	0.074	2.96 (*R*)
Proteases							
Aspergillus oryzae	29	7.0	[j]	0.097	0.157	0.032	4.91 (*R*)
Aspergillus satoi	32	0.40	[j]	0.748	3.17	3.13	1.01 (*R*)
Bacillus licheniformis	5.2	2.1	[g]	0.335	0.261	0.083	3.14 (*R*)
Bac. subtilis var. Biotecus A	**4.2**	**1.7**	[g]	**0.351**	**0.221**	**0.0303**	**7.29 (*R*)**
Subtilisin Carlsberg	9.7	4.4	[j]	0.083	0.213	0.082	2.60 (*R*)
Streptomyces griseus	20.1	7.2	[o]	0.012	0.032	0.0135	2.37 (*R*)
Thermolysin, Type X	2.4	0.15	[j]	0.095	0.255	0.738	2.89 (*S*)
Proteinase, bacterial	**4.7**	**2.1**	[g]	**0.186**	**0.154**	**0.028**	**5.50 (*R*)**
Proteinase K	0.42	0.07	[g]	2.14	3.08	2.29	1.34 (*R*)

[a] The following hydrolases showed no detectable activity (<0.01 μmol min^{-1} mg^{-1} protein) towards racemic solketal butyrate: Lipases : Amano lipases from *Rhizopus stolonifer*, *Mucor javanicus* and *Mucor miehei*, wheatgerm lipase (Sigma), porcine pancreatic lipase (Biocatalysts), lipase from *Rhizopus niveus* (Boehringer Mannheim); Esterases: ThermoGen esterases E008, E012 and E015, Fluka esterases from *Thermoanaerobium brockii* and *Mucor miehei*; Proteases: α-chymotrypsin (Sigma), pepsin from porcine stomach (Fluka), subtilisin from *Bacillus licheniformis* (Fluka), thrombin from human plasma (Fluka), trypsin (Worthington, Freehold, NJ), Sigma proteases from *Bacillus polymyxa*, bovine pancreas type 1, papaya, *Streptomyces caespitosus*. [b] Amount (mg) of solid enzyme per mL of buffer in the stock solutions. [c] Protein concentration of stock solutions in mg protein mL^{-1} determined by the Bio-Rad assay using BSA as a standard. [d] Observed rate of hydrolysis in μmol min^{-1} mg^{-1} protein. Rates calculated by Equation (2) were divided by the protein content in the well. The values are an average of four measurements, which typically varied by less than 2%. [e] Ratio of the separately measured initial rates for the enantiomers. This ratio is *not* the true enantioselectivity, but is a useful estimate of the enantioselectivity. The absolute configuration of the faster reacting ester is in parentheses. Note that hydrolysis of the *R* ester yields the *S* alcohol due to a change in the priority of the substituents. [f] Amano Enzyme USA (Troy, VA). [g] Fluka Chemie (Oakville, ON). [h] Boehringer-Mannheim (Mannheim, Germany). [i] Novo-Nordisk (Baagsverd, DK). [j] Sigma-Aldrich (Oakville, ON). [k] Biocatalysts (Pontypridd, Mid-Glamorgan, Wales, UK). [l] Genzyme (Cambridge, MA). [m] Unilever Research Labs (Vlardingen, the Netherlands). [n] ThermoGen (Chicago, IL). [o] Calbiochem/Behrig Diagnostics (La Jolla, CA).

favored the *R* ester, while the two esterases favored the *S* ester. The highest estimated enantioselectivities were found with horse-liver esterase (HLE, estimated enantioselectivity = 12), *Rhizopus oryzae* lipase (ROL, estimated enantioselectivity = 11), and protease from *Bacillus subtilis*, variation *Biotecus A* (BSP, estimated enantioselectivity = 7). Previous workers identified *Aspergillus oryzae* protease (AOP) as an enantioselective hydrolase.[16] This hydrolase was also among the nine enantioselective hydrolases (estimated enantioselectivity = 5). The identification of HLE, ROL, and BSP as enantioselective hydrolases for solketal butyrate are new results from this screening.

Although we used only the first 3–4 minutes of data in the calculations, we monitored the reactions for one hour to ensure that we did not miss slow hydrolases or hydrolases that show a lag time. All substrate/hydrolase solutions were prepared and measured in quadruplicate to ensure accuracy. The total screening time for seventy-two hydrolases in quadruplicate was 180 minutes plus several minutes between each plate to fill the 96-well plates. This time could be easily reduced to less than an hour with a shorter screening time. Complete screening of the library towards the racemate and its enantiomers is easily completed in an afternoon.

We also changed reaction conditions in the assay in an attempt to increase the estimated enantioselectivity for solketal butyrate. For example, the activity of hydrolases, especially lipases, often increases in the presence of an interface. We reasoned that this interfacial activation may also change the enantioselectivity. We screened the hydrolase library with Triton X-100 (a nonionic detergent) added to create micelles. The reaction rates increased for eight hydrolases (three lipases and five esterases), decreased for thirty-two hydrolases, and stayed constant for twelve hydrolases. Unfortunately, the estimated enantioselectivities remained unchanged or decreased slightly for the best hydrolases: AOP (decrease from 4.9 to 4.2), ROL (decrease from 11.3 to 9.6), HLE (decrease from 12.2 to 7.6), and BSP (decrease from 7.3 to 5.4). With nonselective hydrolases, the estimated enantioselectivities showed small increases or decreases. For example, subtilisin Carlsberg increased from 2.6 to 2.8, esterase from *Bacillus stearothermophilus* increased from 1.7 to 2.4, and *Aspergillus oryzae* lipase decreased from 4.95 to 1.8. Overall, the selectivities for solketal butyrate did not significantly change upon addition of Triton X-100.

To confirm these screening results, we measured the enantioselectivity of three selective hydrolases and three poorly selective hydrolases using the conventional endpoint method[3] (Table 2). Under conditions similar to those in the screening solutions (1 mM substrate, 7% acetonitrile as cosolvent), the true enantioselectivity and the estimated enantioselectivity agreed to within a factor of 2.3. Since 1 mM solketal butyrate is too dilute for practical preparative reactions, we also measured the enantioselectivity of these hydrolases at 50 mM solketal butyrate, where the reaction mixture contained insoluble droplets of substrate. The enantioselectivity under these conditions also agreed with the enantioselectivity estimate from screening to within a factor of 2.6.

The most enantioselective hydrolase was HLE, $E = 14.8$ and 9.7 at substrate concentrations of 1 mM and 50 mM, respectively. At 50 mM substrate without acetonitrile, the enantioselectivity of HLE declined slightly again to $E = 8.7$ ($c = 17.1\%$ after 2.5 h). These values agree well with the estimated enantioselectivities of 12.1 (without Triton X-100) and 7.6 (with Triton X-100). Although Partali et al. reported an enantiomeric ratio of 9 for AOP,[16] we measured an estimated enantioselectivity of 4.9 and a true enantioselectivity of 4.8 under our conditions. Although the estimated enantioselectivity for ROL was also high (11.3), the true enantioselectivity was lower. $E = 4.8–4.9$. Hydrolases with low estimated enantioselectivities (CRL, Esterase E013, cutinase) also showed low true enantioselectivities. Thus, hydrolases identified as enantioselective were indeed enantioselective and hydrolases identified as nonselective were not enantioselective.

Our screening procedure quickly identified HLE as a new hydrolase for the resolution of solketal butyrate with modest enantioselectivity. It is the most selective hydrolase reported in the literature to date for the hydrolysis of an ester of solketal.

Table 2. True enantioselectivities of hydrolases towards solketal butyrate measured by the endpoint method.

Hydrolase	S[a] [mM]	Time[b] [h]	ee_s[c] [%]	ee_p[c] [%]	C[d] [%]	True E[e]	Estimated E[f]
Rhizopus oryzae lipase	1	16.5	95.4	23.6	80.2	5.0 ± 0.1 (*R*)	11.3 (*R*)
Rhizopus oryzae lipase	50	1.25	37.8	51.2	42.5	4.4 ± 0.1 (*R*)	
Horse-liver esterase	1	4.0	40.3	81.8	33.0	14.8 ± 0.7 (*S*)	12.2 (*S*)
Horse-liver esterase	50	2.5	22.1	77.3	22.2	9.7 ± 0.1 (*S*)	
Cutinase	1	2.0	92.8	27.4	77.2	5.0 ± 0.02 (*R*)	2.28 (*R*)
Cutinase	50	0.42	70.3	41.8	62.7	4.8 ± 0.04 (*R*)	
Aspergillus oryzae protease	1	4.0	10.5	65.8	13.8	5.4 ± 0.1 (*R*)	4.91 (*R*)
Aspergillus oryzae protease	50	14	11.1	62.0	15.2	4.8 ± 0.1 (*R*)	
Candida rugosa lipase	1	2.5	86.3	14.4	85.7	3.0 ± 0.1 (*R*)	1.73 (*R*)
Candida rugosa lipase	50	0.1	15.7	40.5	27.8	2.7 ± 0.02 (*R*)	
Esterase E013	1	9.5	0	0	20	1.0	1.02
Esterase E013	50	2.0	0	nr[g]	nr	nr	

[a] Substrate concentration in the reaction mixture. [b] Reaction time. [c] Measured enantiomeric purity of the starting material (ee_s) or product (ee_p). [d] Degree of conversion calculated by $ee_s/(ee_s + ee_p)$. [e] The true enantioselectivity was calculated from ee_s and ee_p according to ref. [3]. The absolute configuration of the fast-reacting ester is in parentheses. The error limits were estimated from enantioselectivities measured from three separate GC injections. [f] Values from Table 1. [g] nr = no reaction detected by GC.

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Discussion

The most important part of the assay design was to ensure that it accurately measured the rates of hydrolysis. The first requirement is that the buffer and indicator have pK_as within 0.1 units. A difference in pK_a of 0.3 units causes an 8% error when the pH changes by 0.1 unit.[12] In a typical assay, the pH changed by 0.05 units (10% hydrolysis of the substrate); thus, differences in pK_as can lead to nonlinear and inaccurate rates. If different pK_as cannot be avoided, one can still get accurate results by using calibration experiments[10] or a more complex equation.[12] The linear relationship between the amount of acid added and the color change, as shown in Figure 2, confirms that the pK_as lie within the acceptable range in our experiment. Further, the agreement of the theoretical and experimental slopes to within 5% establishes that the assay is quantitative.

The assay tolerates small changes in reaction conditions, such as the addition of 7% acetonitrile. Indeed, the pK_a of 4-nitrophenol changes only slightly from 7.15 to 7.17 upon addition of 10% ethanol.[26] This result suggests that cosolvent concentrations below 10% do not compromise the accuracy of the assay. Also, small amounts of salts present in the hydrolase solutions (buffer salts in commercial hydrolase preparations, 2 mM $CaCl_2$ in the protease solutions) did not affect the accuracy.

This assay is approximately seven times less sensitive than the one using hydrolysis of 4-nitrophenyl esters. For example, if the rate of hydrolysis for a nonchromogenic ester and a 4-nitrophenyl ester were identical, then our assay would require seven times more hydrolase to observe the same change in absorbance. The assay with 4-nitrophenyl esters releases one molecule of 4-nitrophenol (53% of these will be deprotonated at pH 7.2), while our assay protonates one 4-nitrophenoxide for every twelve protons released.

There are several advantages to this screening method. First, it is hundreds of times faster than conventional screening. The 96-well format allows the analysis of large numbers of samples simultaneously. However, speed is not gained at the expense of accuracy; variation between quadruplicate measurements for a reaction is typically <2%. Moreover, our method is quantitative, unlike screening for hydrolytic activity by TLC. Second, since all the reactions and analyses take place in the microplate wells, workup and analysis by GC, HPLC, or NMR is avoided. Third, it requires hundreds to thousands of times less substrate (typically 20 μg/well) and hydrolase (we used between 0.6–35 μg protein/well). For this reason, it may be useful in the screening of mutant hydrolases in directed evolution experiments. Fourth, this assay measures the hydrolysis of any ester, not just chromogenic esters. The most important rule of screening is "You get what you screen for", so the ability to screen the target compound, not an analogue of the target compound, is an important advantage. For speed, we screened in microplates, but one could also adjust the concentrations to use cuvettes and a conventional UV-vis spectrophotometer.

There are a few disadvantages with our screening method. First, it requires pure enantiomers, albeit in small amounts. We screened the hydrolase library in quadruplicate with only six milligrams of each enantiomer of solketal butyrate. Second, it provides only an estimated enantioselectivity. This method ignores some or all of the differences in K_M of the enantiomers. Third, it requires clear solutions. To obtain clear solutions with water-insoluble substrates, experimentation is sometimes required to find the best cosolvent or emulsion conditions.

In this paper we assayed for hydrolase activity at pH 7.2, but other buffer indicator pairs may be suitable for screening at other pH values. For example, at pH 6 chlorophenol red (pK_a = 6.0) and MES [2-(*N*-morpholino)ethanesulfonic acid, pK_a = 6.1] may be suitable; at pH 8 phenol red (pK_a = 8.0) and EPPS [*N*-(2-hydroxylethyl)piperazine-*N*'-(3-propanesulfonic acid), pK_a = 8.0] may be suitable; at pH 9 thymol blue (pK_a = 9.2) and CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid, pK_a = 9.3] may be suitable.

We are currently adapting this acid–base indicator assay to measure true enantioselectivity by extending our Quick E method for measuring true enantioselectivity.[23]

Experimental Section

General: Chemicals were purchased from Sigma-Aldrich (Oakville, ON) and were used without further purification unless otherwise stated. Triton X-100 was purchased from ESA (Chelmesford, MA). Standardized acid was purchased from A&C American Chemicals (Montréal, QC). Enzyme suppliers are noted in the footnotes of Table 1. All microplate assays were performed on a Spectramax 340 microplate reader with SOFTmax PRO version 1.2.0 software (Molecular Devices, Sunnyvale, CA). Polystyrene 96-well flat-bottomed microplates (maximum volume 360 μL/well, Corning Costar, Acton, MA) were filled using Eppendorff 8-channel pipettes (5–100 μL, 50–1200 μL) and solution basins for multichannel pipettes (Fisher Scientific, Nepean, ON). The initial rate of the small-scale horse-liver esterase-catalyzed ester hydrolysis reaction was measured with a Radiometer RTS 822 pHstat.

(±)-Solketal butyrate (1): Butyric anhydride (2.87 mL, 17.5 mmol, 1.5 equiv), 4-dimethylaminopyridine (0.071 g, 0.58 mmol, 0.05 equiv), and anhydrous sodium carbonate (1.86 g, 17.6 mmol, 1.5 equiv) were added to a solution of (±)-solketal (1.54 g, 11.7 mmol, 1.0 equiv) in ethyl acetate (40 mL) and stirred overnight. The reaction mixture was washed several times with water, then with brine, and the organic extract was dried with magnesium sulfate. Flash chromatography (3:1 hexanes/ethyl acetate) afforded the pure butyryl ester as a yellow oil in 91% yield. R_f = 0.56 (3:1 hexanes/ethyl acetate): ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.98 (t, ^3J(H,H) = 7.4 Hz, 3H, CH_3), 1.37 (s, 3H, CH_3), 1.43 (s, 3H, CH_3), 1.67 (sextet, ^3J(H,H) = 7.4 Hz, 2H, CH_2), 2.33 (t, ^3J(H,H) = 7.3 Hz, 2H, CH_2), 3.7 (m, 1H, 1H of CH_2), 4.05–4.16 (m, 3H, 1H of CH_2, CH_2), 4.27–4.32 (m, 1H, CH); ^{13}C NMR (200 MHz, $CDCl_3$): δ = 15.3 (CH_3), 19.9 (CH_2), 26.9 (CH_3), 28.1 (CH_3), 37.3 (CH_2), 65.5 (CH_2), 67.3 (CH_2), 74.6 (CH), 110.3 (C), 173.1 (C=O).

(*S*)-Solketal butyrate and (*R*)-solketal butyrate: Samples were prepared from enantiomerically pure solketal as outlined above for the racemate. The enantiomeric purities of the butyrates measured by GC (see below) were 99.2% and 99.8%, respectively. No contaminating butyric acid or solketal were detected by GC or ^{1}H NMR.

Hydrolase library: The hydrolases were dissolved in BES buffer (5.0 mM, pH 7.2 containing 0.02% NaN_3 as preservative) at the concentrations listed in Table 1 (0.5–40 mg solid/mL solution). $CaCl_2$ (2 mM) was added to the protease solutions since some proteases require calcium ions to maintain their structure. For hydrolase samples with low protein content, we used saturated solutions (up to 40 mg solid/mL), and for hydrolase samples with high protein content, we chose lower concentrations (typically, 1 mg solid/mL). Each solution was centrifuged to remove insoluble material (5 min, 2000 rpm) and titrated to a final pH of 7.2. The protein concentrations were determined using a dye-binding assay from Bio-Rad (Mississauga, ON)

with bovine serum albumin (BSA) as the standard. Solutions were stored in a 96-well assay block mother plate equipped with aluminum sealing tape (2 mL maximum volume in each well, Corning Costar, Acton, MA) at −20 °C. This mother plate speeds up repeated screens that use the same hydrolases, and is a convenient way to store large libraries of hydrolases. Hydrolytic activity of the libraries is maintained over several months.

Screening of commercial hydrolases with pH indicators: The assay solutions were prepared by mixing solketal butyrate (420 μL of a 30.0 mM solution in acetonitrile), acetonitrile (470 μL), 4-nitrophenol (6000 μL of a 0.9115 mM solution in 5.0 mM BES, pH 7.2), and BES buffer (5110 μL of a 5.0 mM solution, pH 7.2). Hydrolase solutions (5 μL/well) were transferred from the mother plate to a 96-well microtiter plate using an 8-channel pipette. Assay solution (100 μL/well) was quickly added to each well using a 1200 μL 8-channel pipette. The final concentrations in each well were 1.0 mM substrate, 4.65 mM BES, 0.434 mM 4-nitrophenol, 7.1% acetonitrile. The plate was quickly placed in the microplate reader and shaken for 10 s to ensure complete mixing, and the decrease in absorbance at 404 nm was monitored at 25 °C as often as permitted by the microplate software, typically every 11 seconds. The starting absorbance was typically 1.2. Data were collected for one hour to ensure we detected slow reactions and reactions with a lag time. Each hydrolysis was carried out in quadruplicate and was averaged. The first 10 s of data were sometimes erratic, possibly due to dissipation of bubbles created during shaking. For this reason, we typically excluded the first 10 s of data from the calculation of the initial rate. Activities were calculated from slopes in the linear portion of the curve, usually over the first two hundred seconds. The initial rates were calculated from the average dA/dt by means of Equation (2), where $\Delta\varepsilon = 17300\,\text{M}^{-1}\text{cm}^{-1}$ (experimentally determined for our conditions) and $l = 0.306$ cm. To calculate specific activity (μmol min^{-1} mg^{-1} protein), we took into account the total amount of protein in each well.

Screening of commercial hydrolases with pH indicators under interfacial activation conditions: The procedure was the same as outlined above except that the BES buffer (5 mM, pH 7.2) contained Triton X-100 (8.45 mM). The final concentration of Triton X-100 in the wells was 2.8 mM.

Small-scale reactions with 1 mM (±)-solketal butyrate: These small-scale reactions mimic the conditions in the microplate during pH indicator activity screening except that no indicator is present. Hydrolase solutions (50 μL) were added to solutions of (±)-solketal butyrate (3.50 mL of a 14.4 mM solution in acetonitrile) and BES buffer (46.45 mL of a 5.0 mM solution, pH 7.2) for a final reaction volume of 50 mL (1.0 mM substrate, 4.65 mM BES, 7% acetonitrile). After stirring at room temperature for a time estimated from the pH indicator screening, the mixture was extracted with diethyl ether (3 × 20 mL). The extracts, which contained both the ester substrate and the alcohol product, were combined, washed with water and dried with magnesium sulfate, filtered, and evaporated to dryness.

Small-scale reactions with 50 mM (±)-solketal butyrate: Hydrolase solutions (250 μL for CRL, ROL, HLE, AOP, E013; 50 μL for cutinase) were added to solutions of (±)-solketal butyrate (352 μL of a 0.715 M solution in acetonitrile) and BES buffer (4.398 μL of a 5.0 mM solution, pH 7.2) for a final reaction volume of 5.0 mL (50 mM substrate, 4.65 mM BES, 7% acetonitrile). Reactions were worked up as outlined above.

Determination of enantiomeric purity by GC: Gas chromatography analysis was performed on a Hewlett Packard 5890 Series II Gas Chromatograph equipped with a Chirasil-DEX CB chiral stationary phase (25 m × 0.25 mm × 0.25 μm Chrompack, Raritan, NJ). For analysis, solketal was converted to the acetate by dissolving the mixture of solketal and solketal butyrate in ethyl acetate (5 mL) containing acetic anhydride, 4-pyrrolidinopyridine, and anhydrous potassium carbonate. The solution was stirred for one hour at room temperature, then filtered, washed with brine, then water, dried with magnesium sulfate and evaporated to dryness. Both the starting material, solketal butyrate, and the acetate of the product were simultaneously separated with baseline resolution by means of a temperature gradient (100 °C to 130 °C, 2 °C min^{-1}). Solketal butyrate: $k'_1 = 8.11$ (*S*), $\alpha = 1.04$; solketal acetate: $k'_1 = 4.21$ (*S*), $\alpha = 1.10$. The *ee* values reported in the tables are the mean of three injections. We did not observe any racemization of solketal or its esters during derivatization.

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