Expanding Hydrolase Catalyzed Reactions to New Substrates and Reactions: Subtilisin Catalyzed Hydrolysis of Sulfinamides and Sterically Hindered Substrates

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Your file Votre référence
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Our file Notre référence
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For my brother,
Thomas William Mugford
December 16, 1976 – December 8, 2000

Abstract

Enzymes are increasingly applied to organic synthesis because of their high enantioselectivity, chemoselectivity, and environmental friendliness. Hydrolytic enzymes are the largest class used for biotransformations, and subtilisin is one of the commonly used serine proteases for organic synthesis. We hypothesized that we could expand the usefulness of subtilisin to hydrolyze unnatural substrates. The X-ray crystal structure of subtilisin shows that it has a large open active site. Previous workers focused on hydrolysis of natural substrates and close analogs, but the open active site suggests that subtilisin should accept bulky substrates that do not resemble peptides.

The Diels-Alder reaction is important in organic synthesis because it has the potential to create several stereocenters in a single step. We resolved a bulky secondary alcohol ester of a spiro chiral auxiliary for the Diels-Alder reaction on a gram scale using subtilisin Carlsberg. The reaction proceeded with high enantioselectivity and yield. Cholesterol esterase showed high enantioselectivity but with the opposite enantiopreference, consistent with mirror image arrangement for the active sites of subtilisins and lipases/esterases. We also used molecular modeling to identify the molecular basis of enantioselectivity of subtilisin Carlsberg toward this secondary alcohol.

Resolution of tertiary alcohol esters is difficult because they are also very bulky, but important because there are only a few synthetic and biocatalytic methods to prepare enantiopure tertiary alcohols. We discovered several proteases that hydrolyze esters of tertiary alcohols, one of which was subtilisin Carlsberg. This is the first examination of protease hydrolysis of tertiary alcohol esters. Substrate studies and molecular modeling explained their reactivity and enantioselectivity.

Sulfinamidies are also bulky substrates, but surprisingly subtilisin catalyzed a catalytic promiscuous reaction of *N*-acyl sulfinamides. Subtilisin Carlsberg-catalyzed hydrolysis of *N*-acyl sulfinamides favored cleavage of the sulfinamide (S(O)-N) bond with a minor amount of the expected carboxamide (C(O)-N) bond. The sulfinamide hydrolysis was enantioselective and confirmed by product isolation from the S-N sulfinamide cleavage. In contrast, the related subtilisins BPN' and E favored the C-N carboxamide hydrolysis.

Further examination using electrospray-mass spectrometry revealed a sulfinyl-enzyme intermediate located at the active site, analogous to an acyl-enzyme. This suggested an analogous mechanism to the amide/ester hydrolysis. Substrate variation indicated a substrate reversal was responsible for the change in reactivity by binding in the S_1 acyl pocket. Three mutations of subtilisin BPN` towards subtilisin Carlsberg increased the S-N hydrolysis by 14-fold, indicating that only a few mutations were responsible for the catalytic promiscuity. The substrate specificity and mutagenesis were consistent with a reversed orientation for the sulfinyl hydrolysis reactions.

The active site of subtilisin is indeed versatile for unnatural substrates and unnatural reactions. We expanded the applications of subtilisin to resolve bulky substrates, such as chiral auxiliaries and tertiary alcohol esters, and also to perform a new catalytic promiscuous sulfinamide S-N bond hydrolysis. Based on these results, subtilisin should prove useful for future resolutions of bulky substrates, and also in catalytic promiscuous reactions where unusual reactive centers are hydrolyzed.

Résumé

Les enzymes sont de plus en plus utilisées en synthèse organique du fait de leurs grandes énantio et chimiosélectivités ainsi que pour leur milieu réactionnel relativement doux. Les enzymes hydrolytiques représentent la classe d'enzyme la plus utilisée pour les biotransformations et la subtilisine est une des protéases à sérine le plus fréquemment utilisée en synthèse organique. Nous avons supposé la possibilité d'étendre l'utilisation de la subtilisine à l'hydrolyse de substrats non naturels. La structure tridimensionnelle déterminée par rayons X de la subtilisine a permis d'observer un site actif largement ouvert. Des travaux antérieurs portant sur l'hydrolyse de substrats naturels et de proches analogues ont été réalisés, cependant le site actif ouvert suggère que la subtilisine devrait être en mesure d'accepter des substrats encombrés ne ressemblant pas à des peptides.

La réaction de Diels-Alder est importante en synthèse organique du fait de la possibilité de créer différents stéréocentres en une seule étape. Grâce à la réaction de Diels-Alder nous avons obtenu un ester d'alcool secondaire encombré à partir d'un auxiliaire chiral spirocylclique à l'échelle du gramme et ce en utilisant la subtilisine Carlsberg. La réaction est réalisée avec une haute énantiosélectivité et un fort rendement. La cholestérol estérase montre quant à elle toujours une haute énantiosélectivité mais avec une énantio-préférence inverse, cohérent des subtilisines et des lipases/estérases ayant des sites actifs image miroir l'un de l'autre. Nous avons donc utilisés la modélisation moléculaire afin d'identifier les bases moléculaires de l'énantiosélectivité de la subtilisine Carlsberg en ce qui concerne les alcools secondaires.

L'obtention d'esters d'alcools tertiaires est difficile à cause de leur encombrement stérique mais importante du fait du peu de méthodes de synthèse et de biocatalyse permettant d'obtenir des alcools tertiaires énantiosélectivement purs. Nous avons découvert un grand nombre de protéases permettant l'hydrolyse d'esters d'alcools tertiaires parmi lesquelles la subtilisine Carlsberg. C'est la première étude portant sur l'hydrolyse d'esters d'alcools tertiaires grâce à une protéase. Les études de substrats et de modélisation moléculaire ont permis d'expliquer la réactivité et l'énantiosélectivité.

Les sulfinamides sont aussi des substrats encombrés stériquement et de façon étonnante la subtilisine catalyse une réaction hétérogène des *N*-acyl sulfinamides. L'hydrolyse de *N*-acyl sulfinamides catalysée par la subtilisine Carlsberg favorise le

clivage du lien sulfinamide (S(O)-N) par rapport à la rupture du lien carboxamide (S(O)-N). L'hydrolyse de la sulfinamide est énantiosélective et est confirmée lors de l'isolation du produit après la rupture du lien sulfinamide S-N. De manière opposée, il a été montré que les subtilisines BPN' et E favorisaier quant à elles l'hydrolyse du lien carboxamide (C-N).

De plus des études de spectrométrie de masse-Electrospray ont montré que l'intermédiaire sulfinyl-enzyme était situé au sein du site actif de manière analogue à l'acyl-enzyme. Ceci suggère un mécanisme similaire pour l'hydrolyse de l'amide/ester. Le changement de substrat indique qu'une inversion de substrat est responsable du changement de réactivité lors de la liaison dans la poche acyl S1. Trois mutations de la subtilisine BPN' dans le sens de la subtilisine Carlsberg augmentent l'hydrolyse S-N d'un facteur 14, ceci indiquant que peu de mutations sont responsables de l'hétérogénéité catalytique. La spécificité du substrat et la mutagénèse sont en accord avec une orientation inversée des réactions d'hydrolyse des groupements sulfinyl.

Le site actif de la subtilisine est en effet flexible pour les substrats et les réactions non naturels. Nous avons étendu les applications de la subtilisine pour obtenir des substrats encombrés tels que les auxiliaires chiraux et les esters d'alcools tertiaires et ainsi améliorer une nouvelle catalyse hétérogène de l'hydrolyse des liens S-N sulfinamide. En se basant sur ces résultats, la subtilisine pourrait être utilisée de manière pratique pour de futures obtentions de substrats encombrés lors de réactions catalytiques hétérogènes où le centre réactif rare est hydrolysé.

Acknowledgements

I would like to thank my research supervisor, Dr. Romas J. Kazlauskas, for his support and guidance throughout the duration of this work. I thank all of my colleagues, past and present, in the Kazlauskas lab and in the Chemistry Department. In particular, I would like to thank Christopher Savile, Krista Morley, previous students Seongsoon Park, Vladimir Magloire and Geoff Horsman, who were all excellent colleagues and friends. I thank Mr. Nadime Saade for obtaining mass spectra at McGill University and Chantal Marotte and Renee Charron for all their help.

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Finally, I would like to thank my family for their support. Many thanks go to my wife Rebecca, for her support and encouragement. I also thank my parents for their support throughout my education.

Contribution of authors

This thesis represents a collection of four manuscripts. Two have already been published, and two 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. On each manuscript I am the first author, and have written the manuscripts in entirety under the supervision of Romas J. Kazlauskas, my supervisor. Dr. Kazlauskas and I edited these manuscripts together.

Each manuscript is presented as a separate chapter (Chapters 2-5) in the thesis, with its own introduction and discussion. The common theme is the application of the hydrolase subtilisin to resolve new sterically challenging molecules, and to perform new catalytically promiscuous reactions. Chapter one serves as a general introduction to current synthetic methods, enzymology, and subtilisin.

Co-Authorship of Manuscripts

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

Chapter 2: A collaboration with Susan Lait and Dr. Brian Keay of the University of Calgary. Susan synthesized the compounds for this chapter.

Chapter 3: Chris Savile worked as a partner on this project, performed some of the synthesis and analysis. He also performed the molecular modeling on the tertiary alcohol substrate.

Chapter 4: Vladimir Magloire, a former Masters student in our lab, was the first to notice the S-N hydrolysis reaction while testing for C-N hydrolysis. He did not quantify the reaction but performed the isolation of sulfinic acid from a small scale-up reaction.

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Chapter Five:

Subtilisin-Catalyzed Hydrolysis of *N*-Acyl Sulfinamides and Sulfinates

Glossary of Frequently Used Symbols and Abbreviations

α	separation factor
acac	Acetylacetone (2,4-pentanedione)
BES	*N, N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid
br	broad (in NMR)
c	conversion
C	Celcius
CALB	*Candida antarctica* lipase B
CE	Cholesterol esterase
CI	chemical ionization
$CDCl_3$	deuterated chloroform
CRL	*Candida rugosa* lipase
d	doublet (in NMR)
δ	chemical shift
Da	Dalton
de	diastereomeric excess
ε	extinction coefficient
E	enantiomeric ration
E.C.	enzyme commission number
EI	electron ionization
ee	enaniomeric excess
Et_2O	diethyl ether
EtOH	Ethanol
g	gram
h	hour
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectroscopy
Hz	Hertz
J	coupling constant
K_M	Michaelis-Menten constant
k_{cat}	enzyme turnover number
l	pathlength
L	liter
m	meter
M	molar
MeOH	methanol
MES	2-(*n*-morpholino)ethaniesulfonic acid
min	minute
mol	mole(s)
MS	mass spectroscopy
m/z	mass-to-charge ratio
n.d.	not determined
%	percent
p	para
P	product

pH	hydrogen ion concentration
pK_a	Negative logarithm of equilibration constant for association
q	quartet (in NMR)
R	alkyl or aryl group
s	singlet in NMR
	second
S	substrate
t	triplet (in NMR)
T_d	tetrahedral intermediate
TLC	thin-layer chromatography
TRIS	tris(hydroxymethyl)-aminomethane
U	unit
υ	initial velocity
V_{max}	maximum velocity

Chapter 1
General Introduction

1.1.1 Chirality

Any three non-linear points define a plane. Adding a fourth point off the plane creates three-dimensional shape, and another feature called chirality. Consider two cubes with four corners labeled (Figure 1). The mirror image of this cube is different - it cannot be rotated to align the labeled corners. The word 'chiral', from the Greek for hand, was first introduced by Lord Kelvin (William Thomson), Professor of Natural Philosophy at the University of Glasgow from 1846-1899: "*I call any geometrical figure, or group of points, chiral, and say that it has chirality, if its image in a plane mirror, ideally realized, cannot be brought to coincide with itself.*"[1] Two objects that are non-superimposable mirror images are termed enantiomers, and in chemistry this arises from the three dimensional shape of chiral molecules. Pure enantiomers have equal physical properties, such as boiling point and density, with the exception of the direction in which they rotate plane polarized light, and equal chemical properties, except when interacting with a chiral environment.

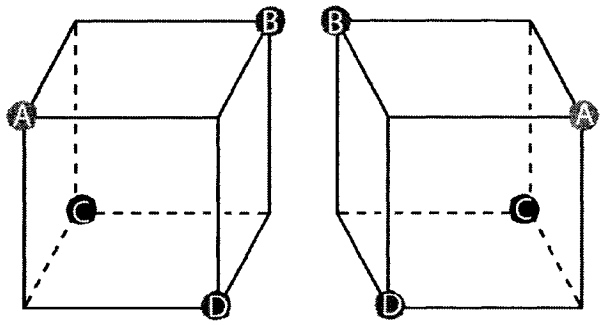


Figure 1: A three-dimensional object with a non-superimposable mirror image; the two labeled cubes are enantiomers and cannot be overlaid by rotation.

There are several real world examples of enantiomers. The standard example is the relationship between left and right hands (Figure 2, A). Cars are another example, with respect to the placement of the steering wheel (Figure 2, B). North American cars place the steering wheel on the left; the British drive the enantiomers

with the wheel on the right. An unoccupied (achiral) road can be driven down equally as well by either type of car. When other cars are present, however, the interaction of different types becomes increasingly important. For instance, with multiple cars a convention as to which side to drive on becomes necessary. That is, the major significance of chirality results when two chiral things interact to form diastereomers.

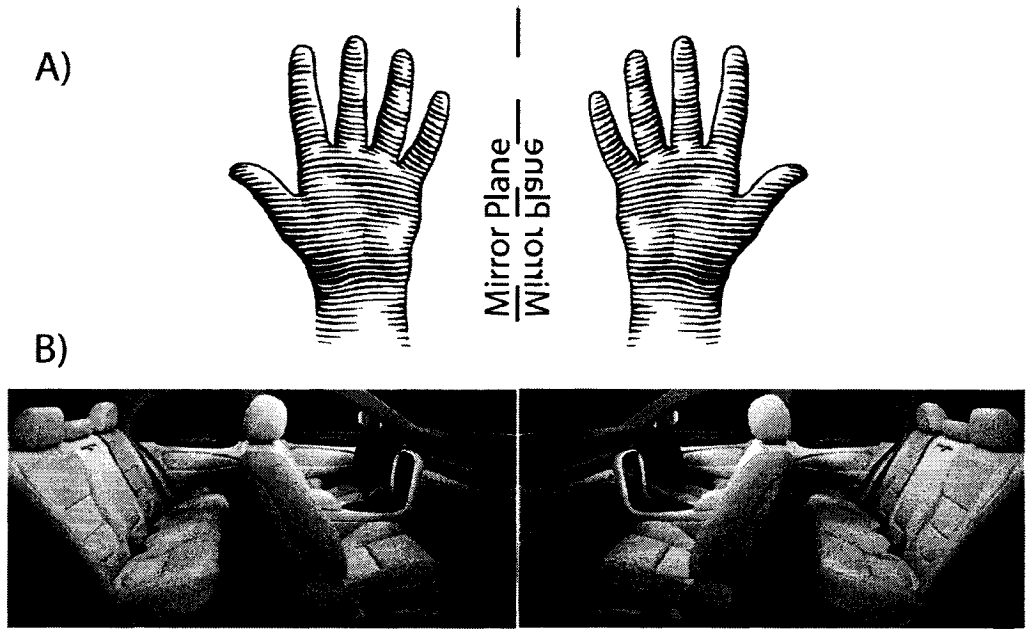


Figure 2: Examples of enantiomers: (A) left and right hands, and (B) North American (left) and British (right) cars. The objects are all related by the mirror plane and are not superimposable with each other. A left hand cannot become a right by rotating it, or a North American car become a British car by turning it around or upside down.

1.1.2 Diastereomers

The joining of enantiomeric elements leads to diastereomers. A handshake combines chiral hands, and there are four possible ways for people A and B to shake hands; two sets of diastereomers. In this case the $Right_A$ + $Right_B$ and the $Left_A$ + $Left_B$ fit better for a handshake, while diastereomers $Left_A$ + $Right_B$ and $Right_B$ + $Left_A$ do not fit as well.

In chemical interactions, the favourable and unfavourable interactions (or the "fit") of the diastereomers are thought of in terms of energy. Thus the combination of two chiral molecules produces diastereomers of different energy, and they also have different physical properties. In the same way, the interaction of enantiopure molecules with chirality from nature leads to interactions of different energy. This energy difference allows enantiomers to be separated when they interact with a resolving agent to form diastereomers. More importantly, it is also the basis for chiral pharmaceuticals. Enantiomers of a potential drug bind and inhibit a chiral target by forming diastereomers with different energy; the one bound more tightly is usually a better drug. Enantiomers may also cause unwanted side reactions - one enantiomer may be an effective drug, while its enantiomer is toxic. Examples of enantiomers along with their differing biological effects are shown in Figure 3.

The commercial market for single enantiomer drugs is valued at over 100 billion, representing approximately 40% of pharmaceuticals.[2] The US Food and Drug Administration (FDA) and its European counter part both recognize the different biological properties of enantiomers and require that each enantiomer of a drug be individually characterized.[3] Racemic drugs may cause problems because of the differences in the pharmacokinetics of enantiomers. For instance the racemic form of Perhexiline, a drug used to treat abnormal heart rhythms, lead to several deaths after a buildup of the slowly metabolized enantiomer.[4] Thus methods to prepare enantiomerically pure compounds for the pharmaceutical industry are increasingly important.

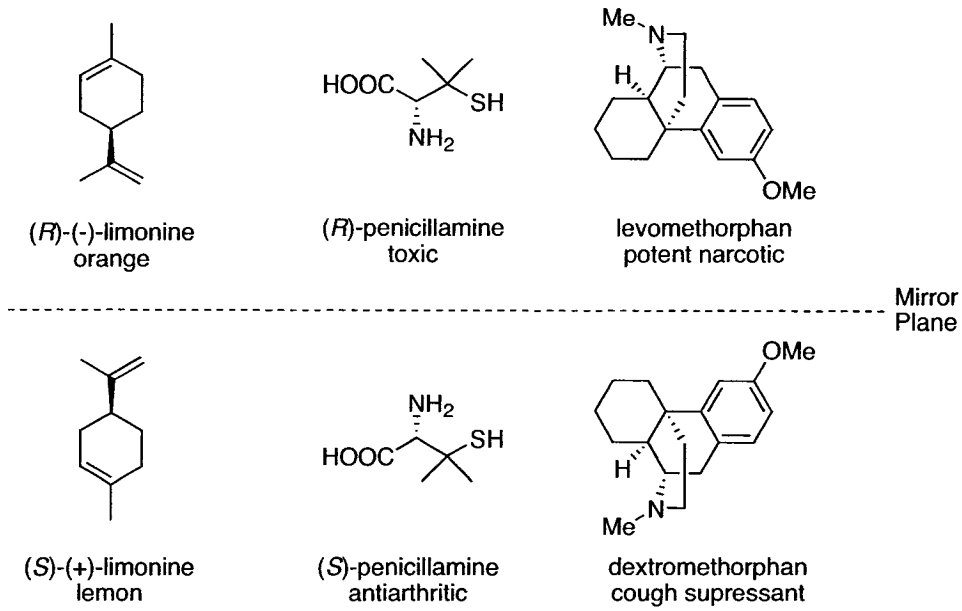


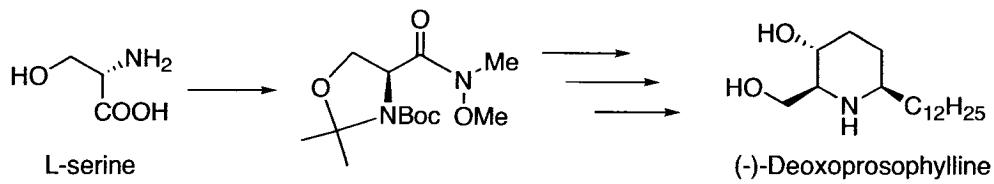
Figure 3: Enantiomers have very different biological properties. The (*R*)-enantiomer of limonine has an orange smell, while the (*S*)-enantiomer smells of lemons. In pharmaceuticals, one enantiomer of penicillamine is used to treat arthritis, while the other is toxic. The cough suppressant dextromethorphan's enantiomer is a potent narcotic.[4]

1.1.3 Controlling chirality: everyone borrows from nature

Chemists need to be able to control the stereochemistry of compounds that will interact with a fundamentally chiral nature. Chirality is important because the basic building blocks of nature are chiral. Sugars, amino acids, and other compounds combine to form larger chiral molecules such as DNA or enzymes. These in turn are involved in the synthesis of enantiopure natural products. It is a fundamental property that an asymmetric transformation requires a chiral influence, which must be borrowed from nature. Synthetic chemistry and biotransformations both take different parts of nature to perform stereoselective transformations. Synthetic chemistry uses small molecules from nature for chiral building blocks, ligands, and auxiliaries. Biotransformations use larger and more complex pieces: enzymes, and even whole-cells.

1.2.1 Chemical methods to enantiopure compounds

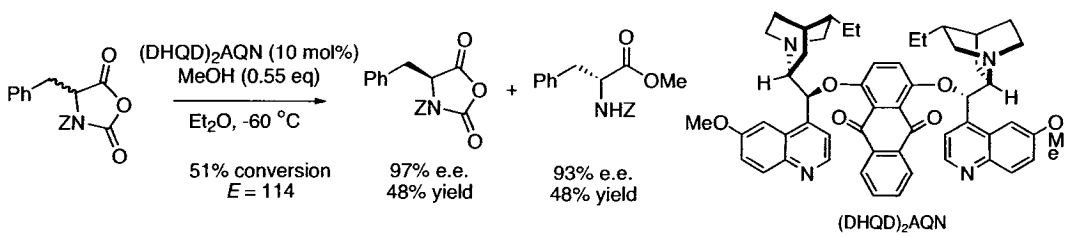
Asymmetric chemistry uses one of three methods to prepare enantiopure compounds. Enantiopure compounds may be taken from the chiral pool, be obtained by a chemical resolution, or be prepared using chiral auxiliaries and catalysts. The first method, the chiral pool, refers to chiral materials such as carbohydrates, amino acids, lipids, terpenes and alkaloids from plant and animal sources. L-Serine was used as the only chiral material in the synthesis of the alkaloid (-)-deoxoprosophylline from the *Prosopis afrikana* plant (Scheme 1).[5] The chiral pool is limited in the type and amount of compounds available, and often they are only readily available in one enantiomer, the natural one.



Scheme 1: The alkaloid (-)-deoxoprosophylline from L-serine, a member of the chiral pool.

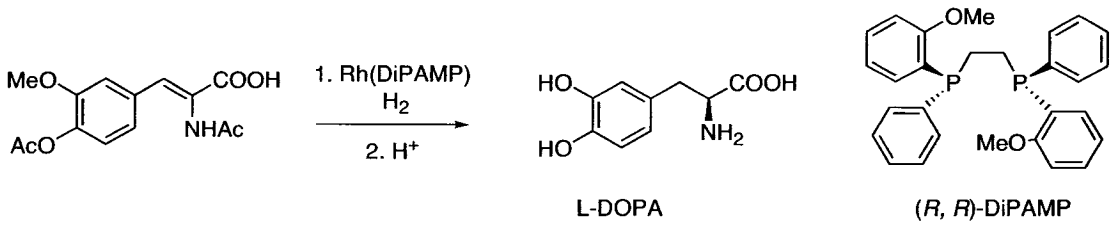
The second method to prepare single enantiomer compounds is a resolution, which separates enantiomers. A common method is separation of racemates through diastereomeric salt formation. For example, racemic mandelic acid treated with (-)-(1*R*, 2*S*)-ephedrine produces diastereomeric salts with over 10-fold difference in aqueous solubility.[6] The less soluble (-)-ephridine (-)-(2*R*)-mandelate salt is readily separated by filtration. Resolution by crystallization is highly empirical and often requires extensive testing to obtain solvent, resolving agent, and other conditions.

Kinetic resolutions with chemical catalysts are also useful to obtain enantiopure compounds. Like an enzymatic resolution, a chiral catalyst acts on a racemic mixture to preferentially transform one enantiomer. Modified cinchona alkaloids catalyze the enantioselective ring opening of urethane-protected amino acids (Scheme 2).[7] The chiral Lewis base promotes a selective alcoholysis of only one enantiomer with high enantioselectivity (E = 114) and yield (48% for product and remaining starting material). A variety of natural and unnatural amino acids were resolved using this method.



Scheme 2: Chemical kinetic resolution of urethane-protected amino acids catalyzed by cinchona alkaloid derivatives occurs with high enantioselectivity.

The final method chemists employ to obtain chiral compounds is asymmetric synthesis using chiral auxiliaries or catalysts. A chiral auxiliary is a temporary stereocenter incorporated into a molecule to direct the asymmetric formation of further stereocenters before its removal. Chiral catalysts favour formation of one enantiomer from achiral starting materials but are employed in a catalytic amount. The production of L-Dopa, used in the treatment of Parkinson's disease, uses an asymmetric hydrogenation with quantitative yield and 95% enantiomeric excess (Scheme 3).[8] The 2001 Nobel Prize in chemistry was awarded in part to Knowles for this first industrial catalytic asymmetric synthesis.[9]



Scheme 3: Key asymmetric hydrogenation in the synthesis of L-DOPA by Monsanto.

1.2.2 Sulfinic acid derivatives

Organosulfur compounds can be classified based on the coordination around the sulfur atom. Some examples of divalent sulfur species are thiols, thiol esters and episulfides. These have analogous oxygen compounds – alcohols, esters and epoxides. Tricoordinate sulfur compounds include sulfoxides, sulfites, sulfinates, and sulfinamides (Figure 4), while tetracoordinate species include sulfonic acids, sulfones, and sulfonamides.

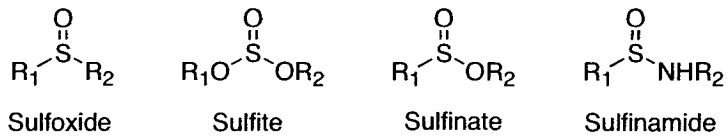


Figure 4: Examples of tricoordinate sulfur compounds, which contain a stereocenter at the sulfur atom.

Sulfoxides contain a stereogenic center and can be used as chiral auxiliaries. The pyramidal configuration at the sulfur atom (tetrahedral if the lone pair is included) has a high thermal stability of approximately 200 °C,[10] allowing sulfoxides to be used as both chiral reagents and substrates. Several pharmaceuticals and natural products contain a stereogenic sulfinyl sufur atom. Omeprazole, marked by Astra Zeneca, is gastric proton pump inhibitor used as an antiulcer agent and now marketed as a single enantiomer, while the immunosupressor oxisurane also contains a chiral sulfoxide (Figure 5).[11]

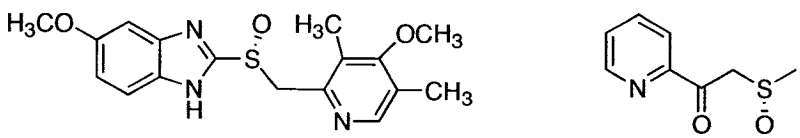
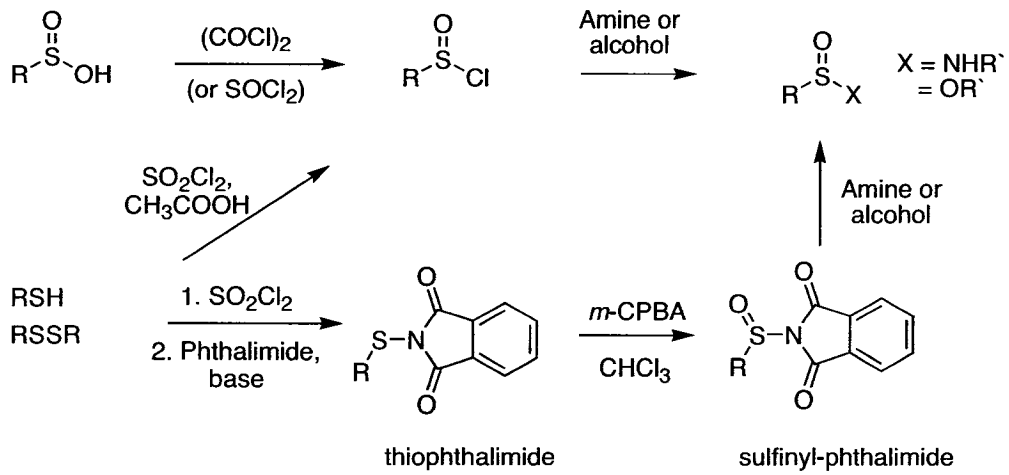


Figure 5: Pharmaceuticals that contain chiral sulfinyl functionality; omeprazole is an antiulcer agent and oxisurane is an immunosupressor.

1.2.3 Racemic synthesis of sulfinyl compounds

Sulfinates and sulfinamides are readily prepared from sulfinic acids, thiols, disulfides, or sulfinyl phthalamides.[12] Sulfinic acids are first converted to the corresponding halide using oxalyl chloride or thionyl chloride, and then condensed with the appropriate amine or alcohol. As only a few sulfinic acids are commercially available, sulfinyl chlorides can also be prepared from thiols or disulfides using chlorine or sulfuryl chloride, and acetic acid.[13] Sulfinyl chlorides are less stable than the corresponding sulfonyl chlorides and disproportionate into sulfonyl and sulfenyl chlorides.[14]

To avoid the instability of sulfinyl chlorides, Harpp and coworkers developed sulfinyl phthalimides as stable sulfinyl transfer agents.[15] Preparation from thiols or disulfides first involves formation of a thiophthalimide, in itself an efficient sulf*en*yl transfer agent.[16] Thiophthalimides are then oxidized to the sulfinyl-phthalimide using *m*-chloroperbenzoic acid (*m*-CPBA) in excellent yield (80-100%). Displacement with amines or alcohols provides sulfinamides or sulfinates in 77-95% yield, and the insoluble phthalimide by-product is easily filtered. Both *N*-(aryl- and alkylsulfinyl)phthalimides are accessible using this method and serve as a stable equivalent to the sulfinyl chlorides.

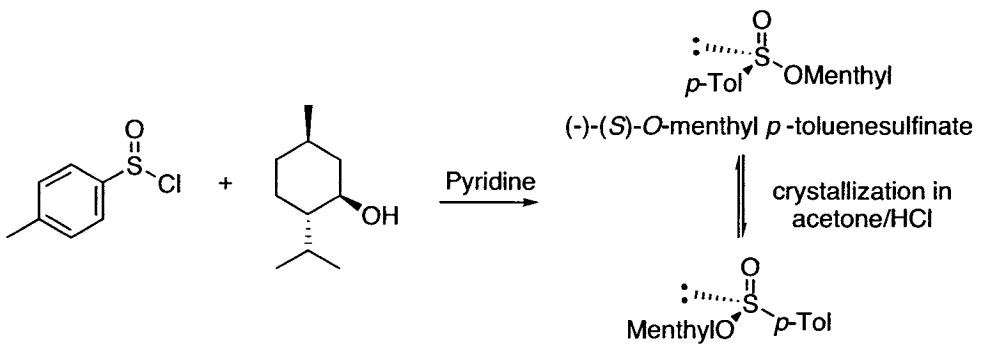


Scheme 4: Preparation of sulfinates and sulfinamides from sulfinic acids, thiols and disulfides via sulfinylchlorides or sulfinylphthalimides

1.2.4 Asymmetric synthesis of sulfinyl compounds

There are several strategies to prepare chiral compounds containing the sulfinyl functionality. The first method involves resolution of diasteromers formed by attaching a chiral auxiliary to give a sulfinate. The second also involves a chiral auxillary, but attached to give a sulfite or aminosulfite derivative (attached in two positions to the sulfur atom). The final method is asymmetric oxidation to give a chiral sulfur species. Emphasis is given to cases in which a heteroatom is attached to the sulfinyl center; these are possible targets for hydrolase chemistry.

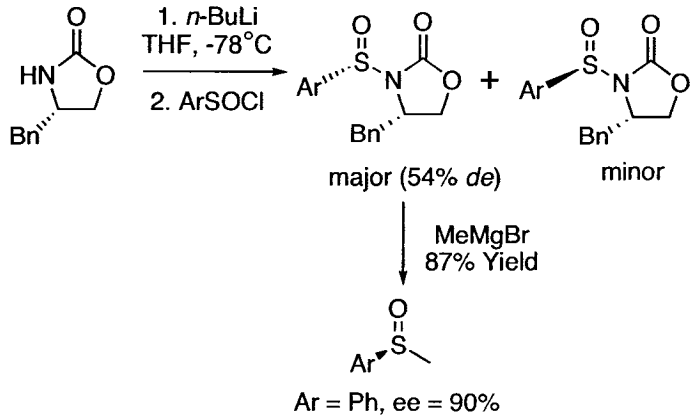
Diastereomeric sulfinic esters are prepared by the reaction of a sulfinyl chloride with a chiral auxillary. The Anderson method is the most common, and is based on formation of (*S*)-menthylsulfinate diastereomers with menthol and pyridine, and separation by crystallization in acetone (Scheme 5). The resulting diastereomerically pure sulfinates react with Grignard reagents with inversion of configuration to give sulfoxides.[17] The method was improved when crystallization in HCl/acetone catalyzed epimerization of the more soluble diastereomer to the crystalline diastereomer, with yields up to 90%.[18] This method is limited to *para*-substituted aryl sulfinates, as other aryl and alkyl sulfinates are liquids and difficult to separate. Similar strategies make use of compounds other than menthol as the resolving agent. The alcohol *trans*-2-phenylcyclohexanol provides access to some aliphatic sulfinates,[19] while the diacetone-D-glucose (DAG) methodology uses a protected sugar as the auxiliary.[20] In all cases the resolved sulfinyl diastereomoers can be treated with a Grignard reagent to prepare enantiopure sulfoxides with inversion at the sulfur center.



Scheme 5: Synthesis of the Anderson reagent, (*S*)-menthyl-*p*-toluenesulfinate, by diastereomer formation with menthol and crystallization in acetone/HCl

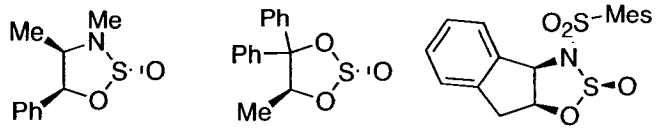
The Evans chiral sulfinyl transfer reagents make use of chiral oxazolidinones derived from ephedrine or phenylalanine.[21] Preparation of *N*-sulfinyloxazolidinones involved either reaction of oxazolidinones with *n*-butyllithium, followed by a sulfinyl chloride, or alternatively oxidation of *N*-sulfenamides. Both cases gave low

diastereomeric ratios (16-54%), but the resulting diastereomers were readily separated by chromatography. Treatment of the purified *N*-sulfinyloxazolidinones with Grignards provided sulfoxides in excellent yields (78-92%) and inversion with high enantiopurity (90-99%).



Scheme 6: Evans' *N*-sulfinyloxazolidinone methodology for chiral sulfinyl transfer.

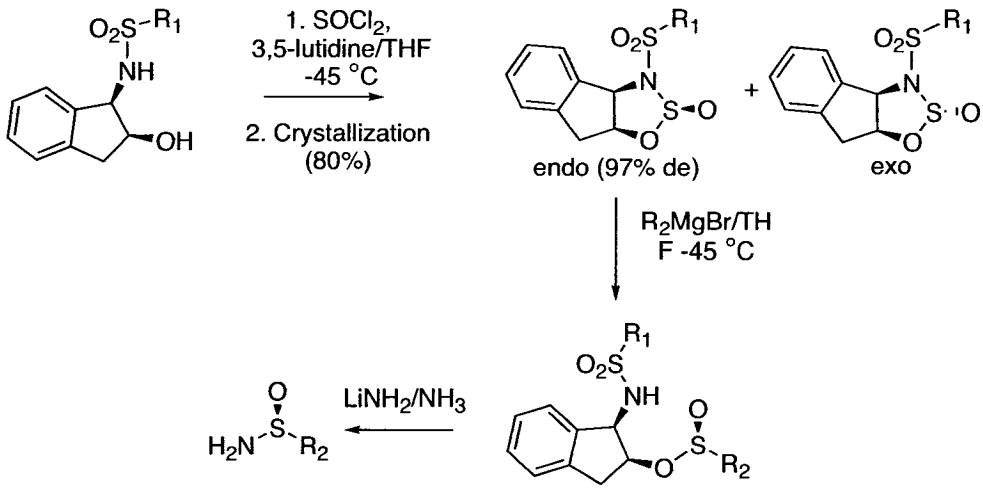
Another strategy for chiral sulfinyl transfer uses sulfites or aminosulfites. Here the auxiliary is attached to the chiral sulfur in two positions, and can be displaced by two successive nucleophilic attacks to give sulfinamides and sulfoxides. The aminosulfite methodology reported recently by Senanayake[22] improves upon previous reagents reported by Wudl and Lee (an aminosulfite)[23] and Kagan (a sulfite, Figure 6).[24] Wudl and Lee's reagent is limited because of low reactivity and cannot prepare *tert*-butyl or diaryl sulfoxides. Kagan's reagent can accept many substrates, but has poor chemoselectivity in ring opening, and therefore low stereoselectivity. Senanayake's reagent overcomes the limitations of the previous reagents as it accepts a wide variety of nucleophiles and has high selectivity. Thus, Senanayake's method can be used to make a wide range of sulfinamides, and possibly sulfoxides in the future.



Wudl and Lee, 1973 | Kagan, 1991 | Senanayake, 2002

Figure 6: Sulfite and aminosulfite derived chiral sulfinyl transfer reagents.

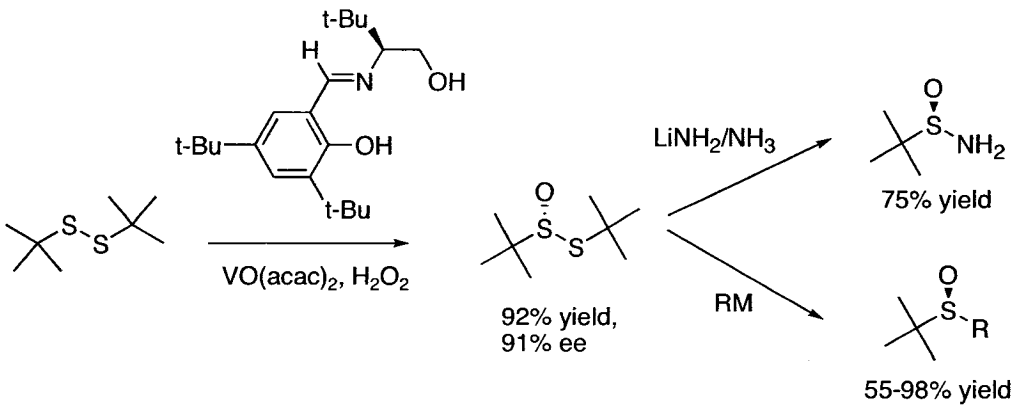
To prepare Senanayake's reagent, thionyl chloride is reacted with the *N*-sulfonyl-derived (1*R*, 2*S*)-aminoindanol to give primarily the endo product (97% d.e.) in 80% yield after crystallization (Scheme 7). The reagent can be prepared on the kilogram scale. The activating sulfonyl group on the nitrogen allows chemoselective attack to break the S-N bond before the S-O bond, with inversion of configuration, giving the sulfinate ester intermediate. A second attack allows production of various sulfinamides, including *tert*-buyl sulfinamide, in high enantiomeric excess.



Scheme 7: Preparation of Seyanayake's aminosulfite reagent. The *N*-sulfonyl group activates the nitrogen so that the S-N bond is cleaved chemoselectively by the Grignard reagent.

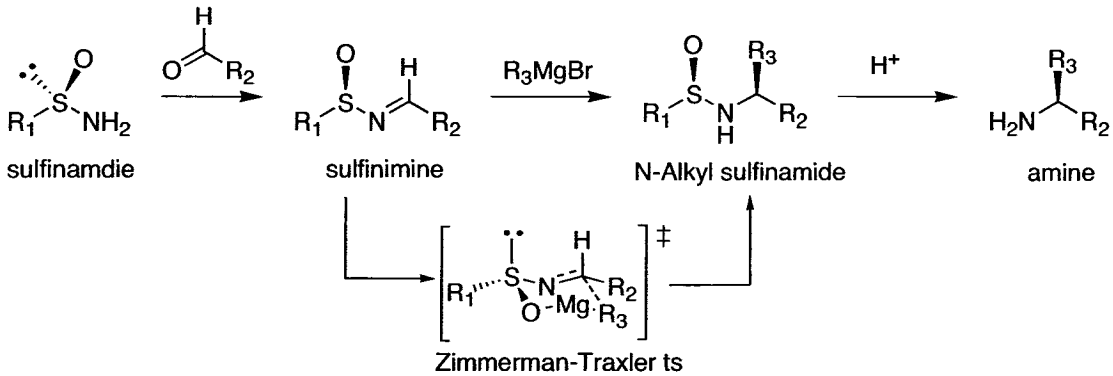
The final method to prepare chiral sulfinyl compounds is by oxidation, and there are a plethora of methods for the asymmetric oxidation of sulfur. Most recently, Ellman developed the asymmetric oxidation of *tert*-butyl disulfide to prepare *tert*-butyl sulfinamide.[25] Using hydrogen peroxide, $VO(acac)_2$, and a chiral ligand gave

(*R*)-*tert*-butanethiolsulfinate in 92% yield and 91% ee on a 1 mole scale (Scheme 8). Reaction with Grignards and lithium amides provide enantiomerically pure sulfoxides and sulfinamides. The reaction is limited to the *tert*-butyl disulfide; smaller groups give racemization at the thiosulfinate stage.



Scheme 8: Enantioselective oxidation of *tert*-butyl disulfide for the preparation of sulfinamides and sulfoxides.

The variety of methods to produce optically active sulfinyl compounds indicates their value. Enantiomerically pure sulfinamides are widely used as auxiliaries providing a chiral ammonia equivalent. Condensation of a sulfinamide with an aldehyde provides an *N*-sulfinyl imine, (or sulfinimine). The electron withdrawing *N*-sulfinyl group activates the C=N bond for nucleophilic addition and controls the stereochemistry of the newly formed stereocenter.[26] Addition of Grignard reagents proceeds via a Zimmerman-Traxler transition state with the large R_1 (usually *p*-tolyl or *tert*-butyl) and R_2 groups in the less hindered equatorial position, and the Mg coordinated to the oxygen of the sulfinyl group (Scheme 9).[27] Enantiopure amines such as α- and β-amino acids[28] and β-hydroxy α-amino acids[29] were synthesized using this method.



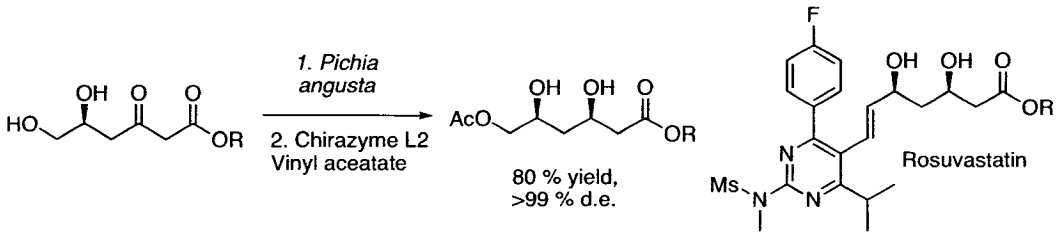
Scheme 9: Nucleophilic Grignard addition to sulfinimines proceeds via a Zimmerman-Traxler transition state in the synthesis of enantiopure amine derivatives.

Chapters four and five will focus on new ways to make chiral sulfinyl compounds using enzymatic hydrolysis of *N*-acyl sulfinamides.

1.3.1 Biocatalysts

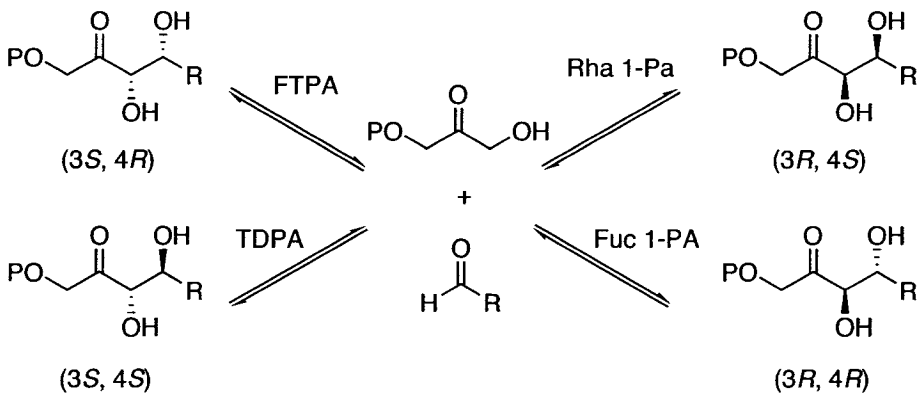
Enzymes are also efficient catalysts for organic transformations. Enzymes are widely applied in food and pharmaceutical industries, and their use is expected to increase.[30] Some of the advantages of enzyme-catalyzed reactions include their enantioselectivity, regioselectivity, large rate accelerations, environmentally friendliness, and mild reaction conditions. There are also enzymatic equivalents to many types of organic transformations such as oxidation/reduction, addition/elimination, hydrolysis, aldol reactions, and others.[31]

An example of an enantioselective reduction is found in the synthesis of cholesterol lowering statins, which inhibit the enzyme 3-hydroyl-3-methylglutaryl reductase and prevent the biosynthesis of cholesterol. All statins contain a common 3,5-dihydroxy acid side chain, and enzymes have been extensively used in the stereoselective synthesis of this group. The reduction of ketone by the yeast *Pichia angusta* and regioselective acylation of the primary hydroxyl group provided the dihydroxy statin side chain in high yield and diastereomeric purity (Scheme 10).[32]



Scheme 10: The cholesterol-lowering statins contain a common 3,5-dihydroxy side chain, as found in Rosuvastatin. One synthetic method involves bioreduction of the ketone followed by regioselective acylation to produce a key intermediate.

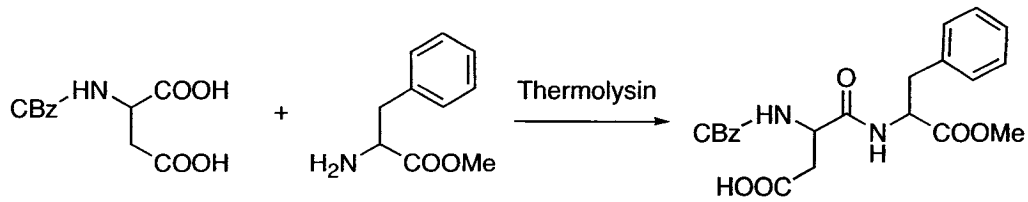
Carbon-carbon bond forming reactions are valuable in organic synthesis. Aldolases catalyze the formation of a C-C bond between an aldehyde and a ketone with stereochemical control. The dihydroxyacetone phospate (DHAP) aldolases catalyze a condensation between dihydroxyacetone phosphate and an aldehyde to create two stereocenters (Scheme 11).[33] The enantiomeric and diastereomeric products are also available by switching to one of the four complementary aldolases.



Scheme 11: Carbon-carbon bond formation with four complementary DHAP-dependant aldolases provides all of the four possible stereoisomers. P = PO_3^{2-}.

Regioselectivity is another valuable feature of enzyme catalysis. An enzyme can selectively modify one of several functional groups within a molecule. Thermolysin reacts with only one of the carboxyl groups in aspartic acid when coupled with the methyl ester of phenylalanine to produce a precursor to aspartame, a

low-calorie sweetener (Scheme 12).[34] This avoids a protection step and the formation of the unwanted β-isomer.



Scheme 12: Regioselective amide bond formation by thermolysin to produce CBz-protected aspartame.

A limitation of resolutions is a maximum 50% yield. The recently developed dynamic kinetic resolutions (DRK) avoid this problem by racemization of the starting material, followed by an enantioselective enzymatic reaction. There are several requirements for an efficient dynamic resolution. The rate of racemization should be faster than the acylation step, that is, k_{rac} should be larger than k_R or k_S (especially the rate of the slow enantiomer, Figure 6). An enantioselective enzyme is necessary, and both the racemization catalyst and conditions must be compatible with the enzyme.

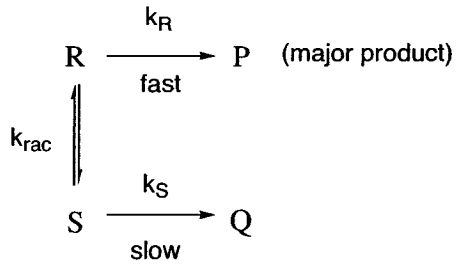
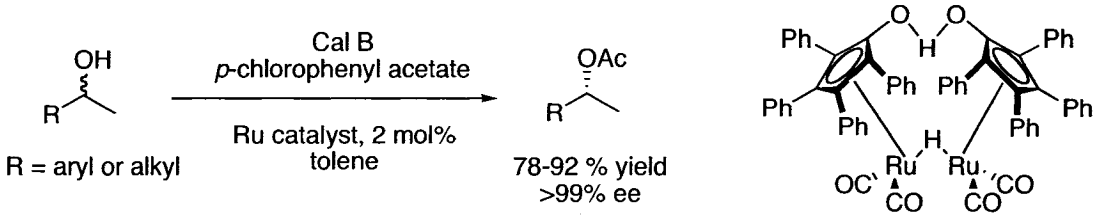


Figure 6: *R*-selective dynamic kinetic resolution allows a theoretical yield of 100%. R and S represent the two enantiomers of starting material; P and Q represent the two possible enantiomers of product. k_R and k_S are rate constants for each enantiomer in the enzymatic reaction, and k_{rac} is the racemization. The starting material is constantly racemized so the slow enantiomer, S, is eventually converted to P.

Secondary alcohols have been successfully resolved using dynamic kinetic resolutions. A transition metal catalyst, such as the ruthenium complex in scheme 13,

is used to chemically oxidize and reduce the alcohol leading to racemization. Bäckvall and coworkers developed and efficient system using *Candida antartica* lipase B (Cal B) to give the resolved ester in good yield and excellent enantiopurity, scheme 13.[35] To access the opposite enantiomer, an enantiocomplementary enzyme (see section 1.3.2 below) was used for the dynamic kinetic resolution. Subtilisin has the opposite stereopreference towards secondary alcohols, and thus gave an *S*-selective resolution.[36]



Scheme 13: Dynamic kinetic resolution of secondary alcohols. The ruthenium complex constantly racemizes the starting alcohol, followed by an enantioselective acylation of the enzyme. This allows for yields greater than 50%.

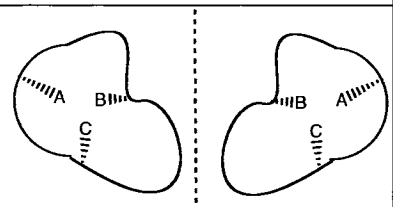
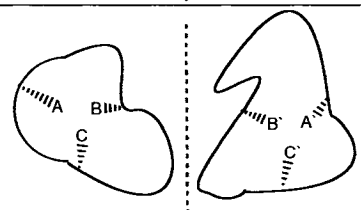
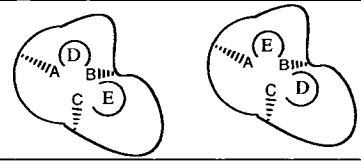
1.3.2 Enantiocomplementary enzymes

An objection to the use of enzymes is that they are available in only one enatiomeric form, being made from L-amino acids. In asymmetric synthesis switching to the ligand or starting material with the opposite configuration yields the opposite enantiomer. The equivalent enzyme, prepared from D-amino acids, is not a practical solution, although some have been made by chemical synthesis. For example, the 99 D-amino acid HIV-1 protease was prepared by peptide coupling, and displayed the opposite enantiopreference.[37] Related examples include a 62 amino acid oxalocrotonate tautomerase from D-amino acids,[38] and of a 49-mer ribozyme prepared from unnatural L-nucleotides that catalyzes a Diels-Alder cycloaddition reaction with opposite specificity from the natural D-nucleotide ribozyme.[39] Although proven in principle, enantiomeric enzymes can only be prepared on a very small scale with great expense. Practical biocatalytic routes to enantiomeric products employ enantiocomplementary enzymes.

Enantiocomplementary enzymes are pairs of enzymes from natural L-amino acids that catalyze mirror image (enantiomeric) biotransformations. The starting

materials are enantiomers or achiral, but the products of the transformations are enantiomers. The catalyst acts in a mirror image fashion, and the mechanism and type of transformation is the same. In principle there are two types of enantiocomplementary enzymes based on the structural basis by which they produce opposite enantiomers (Table 1). The first involves a mirror image arrangement of the catalytic machinery to provide the opposite stereopreference. The second involves a reversal of the substrate orientation relative to the catalytic machinery to reverse the enantioselectivity. For many practical synthetic examples of enantiocomplemtary enzymes there is no structural information available to determine the basis of reversal, but they likely function by one of the two methods. Knowledge of the crystal structure, binding sites and mechanism is necessary before the molecular basis of enantiocomplementary enzymes can be established. For some whole-cell catalysis, the active enzyme is not even determined.

Table 1 Possible ways to create enantiocomplementary enzymes: moving the catalytic groups vs. moving the substrate binding pockets

Category	Schematic	Features	Examples
Enantiomeric enzymes from D- vs. L- amino acids		Exact mirror image by D-amino acids synthesis; impractical	HIV protease, oxalocrotonate tautomerase, Diels-Alder ribozyme
Type 1: Mirror image enantiocomplementary enzymes		Altered orientation of the catalytic machinery	Lipase/protease, methionine sulfoxide reductase
Type 2: Substrate reversal enantiocomplementary enzymes		Altered orientation of the substrate-binding pockets	Tropinone reductase phosphotriesterase mutants

There are several examples of enantiocomplementary enzymes with mirror image catalytic machinery held in position by a different overall fold. Chemical

oxidation of methionine yields both epimers of methionine sulfoxide. To rectify this requires either a non-specific enzyme or two enzymes with opposite stereospecificity. In *Escherichia coli*, separate enantiocomplementary enzymes reduce the enantiomeric sulfoxide configurations in methionine sulfoxide epimers.[40] In *Neisseria gonorrhoeae,* one protein containing mirror-image active sites in separate domains reduces these enantiomeric sulfoxide configurations, as shown by x-ray crystallography.[41]

Another example of enantiocomplementary enzymes resulting from mirror image active sites is the opposite enantiopreference of lipases and proteases. Lipases and subtilisin, both serine hydrolases, contain a catalytic triad, an oxyanion hole, and follow an acyl enzyme mechanism (see section 1.4.2). However, the three-dimensional arrangement of the catalytic triad in subtilisin is the mirror image of that in lipases, even though the overall fold is not related.[42] Folding the protein to assemble the catalytic machinery creates a pocket with restricted size, the 'M' or stereoselectivity pocket for lipases and the S_1' pocket for subtilisin. The mirror image relationship places the catalytic histidine on opposite sides of this pocket in lipases and subtilisin (Figure 7).[43] This difference likely accounts for the opposite enantiopreference. The dynamic kinetic resolution of secondary alcohols using *Candida antartica* lipase provided the *R*-alcohol, while subtilisin gave the *S*-alcohol (Section 1.3.1, above). The empirical secondary alcohol rules, developed from substrate preference, are also related to these differences in structure (section 1.4.6, below). There are several other examples of mirror image active sites. The active site of D-amino acid oxidase is a mirror image of that in flavocytochrome-b_2.[44] Two mirror image hydratases catalyze addition of water from opposite sides of a double bond.[45] Although not important to substrate chirality, mirror image active sites exist in disulfide reductases[46] and carbonic anhydrase.[47]

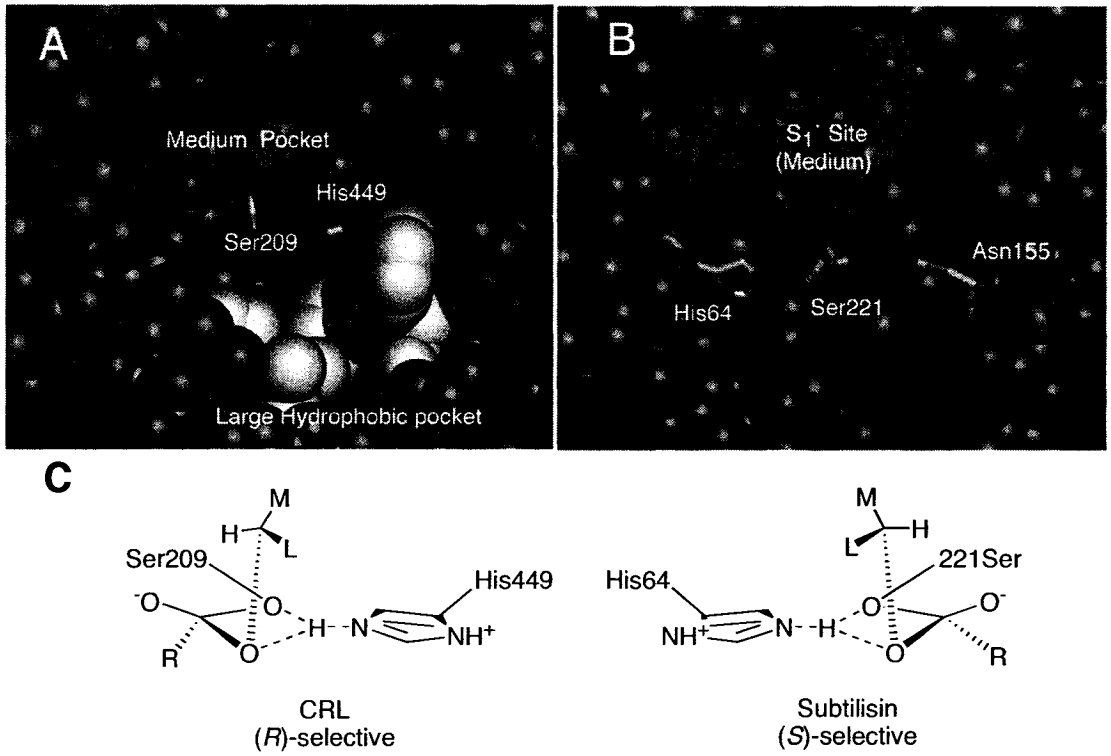
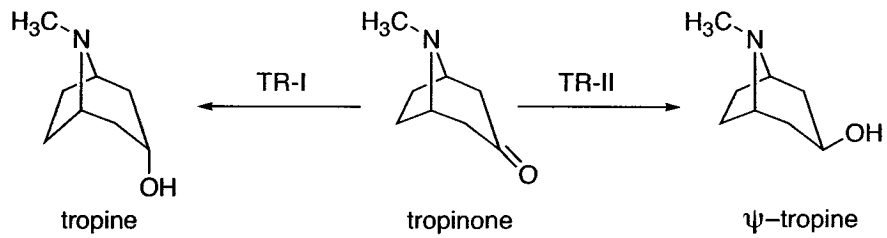


Figure 7: Lipases and subtilisin have mirror image catalytic machinery resulting in opposite enantiopreference towards secondary alcohols. (A) *Candida rugosa* lipase (PDB code 1crl), shown with catalytic residues Ser 209 and His 449, and two regions of the alcohol binding site: the large substituent binds in the large hydrophobic pocket (in yellow), while the small substituent binds in the medium pocket (in green). (B) Subtilisin (PDB code 1sbn) has a mirror image arrangement; the catalytic residues Ser 221 and His 64 are shown, along with the oxyanion stabilizing Asn 155. The S_1' medium binding site (in green) positions the medium substituent, while the large substituent is oriented towards solvent. (C) Schematic of tetrahedral intermediates for the favoured secondary alcohols for lipase and subtilisin.

In addition to mirrored active sites, enantiocomplementary enzymes may reorient the substrate to favour opposite enantiomers through a switch in binding pocket preference. A reverse binding can occur when two enantiocomplementary enzymes have both a high sequence homology and a conserved location of catalytic residues. Enzymes are unlikely to have major structural changes with very similar

sequences, and conserved catalytic residues eliminate mirror image machinery, so it is likely the preference for placement of substrate has reversed.

There are several examples of complementary reductions. Two tropinone reductases, TR-I and TR-II, from the plant species *Datura stramonium* give opposite configurations of the product hydroxyl group (Scheme 14).[48] The two enzymes are 260-274 amino acids in length and are 64% identical. In addition, crystal structures revealed the hydride donor (NADPH) binding site and catalytic residues are conserved. The configuration of the product is therefore dependant on the binding orientation of the tropinone to the enzyme. The two enzymes' substrate binding sites differ in a small number of charged amino acids, resulting in a reversed orientation for the tropinone and reduction from the opposite face. This was confirmed when only five amino acid substitutions in the binding site of either TR-I or TR-II switched the stereospecificity to the opposite enzyme. [49]



Scheme 14: Reduction of tropinone by two similar reductases, TR-I and TR-II, to give opposite hydroxyl configurations occurs by binding of the tropinone in a reversed orientation. Five mutations of either TR-I or TR-II inverted their stereoselectivity to that of the opposite enzyme.

As in the tropinone example, mutagenesis resulting in reversed enantioselectivity often involves modifying binding sites to reposition the substrate; the catalytic residues are seldom moved. Mutagenesis of the phosphotriesterase from *Pseudomonas diminuta* produced two enzymes that clearly demonstrate switching substrate orientation to reverse enantioselectivity. The phosphotriesterase has three binding sites with which it binds phosphotriesters, referred to as small, large, and leaving group subsites based on which part of the substrate occupies the site.[50] The enantioselectivity was altered, and then reversed, by making changes to the size of the

small and large subsites. The wild type enzyme hydrolyzed the substrate ethyl phenyl *p*-nitrophenyl phosphate with an enantioselectivity of 21 favouring the *S*-enantiomer. Mutation of the small subsite to reduce its size increased the enantioselectivity, *E*, from 21 to >1000, probably making it impossible for the large substituent of the slow enantiomer to bind. Enlargement of the small subsite decreased *E* from 21 to 1.3 because now the large substituent can bind in either site. Simultaneously enlarging the small subsite while the large subsite was reduced in size gave a mutant that favoured the opposite *R*-enantiomer by a factor of 80. Thus binding the substrate in a reversed manner reverses the enantioselectivity.

Other examples in this category include 8*R*- and 8*S*-lipoxygenases whose conserved active site and other essential residues suggest a substrate reversal leading to opposite selectivity, and not a mirror image active site.[51] Likewise, two cyclooxygenase enzymes with 80% identity and conserved catalytic residues produce 15*R* and 15*S* prostaglandins, due to a substrate reversal.[52] Subtilisin and cholesterol esterase are enantiocomplementary enzymes in the resolution of a chiral auxiliary for the Diels-Alder reaction, discussed in chapter two of this thesis.

1.3.3 Catalytic promiscuity: multiple enzyme functions

Another frontier of biocatalysts is catalytic promiscuity, the ability of some enzyme active sites to catalyze two chemically distinct transformations.[53] Catalytic promiscuity is important in enzyme evolution and developing new reactions for organic synthesis. The type of bond broken, or the reaction mechanism, or both, may change in the promiscuous function. Gene duplication followed by modification is thought to be responsible for the evolution of new activities.[54] Adaptation of new functions is also important in understanding pesticide and drug resistance.

Atrazine, a herbicide used since the 1950's, initially was not metabolized by microorganisms in the soil. Recently, several groups discovered bacteria with the ability to degrade atrazine using a modified form of the enzyme melamine hydrolase. Melamine hydrolase shows low activity towards atrazine, but 9 amino acid mutations produces a new enzyme, atrazine chlorohydrolase, which cleaves the C-Cl and not the original C-N bond (Figure 8).[55] Interestingly, several groups independently

discovered the same chlorohydrolase in different types bacteria isolated from four continents, suggesting an evolutionary relationship between the two activities.[56]

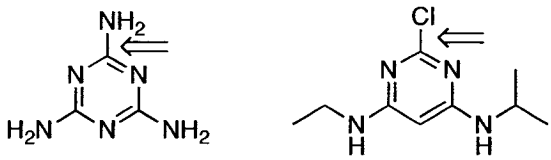


Figure 8: Mutation of nine amino acids in melamine hydrolase creates a new enzyme capable of C-Cl bond cleavage to degrade the herbicide, atrazine.

The hydrolysis of sulfite esters by the protease pepsin highlights a single active site that can hydrolyze two mechanistically different reactions. Pepsin is an aspartate protease active at low pH in the stomach. It also catalyzes the hydrolysis of sulfite esters rapidly, and with some stereoselectivity (Figure 9).[57] The sulfur reaction center is trigional, unlike the natural sp^2 carbonyl of the amide bond. Therefore the reaction must proceed through transition state with a different shape. A catalytic promiscuous reaction of subtilisin is discussed in chapters four and five.

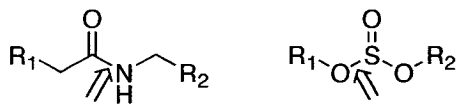


Figure 9: The protease pepsin hydrolyzes both amide and sulfite bonds.

1.3.4 Enzymes accelerate reactions by stabilizing the transition state

Enzymes are efficient catalysts that accelerate a reaction, sometimes by a factor as high as 10^{19}.[58] Many uncatalyzed reactions would occur on a time scale too long for biological processes. For example, peptide hydrolysis in water has a half-life of 450 years, highlighting the need for catalysis.[59] As seen in Figure 10, enzymes decrease the activation energy the reaction compared to the uncatalyzed process. Pauling proposed that the lowering of the activation energy results from a preferential binding of the transition state over the substrate.[60] Two kinds of enzyme-substrate interactions lead to a stronger binding of the transition state: electrostatic forces, such

as hydrogen bonding and dipole interactions, and van der Waals interactions. In addition to these effects, others have proposed entropic factors make a large contribution. Pre-organization to bring reacting groups together may assist catalysis. Bruice has proposed Near Attack Conformers (NAC), which are ground state conformers that closely resemble the transition state.[61] These involve arranging the reacting groups so that distances and angles are similar to those in the transition state. Menger has proposed that holding the substrate in a reactive conformation contributes to the catalytic efficiency of enzymes.[62]

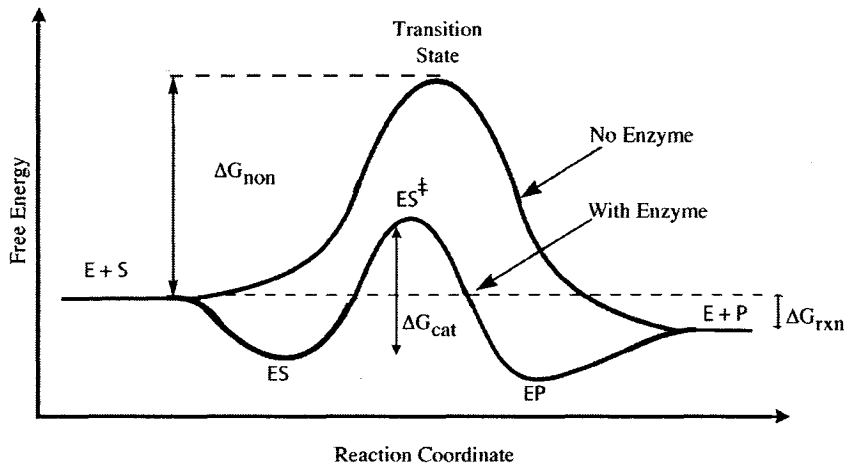


Figure 10: Energy diagram an enzyme catalyzed reaction. The energy of the enzyme catalyzed reaction, ΔG_{cat}, is less than the uncatlyzed reaction, ΔG_{non}, while the overall energy of the transformation is unchanged, ΔG_{rxn}.

1.3.5 Enzyme kinetics

The kinetic behaviour for many enzymes, including the serine proteases, is described by Michaelis-Menten kinetics and refined further by Briggs and Haldane.[63] The enzyme (E) and substrate (S) combine in a rapid, reversible equilibrium with no chemical changes taking place to form an enzyme-substrate (Michaelis) complex, ES, with rate constants of k_1 and k_{-1}. This complex can then react to give free enzyme and product (P) with rate constant k_{cat}.

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

The reverse reaction, E + P, can be ignored because when measuring initial rates the concentration of product is low. It is also assumed that the concentration of substrate is both constant (as in the initial stages of the reaction) and much greater than that of enzyme. The initial rate (v) of formation of product with time is given by:

$$v = k_{cat}[ES]. \qquad 1.1$$

Initial rates are measured so that changes in substrate concentration are negligible. During the initial portion of the reaction, the reaction velocity is constant and the rate of formation of the ES complex is equal to its rate of destruction. The steady state assumption gives:

$$k_1[E][S] = (k_{cat} + k_{-1})[ES]. \qquad 1.2$$

Rearranging this equation to group the rate constants provides:

$$[ES] = \frac{k_1[E][S]}{k_{cat} + k_{-1}}. \qquad 1.3$$

The three rate constants can be redefined as one constant, K_M, termed the Michaelis constant.

$$K_M = \frac{k_{cat} + k_{-1}}{k_1} \qquad 1.4$$

The enzyme may be either free enzyme [E] or in a bound form [ES], such that the total amount of enzyme [E_T] less that which is bound to substrate gives the amount of free enzyme,

$$[E] = [E_T] - [ES]. \qquad 1.5$$

Substituting equation 1.5 into 1.3 gives

$$[ES] = \frac{[E_T][S]}{[S] + K_M} \qquad 1.6$$

Substitution of this expression for [ES] into 1.1 gives:

$$v = \frac{k_{cat}[E_T][S]}{K_M + [S]} \qquad 1.7$$

The maximum rate (v_{max}) is achieved when the enzyme is saturated with substrate, such that all enzyme is in the ES form. This occurs at concentrations of [S] >> K_M, allowing cancellation in 1.7 to give,

$$v_{max} = k_{cat}[E_T]. \qquad 1.8$$

Substituting 1.8 into 1.7 gives the Michaelis-Menten equation,

$$v = \frac{v_{max}[S]}{K_M + [S]} \qquad 1.9$$

Graphically a plot of v vs. [S] is a hyperbola, with an asymptote corresponding to v_{max}, representing the velocity at high substrate concentrations (Figure 11). The value K_M corresponds to the concentration of substrate at which the velocity is half the maximum velocity.

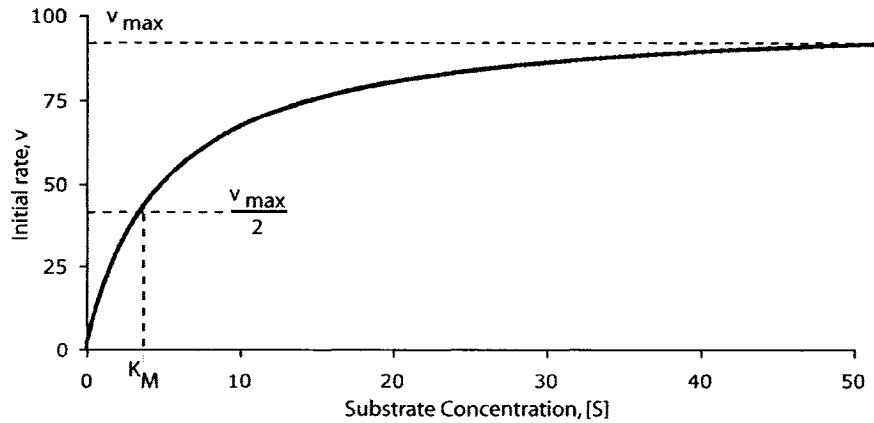


Figure 11: Plot of v vs. [S] illustrating v_{max} and K_M values.

A competitive, reversible inhibitor (I) binds to the enzyme active site and prevents substrate binding. The affinity for the inhibitor (I) is defined by K_I, the dissociation constant of the enzyme-inhibitor complex.

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

$$K_I \quad \Vert \quad I$$

$$EI$$

Competitive inhibition does not affect the k_{cat} value (at high substrate concentrations, all inhibitor will be displaced), but gives an apparent increase in the Michaelis constant, K_M. The apparent K_M value is increased by a factor of $(1+[I]/K_I)$.[64]

$$v = \frac{v_{max}[S]}{\left(1 + \frac{I}{K_I}\right)K_M + [S]} \qquad 1.10$$

1.3.6 Kinetic resolution and enantioselectivity, *E*[65]

In a kinetic resolution, two enantiomeric substrates, A and B, compete for an enzyme's active site.

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

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

The initial rate for each enantiomer is given by:

$$v_A = (k_{cat})_A[EA] \text{ and } v_B = (k_{cat})_B[EB]. \qquad 1.11$$

When k_{cat} is slow relative to the binding equilibrium, then the Michaelis constant, K_M, simplifies to a true binding constant for each enantiomer is:

$$(K_M)_A = \frac{[E][A]}{[EA]} \text{ and } (K_M)_B = \frac{[E][B]}{[EB]}. \qquad 1.12$$

Substituting 1.12 into rate equations 1.11 gives

$$v_A = \left(\frac{k_{cat}}{K_M}\right)_A [E][A] \text{ and } v_B = \left(\frac{k_{cat}}{K_M}\right)_B [E][B] \qquad 1.13$$

The enantiomeric ratio, *E* (or, enantioselectivity), is defined the ratio between the rates of each enantiomer,

$$E = \frac{v_A}{v_B} = \frac{\left(k_{cat}/K_M\right)_A [A]}{\left(k_{cat}/K_M\right)_B [B]} \qquad 1.14$$

Integration of 1.14 gives the E value as a function of k_{cat} and K_M for each enantiomer,

$$E = \frac{\ln\left([A]/[A]_0\right)}{\ln\left([B]/[B]_0\right)} = \frac{\left(k_{cat}/K_M\right)_A}{\left(k_{cat}/K_M\right)_B}, \qquad 1.15$$

where $[A]_0$ and $[B]_0$ are initial concentrations for each enantiomer. The enantiomeric ratio measures the performance of an enzyme to produce enantiomerically pure compounds, making it important to quantitate for an enzyme catalyzed resolution. The enantioselectivity, *E*, is dimentionless and is dependant only on the ratio of constants and remains constant throughout an enzymatic resolution. Measuring initial rates and determining the necessary kinetic parameters for each pure enantiomer is

tedious. An alternate method employs the conversion of the reaction along with either the enantiopurity of the substrate or product;

$$E = \frac{\ln[(1-c)(1-ee_S)]}{\ln[(1-c)(1+ee_S)]} \text{ or } E = \frac{\ln[1-c(1+ee_P)]}{\ln[1-c(1-ee_P)]} \qquad 1.16$$

where c is the conversion, ee = enantiomeric excess of substrate (ee_S) or product (ee_P). The conversion and enantiopurity are obtained by:

$$c = 1 - \frac{A+B}{A_0+B_0}, \; ee = \frac{A-B}{A+B}. \qquad 1.17$$

Enantioselectivity values determined using conversion are generally less accurate due to experiential errors. Alternatively, the enantioselectivity is determined from the enantiomeric excess of substrate and product via:

$$E = \frac{\ln\left[\frac{ee_P(1-ee_S)}{ee_P+ee_S}\right]}{\ln\left[\frac{ee_P(1+ee_S)}{ee_P+ee_S}\right]}. \qquad 1.18$$

Plots for three different enantioselectivities are shown in Figure 12, and illustrate the change in both ee_S and ee_P as a function of conversion. In the first plot, a low enantioselectivity of 5 demonstrates that the product cannot be obtained in high enantiopurity, while remaining starting material can be obtained in high enantiopurity only at conversions >80% (therefore with low yield). For synthetic purposes, an enantioselectivity of greater than 20 is considered useful, shown in the second plot. A reaction with E = 20 at 50% conversion gives an enantiomeric excess of 79% for the substrate (ee_S) and product (ee_P). Stopping the reaction at the midpoint maximizes the yield of the starting material and product. The final graph shows a high enantioselectivity (E=200), and at 50% conversion $ee_S = ee_p = 97\%$. An enantioselectivy above 200 is difficult to measure accurately because of experimental errors.

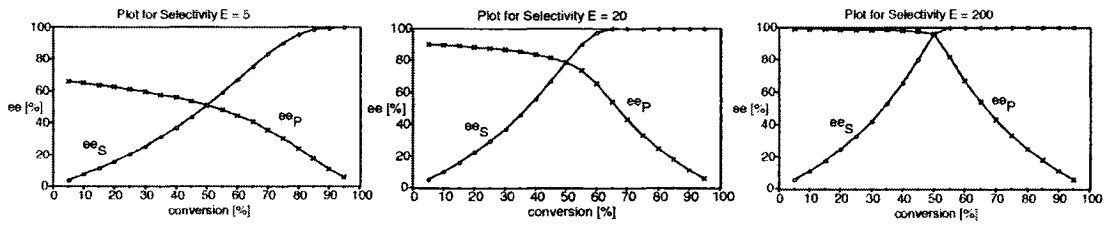


Figure 12: Enantiomeric excess of starting material (ee_S) and product (ee_P) as a function of conversion for enantioselectivity 5, 20, and 200. Produced using the computer program Selectivity.[66]

1.3.7 Thermodynamic description of selectivity

The success of a resolution depends on the energy difference at the transition state in the rate determining step(s). The enantioselectivity, a ratio of rates, is related to the energy difference, $\Delta\Delta G^{\ddagger}$, by:

$$\Delta\Delta G^{\ddagger} = \Delta G^{\ddagger}_{R} - \Delta G^{\ddagger}_{S} = -RT\ln E. \qquad 1.19$$

The energy difference arises from the interaction of two enantiomers with a chiral environment, the enzyme. The resulting diastereomers, the enzyme-substrate complexes, have different energies (Figure 13). Surprisingly, the energy difference to obtain a high selectivity is not extremely large. For enantioselectivities of 20 and 200, the energy difference in transition states is 1.7 and 3.1 kcal mol^{-1}. For comparison a hydrogen bond contributes ~1 to 5 kcal mol^{-1}, while a peptide bond has a bond strength of ~96 kcal mol^{-1}.[67] The relatively small energy difference is one reason why molecular modeling to quantitate enantioselectivity remains elusive.

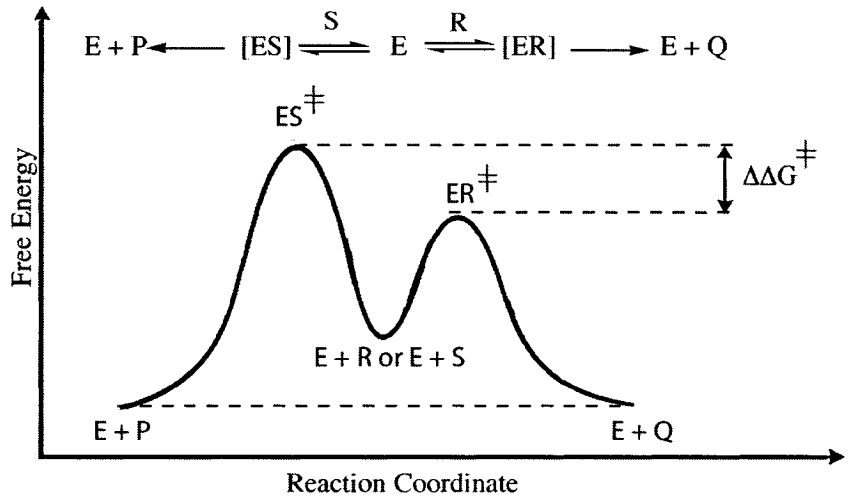


Figure 13: Enzyme (E) catalyzed enantioselective reaction has a difference in energy at the transition state (‡) for the substrate enantiomers (R and S) reacting to product enantiomers (P and Q).[31]

1.3.8 Molecular modeling to rationalize enantioselectivity

Combining three dimensional protein structures and computer modeling allows an enhanced understanding of enzymatic reactions by predicting and explaining experimental results.[68] Molecular mechanics (MM) uses empirically derived parameters to describe bonding, where atoms are treated as balls and bonds are treated as springs. A force field is used to calculate the energy and geometry of a molecule. It is a collection of atom types, parameters such as bond lengths, bond angles, etc., and equations to calculate the energy of a molecule using these parameters. Amber is one common force field in MM calculations for large proteins and nucleotides.

Modeling has several applications, such as docking or explaining experimental results. Docking plays an important role in drug discovery programs to identify potential drugs by predicting the conformation and orientation of a small molecule in a binding site. Inhibition of tyrosine kinase activity is important in the treatment of leukemia. Peng and coworkers identified 15 inhibitors from a library of 200 000 by virtual screening, several of which had IC_{50} values of 10-200 μM.[69]

Molecular modeling can also explain the behavior of an enzyme or suggest modifications to the substrate or enzyme for an improved reaction. For example Hult and coworkers used molecular modeling to rationally design lipase mutants with improved enantioselectivity.[70] *Candida antarctica* lipase B (CALB) has high enantioselectivity towards secondary alcohols (E = 200), but shows low enantioselectivity toward isosteric halohydrins such as 1-bromo-2-octanol (E = 6.5) and 1-chloro-2-octanol ((E = 14, Figure 14). Molecular modeling revealed an unfavourable interaction between the halogen of the fast reacting enantiomer and the medium size stereoselectivity pocket. Virtual mutation of three residues which define this pocket predicted amino acid substitutions with improved interactions. Based on this molecular modeling single point mutations were prepared and evaluated in kinetic resolutions. Replacing the electronegative serine in a Ser47Ala mutation doubled the enantioselectivity for both 1-chloro and 1-bromo-2-octanol. Another mutation, Tyr104His, increased the size of the medium pocket so that it could now accommodate both the large and medium size substituents, and the enantioselectivity decreased as expected (E <2). Thus molecular modeling predicted steric and electrostatic interactions explaining enantioselectivity and predicting mutants for rational protein engineering.

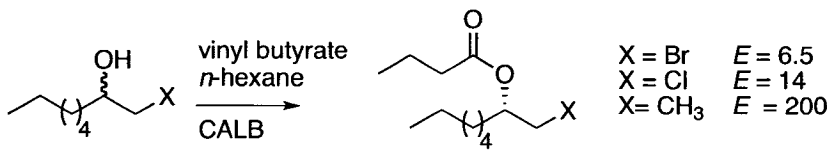


Figure 14: *Candida antarctica* lipase B exhibits high enantioselectivity with secondary alcohols such as 3-nonanol, but low enantioselectivity with the isosteric halohydrins.

Modeling with force fields is limited; it cannot study bond making and breaking processes or examine transition states. The combined quantum mechanical/molecular mechanics (QM/MM) overcomes this by treating the reactive portion at the active site with quantum mechanics, and the remaining unreactive portion with molecular mechanics. Alternatively, a stable transition state analogue

can also be used to approximate the transition state. For instance, phosphonates serve to mimic the transition state in modeling of ester hydrolysis.[71]

Finding the global minimum, rather than a local minimum, is another challenge, particularly in substrates with a large amount of flexibility. In these cases, molecular dynamics simulations use an increase in temperature to cause conformational changes and cross small energy barriers. Another limitation is a quantative determination of an enzymes' enantioselectivity, which is currently not possible for any computational method because of the relatively small energies involved.

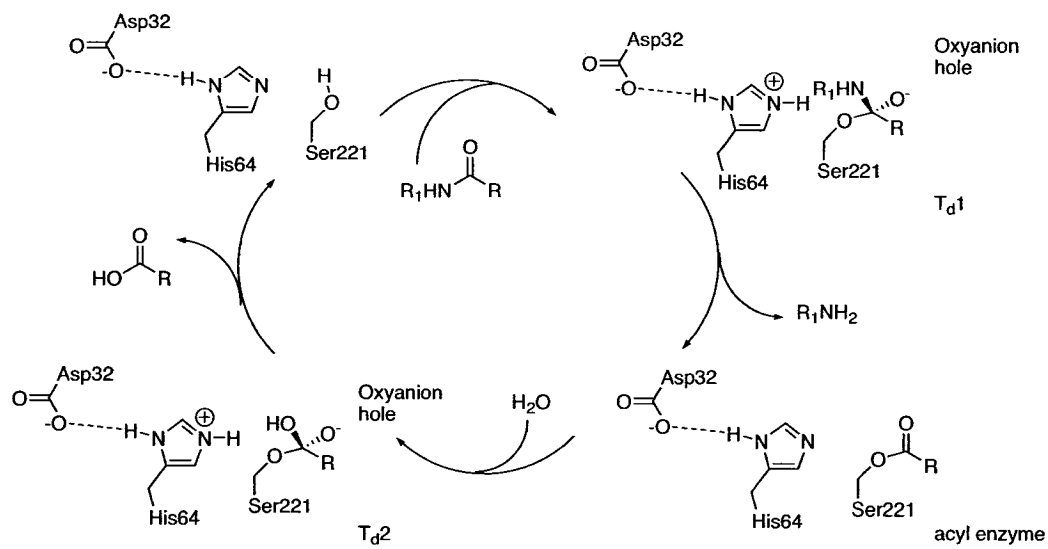
1.4.1 Hydrolases

Hydrolyases (E.C. 3.X) encompass a wide range of enzymes including lipases, esterases, and proteases. They are the largest class of enzymes used in applications such as food, dairy, detergent, and pharmaceutical industries, accounting for two-thirds of biotransformations.[72] Hydrolases are widely used because they are readily available, they do not require cofactors, and they are stable to the addition of organic co-solvents. Hydrolases catalyze the addition of water to cleave a bond, often in an amide or ester. They are also used in the reverse reaction to form esters and amides in solvents with low water content, or in transesterification reactions.

1.4.2 Serine hydrolase mechanism

The serine hydrolase mechanism is well understood. It was first reported for chymotrypsin, and later in many other hydrolases.[73] The catalytic triad consists of a charge relay system between serine, histidine and aspartate group. The spatial arrangement of the catalytic triad is conserved among serine proteases despite a lack of sequence homology and differences in overall fold. The mechanism involves nucleophilic attack of the serine hydroxyl on the carbonyl carbon of an amide, or ester, of the non-covalently bound substrate to provide the first tetrahedral intermediate (T_d1, Scheme 15). The catalytic histidine forms a hydrogen bond to the catalytic serine during attack, reducing the pK_a of the hydroxyl group, and then receives a proton from the serine hydroxyl. The imidazole, now with a positive

charge, is stabilized by the negatively chargee aspartate residue. The negative charge on the oxyanion of the tetrahedral intermediate is stabilized by hydrogen bonds. In subtilisin the backbone NH of the catalytic serine and the NH_2 of aspargine 155 form the oxyanion hole to stabilize the oxyanion charge. Transfer of the histidine proton to the leaving group amine, or alcohol, releases the leaving group to give the acyl enzyme intermediate. Any enantioselectivity for amines or alcohols must have occurred by this point because the alcohol or amine is released from the enzyme. Attack of water on the acyl enzyme produces the second tetrahedral intermediate (T_d2), with the catalytic histidine transferring a proton from the attacking water back to the serine. Release of the carboxylic acid regenerates free enzyme. For amide hydrolysis the acylation step is usually rate limiting, and for ester hydrolysis deacylation of the acyl enzyme intermediate is rate limiting.[74] This agrees with amides being much less reactive than esters, but may not apply to amides such as reactive peptides and *para*-nitroanilides.[75]



Scheme 15: The catalytic mechanism for serine hydrolases. The catalytic triad is composed of Asp32, His64, and Ser221; the residues are numbered according to subtilisin. The mechanism involves attack by the catalytic serine on an ester or amide to produce the first tetrahedral intermediate, T_d1, which breaks down to give an acyl enzyme. Hydrolysis by water through the second tetrahedral intermediate, T_d2, completes the catalytic cycle.

Subtilisin was used to definitively determine the effect of each catalytic residue on the serine protease mechanism. Replacement of either of the active site residues Ser221, His64, and Asn155 with alanine decreases k_{cat} by 10^4-10^6 towards the peptide substrate, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide.[76] Removing active site residues was particularly difficult because subtilisin is synthesized containing a propeptide segment, usually processed by the active site. Active site mutants were co-cultured with a small amount of wild-type enzyme to remove the propeptide. An additional Ser24Cys mutant away from the active site allowed separation of the mutants by binding to a thiol sepharose column. Even with the catalytic triad disabled, the mutant subtilisin hydrolyses the peptide substrate at a 10^3 higher rate than the uncatalyzed process. This residual activity may reflect contributions from the oxyanion hole, desolvation and binding.

The existence of the acyl enzyme is also well established. If the substrate and reaction conditions favor fast acylation, but slow deacylation, an acyl enzyme accumulates and can be detected by spectroscopic techniques,[77] mass spectrometry[78] and even x-ray crystallography.[79] At low pH the histidine is protonated and prevents catalytic turnover of the enzyme.

A recent paper suggested that the attack on inhibitor peptides at the carbonyl center occurs at approximately 90°, based on crystallographic, kinetic and mass spectrometry evidence with subtilisin BPN`.[80] This is significantly different from Bürgi-Dunitz trajectory of 105 ± 5° from small molecule crystallization and calculations.[81] In this example, the attack angle for enzymatic peptide hydrolysis is not exactly analogous to nucleophilic attack on a carbonyl center, but it is not clear why the two are different.

1.4.3 Screening to discover active enzymes

Hydrolase screening is easily accomplished using spectrophotometric methods. Hydrolysis of an ester produces a carboxylic acid and an alcohol (Figure 15). Above the pKa of the carboxylic acid (typically 3-5), the acid is ionized to give a proton, which is detectable by a pH indicator, such as *para*-nitrophenol.[82] When

protonated, the decrease in absorbance at 404 nm (yellow) with time gives the rate of hydrolysis. Some protons are lost to the buffer but small changes in pH give large changes in absorbance, and under the correct conditions the colour change is proportional to the number of protons released. The pK_a of the indicator and the buffer are ideally within 0.1 units so that the relative amount of protonated buffer to protonated inhibitor stays constant as the pH shifts during the reaction, and gives improved linearity.[83] The highest assay sensitivity is given with a low buffer concentration and high indicator concentration. Screening for enantioselectivity requires more in depth screening methods: Quick E[84], chiral CG or HPLC, or deuterium labeled mass spectrometry[85] are some options.

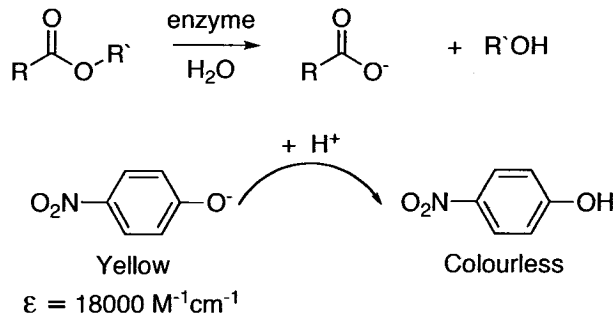


Figure 15: Activity screening for hydrolysis with the pH indicator *p*-nitrophenol: protons released upon ester hydrolysis can protonate the highly coloured *p*-nitrophenoxide to give a decrease in absorbance at 404 nm.

1.4.4 Subtilisin

Subtilisin (E.C. 3.4.21.62) is a 275 amino acid single chain protease that has several variants from various *bacillus* species: subtilisin E (*Bacillus subtilis*), subtilisin BPN` (*Bacillus amyloliquefaciens*), subtilisin Carlsberg (*Bacillus lichenformis*), and subtilisin BL (*Bacillus lentus*). Subtilisins are produced as precursors, called preprosubtilisins. The pre-region, a signal peptide, is required for protein secretion. The pro-region, or the propeptide, is located between the signal peptide and the mature protein and functions as a chaperone for folding before undergoing autolysis. A schematic for the subtilisin BPN' plasmid with restriction sites, subtilisin gene, and resistance is shown in figure 16.[86] Subtilisin-like motifs,

termed subtilases, are found in other organisms, such as archaea, fungi and yeasts.[87] Due to its stability, wild-type or genetically modified enzyme is widely used in detergents for degrading peptide based stains.[88]

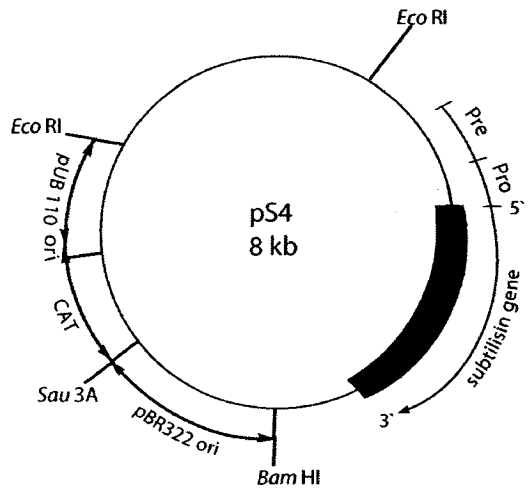


Figure 16: Plasmid pS4 containing the subtilisin BPN` gene. Select restriction sites are shown (*Eco* RI, *Bam* HI and *Sau* 3a).[86] Pre: Signal peptide, Pro: pro-peptide, pUB 110 ori: origin of replication, CAT: chloramphenicol acetyltransferase gene, pBR322 ori: origin of replication in *E. coli.*

Protease specificity is described in a series of binding sites based on peptide binding, as described by Schechter and Berger.[89] The substrate is divided into two at the scissile bond. Binding sites for the carboxy, or acyl, portion of the substrate are termed S_1, S_2 etc., increasing away from the active site (Figure 17). The amino acids of the substrate are correspondingly P_1, P_2. Etc. The amino portion (or leaving group) of the substrate is denoted P_1`, $P_2$`, binding to the corresponding S_1` and $S_2$` binding sites of the protease.

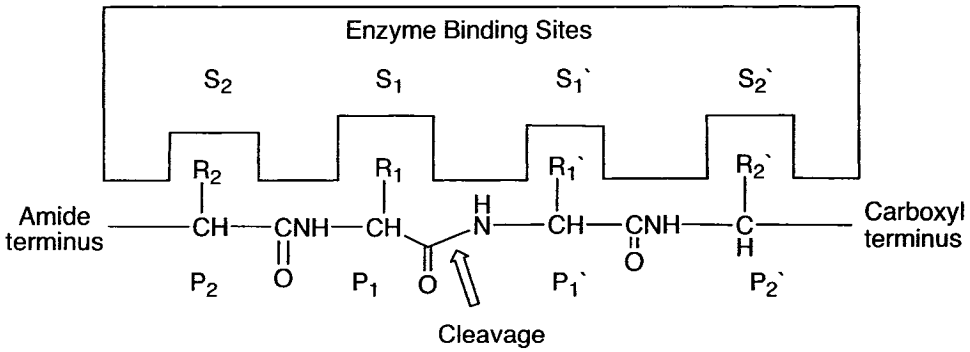


Figure 17: Protease active site nomenclature for peptide hydrolysis. An arrow indicates the position of hydrolysis. Peptide residues on the acyl portion of the peptide (left) are numbered P_1, P_2 etc, with numbers increasing away from the cleavage site. Residues in the amino portion (leaving group) of the peptide are numbered P_1`, $P_2$`, etc. The corresponding enzyme binding sites are S_2, S_1, S_1` $S_2$`. etc.[89, 90]

1.4.5 Structure

The subtilisin BPN` crystal structure was first reported in 1969,[91] and since then crystal structures of Carlsberg,[92] E,[93] and others in the subtilisin family have been reported. To prevent autodigestion subtilisin is crystallized in an inactive form, usually complexed with a small, non-covalent peptide inhibitor, or with the active site serine mutated to a cysteine. The overall folding is termed the α/β-subtilase protein fold, which differs from other serine proteases such as chymotrypsin. The secondary structure consists of seven parallel β-strands that form the core of the enzyme. These are surrounded by eight α-helices, figure 18. The active site residues are located within the secondary structure, Asp32 is located on the carboxyl terminal end of β-strand E1, His64 is located on helix HC, and the catalytic Ser221 is positioned near the amino terminus of helix HF. In contrast the active site residues of lipases (α/β-hydrolase fold) are located in loop regions. The variety of reactions catalyzed by the α/β-hydrolase fold family may reflect the flexibility of these loops.[94]

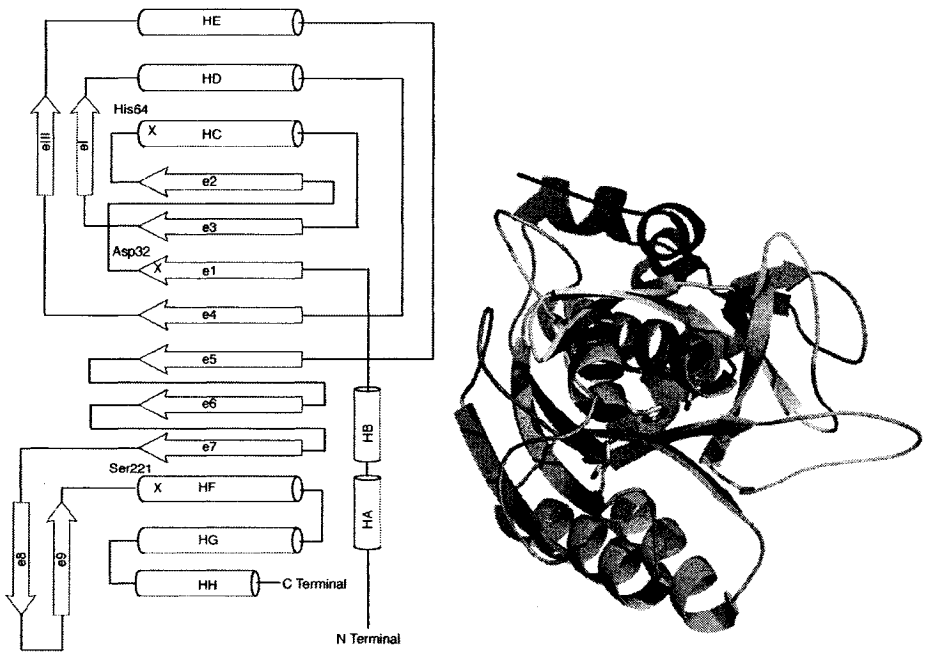


Figure 18: Secondary structure of the α/β-subtilase fold. α-Helices are shown as cylinders and β-sheets strands as arrows, along with the position of the catalytic triad. (Adapted from Siezen et. al.[87]) Ribbon diagram of subtilisin BPN` (PDB 1SBN[95]) in which each of the structural elements are coloured differently.

1.4.6 Substrate specificity

The active site of subtilisin is on the surface of the enzyme so that it is easily accessible to degrade long peptides. Peptides bind to subtilisin in an extended conformation.[96] The substrate makes several hydrogen bonds along the peptide channel in addition to specific side chain – binding pocket interactions.[97] Substrate specificity is generally broad. For small molecules, the S_1 acyl and S_1` leaving group pockets are the most significant because they are close to the active site residues (Figure 19).

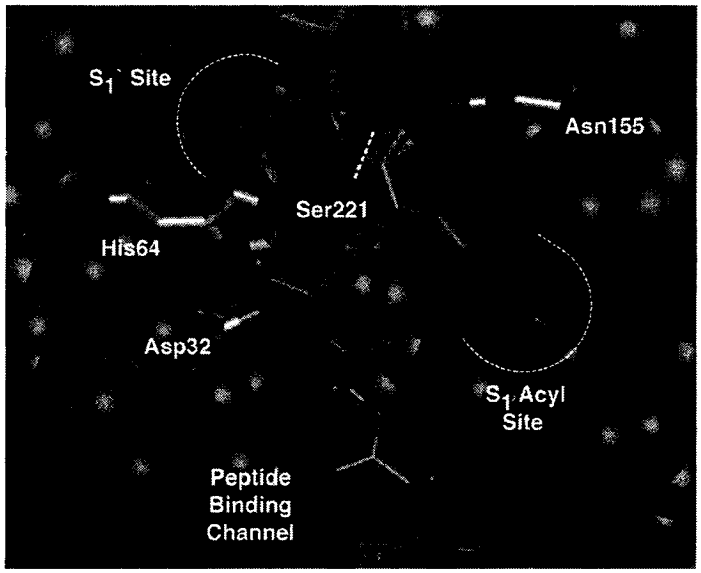


Figure 19: X-ray crystal structure of subtilisin BPN` with the non-covalent eglin-C inhibitor from the leech *Hirudo medicianlis* (PDB code1SBN).[95] The inhibitor is shown in green binding along the peptide channel and some inhibitor residues not in direct contact with the enzyme are removed for clarity. The catalytic triad of Asp32, His64, and Ser221, along with the oxyanion stabilizing Asn155, is shown as sticks, while the remained of the enzyme is spacefill. The location of the scissile bond is shown with a dashed line. Occupying the S_1 acyl site is an arginine (P_1) of the inhibitor, and in the S_1` site is an aspartate residue ($P_1$`) of the inhibitor.

The S_1 site, or the acyl-binding site, can distinguish many types of acyl groups. For peptide substrates, subtilisin generally prefers large, nonpolar amino acids such as phenylalanine, tyrosine or leucine. For example, BPN` cleaves the peptide succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide peptide with a k_{cat}/K_m value of 0.36 $s^{-1}mM^{-1}$(k_{cat} = 50 s^{-1}, K_m = 0.14 M).[98] The analogous peptide with alanine is cleaved 25-fold slower; succinyl-Ala-Ala-Pro-Ala-*p*-nitroanilide reacts with a k_{cat}/K_m value of 0.014 $s^{-1}mM^{-1}$ (k_{cat} = 1.9 s^{-1}, K_m = 0.15 M). In a similar way, hydrolysis of phenyl acetate vs. phenyl hippurate occurs with a 20-fold higher k_{cat}/K_m value, highlighting the preference for non-polar groups in the acyl portion.[99]

The S_1` pocket binds the leaving group of the substrate, usually an amine or alcohol. The enantioselectivity is determined by the first step in the reaction -

formation of the first tetrahedral intermediate. Empirical rules predict the enantiopreference for subtilisin reactions of secondary alcohols and primary amines in organic solvent and water based on the sizes of the substituents at the stereocenter (Figure 20).[100] These are similar to rules developed for lipases/esterases to predict the fast reacting enantiomer of secondary alcohols.[101] For lipases, the rule successfully predicted the fast reacting enantiomer of over 130 esters of secondary alcohols with ~ 95% accuracy. As noted in the enantiocomplementary enzyme section (Section 1.3.2), lipases have an approximate mirror image arrangement of the catalytic machinery, and thus show the opposite stereopreference to subtilisin. The rule for subtilisin is slightly less accurate, and predicted the correct enantiomer approximately 85% of the time. It is reliable for reactions in organic solvent, but sometimes predicts the incorrect enantiomer for small substrates in water.

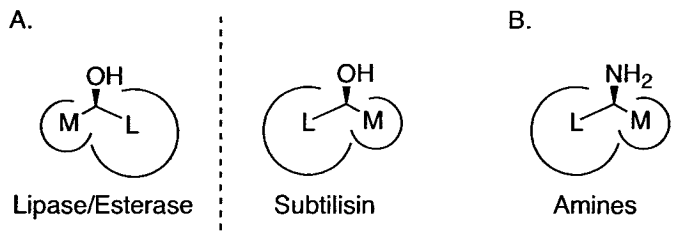


Figure 20: Empirical rules to predict the fast reacting enantiomer of secondary alcohols base on substituent sized where L is the large substituent and M is the medium substituent. A. Lipases/esterases favour opposite enantiomers of secondary alcohols than subtilisin. B. The rule can also be extended to primary amines and subtilisin.

Subtilisin typically shows low to moderate enantioselectivity towards simple substrates when compared with lipases and esterases. Lipases have two pockets of different size with which they bind both substituents of a secondary alcohol. Subtilisin has only one pocket, the S_1` pocket, which binds one portion of the secondary alcohol, while the other portion is orientated towards the solvent. Therefore both substituent size and solvation can contribute to enantioselectivity. The open $S_1$` binding pocket also accounts for the inaccuracy of the rule in solvents of differing polarity. For example, the *R*-enantiomer of esters of *sec*-phenethyl alcohol

reacts faster in water (against the rule), while in organic solvent the *S*-enantiomer reacts faster.[102] The S_1` site is hydrophobic, and will bind the larger, non-polar phenyl group in a polar solvent such as water, while the medium methyl group is oriented towards the solvent. In a non-polar organic solvent, this hydrophobic effect is diminished, and steric interactions dominate to give the opposite enantiomer. Altering the polarity of the substrate has a similar effect. Adding charged residues in the *para*-position makes it favourable to orient the aryl group toward a polar solvent, while the highly polar sulfinamides react with high enantioselectivity.[103] Solvation effects disappear with groups larger than *para*-substituted aryl groups because they are too large to fit in the S_1` pocket and the *S*-enantiomer is favoured.

1.4.7 Mutation of subtilisin

Extensive mutation of subtilisin has modified almost all its properties. Altering over half of the 275 amino acids has improved various features.[104] Subtilisin is a model system for mutation studies because the mechanism and crystal structure are well known, and the enzyme is easily cloned, expressed and purified. Mutations of the specific sites, the entire enzyme, or residues close to the active site have all been used to improve performance. Generally, global effects that are poorly understood, such as thermostabilty, are best solved with the irrational approach of mutation of the entire enzyme. Problems in which some structure/function information is known (such as the binding site of a substrate) are most efficiently treated with a "closer is better" approach – that is, mutations close to the active site have a larger effect than those distant from the active site.[105]

In detergent applications, bleach inactivates subtilisin by oxidizing the Met222 residue, adjacent to the active site in the S_1` pocket.[106] Site directed mutagenesis replaced position 222 with all other amino acids, and subsequent tests revealed both Met222Ser and Met222Ala were resistant to peroxide oxidation.[107] In another study, modification of the S_1 acyl site by replacing the small glycine 166 residue at the bottom of the S_1 site with 12 larger amino acids changed the substrate specificity.[108] Wild-type subtilisin generally prefers large, non-polar groups in the P_1 position, but the catalytic efficiency of the enzyme increased up to 16-fold towards

small, non-polar substrates, due to improved binding in the now smaller S_1 pocket. Engineering of subtilisin has also provided mutants with improved thermostability,[109] calcium ion stability,[110] pH stability,[111] substrate specificity,[112] and organic solvent tolerance.[113]

1.5.1 Thesis outline: Expanding the use of enzymes to new substrates and reactions

This thesis contains new applications of enzymes, in particular subtilisin, to perform novel chemical transformations. The large open active site of subtilisin suggests it may accommodate sterically hindered substrates. In chapter two enantiocomplementary enzymes hydrolyzed a bulky spiro chiral auxiliary for the Diels-Alder reaction with high, opposite enantioselectivity. The molecular basis of subtilisin was examined with molecular modeling. In chapter three we examined the application of proteases to hydrolyze sterically demanding esters tertiary alcohols, along with the molecular basis for the reactivity and enantioselectivity. In chapter four a new catalytically promiscuous reaction was discovered with the protease subtilisin Carlsberg. The bulky *N*-acyl sulfinamides are typically hydrolyzed at the C-N bond by related subtilisins, but subtilisin Carlsberg hydrolyses the S-N bond. In chapter five the mechanism of this S-N reaction was explored and determined to proceed through a sulfinyl enzyme intermediate at the active site. Substrate variation indicated that a substrate reversal was responsible for the alternate bond hydrolysis. Mutations also demonstrated the basis for C-N vs. S-N selectivity differences between the subtilisins.

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Chapter 2

Expanding the role of biocatalysts provides alternate, environmentally friendly, routes to enantiopure compounds. Subtilisin is a well-known protease used in detergents and commonly in organic synthesis for resolution of amines and alcohols, and carboxylic acids. We hypothesized that the large open active site of subtilisin could accommodate sterically hindered substrates.

Enantiocomplementary enzymes (section 1.3.2) are two enzymes that favor opposite enantiomers. In this chapter, we describe two enantiocomplementary enzymes, subtilisin and cholesterol esterase, found by screening an enzyme library. The enzymes hydrolyze different enantiomers of a bulky secondary alcohol Diels-Alder chiral auxiliary with high enantioselectivity. The opposite enantiopreference was in agreement with the secondary alcohol rules for proteases and lipases/esterases. We used subtilisin to resolve the auxiliary on a gram scale. Molecular modeling using the crystal structure of subtilisin revealed that the slow enantiomer bumps into the catalytically essential residues.

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Enantiocomplementary Enzymatic Resolution of the Chiral Auxiliary *cis, cis*-6-(2,2-Dimethylpropanamido)spiro[4.4]nonan-1-ol and the Molecular Basis for the High Enantioselectivity of Subtilisin Carlsberg

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Abstract: *cis, cis*-(±)-6-(2,2-Dimethylpropanamido) spiro[4.4]nonan-1-ol, **1**, a chiral auxiliary for the Diels-Alder additions, was resolved by enzyme-catalyzed hydrolysis of the corresponding butyrate and acrylate esters. Subtilisin Carlsberg protease and bovine cholesterol esterase both showed high enantioselectivity (*E* >200), but favored opposite enantiomers. Subtilisin Carlsberg favored esters of (1*S*, 5*S*, 6*S*)-**1**, while bovine cholesterol esterase favored esters of (1*R*, 5*R*, 6*R*)-**1**, consistent with the approximately mirror-image arrangement of the active sites of subtilisins and lipases/esterases. A gram-scale resolution of **1**-acrylate with subtilisin yielded (1*S*, 5*S*, 6*S*)-**1** (1.1 g, 46% yield, 99% ee) and (1*R*, 5*R*, 6*R*)-**1**-acrylate (1.3 g, 44% yield, 99% ee) although the reaction was slow. The high enantioselectivity combined with the conformational rigidity of the substrate made this an ideal example to identify the molecular basis of enantioselectivity of subtilisin Carlsberg toward secondary alcohols. The favored (1*S*, 5*S*, 6*S*) enantiomer adopted a catalytically productive conformation with two longer-than-expected hydrogen bonds, consistent with the slow reaction. The slow-reacting (1*R*, 5*R*, 6*R*) alcohol moiety encountered severe steric interactions with catalytically essential residues. It either distorted the catalytic histidine position or encountered severe steric strain with Asn155, an oxyanion-stabilizing residue.

Introduction

The enantiomer preference (which enantiomer is favored) is a key characteristic of enantioselective catalysts. For asymmetric syntheses, desymmetrizations, and dynamic kinetic resolutions, the enantiomer preference of the catalyst determines which enantiomer forms. Since most applications (e.g. preparation of a pharmaceutical or pharmaceutical precursor) require only one enantiomer, it is important that the enantiomer formed be the desired one. Although kinetic resolutions yield both enantiomers, the enantiomer preference still determines which enantiomer will be the unreacted starting material and which will be product of the reaction. This choice can simplify the next synthetic steps or, in a moderately enantioselective kinetic resolution, give higher enantiomer purity because the remaining starting material can be recovered in higher enantiomeric purity than the product.[1]

To reverse the enantiomer preference of a chemical catalyst, one uses the enantiomeric catalyst; for example, a D-tartrate-derived epoxidation catalyst in place of an L-tartrate-derived catalyst. For enzymes, the switch to an enantiomeric form is possible, but not practical because it requires chemical synthesis of the enzyme from D-amino acids.[2] The practical solution for enzyme-catalyzed reactions is to use an enantiocomplementary enzyme, that is, one that favors the opposite enantiomer. Enantiocomplementary enzymes favor opposite enantiomers because either the substrate or the active site machinery orients differently.

Researchers have discovered many examples of enantiocomplementary enzymes. For example, dehydrogenases as well as yeast reductases with opposite stereopreferences are common.[3,4] In *Escherichia coli*, separate enantiocomplementary enzymes reduce the enantiomeric sulfoxide configurations in methionine sulfoxide epimers.[5] In *Neisseria gonorrhoeae,* one protein containing mirror-image active sites in separate domains, reduces these enantiomeric sulfoxide configurations.[6] In an oxidation example, the active site of D-amino acid oxidase is a mirror image of that in flavocytochrome-b_2. Both enzymes catalyze amino acid oxidation, but with opposite enantiomer preference.[7] Toluene dioxygenase (TDO) and naphthalene dioxygenase (NDO) are enantiocomplementary.[8] Researchers reported diastereocomplementary

aldolases[9] and enantiocomplementary aldolase catalytic antibodies.[10] Enantiocomplemtary pinene synthases cyclize geranyl diphosphate to give either (+)-(3R,5R)-α-pinene or (-)-(3S,5S)-α-pinene.[11] Researchers have also used protein engineering to reverse the enantioselectivity of enzymes.[12]

Enantiocomplementary hydrolases include esterases, lipases and proteases,[13] epoxide hydrolases,[14] hydantoinases,[15] and lactamases.[16] Serine proteases and lipases have approximately mirror image active sites and thus usually prefer opposite enantiomers of secondary alcohols and primary amines, Figure 1.[17] In this paper, we report an enantiocomplementary resolution of a secondary alcohol useful as a chiral auxiliary using subtilisin or cholesterol esterase.

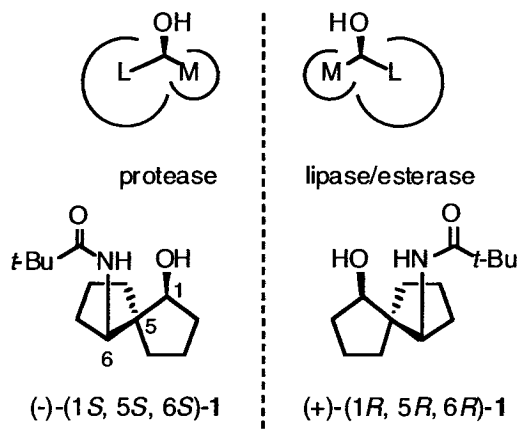
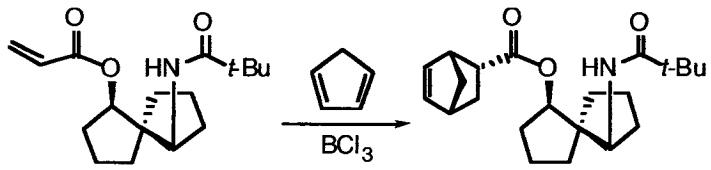


Figure 1: Empirical rule to predict the fast reacting enantiomer in hydrolysis reactions. M represents a medium-size substituent – in **1** this corresponds to the methylene (C2). L represents a large substituent – in **1** this corresponds to the spiro center (C5).

The *cis,cis* 1,3-amino alcohol **1** is an excellent chiral auxiliary for the Diels-Alder reaction because it shows excellent stereocontrol, high reactivity, and is easily removed. The acrylate of **1** forms a Diels-Alder adduct with cyclopentadiene in >98% de with an *endo:exo* ratio of >99:1, Scheme 1.[18] In addition, it reacts with the less reactive dienes isoprene, furan, 1-vinylcyclopentene, and 1-vinylcyclohexane with good diastereoselectivities and yields. Removal of auxiliaries linked via an amide bond can be difficult, but the ester link of this auxiliary cleaves readily by saponification.



(1*R*, 5*R*, 6*R*)-**1**-acrylate

84% yield, > 98% de
> 99:1 *endo:exo*

Scheme 1: Highly diastereoselective Diels-Alder addition of cyclopentadiene to (*1R, 5R, 6R*)-**1**-acrylate.

The synthesis of racemic **1** controls the relative configuration of the three stereocenters to give the *cis,cis* stereoisomer.[18] Fractional crystallization of the amine precursor of **1** (lacking the *N*-pivaloyl group) as the mandelate salt provided pure enantiomers with a yield of only 28% for each enantiomer, plus 28% recovered starting material. Here we report a kinetic resolution of (±)-**1** by two enantiocomplementary enzymes, both with an enantioselectivity greater than 200.

Results

We screened approximately one hundred commercially available hydrolases for their ability to catalyze hydrolysis of racemic **1**-acetate. For the initial screen, we used *p*-nitrophenol as the pH indicator to detect proton release upon hydrolysis.[19] At the second stage, we monitored 1-5-mg scale reactions using gas chromatography. This screening identified only two hydrolases that catalyzed hydrolysis of **1**-acetate – subtilisin Carlsberg (also called protease from *Bacillus licheniformis*) and proteinase N from *Bacillus subtilis*, Table 1. Even these two hydrolases were inefficient catalysts as the mass of enzyme required was 12-19 times higher than the mass of substrate. Subtilisin Carlsberg was approximately four times faster than proteinase N. The slow reaction with these two hydrolases and the inability of most hydrolases to catalyze hydrolysis of **1**-acetate is likely due to the rigid and hindered nature of this substrate.

Even though it showed no activity in the initial screen, we also checked the ability of bovine cholesterol esterase (CE) to catalyze hydrolysis of **1**-acetate because this esterase previously resolved several spiro compounds.[20] We expected the reaction, if any, to be slow. Indeed, the reaction was very slow – with a 14-fold greater mass of esterase over substrate, the reaction reached 36% conversion after 30

h. Even after 110 h it did not proceed beyond 38% conversion. It is possible that our screen missed other slow reacting enzymes. Thus, we identified three hydrolases that could catalyze hydrolysis of this unnatural substrate.

All three hydrolases showed excellent enantioselectivity, Table 1. We calculated the enantioselectivity of the candidate enzymes from the enantiomeric purity measured by gas chromatography on a chiral stationary phase. For **1**-acetate, subtilisin Carlsberg gave an *E* greater than 200 while proteinase N gave an *E* of 100. CE was also highly enantioselective towards **1**-acetate with *E* greater than 200. The absolute configuration of the favored enantiomer was determined by comparison with a sample of **1** with known configuration.[18,21] The two proteases favored esters of (1*S*, 5*S*, 6S)-**1**, while CE favored esters of (1*R*, 5*R*, 6*R*)-**1**. This opposite enantiopreference is consistent with the secondary alcohol rules for proteases and lipases/esterases, Figure 1 above. The two proteases and CE are enantiocomplementary enzymes and all show high enantioselectivity.

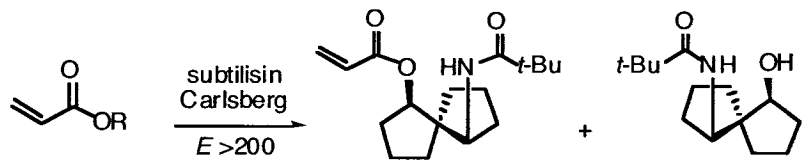
We tested resolution of **1**-butyrate ester since separation of **1** and **1**-butyrate by column chromatography is easier than for the acetate and also tested resolution of **1**-acrylate since this yields the reactant for the Diels-Alder addition directly, Table 1. Upon changing the acetate to a butyrate or acrylate, the enantioselectivity remained unchanged. The rate for the acetate and butyrate/acrylate reactions in Table 1 should not be compared as these reactions were under different conditions. Butyrate reactions were carried out on a slightly larger 20 mg scale and the relative amount of enzyme was reduced. Again we found that a high conversion could not be obtained using CE and the reaction temperature of 37 °C was crucial for any reaction with this enzyme. In contrast the subtilisin-catalyzed hydrolysis of **1**-butyrate proceeded to 47% conversion in 24 h at room temperature.

Table 1. Enzymatic resolution of esters of (±)-**1**

Enzyme	Ester	Time (h)	Ratio enzyme/ester[a]	ee_S [b, c] (yield)[d]	ee_P[b] (yield)[e]	Conv. %[f]	E[g, h]
Subtilisin Carlsberg	Acetate[i]	48	12[j]	37	99	27	>200 (*S*)
From	Butyrate[i]	24	1.7	89	99	47	>200 (*S*)
Bacillus licheniformis	Acrylate	48	1.5	99 (44%)	99 (46 %)	50	>200 (*S*)
Proteinase N from	Acetate	46	19[j]	20	98	17	100 (*S*)
Bacillus subtilis	Butyrate	96	3.9	69	96	42	102 (*S*)
Cholesterol	Acetate	30	14[j]	55	99	36	>200 (*R*)
Esterase	Butyrate	24[k]	2.6	61	98	38	>200 (*R*)

[a] Ratio of mass of enzyme to mass of ester of (±)-**1**. [b] Determined by GC. [c] ee_s for the butyrate determined after column chromatography and hydrolysis to the alcohol **1**. [d] Isolated yield of **1**-acrylate. [e] Isolated yield of **1**. [f] Conversion % was determined from ee_s and ee_p by the formula $c = (ee_S)/(ee_S + ee_P)$. [g] The enantiomeric ratio E measures the relative rate of hydrolysis of the fast enantiomer as compared to the slow enantiomer $E = \ln[ee_P(1 - ee_S)/(ee_P + ee_S]/\ln[ee_P(1 + ee_S)/(ee_P + ee_S]$ as defined by Sih.[22] [h] Absolute configuration was determined by comparison to an authentic sample of (1*R*, 5*R*, 6*R*)-**1**.[18, 21] [i] The rate for **1**-acetate should not be compared with the butyrate/acrylate as the reactions were under different conditions. [j] When the amount of enzyme is more than twice the weight of substrate, Michaelis-Menten kinetics and the equations used to calculate enantioselectivity may not be valid because the molar amount of enzyme is no longer negligible compared to the amount of substrate. Nevertheless, the high enantiomeric purity of the product and experiments with smaller amounts of enzyme clearly indicate that the enantioselectivity is high. [k] CE butyrate reaction at 37 °C; other butyrate reactions at 25 °C.

For a practical resolution, we chose the subtilisin-catalyzed hydrolysis of **1**-acrylate, Scheme 2. Subtilisin Carlsberg is stable and inexpensive and this reaction directly yields the reactant needed for the subsequent Diels-Alder reaction. A 2.6-gram resolution using subtilisin Carlsberg (4.3 g) in approx 600 mL of buffer reached 50% conversion after 48 h at room temperature. Isolation and separation by column chromatography yielded unreacted (*1R, 5R, 6R*)-**1**-acrylate in 44% yield and 99% ee and product (*1S, 5S, 6S*)-**1** in 46% yield and 99 % ee.



Scheme 2: Preparative-scale resolution of **1**-acrylate using subtilisin Carlsberg.

Molecular Basis for High Enantioselectivity of Subtilisin Toward 1-butyrate. Starting with the X-ray crystal structure of subtilisin Carlsberg, we added **1**-butyrate to the active site using computer modeling.[23] We further replaced the reacting ester carbonyl of **1**-butyrate with a phosphonate to mimic the transition state, Figure 2a, and linked this phosphonate to the catalytic serine. The chiral alcohol moiety has only a few accessible conformations because it has only three rotatable bonds and its large size excludes many conformations within the active site pocket. We manually searched conformations by rotating the phosphorus-oxygen bond, the oxygen-C1 bond, and the C6-N bond. Catalytically productive structures were those that contained all five catalytically essential hydrogen bonds,[24] Figure 2b, and avoided steric clashes with the protein.

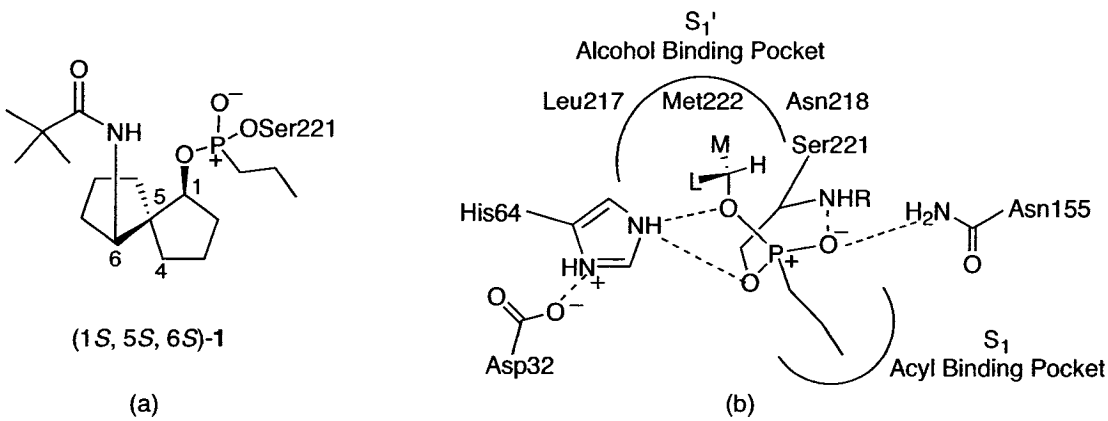


Figure 2: Phosphonate transition state analog for hydrolysis of **1**-butanoate. a) Analog containing the fast reacting (1*S*, 5*S*, 6*S*)-**1**. b) Key hydrogen bonds between the phosphonate and the catalytic residues of subtilisin Carlsberg.

The phosphonate mimicking hydrolysis of the fast reacting (1*S*, 5*S*, 6*S*) enantiomer adopted a catalytically productive orientation, Figure 2b, but the alcohol

substituents made little contact with the substrate binding site. The alcohol moiety rested above the active site with the medium substituent (C2 methylene, Figure 1) slightly above the right side of the shallow S_1' binding site (defined by Met222, yellow, at the bottom, Leu217, red, on the left, and the backbone of Asn218, green, on the right), Figure 3(I). The large substituent (C5 spiro center and ring, Figure 1) was unbound and pointed toward the solvent and to the left in Figure 3,(I). The closest contact between the medium substituent and the S_1' binding site was longer than the van der Waals contact distance (4.52 Å between C3 of the **1** moiety and the sulfur of Met 222 vs. 4.09 Å,[A] for a van der Waals contact). But this distance is close enough to exclude water and create a favorable hydrophobic interaction. Thus, the substituents in the alcohol moiety were only partly in the substrate-binding site.

Two of the catalytically essential hydrogen bonds were longer than expected. The alcohol orientation pointed the alcohol oxygen toward the catalytic histidine to form a hydrogen bond. This bond was longer than expected: Nε2 to O distance 3.33 Å, N-H-O angle 135° while normal limit for a hydrogen bond is ~3.1 Å and an angle greater than 120°. Of the remaining four catalytically essential hydrogen bonds, Table 2, the hydrogen bond from the catalytic histidine 64 Nε2 to the catalytic serine 221 Oγ was also longer than expected: 3.42 Å (N-H-O angle 133°). These two long hydrogen bonds may explain the very slow reaction for this substrate.[B]

We examined three different conformations for the slow reacting (1*R*, 5*R*, 6*R*) enantiomer, but they either lacked key hydrogen bonds or encountered severe steric clash with the protein. The first conformation for the (1*R*, 5*R*, 6*R*) enantiomer (Figure 3, II, Conformation A) had a severely distorted histidine orientation. It placed the medium substituent of **1** into (deeper than the fast-reacting enantiomer) the left side

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[A] Estimated from the C-H bond length (1.09 Å) and van der Waals radii for sulfur (1.80 Å) and hydrogen (1.20 Å) from A. Bondi, *J. Phys. Chem*, **1964**, *68*, 441-451.

[B] Although nonproductive orientations may also exist for the fast enantiomer we did not consider them because only productive orientations lead to product. Nonproductive orientations only slow the rate of reaction. Nonproductive orientations alter k_{cat} and K_M in a compensating manner so that k_{cat}/K_M and the enantioselectivity are unaffected (A. Fersht, *Structure and Mechanism in Protein Science*, Freeman Freeman, New York, **1999**, pp. 114-117).

of the shallow S_1' pocket; the distance from C3 to the sulfur of Met222 was 4.20 Å. The large substituent pointed directly out of the active site and toward the solvent. The different absolute configuration of the alcohol stereocenter pointed the alcohol oxygen away from the catalytic histidine. In addition, the medium substituent hit the catalytic histidine causing the plane of the imidazole ring to rotate by 60° down into the active site as compared with the fast enantiomer. The resulting distance between C2 of the substrate and the Nε2 of His 64 was 3.44 Å. This shift severely disrupted all three of the catalytically essential hydrogen bonds involving the catalytic histidine, Table 2. The distance from the catalytic histidine 64 Nε2 to the catalytic serine 221 Oγ was too long form a hydrogen bond (4.04 Å, N-H-O angle 124°) as was the distance and to the alcohol oxygen (5.17 Å, N-H-O angle 96°). Although the distance between aspartate 32 Oδ and histidine 64 Nδ1 remained close (2.95 Å), the O-H-N angle decreased to 66°, which is too acute to form a hydrogen bond. These disruptions of the catalytic hydrogen bond network make it extremely unlikely that the slow enantiomer could react via this Conformation A.

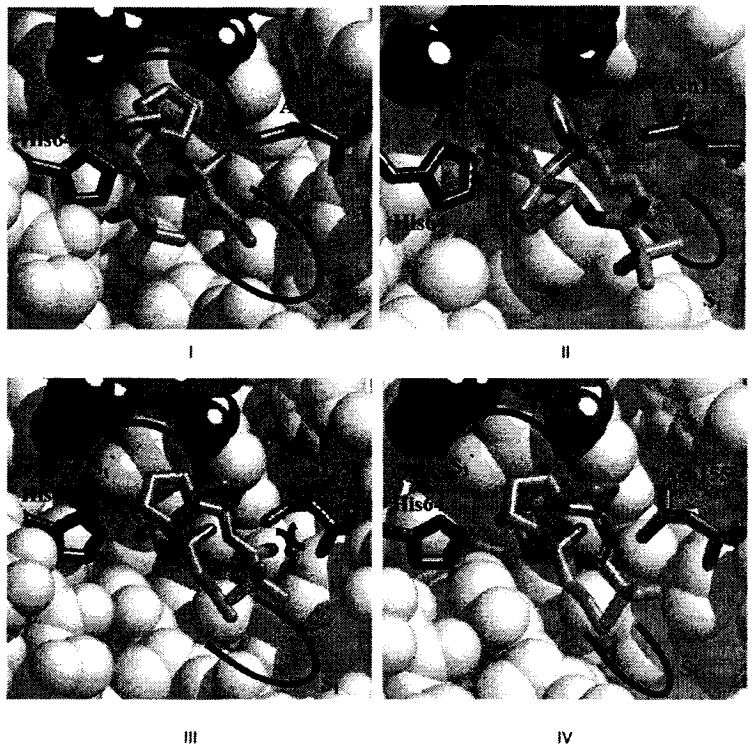

Figure 3: Geometry optimized conformations of the tetrahedral intermediates for hydrolysis of **1**-butyrate in the active site of subtilisin Carlsberg. Residues defining the S_1` site are: Met222 in yellow, Leu217 in red, and Asn218 in green. (I) Catalytically productive conformation of the fast-reacting enantiomer (1*S*, 5*S*, 6*S*)-**1**-butyrate. Three non-productive conformations of the slow-reacting enantiomer (1*R*, 5*R*, 6*R*)-**1**-butyrate: (II) Conformation A is missing key hydrogen bonds because of the distorted histidine orientation, (III) Conformation B has several distorted bond angles as a result of steric interactions with Asn155, and (IV) Conformation C has a *syn*-pentane like interaction and several other distorted bond angles.

In the second slow reacting (1*R*, 5*R*, 6*R*) conformation, conformation B, the alcohol moiety rotated clockwise along the P-O bond, to relieve the histidine distortion and restore the catalytically essential hydrogen bonds (Table 2). This rotation placed the medium substituent above the left side of the S_1` pocket and the closest distance was significantly longer than the van der Waals contact distance (5.06 Å between C3 of the alcohol moiety and sulfur of Met 222). This rotation also pointed the large group to the right (Figure 3, III) where its bulky pivalamide group hit the side of the active site at Asn155. Asn155 also has a catalytic role – the N_α-H stabilizes the oxyanion of the transition state by forming a hydrogen bond. The pivalamide carbonyl group made hydrogen bonds to the NH_2 ($N_{\delta 2}$) of Asn 155 (2.92

Å N-O distance, O-H-N angle of 163°) and to the backbone NH (N_α)(3.07 Å, N-H-O angle of 125°) as well as a close steric contact with the β-carbon (3.27 Å). This pivalamide-Asn155 contact also distorted bond angles in the alcohol moiety: P-O-C1 angle 128° vs. 120° in the ideal case and the O-C1-C5 angle was 116° vs. 109° (Table 3). Restoring these two angles to their ideal values dramatically increased the steric interactions between the pivalamide carbonyl and Asn 155: $N_{\delta 2}$-O = 2.29 Å, N_α-O = 3.04 Å, and C_β-O = 2.54 Å. To confirm that conformation B is a strained conformation, we calculated its energy (AM1, heat of formation) after removal from subtilisin to be 16 kcal/mol higher than the conformation for the fast-reacting enantiomer. In summary, although conformation B contains the catalytically essential hydrogen bonds, steric clash between the pivalamide carbonyl and Asn155 make this a highly strained conformation.

Table 2. Catalytic Essential Hydrogen Bond Distances and Angles[a]

	(1*S*, 5*S*, 6*S*)		(1*R*, 5*R*, 6*R*)	
		Conformation A	Conformation B	Conformation C
		Hbond distance, Å (angle)		
His64-Oalc	3.33 (135°)	**5.17 (96°)**	3.30 (146°)	3.30 (147°)
His64-Oser221	3.42 (133°)	**4.04 (124°)**	3.36 (137°)	3.39 (138°)
Oxyanion-NH Ser221	2.80 (154°)	2.72 (159°)	2.86 (149°)	2.85 (151°)
Oxyanion-NH_2Asn155	2.79 (164°)	2.78 (129°)	2.76 (128°)	2.75 (140°)
Asp32-His64	2.76 (162°)	2.95 (66°)	2.78 (173°)	2.78 (174°)
	Productive conformation	Key hydrogen bonds missing	High energy structure	*syn*-pentane interaction

[a]Bold font indicates distances that are too long to form a hydrogen bond.

Rotation of the pivalamide group along the C6-N bond to avoid the steric clashes with Asn155 yields conformation C. Unfortunately, this rotation creates a new steric strain - a *syn*-pentane like interaction between the oxygen atom of the pivalamide and the spiro stereocenter: O-C5 distance = 3.00 Å (Figure 3, IV). Typically a *syn*-pentane interaction increases the energy by ~3.6 kcal/mol.[25] The main close contact with the enzyme was the pivalamide NH to the NH_2 of Asn 155: 3.16 Å.

The significantly distorted angles in this structure were: the N-C6-C5 angle (117°), the P-O-C1 angle (128°), and the O-C1-C5 angle (116°), Table 3. Restoring these angles to their ideal values dramatically shortened the distance from the NH of the pivalamide to the NH_2 of Asn 155: 2.51 Å. The calculated energy of the transition state analogue removed from the protein was 17 kcal/mol higher than the fast reacting enantiomer, indicating that this conformation is highly strained.

Table 3: Energy of substrate and substrate angles for tetrahedral intermediates

	(1*S*, 5*S*, 6*S*)	(1*R*, 5*R*, 6*R*)		
		Conformation A	Conformation B	Conformation C
Heat of Formation[a]	0 kcal/mol	+ 2 kcal/mol	+16 kcal/mol	+17 kcal/mol
C1-C5-C4	102°	103°	100°	99°
C1-C5-C6	116°	116°	117°	119°
C1-C5-C9	113°	113°	116°	117°
N-C6-C5	112°	110°	110°	**117°**
O-C1-C5[b]	109°	109°	**116°**	**116°**
P-O-C1[b]	122°	123°	**127°**	**128°**

[a] AM1 heat of formation of the substrate removed from subtilisin. Bold font indicates bond angles distorted from their ideal values.[b] Restoring the angles in bold font to their ideal values in Conformation B and C gave severe steric clashes with the Asn155.

Discussion

Two enantiocomplementary enzymes – subtilisin and cholesterol esterase - show high enantioselectivity toward esters of (±)-**1**. The favored enantiomer for both follows the secondary alcohol rules developed to predict the enantioselectivity of serine hydrolases.[17] Kim and coworkers also recently used subtilisin and lipases as enantiocomplementary enzymes in the dynamic kinetic resolution of secondary alcohols.[26]

The gram-scale resolution of **1**-acrylate with subtilisin gave high yield and enantiomeric purity, but was very slow. We used 1.7 times higher mass of subtilisin than of substrate. Nevertheless, this resolution is a practical procedure because subtilisin Carlsberg is inexpensive enzyme (US $12/gram from Sigma). In addition, subtilisin is stable and could be reused. Given the bulky nature of this substrate, it is not surprising that it might react slowly. Other bulky substrates such as esters of α-branched amino acids also reacted slowly with subtilisin.[27] The advantage of this

resolution over resolution by crystallization of the amine precursor as the mandelate salt is that this kinetic resolution yields the desired acrylate ester directly.

Subtilisin usually shows only low to moderate enantioselectivity toward secondary alcohols, but in this case the enantioselectivity is very high. Molecular modeling revealed the molecular basis for this high enantioselectivity. The fast-reacting (1*S*, 5*S*, 6*S*) enantiomer fits in the active and makes all five catalytically essential hydrogen bonds, although two of these are longer than normal, likely reflecting the low reactivity for the substrate. On the other hand, the slow-reacting (1*R*, 5*R*, 6*R*) alcohol moiety encounters severe steric interactions with catalytically essential residues. These interactions either distort the catalytic histidine position or cause severe steric strain with Asn155, an oxyanion-stabilizing residue.

Ema and coworkers proposed an alternative explanation for the enantioselectivity of lipases[28] and subtilisin[29] toward secondary alcohols. They suggest that the fast reacting enantiomer adopts a conformation where stereoelectronic effects favor reaction, while the slow enantiomers cannot adopt this conformation. Thus, the fast enantiomer should adopt a *gauche* (*g*- for proteases) conformation along the $C_{alcohol}$-$O_{alcohol}$-$C_{C=O}$-Oγ dihedral, allowing overlap of the antiperiplanar lone-pair orbital of $O_{alcohol}$ with the σ^* orbital of the breaking C-Oγ bond. They propose that the slow reacting enantiomer cannot adopt this favorable orientation because the large substituent would hit the catalytic histidine.

Our modeling does not support this proposal because the fast reacting enantiomer cannot adopt a gauche orientation along the $C_{alcohol}$-$O_{alcohol}$-$C_{C=O}$-Oγ dihedral. The fast-reacting enantiomer adopted an *anti* conformation along this bond (-172°) in order to make the catalytically essential hydrogen bonds. Imposing a gauche conformation (-60°) along this dihedral caused a severe steric clash between the medium substituent (C2) and His 64 (1.78 Å, with the C1 hydrogen *syn* to the oxyanion). The slow-reacting enantiomer, predicted to adopt an *anti* conformation by the stereoelectronic effects model, indeed adopted the *anti* conformation in conformations B (151°) and C (159°); however, because this orientation is also *anti* for the fast-reacting enantiomer, it cannot contribute to enantioselectivity. Conformation A of the slow enantiomer adopted a *gauche* conformation, ((63°)g+,

rather than *g*-,) but still hit the catalytic His 64. Thus, we find no evidence to support the notion that stereoelectronic effects along the $C_{alcohol}$-$O_{alcohol}$-$C_{C=O}$-Oγ dihedral contribute to the enantioselectivity of subtilisin toward alcohol **1**.

Previous X-ray crystal structures of phosphonate transition state analogs containing secondary alcohols also do not support the notion that stereoelectronic effects along the $C_{alcohol}$-$O_{alcohol}$-$C_{C=O}$-Oγ dihedral contribute to enantioselectivity. The fast reacting enantiomer of menthol bound to lipase from *Candida rugosa* did not adopt the predicted gauche conformation (130°).[30] On the other hand, the slow reacting enantiomer did adopt a gauche conformation (71°), again contrary to predictions.

The X-ray crystal structures of bovine cholesterol esterase are only of inactive forms,[31] so we did not model their interactions with alcohol **1**.

Acknowledgements. We thank the Natural Sciences and Engineering Research Council of Canada for financial support.

Experimental Section

General

Chemicals were purchased from Sigma-Aldrich (Oakville, ON) and were used without further purification. Subtilisin from *Bacillus licheniformis* (E.C. 3.4.21.62, catalog no. P5459) was obtained from Sigma (Oakville, ON) and Proteinase N from *Bacillus subtilis* (catalog no. 82458) was obtained from Fluka (Oakville, ON). Cholesterol esterase from beef pancreas (EC 3.1.1.13, catalog no. 1081) was obtained from Genzyme (Cambridge, MA).

Synthesis

The alcohol **1** and the acrylate ester were synthesized according to a previous method.[18] The **1**-acetate and **1**-butyrate were made by a similar method.

(±)-1-Acetate

(±)-**1** (62.0 mg, 0.259 mmol) and LHMDS (100 mg, 0.598 mmol) were dissolved in THF (6 ml) and stirred for 1 h to give a gold-colored suspension. Acetic anhydride (60 µl, 0.64 mmol) was added, and the solution was stirred for 16 h. The resulting gold solution was quenched with water (10 ml) then $HCl_{(aq)}$ (4 M, 10 ml), extracted with CH_2Cl_2 (3x20 ml), washed with saturated $Na_2CO_{3(aq)}$ (20 ml) and dried over $MgSO_4$. Concentration *in vacuo* followed by flash column chromatography (silica, 4 : 1 hexanes : EtOAc) gave (±)-1-Acetate (47.6 mg, 0.169 mmol, 65.3%) as a white solid: mp 122-123 °C; $[\alpha]_D^{22}$ -47.2 (*c* 1.08, $CHCl_3$); IR (film) λ_{max} 3328 (N-H), 2956 (C-H), 2873 (C-H), 1727 (ester C=O), 1625 (amide C=O), 1538 (C=C), 1373, 1245, 1100, 1016 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 6.00 (br d, 1H, HN), 5.02-4.96 (m, 1H, H1), 4.28-4.20 (m, 1H, H6), 2.03 (s, 3H, H14), 2.05-1.45 (m, 12H, H2-H4, H7-H9), 1.16 (s, 9H, H12); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.4 (C, C10), 170.4 (C, C13), 81.2 (CH, C1), 56.2 (CH, C6), 56.1 (C, C5), 38.7 (C, C11), 34.8 (CH_2), 34.7 (CH_2), 32.2 (CH_2), 31.8 (CH_2), 27.5 (CH_3, C12), 21.5 (CH_3, C14), 21.1 (CH_2), 20.2 (CH_2); MS: *m/z* 281 (6, M^+), 238 (12, $[M\text{-}CH_3CO]^+$), 196 (30, $[M\text{-}(CH_3)_3CCO]^+$), 121 (64, $[M\text{-}\{(CH_3)_3CCONH_2\text{+}CH_3CO_2\}]^+$), 120 (100, $[M\text{-}\{(CH_3)_3CCONH_2\text{+}CH_3CO_2H\}]^+$), 102 (75, $[(CH_3)_3CCONH_3]^+$), 57 (86); HRMS calcd for $C_{16}H_{27}NO_3$ 281.19909, found 281.20062.

(±)-1-Butyrate

(±)-**1** (810 mg, 3.38 mmol) and LHMDS•OEt_2 (1.72 g, 7.12 mmol) were dissolved in THF (40 ml) and stirred for 1 h to give a gold-colored suspension. Butyryl chloride (750 µl, 7.22 mmol) was added, and the solution was stirred for 1 h. The resulting clear yellow solution was quenched with water (20 ml) then $HCl_{(aq)}$ (4 M, 50 ml), extracted with CH_2Cl_2 (3x50 ml), washed with saturated $Na_2CO_{3(aq)}$ (50 ml) and dried over $MgSO_4$. Concentration *in vacuo* followed by flash column chromatography (silica, 4 : 1 hexanes : EtOAc) gave (±)-**1**-butyrate (901 mg, 2.91 mmol, 86.1%) as a white solid: mp 107-108 °C; $[\alpha]_D^{22}$ -44.6 (*c* 1.01, $CHCl_3$); IR (film) λ_{max} 3339 (N-H), 2962 (C-H), 2872 (C-H), 1730 (ester C=O), 1632 (amide C=O), 1537 (C=C), 1453, 1435, 1416, 1394, 1367, 1305, 1203, 1186, 1100, 994, 945 cm^{-1}; ^1H NMR (400 MHz,

$CDCl_3$): δ 6.04 (br d, 1H, HN), 5.01 (dd, J = 5.5, 2.5 Hz, 1H, H1), 4.26-4.18 (m, 1H, H6), 2.26 (t, J = 7.5 Hz, 2H, H14), 2.08-1.45 (m, 14H, H2-H4, H7-H9, H15), 1.16 (s, 9H, H12), 0.94 (t, J = 7.4 Hz, 3H, H16); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.5 (C, C10), 172.9 (C, C13), 81.0 (CH, C1), 56.4 (CH, C6), 55.8 (C, C5), 38.7 (C, C11), 36.6 (CH_2), 35.0 (CH_2), 34.9 (CH_2), 32.4 (CH_2), 31.7 (CH_2), 27.6 (CH_3, C12), 21.1 (CH_2), 20.2 (CH_2), 18.4 (CH_2), 13.7 (CH_3, C16); MS: *m/z* 309 (4, M^+), 238 (15, [M-$C_3H_7CO]^+$), 224 (35, [M-$(CH_3)_3CCO]^+$), 121 (64, [M-{$(CH_3)_3CCONH_2$+$C_3H_7CO_2$}]$^+$), 120 (100, [M-{$(CH_3)_3CCONH_2$+$C_3H_7CO_2H$}]$^+$), 102 (82, [$(CH_3)_3CCONH_3]^+$), 57 (78); HRMS calcd for $C_{18}H_{31}NO_3$ 309.23039, found 309.23114.

Identifying the active hydrolases. An enzyme library of commercial hydrolases was prepared as previously described.[19] Hydrolase solutions (20 μL $well^{-1}$) were placed in a 96 well microplate followed by addition of assay solution (80 μL $well^{-1}$). The final concentrations were **1**-acetate (5.1 mM), BES (N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 3.6 mM), 4-nitrophenol (0.36 mM), and acetonitrile (8%). The plate was then 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 every 11 s for 30 min. Each hydrolysis was carried out in triplicate and averaged. The normalized initial decrease in absorbance for the interval 20-300 s was –13 s^{-1} for subtilisin from *Bacillus licheniformis* (initial hydrolase solution of 22 mg mL^{-1}) and –6 s^{-1} for Proteinase N (initial hydrolase solution of 60 mg mL^{-1}). These enzymes were then tested for enantioselectivity in small-scale reactions.

Measuring hydrolase enantioselectivity. BES buffer (450 μL, 50 mM, pH 7.2) and **1**-acetate (50 μL, 76 mM in MeCN) were placed in a 1.5 mL centrifuge tube along with 12-19 mg of enzyme. Reactions were shaken at 600 rpm and 37 °C for 12 h in an Eppendorf Thermomixer R, extracted with EtOAc (0.5 mL), dried with $MgSO_4$, and analyzed by gas chromatography on a Chrompack Chirasil-DEX CB column (25 m x 0.25 mm) using He as a carrier gas (120 °C for 5 min, 2.5 °C min^{-1}, 195 °C, 10 psi): **1**-acetate: k'_1 = 15.94 (S), α =1.01; 1: k'_1 = 17.10 (R), α = 1.02. Both **1**-acetate and **1**

showed similar retention on thin layer chromatography (silica gel): R_f 0.65 and 0.57 respectively in 1:1 hexanes: ethyl acetate. To measure enantiomeric purity of reactions involving **1**-butyrate, the remaining **1**-butyrate and product **1** were separated by TLC (R_f 0.81 and 0.57, respectively in 1:1 hexanes: ethyl acetate). The product **1** as analyzed by GC as above, but enantiomers of **1**-butyrate did not separate under these conditions, so it was hydrolyzed to **1** with NaOH (1 mL, 6 M) in MeOH (4 mL), extracted with EtOAc (2 mL)and dried. To measure enantiomeric purity of reactions involving **1**-acrylate, the remaining **1**-acrylate and product **1** were separated by chromatography (**1**-acrylate R_f 0.77 in 1:1 hexanes: ethyl acetate) and analyzed by gas chromatography (130 °C, 0.5 °C min^{-1}, 185 °C, 10 psi): **1**-acrylate: $k'_1 = 34.71$ (*S*), $\alpha = 1.01$; 1: $k'_1 = 34.62$ (*R*), $\alpha = 1.04$, where k_1' is the capacity factor given by $k_1' = (t_{RT}-t_M)/(t_M)$ where t_{RT} is the retention time of the sample and t_M is the dead time. α is the selectivity factor given by the ratio of the R and S capacity factors). For **1**-acetate the (*S, S, S)* enantiomer elutes first while for the alcohol **1** the (*R, R ,R*) enantiomer elutes first. Separation of **1**-acrylate and **1** was needed because they overlap in the GC. Enantioselectivity was calculated from the measured ee_p and ee_s according to $E = \ln[ee_P(1 - ee_S)/(ee_P + ee_S]/\ln[ee_P(1 + ee_S)/(ee_P + ee_S]$ as defined by Sih.[22]

Resolution of 1-Acrylate. Protease from *Bacillus licheniformis* (60 mL aqueous propylene glycol solution, 73 mg mL^{-1}, 9.1 μ mg^{-1}) was placed in BES buffer (2 mM, 560 mL) and MeCN (25 mL) and adjusted to pH 7.2 using a pH stat. **1**-Acrylate (2.88 g, 98.2 mmol) in MeCN (25 mL) was then added and the pH was maintained at 7.2 by the addition of NaOH (0.1 M). At 50% conversion the reaction was extracted with EtOAc (3 x 200 mL), washed with water (200 mL), brine (200 mL), dried over $MgSO_4$, and the solvent removed under reduced pressure. Chromatographic separation (5:1 hexanes : EtOAc) yielded (1*S*, 5*S*, 6*S*)-**1** (1.07 g, 44.7 mmol, 46 %, 99% ee) as a white solid: mp 102-104 °C and (1*R*, 5*R*, 6*R*)-**1**-acrylate (1.27 g, 43.3 mmol, 44 %, 99% ee) as a white solid: mp 126-127 °C.

Computer Modeling. Molecular modeling was performed using the Biosym/MSI *InsightII 97.0/Discover* software (San Diego, CA) using the Amber[32] 95.0 force field.

A distant-dependent dielectric constant of 4.0 was used and 1-4 van der Waals interactions were scaled by 50%. The starting subtilisin Carlsberg structure was obtained from the Brookhaven protein data bank[33] (1cse).[23] The eglin c inhibitor was removed and hydrogens were added to correspond to a pH of 7.0 and the catalytic histidine (His64) was protonated. Initial relaxation of the enzyme was performed with a simple phosphonate transition state analogue. With the backbone constrained, 200 iterations of steepest descent algorithm were preformed followed by 200 iterations of conjugate gradients algorithm with the backbone tethered by a 10 kcal $mol^{-1}A^{-1}$ force constant and finally the 200 iterations without any constraints. The substrate was then added and minimizations were preformed in an analogous manner. Crystallographic water molecules were included in all minimizations. Final minimization was continued until the RMS value was less than 0.0001 kcal/mol.

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Chapter 3

In the previous chapter we described the enantiocomplementary resolution of a bulky secondary alcohol ester of a chiral auxiliary for the Diels-Alder reaction. Molecular modeling revealed the basis for the high enantioselectivity of subtilisin towards this auxiliary.

In this chapter, we expand the hydrolysis to new bulky substrates – tertiary alcohol esters. Subtilisin was one of the principal enzymes, along with other common proteases, in this first investigation of the protease-catalyzed resolution of tertiary alcohol esters. Tertiary alcohols are found in many natural products, but currently there are only a few synthetic and biocatalytic methods to prepare enantiopure tertiary alcohols. Substrate studies and molecular modeling were also used to further understand the reaction.

Chapter 3

Protease Catalyzed Hydrolysis of Tertiary Alcohol Esters

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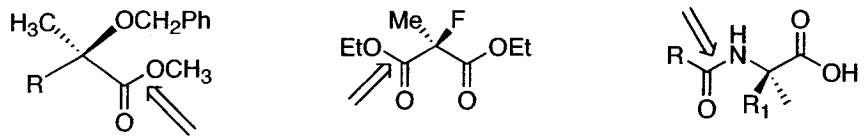
Abstract

Currently there are only a few synthetic and biocatalytic methods to prepare enantiopure tertiary alcohols. We discovered several proteases that hydrolyze esters of tertiary alcohols. The initial discovery was the subtilisin Carlsberg catalyzed hydrolysis of the ester bond of *N*-(phenylsulfinyl)-*tert*-buylglycine to release *tert*-butanol and resolve the remote sulfinyl stereocenter with high enantioselectivity (E = 116). Further testing revealed six different proteases that catalyzed the hydrolysis of α-trifluoromethyl-α-acetylenic ester with different acyl groups. *Aspergillus melleus* protease reacted slowly with the dihydrocinnamoyl derivative, but showed good reactivity (38% conversion) and enantioselectivity with the acetate (E = 14). In general proteases showed good reactivity but low enantioselectivity. Further, subtilisin Carlsberg and α-chymotrypsin also catalyzed the hydrolysis of more sterically demanding allylic and alkyl tertiary alcohol esters, which are not hydrolyzed by lipases. Molecular modeling suggests their reactivity is limited to tertiary alcohols with a methyl or smaller small substituent because this substituent must adopt an unfavorable *syn*-pentane-like orientation with the oxyanion of the first tetrahedral intermediate, creating internal strain within the substrate. The enantioselectivity is low because the medium-sized substituent and the large substituent compete for the S_1' leaving group pocket, where binding of the methyl group avoids some steric interactions and binding of the phenyl group adds a good hydrophobic interaction.

Introduction

Tertiary alcohol functionalities are found in many natural products. For instance the flavor compounds such as α-terpineol and linalool contain the tertiary alcohol functionality. The preparation of this bulky functional group in enantiopure form is challenging for both enzymes and synthetic organic chemistry.

Enzymatic resolution of sterically crowded stereocenters is challenging. There are many examples for the resolution of smaller racemic esters of chiral primary alcohols, secondary alcohols, and carboxylic esters, but resolution of quaternary stereocenters are rare. Examples include α,α-disubstituted carboxylic acid esters,[1] α-fluorinated malonate diesters[2] and *N*-acylamino acids,[3] Figure 1.



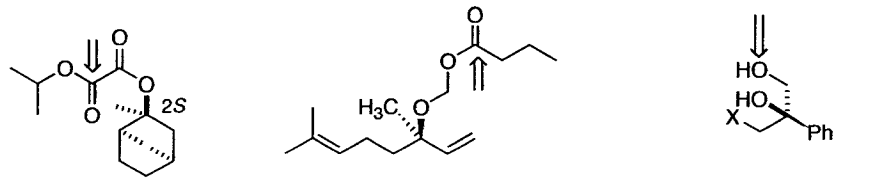
lipase OF
R = Et, E = 10
R = *n*-Pr, E = 20

Candida cylindracea lipase, ee 91%

porcine kidney acylase
R_1 = aryl, E = 15

Figure 1: Resolution of sterically demanding substrates containing a quaternary stereocenter. The reaction site is indicated with an arrow.

There are several approaches to tertiary alcohol ester hydrolysis. One approach is to attach a linker to place the sterically hindered portion of the tertiary alcohol further from the reactive center. Substrates in which the reactive center was moved by two bonds in the acyl portion gave low enantioselectivity (E = 7 or 9.7, Figure 2).[4] A distant chiral center reduces the potential for a highly enantioselective reaction. The tertiary alcohol can also be modified to include an adjacent primary alcohol. Acylation occurred with an improved enantioselectivity (E = 12-70), but this approach is limited in substrate range, as the tertiary alcohol must contain an adjacent primary alcohol.[5]



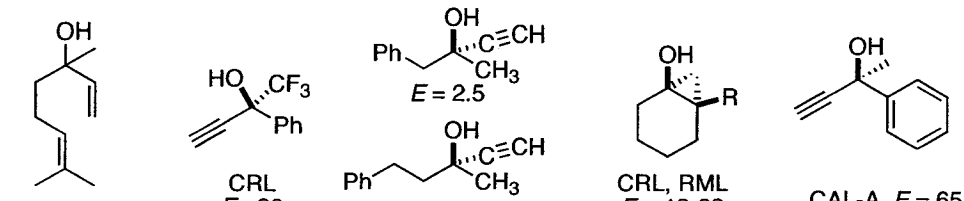
PPL, $E = 7$
hydrolysis

CRL, $E = 9.7$
alcoholysis with n-butanol

PFL (lipase AK), $E = 12\text{-}70$
X = Cl, Br, I
acylation

Figure 2: Resolution of remote tertiary alcohol stereocenters by reaction at a less hindered remote site. The reactive center is indicated with an arrow.

Another approach is screening of enzyme libraries to identify those that accept hindered alcohols esters. A library of over 100 yeast strains was screened for hydrolysis of racemic linalyl acetate.[6] Twelve were found with activity, the best with an enantioselectivity of five. In another example, *Candida cylindracea* (*rugosa*) lipase (CRL) resolved several tertiary acetylenic acetate esters with low to moderate enantioselectivity[7] and several lipases resolved 1-bicyclo[4.1.0]-heptyl acetate and chloroacetate with moderate enantioselectivity.[8] The only practical resolution was a *Candida antarctica* A (CAL-A) transesterfication of 2-phenylbut-3-yn-2-ol with vinyl acetate.[9]



yeast cultures
$E = 5$

CRL
$E > 20$

CRL $E \sim 4$,
abs. config. assumed

CRL, RML
$E = 13\text{-}38$

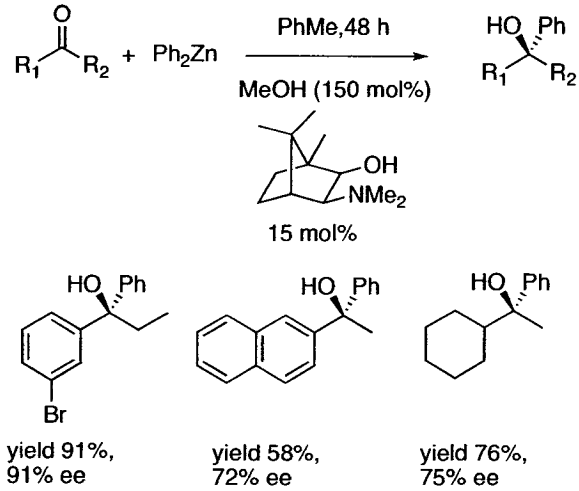
CAL-A, $E = 65$

Figure 3: Screening revealed lipases that resolve tertiary alcohol stereocenters.

Bornscheuer and coworkers noted lipases such as CRL that hydrolyze tertiary alcohol esters contain a GGGX sequence motif at the active center.[10] In contrast, most commercial lipases have a GX motif at the active site.[11] Structural analysis revealed that the alcohol binding pocket was wider in the CRL than in other lipases, possibly allowing the tertiary alcohol to bind. The extra glycines in the GGGX motif provide more space for the bulky substrate. Database searching for hydrolases containing the GGGX motif identified four esterases that later showed activity towards three model

tertiary alcohol acetates (linalyl acetate, methyl-1-pentin-1-yl acetate, 2phenyl-3-butin-2-yl acetate). However, recent unpublished work suggests that the GGGX motif may be only part of the story because adding this motif to an esterase by site-directed mutagenesis did not enable it to hydrolyze esters of tertiary alcohols. Thus, researchers have not found a good solution of the problem of resolution of tertiary alcohols.

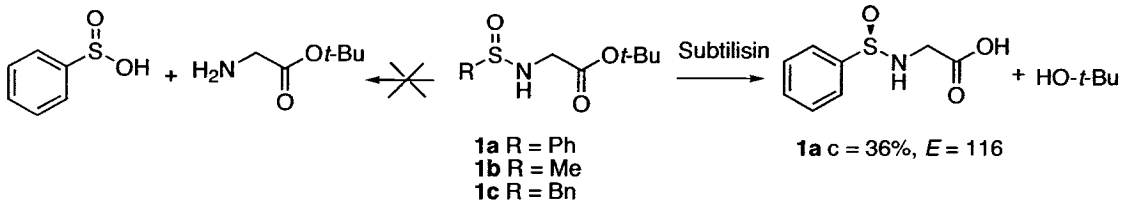
There are also only a few chemical methods to prepare enantiopure tertiary alcohols. The simplest approach is the enantioselective addition of organometallic reagents to a ketone.[12] The high reactivity of organolithium and Grignard reagents require at least one equivalent of chiral ligand, making them impractical. Using less than one equivalent allows reaction of uncoordinated ketone, giving lower enantiomeric excess. To reduce the amount of chiral ligand, an organometallic reagent with lower nucleophilic character is used, supplemented by activation of the carbonyl or nucleophile. The low nucleophilic character of organozinc reagents makes them ideal for this type of reaction. In a noncoordinating solvent, for instance, they will not add to aldehydes, but with a chiral promoter organozinc reagents add to aldehydes with excellent enantioselectivity. Extending this addition to ketones proved difficult because of the lower reactivity of ketones prevented reaction even with promoters or at high temperature. The solution to the low reactivity was a more reactive diphenyl zinc (instead of a dialkyl zinc reagent) along with methanol, which exchanges for one of the phenyl groups to increase reactivity.[13] Thus, addition of a large excess of diphenylzinc to ketones promoted by 3-*exo*-(dimethylamino)isoborneol in the presence of methanol provides phenyl chiral benzyl alcohols in 71-91% enantiomeric excess (Scheme 1). Further improved reagents allowed addition of alkyl and alkynyl reagents to ketones.[14] In general the methodology is limited because large amounts of chiral ligand (10-20%) and organozinc reagents are required, and activated ketones are often necessary.



Scheme 1: Addition of diphenylzinc to ketones with the chiral ligand 3-*exo*-(dimethylamino)isoborneol.

Results

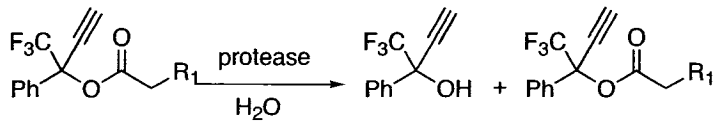
We discovered an unexpected subtilisin catalyzed hydrolysis of a tertiary alcohol ester while testing for S-N sulfinamide cleavage. We made **1a** to test hydrolysis of the S-N sulfinamide bond,[15] and expected no hydrolysis of the *tert*-butyl ester because there were no known examples of subtilisin hydrolysis of tertiary alcohols esters. HPLC analysis revealed an enantioselective disappearance of starting material, but attempts to isolate expected products, sulfinic acid and *tert*-butyl glycine resulting from sulfinamide hydrolysis, were not successful. Following the reaction using NMR in D_2O revealed that a different reaction occurred. The enzyme cleaved the ester bond to produce *tert*-butanol, and gave only a small amount of chemical S-N hydrolysis (Scheme 2). The reaction proceeded with high enantioselectivity, $E = 116$. The related substrates *N*-(methylsulfinyl)- and *N*-(benzylsulfinyl)-*tert*-butylglycine reacted with lower enantioselectivity, $E = 12$ and 6, presumably through ester hydrolysis as well. Thus subtilisin can resolve a remote sulfinyl stereocenter and, more surprisingly, hydrolyze a tertiary alcohol ester.



Scheme 2: NMR demonstrates subtilisin Carlsberg resolves a remote sulfinyl stereocenter by hydrolysis of tertiary alcohol ester, and not the sulfinamide bond.

Encouraged by this result, we investigated the reactivity of several commercial proteases toward α-trifluoromethyl-α-acetylenic esters **2a-b** (Table 1). This tertiary alcohol ester is an ideal substrate because the CF_3 group helps to reduce chemical hydrolysis via alkyl oxygen cleavage.[7] All proteases tested, except *Aspergillus oryzae* protease, catalyzed the hydrolysis of the dihydrocinnamoyl ester **2a** with moderate to high conversion. The dihydrocinnamoyl derivative **2a** showed less than 1% chemical hydrolysis in buffered solutions after 48 h. The reactivity and enantioselectivity of *Aspergillus oryzae* protease and *Aspergillus melleus* protease improved upon using acetate **2b**. The enantioselectivity of *Aspergillus melleus* increased to a moderate $E = 14$. The reactivity of subtilisin Carlsberg, subtilisin BL and *Streptomyces griseus* protease also improved, but there was no significant change in their enantioselectivity. α-Chymotrypsin did not react with **2b**, which is not surprising considering its specificity for large non-polar acyl groups.[16]

Table 1. Reactivity and enantioselectivity of proteases toward dihydrocinnamate **2a** and acetate **2b**.[a]



2a $R_1 = CH_2C_6H_5$
2b $R_1 = H$

	2a		**2b**	
Protease	%c[a]	$E^{b,c}$	%c	$E^{b,c}$
Aspergillus melleus protease	3	2.0 (*R*)	38	14 (*R*)
Aspergillus oryzae protease	n.r.[d]	n.r.	15	6.4 (*R*)
Subtilisin BL	15	1.5 (*R*)	46	2.7 (*R*)
Subtilisin Carlsberg	27	1.3 (*S*)	84	2.5 (*R*)
α-Chymotrypsin	51	1.9 (*S*)	<1	
Streptomyces griseus protease	7	2.4 (*S*)	14	2.4 (*R*)

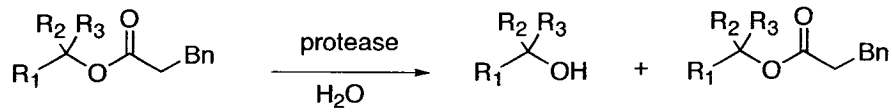
[a]Conversion: reaction for 48 h. [b]Enantioselectivity: the enantiomeric ratio *E* measures the relative rate of hydrolysis of the fast enantiomer as compared to the slow enantiomer as defined by Sih (Chen, C.S.; Fujimoto, Y.; Girdaukas, G.; Sih, C.J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299). The enantioselectivity was determined using the enantiomeric excess of substrate and product, which were determined by GC analysis on a column. [c]Fast reacting enantiomer (enantiopreference) in parenthesis. The absolute configuration was determined by *Candida rugosa* lipase (CRL)-catalyzed hydrolysis of **2b**, which gives (*R*)-**2** (O'Hagan, D.; Zaidi, N. A. *Tetrahedron: Asymmetry* **1994**, *5*, 1111-1118). [d]No reaction.

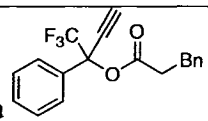
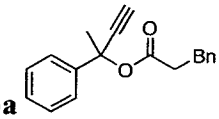
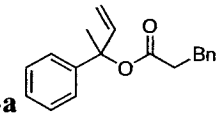
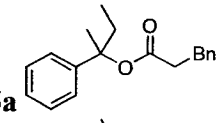
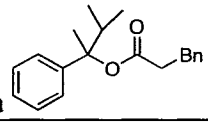
The dihydrocinnamoyl group was chosen for further study because it is stable to chemical hydrolysis. Even though the acetate **2b** showed the highest overall activity and enantioselectivity, it readily hydrolyzes for other tertiary alcohols without an electron-withdrawing group, such as CF_3, on the alcohol portion.[7] Attempted resolution of **5b** (the acetate equivalent of 5a, table 2) with *Aspergillus oryzae* and *Aspergillus melleus* proteases resulted in >50% chemical hydrolysis. These proteases favour the acetate (<3% conversion with the dihydrocinnamoyl group, Table 1) and

may be useful for resolving tertiary alcohols that form stable acetate esters. It is unclear why the acetate esters are unstable while the dihydrocinnamate esters are stable in aqueous solutions because both are unactivated. Not surprisingly the activated chloroacetyl esters were not stable in water and could not be tested.

To establish the substrate range and decipher the molecular basis for protease reactivity and enantioselectivity toward tertiary alcohol esters, we used subtilisin Carlsberg and α-chymotrypsin to resolve a variety of tertiary alcohols with the aqueous-stable dihydrocinnamoyl group. Subtilisin Carlsberg and α-chymotrypsin showed high reactivity, but with low enantioselectivity, toward tertiary acetylenic dihydrocinnamoyl esters **2a**-**3a**. Subtilisin Carlsberg showed its highest enantioselectivity with **3a** ($E = 2.8$) to give (*R*)-**3** and α-chymotrypsin showed its highest enantioselectivity with **5a** ($E = 3.0$). As well, subtilisin Carlsberg and α-chymotrypsin catalyzed the hydrolysis of the allylic derivative **4a** and alkyl derivative **5a** – sterically hindered substrates that show no activity with lipases.[17] Subtilisin Carlsberg showed moderate-low reactivity toward **4a** (c ~5%) and low reactivity toward **5a** (c <1%). Decomposition of the allylic alcohol product from **4a** prevented accurate determination of conversion and *E*. α-Chymotrypsin showed moderate reactivity toward **4a** (c ~ 25%) and low reactivity toward **5a** (c ~ 1%). The increased size of **6a** over **5a** (ethyl to isopropyl) prevented any reaction from occurring.

Table 2. Reactivity and enantioselectivity of subtilisin Carlsberg and α-chymotrypsin toward **2a-6a**.[a]



	Subtilisin Carlsberg				α-Chymotrypsin			
	%c[a]	ee_s (%)[b]	ee_p (%)[b]	E[c,d]	%c[a]	ee_s (%)[b]	ee_p (%)[b]	E[c,d]
2a 	27	4	11	1.3(S^e)	51	23	22	1.9(S^e)
3a 	22	12	42	2.8 (R^f)	94	61	4	1.6(R^f)
4a 	~5	n.d.[h]	n.d.[i]	n.d.	~25	n.d.[h]	n.d.[i]	n.d.
5a 	1	1	64	4.6[j]	1	1	50	3.0[f]
6a 	n.r.[k]	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.

[a]Conversion after reaction for 48 h. [b]Enantiomeric excess: enantiomeric excess of substrate and product were determined by GC analysis on a chiral column. [c]Enantioselectivity: the enantiomeric ratio E measures the relative rate of hydrolysis of the fast enantiomer as compared to the slow enantiomer as defined by Sih (Chen, C.S.; Fujimoto, Y.; Girdaukas, G.; Sih, C.J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299). [d]Enantiopreference in parenthesis. [e]The absolute configuration was determined by *Candida rugosa* (CRL)-catalyzed hydrolysis of **2b**, which gives (*R*)-**2** (O'Hagan, D.; Zaidi, N. A. *Tetrahedron: Asymmetry* **1994**, *5*, 1111-1118). [f]The absolute configuration was determined by *Candida antarctica* lipase A (CAL-A)-catalyzed acylation of **3** with vinyl acetate, which proceeds with R-selectivity (Krishna, S. H.; Persson, M.; Bornscheuer, U. T. *Tetrahedron: Asymmetry* **2002**, *13*, 2693-2696). [g]Not determined. [h]The enantiomers could not be separated by GC or HPLC. [i]No product allylic alcohol was detected as it readily decomposed in aqueous solutions. However, we observed loss of substrate in the presence of enzyme. [j]The enantioselectivity may be inaccurate because of low conversion. [k]No reaction.

Tertiary alcohols react slower than secondary alcohols because they form a *syn*-pentane-like interaction with the oxyanion hole in the first tetrahedral intermediate. For secondary alcohols, the orientation within the enzyme places the smallest group (hydrogen) in a *syn*-pentane-like orientation to the oxyanion (Figure 4a). A hydrogen fits in this orientation easily, but in a tertiary alcohol the hydrogen is replaced with a larger group creating significant steric strain. For instance, a methyl/methyl *syn*-pentane interaction destabilizes a conformation by ~3.6 kcal/mol.[18] Previous work by O'Hagan and coworkers with lipases suggests that acetylene occupies the *syn*-pentane-like position in tertiary alcohols such as **3a** based on absolute configuration.[17] Tertiary alcohols where the smallest substituent was larger than an acetylene did not react. While subtilisin Carlsberg and α-chymotrypsin show high reactivity toward the secondary alcohol 1-phenethyl dihydrocinnamate,[19] and tertiary acetylenic ester **3a**, the conversion decreases with more sterically hindered tertiary alcohols. The allylic ester **4a** reacts two- to five-fold slower, the sterically demanding tertiary alkyl ester **5a** reacts approximately fifty-fold slower, and there is no reaction with **6a**. Molecular mechanics calculations suggest a methyl/oxyanion *syn*-pentane-like interaction is ~2.5 kcal/mol higher in energy than an acetylene/oxyanion *syn*-pentane-like interaction (Figures 4b and 4c). Thus replacing hydrogen of a secondary alcohol with anything larger reduces activity.

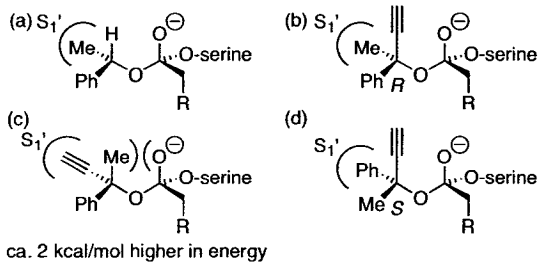


Figure 4. Schematic representation of the proposed conformation of the first tetrahedral intermediate of protease-catalyzed ester hydrolysis. (a) For a secondary alcohol, the hydrogen orients to form a *syn*-pentane-like orientation with oxyanion. (b) For a tertiary acetylenic ester, the smallest substituent (acetylene) forms the *syn*-pentane-like interaction with oxyanion. The fast-reacting (*R*)-enantiomer binds with the medium-sized methyl group in the S_1' pocket and the larger phenyl group open to solvent (c) When the substituent forming the *syn*-pentane-like interaction with oxyanion is methyl or larger the transition state is destabilized and the reaction is slow. (d) The slow-reacting (*S*)-enantiomer binds with the large phenyl group in the S_1' pocket and the medium-sized methyl group open to solvent.

If the acetylene susbtituent of **3a** occupies the *syn*-pentane-like position, then the placement of the two other susbtituents determines the enantioselectivity of subtilisin Carlsberg. Previous modeling of secondary alcohol resolution with subtilisin reveal one pocket – the S_1' pocket – that binds one substituent of the alcohol portion of an ester, while the other points towards solvent.[20] This pocket is narrow and can accommodate substituents approximately the size of a phenyl group. In **3a** either the phenyl or the methyl substituent can occupy the S_1' alcohol pocket. Molecular modeling of the first tetrahedral intermediate for subtilisin Carlsberg-catalyzed hydrolysis of **3a** reveals that (*R*)-**3a** places the methyl group in the S_1' pocket, while (*S*)-**3a** places the phenyl group in this pocket (Figure 5). In both cases the other substituent is orientated towards the solvent, and the acetylene group forms the *syn*-pentane-like orientation with oxyanion. Placing the methyl group of (*R*)-**3a** in the S_1' pocket avoids some steric interactions between the pocket and the phenyl group. However, binding the phenyl group of (*S*)-**3a** has a favourable hydrophobic interaction with the pocket. The enantioselectivity for the subtilisin catalyzed resolution is low because of these competing hydrophobic interactions. A similar result was obtained for the resolution of the secondary phenethyl alcohol, where there was no clear favourite between phenyl and methyl for binding in the S_1' pocket and

the enantioselectivity was only 1.2.[20b] Other non-productive conformations (acetylene not in the syn-pentane like position) encountered severe steric clash with the active site residues of the protein.

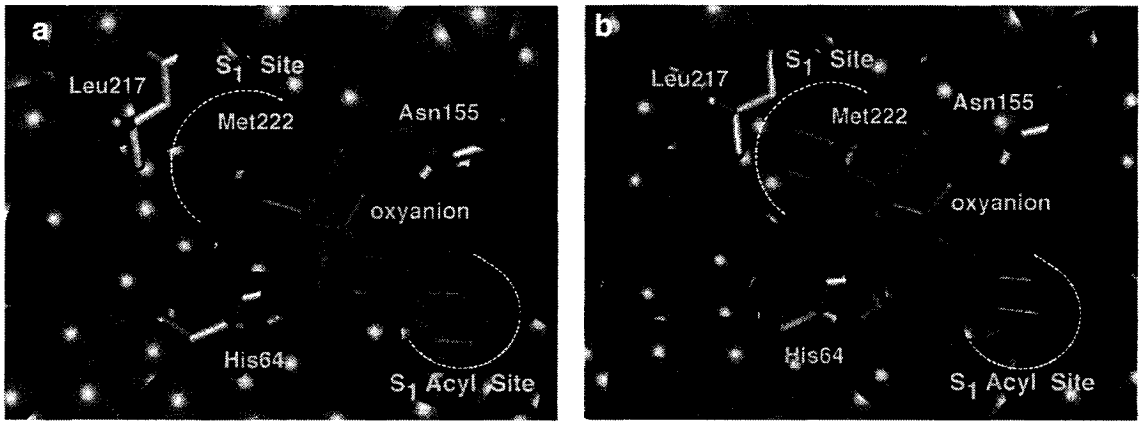


Figure 5. Catalytically productive orientation of the tetrahedral intermediates for the subtilisin Carlsberg catalyzed hydrolysis of (*R*)-**3a** (a) and (*S*)-**3a** (b) as identified by molecular modeling. The substrate atoms (green) and important residues are shown as sticks, and the remaining enzyme in spacefill (blue). The binding modes of a and b maintain all catalytically essential hydrogen bonds and the acyl dihydrocinnamoyl group was bound in the S_1 acyl site. The acetylene occupied the same site as hydrogen of a secondary alcohol, based on molecular mechanics calculations and substrate mapping experiments with **4-6**. (a) In the fast-reacting enantiomer, (*R*)-**3a**, the methyl group is bound in the hydrophobic S_1' pocket and the phenyl group is exposed to solvent, water. (b) In the slow-reacting enantiomer, (*S*)-**3a**, the phenyl group is bound in the hydrophobic S_1' pocket and the methyl group is exposed to solvent, water.

Consistent with this explanation for enantioselectivity, subtilisin-Carlsberg-catalyzed hydrolysis of **3a** increased from E = 2.8 in 90:10 water-acetonitrile to E = 4.2 in 40:60 water-acetonitrile. The high concentration of acetonitrile favors the phenyl group in the solvent; thus, the enantiomer preference shifts toward the (*R*)-enantiomer, which orients with the methyl group in the S_1' pocket and the phenyl group in solvent. This result suggests that the enantioselectivity for acylation of tertiary alcohols in organic solvent would also be higher. Unfortunately, we did not detect any reverse reaction, possibly because binding predominately occurs in the acyl portion for proteases.

Discussion

Here we report the protease-catalyzed hydrolysis of tertiary alcohol esters, and also the resolution of a remote sulfinyl stereocenter by subtilisin. The hydrolysis of tertiary alcohol esters by proteases is an unexplored area in biocatalysis. Many of the proteases tested showed some activity, suggesting this may be a general reaction. There is only one literature example of a protease-catalyzed hydrolysis: thermitase catalyzes the deprotection of *tert*-butyl esters of several *N*-protected peptides.[21]

The resolution of **1a** demonstrates subtilisin's ability to resolve a remote sulfinyl stereocenter. The stereocenter is three bonds from the reacting carbonyl. In a related reaction, a crude lipase preparation from *Pseudonomas sp.* resolved a three-bond-distant sulfoxide with high enantioselectivity.[22] Chiral aldehyde inhibitors of subtilisin Carlsberg, with a stereocenter three bonds from the carbonyl[23] showed a 4.8-fold preference for the *R*-enantiomer of the aldehyde when comparing their inhibition constants (Figure 6, a). Molecular modeling of these aldehydes revealed that the *R*-inhibitor was bound more deeply in the hydrophobic S_1 region than the *S*-enantiomer.

The substrate **1a** also resembles the hippurate acyl group, an excellent acyl group for subtilisin. For example, the hydrolysis of the phenyl hippurate ester is approximately 200 times faster than the corresponding phenyl acetate (Figure 6, b).[24] Their K_M values differ by 100-fold, indicating that better binding for the hippurate accounts for most of the increase in catalytic efficiency. The phenyl ring of the hippurate contributes to binding, as replacing it leads to a 500-fold decrease in catalytic efficiency (Figure 6, c). Thus the ability of **1a** to hydrolyze tertiary alcohol esters and resolve a remote stereocenter may be due to an efficient acyl group. The methyl and benzyl compounds, **1b**-**1c**, do not react with such a high enantioselectivity, possibly due to weaker binding.

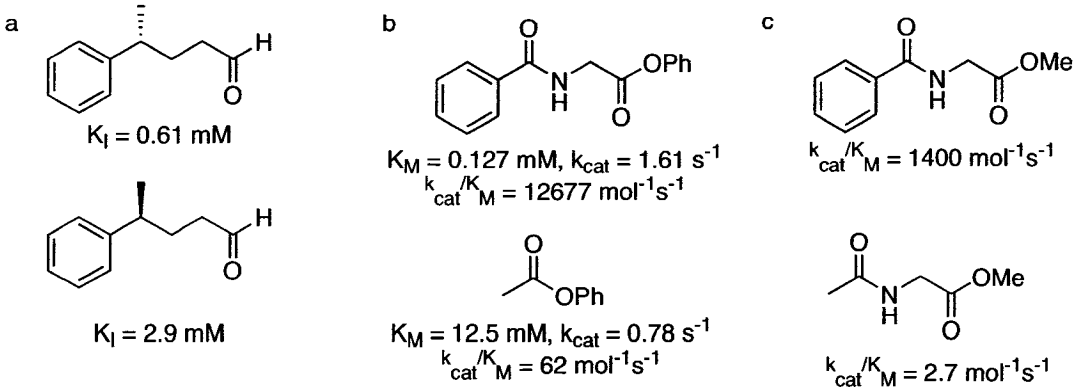


Figure 6: (a) Subtilisin Carlsberg has a 4.8 fold difference in the inhibition constants of chiral aldehyde inhibitors. The stereocenter in **1a** is located in the same relative position. (b) Phenyl hippurate reacts 200 times more efficiently than the corresponding acetate. Most of the difference is in K_M, indicating the hippurate binds preferentially to subtilisin. (c) The phenyl ring of the hippurate group contributes significantly to catalytic efficiency of the reaction, with a 500-fold greater k_{cat}/K_M value compared with the methyl analogue.

It is interesting to note that only a few lipases catalyze hydrolysis of tertiary alcohol esters, whereas for serine proteases it appears to be a more general reaction. Lipases likely show low activity toward tertiary alcohols because of their restricted alcohol-binding site and well defined pockets. This may increase the internal strain arising from the *syn*-pentane-like interaction. While lipases must bind substrate in a folded conformation for reaction,[25] proteases bind substrate in an extended conformation.[26] The active site is on the surface of the enzyme allowing it to accommodate polypeptides, making it more accessible to sterically hindered substrates such as tertiary alcohols.[27]

Proteases may also use acyl group binding to anchor the substrate to the protease active site and help stabilize the transition state.[28] This binding may help overcome the destabilizing *syn*-pentane-like interaction in tertiary alcohols. Enzymes often use an anchoring group to bind and orient a substrate for reaction. For example, orotidine 5'-monophosphate decarboxylase is an efficient catalyst.[29] Although reaction occurs entirely in the orotic acid base, the ribosyl phosphate moiety is essential for activity. Orotic acid (2.5×10^{-5} M^{-1} s^{-1}) alone shows twelve orders of

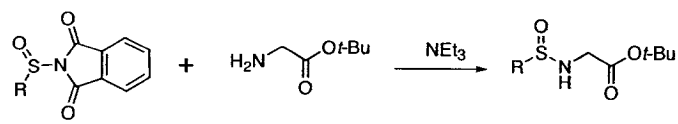
magnitude lower activity than orotidine 5'-monophosphate (6.3×10^7 M^{-1} s^{-1}), even though orotic acid binds to the enzyme active site (K_i = 9.5 mM). In a few cases, researchers have used complementary anchor groups to enhance biocatalytic reactions. For example, the fungus *Beauveria bassiana* did not hydroxylate cyclopentanone, but did hydroxylate the *N*-benzoylspirooxazolidine derivative with moderate yield and diastereoselectivity.[30]

Although the enantioselectivities are low, the enantioselectivity of the reaction might be optimized using mutagenesis. For example, Reetz and coworkers expanded the range of substrates accepted by the lipase from *Pseudomonas aeruginosa*.[31] The wild type lipase hydrolyzes esters of straight chain acids, but cannot hydrolyze more sterically demanding substrates. Mutation of residues spatially close to the active site and screening gave mutants with an increase in hydrolysis rate. One mutant could even hydrolyze a bulky adamantyl carboxyl acid ester with low activity. Thus protein engineering can increase an enzyme's substrate acceptance and may be used in this case to improve reactivity and enantioselectivity for the protease catalyzed resolution of tertiary alcohol esters. Another method to improve the enantioselectivity is to use groups that are easily distinguished, unlike phenyl and methyl. Since only part of the alcohol binds in the active site and the rest remains in solution, the difference in hydrophobicity (or polarity) of the substituents contributes to enantioselectivity. The hydrophobicity, measured by LogP, is similar for phenyl (2.1) and methyl (1.1) contributing to the low enanatioselectivity.[32] Increasing the difference hydrophobicity increased the enantioselectivity of subtilisin towards secondary alcohols,[20] and a similar approach may be successful here.

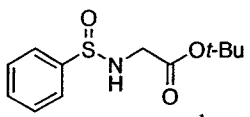
Experimental section

General ^{1}H- and ^{13}C-NMR spectra were obtained as $CDCl_3$ solutions at 300 MHz and 75 MHz, respectively. Chemical shifts are expressed in ppm (δ) and are referenced to tetramethylsilane or solvent signal. Coupling constants are reported in Hertz (Hz). GC analyses were performed on a 25 m x 0.25 mm Chrompack CP-Chiralsil-Dex CB column (Varian Inc., Palo Alto, USA) with He as carrier gas using one of the three following temperature programs: A 17.5 psi, 50 °C, 5 °C min^{-1}, 150

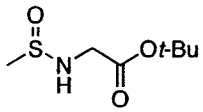
°C held for 5 min, 2.5 °C min^{-1}; 175 °C held for 5 min, 5 °C min^{-1}, 200 °C held for 30 min); B 17.5 psi, 120 °C held for 10 min, 20 °C min^{-1}, 200 °C held for 20 min. HPLC analyses were performed on a 4.6 x 250 mm Daicel Chiralcel OD column (Chiral Technologies, Exton, USA) and monitored at 254 nm. Flash chromatography with silica gel (35-75 mesh) was used to purify all intermediates and substrates. All reagents, buffers, starting materials and anhydrous solvents were purchased from Sigma-Aldrich (Milwaukee, USA) and used without purification. All air- and moisture-sensitive reactions were performed under Ar. Subtilisin Carlsberg, protease from *Streptomyces griseus* and α-chymotrypsin were purchased from Sigma-Aldrich (St. Louis, USA). Lipase from *Candida rugosa* (CRL), lipase A from *Candida antarctica*, protease from *Aspergillus melleus* and protease from *Aspergillus oryzae* were a generous gift from Altus Biologics (Cambridge, USA).



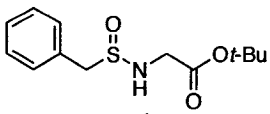
Synthesis of *N*-(sulfinyl)-*tert*-butylglycines, 1a-1c. General procedure.[33] The *t*-butyl ester of glycine (11 mmol, as HCL salt) was added dropwise to a solution of *N*-(sulfinyl)phthalimide (11 mmol) and NEt_3 (18 mmol) in CH_2Cl_2 (50 mL) over 1 h . Stirring was continued for 5 h and the reaction mixture was filtered to remove phthalimide and washed with water (2 x 50 mL), sat. $NaCO_3$ (2 x 50 mL), brine (50 mL), dried over $MgSO_4$ and the solvent evaporated under reduced pressure and purified by column chromatography in EtOAc/hexanes.



***N*-(phenylsulfinyl)-*tert*-butylglycine, 1a** Purification on silica gel (20:80 hexanes/EtOAc/hexanes) gave a clear yellow liquid (1.36 g, 47 %); ^{1}H NMR: 1.35 (s, 9H), 3.15 (d, 1H, J = 12), 3.75 (d, 1H J = 11), 4.95(t, 1H), 7.45 (m, 3H), 7.68 (m, 2H); ^{13}C NMR: δ 28.3, 40.5, 82.8, 126.2, 129.2, 131.2, 143.5, 169.4; HR-EIMS *m/z*: M^+ calcd for $C_{12}H_{17}NO_3S$ 255.09292, found 255.09230. HPLC: 95/5 Hexanes/Ethanol, flow 0.8 mL/min, **1a** t_R = 8.2 min, t_R = 9.5 min; 2-(*p*-nitrophenyl)-ethanol (internal standard) t_R = 16.6 min.



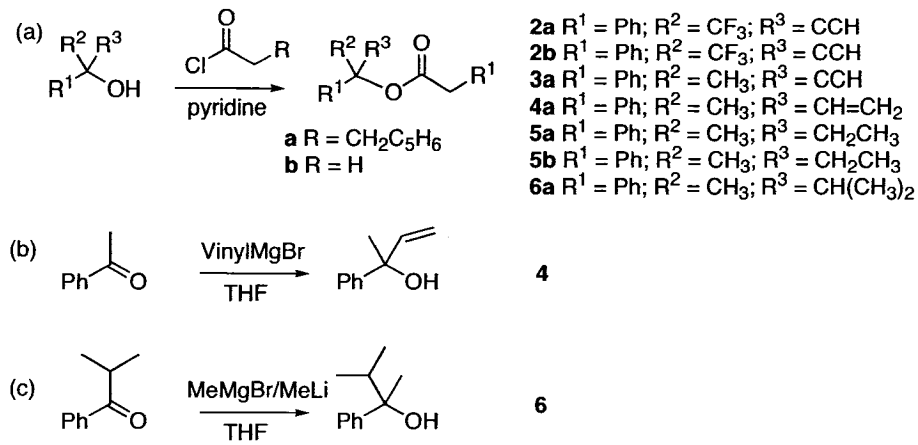
***N*-(methylsulfinyl)-*tert*-butylglycine, 1b** Purification on silica gel (25:75 hexanes/EtOAc/hexanes) gave a clear liquid (710 mg, 39%); ^{1}H NMR: 1.37 (s, 9H), 2.73 (s, 3H), 3.15 (d, 1H, J = 12), 3.75 (d, 1H, J = 11); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 28.1, 38.1, 40.5, 82.8, 169.6; HR-EIMS *m/z*: M^+ calcd for $C_7H_{15}NO_3S$ 193.0773, found 193.0785. HPLC: 95/5 Hexanes/Ethanol, flow 0.8 mL/min, **1b** t_R = 7.6 min, t_R = 8.8 min; 2(*p*-nitrophenyl)-ethanol (internal standard) t_R = 16.6 min.



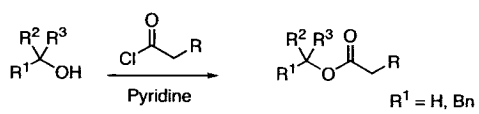
***N*-(benzylsulfinyl)-*tert*-butylglycine 1c** Purification on silica gel (25:75 hexanes/EtOAc/hexanes) gave a clear liquid (610 mg, 50%); ^{1}H NMR: 1.35 (s, 9H), 3.15 (d, 1H, J = 12), 3.75 (d, 1H, J = 11), 4.90 (d, 1H, J = 12), 5.29 (d, 1H, J = 12), 7.40 (m, 3H), 7.62 (m, 2H); ^{13}C NMR: δ 28.5, 40.5, 59.3, 82.8, 128.4, 128.9, 130.5, 134.4, 169.4; HR-EIMS *m/z*: M^+ calcd for $C_{13}H_{19}NO_3S$ 269.1086, found 269.1102. HPLC: 90/10 Hexanes/Ethanol, flow 1 mL/min, **1c** t_R = 6.6 min, t_R = 7.6 min; 2-(*p*-nitrophenyl)-ethanol (internal standard) t_R =12.6 min.

NMR reaction ^{1}H NMR was used to determine the products of the subtilisin Carlsberg-catalyzed hydrolysis of **1a**. Reactions conditions were as follows: D_2O (1 mL) containing phosphate buffer (20 mM, pH 7.4) and subtilisin Carlsberg (10 mg, Fluka 82518), d_3-MeCN (100 μL), **1a** (60 mM) and sodium 4,4-dimethyl-4-silapentane-1-sulfonate,[34] (DSS, 1 mM). The reaction was monitored over the course of 24 h using a 500 MHz instrument. Under these conditions the *tert*-butyl CH_3 group has the following 500 MHz ^{1}H resonances relative to DSS: *tert*-butanol δ = 1.228, *tert*-butylglycine δ = 1.494, and **1a** δ = 1.349. The major product was *tert*-butanol from ester cleavage, while a small amount of *tert*-butylglycine from S-N cleavage (less than 5%) was detected. A blank reaction with no enzyme also showed a small amount of *tert*-butylglycine after 24 h.

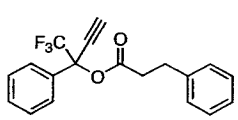
Synthesis of tertiary alcohol substrates. We synthesized esters **2a-6a**, and **2b** by treating the corresponding tertiary alcohol with the appropriate acid chloride in the presence of pyridine (Scheme 3a). We synthesized tertiary alkenyl alcohols via vinyl magnesium bromide addition to the appropriate ketone (Scheme 3b) and tertiary alkyl alcohols via modified alkyl addition of magnesium ate complexes derived from Grignard and alkyllithium reagents[35] to the appropriate ketone (Scheme 3c).



Scheme 3. Synthesis of esters **2a-6a**, **2b**, **5b**, and tertiary alcohols **4** & **6**.

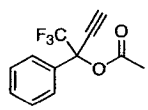


Synthesis of racemic esters 2a-6a, 2b. General procedure. Dihydrocinnamoyl chloride (7.5 mmol) was added drop-wise to a stirred solution of tertiary alcohol (5 mmol) and pyridine (7.5 mmol) in CH_2Cl_2 (25 mL) at 0 °C. The ice bath was removed and stirred until the reaction was complete by TLC (2-3 d). The reaction was quenched with the addition of sat. $NaHCO_3$ (25 mL). The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (2 x 25mL). The combined organic layers were washed with sat. $NaHCO_3$ (2 x 25 mL), sat. NaCl (25 mL) and dried over $MgSO_4$. The organic layer was concentrated *in vacuo* to give the crude ester. All substrates were purified on silica gel. The relevant analytical data are given below:

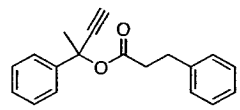


3-Dihydrocinnamic acid 1-phenyl-1-trifluoromethyl-prop-2-

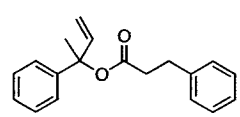
ynyl ester (2a). Purification on silica gel (1:1 hexanes/CH_2Cl_2) white solid (550 mg, 43%): mp 71-72 °C; ^{1}H NMR δ 2.81 (m, 2H), 2.89 (s, 1H), 2.97 (t, J = 7.4, 2H), 7.30 (m, 8H), 7.48 (d, J = 6.9 Hz, 2H); ^{13}C NMR δ 30.5, 36.0, 75.2, 78.9, 120.4, 124.1, 126.6, 126.9, 128.5, 128.6, 128.7, 129.8, 132.3, 139.9, 168.9; HRMS calcd for $C_{19}H_{15}F_3O_2Na$ $[M+Na]^+$ 355.0898. Found: 355.0921. The enantiomers were separated using GC (program A; (*R*)-**2a**, t_R = 42.9 min; (*S*)-**2a**, t_R = 43.1 min).



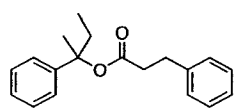
Acetic acid 1-phenyl-1-trifluoromethyl-prop-2-ynyl ester (2b).[36] Purification on silica gel (100% hexanes to 80:20 hexanes/acetone) to give a low melting white solid (288 mg, 51%): ^{1}H NMR δ 2.20 (s, 3H), 2.93 (s, 1H), 7.38-7.44 (m, 3H), 7.62-7.65 (m, 2H); ^{13}C NMR δ 21.3, 75.5 , 78.8, 120.3, 124.1, 126.9, 128.6, 129.9, 132.4, 166.9. The enantiomers were separated using GC (program B; (*R*)-**2b**, t_R = 5.2 min; (*S*)-**2b**, t_R = 5.5 min).



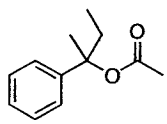
3-Dihydrocinnamic acid 1-methyl-1-phenyl-prop-2-ynyl ester (3a). Purification on silica gel (100% hexanes to 95:5 hexanes/EtOAc) to give a clear liquid (927 mg, 67%): ^{1}H NMR δ 1.85 (s, 3H), 2.65 (m, 2H), 2.79 (s, 1H), 2.92 (t, J = 7.8, 2H), 7.15-7.34 (m, 8H), 7.47-7.49 (m, 2H); ^{13}C NMR δ 30.8, 32.2, 36.5, 75.6, 75.8, 83.1, 124.9, 126.4, 128.0, 128.5, 128.6, 140.6, 142.2, 170.6; HRMS calcd for $C_{19}H_{18}O_2Na$ $[M+Na]^+$ 301.1224. Found: 301.1204. The enantiomers were separated using GC (program A; (*S*)-**2a**, t_R = 46.7 min; (*R*)-**2a**, t_R = 46.9 min).



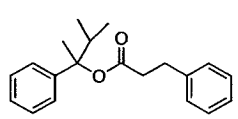
Dihydrocinnamic acid 1-methyl-1-phenyl-allyl ester (4a). Purification on silica gel (100% hexanes to 95:5 hexanes/EtOAc) to give a clear liquid (493 mg, 35%): ^{1}H NMR δ 1.87 (S, 3H), 2.69 (m, 2H,), 2.97 (t, J = 7.5, 2H), 5.24 (dd, J = 0.9, 10.5, 1H), 5.26 (dd, J = 0.9, 17.4, 1H), 6.25 (dd, J = 10.8, 17.4, 1H), 7.21-7.34 (m, 10H); ^{13}C NMR δ 25.5, 31.0, 36.9, 83.3, 114.5, 125.3, 126.3, 127.3, 128.3, 128.5, 128.6, 140.7, 141.5, 143.7, 171.2; HRMS calcd for $C_{19}H_{20}O_2Na$ $[M+Na]^+$ 303.1373. Found: 303.1360. The enantiomers could not be separated using GC or HPLC.



Dihydrocinnamic acid 1-methyl-1-phenyl-propyl ester (5a). Purification on silica gel (1:1 hexanes/CH_2Cl_2) to give a clear liquid (1.13 g, 61%): ^{1}H NMR δ 0.72 (t, J = 7.5, 3H), 1.76 (s, 3H), 1.96 (q, J =7.2, 2H), 2.63 (t, J = 7.5, 2H), 2.92 (t, J = 7.8, 2H), 7.15-7.27 (m, 10H); ^{13}C NMR δ 8.3, 24.5, 31.2, 35.4, 37.0, 84.6, 124.9, 126.4, 127.0, 128.3, 128.6, 128.7, 140.9, 145.0, 171.6; HRMS calcd for $C_{19}H_{22}O_2Na$ $[M+Na]^+$ 305.1523. Found: 305.1517. The enantiomers could not be separated using GC.



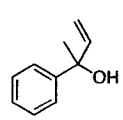
Acetic acid 1-methyl-1-phenyl-propyl ester (5b). Purification on silica gel (100% hexanes to 95:5 hexanes/EtOAc) to give a clear liquid (518 mg, 54%): ^{1}H NMR δ 0.78 (t, J = 7.2, 3H), 1.81 (s, 3H), 2.02 (q, J =7.5, 2H), 2.07 (s, 3H), 7.20-7.35 (m, 5H); ^{13}C NMR δ 8.2, 22.3, 24.5, 35.4, 84.4, 124.7, 126.9, 128.2, 145.0, 169.8; HRMS calcd for $C_{12}H_{16}O_2Na$ $[M+Na]^+$ 215.1049. Found: 215.1047. The enantiomers were separated using GC (program B; (*S*)-**5b**, t_R = 6.6 min; (*R*)-**5b**, t_R = 6.8 min).



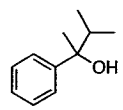
Dihydrocinnamic acid 1,2-dimethyl-1-phenyl-propyl ester (6a). Purification on silica gel (100% hexanes to 90:10 hexanes/EtOAc) to give a clear liquid (536 mg, 36%): ^{1}H NMR δ 0.64 (d, J = 6.9), 0.91 (d, J = 6.9), 1.77 (s, 3H), 2.02 (sept, 1H, J = 6.8), 2.60 (t, J = 7.5, 2H), 2.91 (t, J = 7.5, 2H), 7.10-7.28 (m, 10H); ^{13}C NMR δ 17.2, 17.7, 19.0, 31.1, 37.0, 39.8, 85.6, 125.4, 126.4, 126.9, 128.0, 128.6, 128.7, 140.9, 144.1, 171.5, 171.5; HRMS calcd for $C_{20}H_{24}O_2Na$ $[M+Na]^+$ 319.1662. Found: 319.1673. The enantiomers could not be separated using GC.

Synthesis of racemic tertiary α-acetylenic alcohols 4 & 6. General procedure. Alkynyl magnesium bromide (0.5 M; 48 mL, 24 mmol) was added to a solution of the appropriate ketone (20 mmol) in THF (10 mL) and refluxed for 4 h or until complete by TLC. The solution was cooled to RT and quenched with dH_2O (25 mL) and extracted with EtOAc (3 x 25 mL). The combined organic layers were washed with

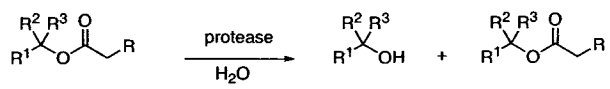
sat. NaCl (25 mL) and dried over $MgSO_4$. The organic layer was concentrated *in vacuo* to give the crude tertiary alcohol. The relevant analytical data are given below:



Synthesis of 2-phenyl-but-3-en-2-ol (4).[37] Vinyl magnesium bromide (0.5 M; 48 mL, 24 mmol) was added to a solution of acetophenone (20 mmol) in THF (10 mL) and refluxed for 4 h. The solution was cooled to RT and quenched with dH_2O (25 mL) and extracted with EtOAc (3 x 25 mL). The combined organic layers were washed with sat. NaCl (25 mL) and dried over $MgSO_4$. The organic layer was concentrated *in vacuo* to give a clear liquid (2.67 g, 90%): ^{1}H NMR δ 1.68 (s, 3H), 5.17 (dd, J = 0.9, 10.5, 1H), 5.32 (dd, J = 1.2, 17.4, 1H), 6.20 (dd, J = 10.8, 17.4, 1H), 7.30-7.51 (m, 5H); ^{13}C NMR δ 29., 74.8, 112.4, 125.3, 127.1, 128.3, 144., 146.6. The enantiomers were separated using HPLC (Chiralcel OD-H column, 98:2 hexanes/*i*-PrOH, 1.0 mL/min, 254 nm; (*R* or *S*)-**7**, t_R = 12.7 min; (*R* or *S*)-**7**, t_R = 16.2 min).

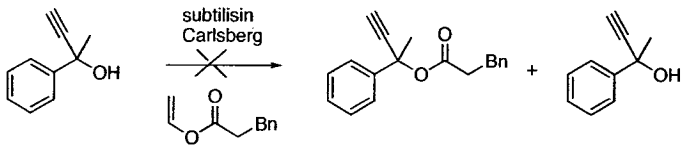


Synthesis of 3-methyl-2-phenyl-butan-2-ol (6).[35] Methyllithium (1.5 M; 50 mmol, 33 mL) was added to methylmagnesium bromide (3.0 M, 10 mL, 30 mmol) at -78 °C and stirred at this temperature for 1 h. A solution of isobutyl acetophenone (3.70 g, 25 mmol) in THF ((40 mL) was then added drop-wise and stirred for 6 h at -78 °C.[35] The reaction was quenched with addition of sat. NH_4Cl (100mL) and extracted with Et_2O (3 x 50 mL). The combined organic layers were washed with sat. NaCl (50 mL) and concentrated *in vacuo* to give give a clear liquid (4.05 g, 96%): ^{1}H NMR δ 0.82 (d, J = 6.9), 0.91 (d, J = 6.9), 1.55 (br s, 1H), 1.65 (s, 3H), 2.04 (sept, 1H, J = 6.9), 7.22-7.45 (m, 5H); ^{13}C NMR δ 17.3, 17.5, 38.7, 125.4, 126.5, 127.9, 147.9. The enantiomers were separated using GC (program B; (*R* or *S*)-**9**, t_R = 9.2 min; (*R* or *S*)-**9**, t_R = 9.9 min).



Protease-catalyzed hydrolysis of esters 1a-6a, 2b. Subtilisin BL, *Aspergillus melleus* protease, *Aspergillus oryzae* protease, subtilisin Carlsberg, α-chymotrypsin

and protease from *Streptomyces griseus* in 50 mM BES buffer (10 mg/mL, pH 7.2, 450 μL) and substrate (100 mM in CH_3CN, 50 μL) were mixed in a 1.5 mL glass vial at 30 °C for 24 h to 48 h. The reaction was terminated with the addition of CH_2Cl_2 (500 μL). The phases were separated by centrifugation, and the organic layers were collected. The aqueous phase was extracted with CH_2Cl_2 (2 x 500 μL) and the combined organics were evaporated under a stream of air. The residue was diluted with EtOAc or EtOH (150 μL) and analyzed by GC or HPLC. The enantiomers were separated using the conditions described above. In cases where GC could not separate enantiomers of the substrate, it was purified using flash chromatography, hydrolyzed in ethanolic KOH and the enantiomeric excess was determined as the alcohol.



Attempted transesterification of 2-phenyl-but-3-yn-2-ol with subtilisin Carlsberg. Subtilisin Carlsberg (50 mg), dihydrocinnamic vinyl ester (18 mg, 100 μmol), 2-phenyl-but-3-yn-2-ol (73 mg, 500 μmol) and 4-Å molecular sieves (100 mg) were added to a glass vial. Anhydrous dioxane (2 mL) was added and the mixture was stirred at 30 °C for at least 48 h. A small amount of the solution was removed, filtered through a 0.45 μm nylon filter and analyzed by GC. The enantiomers were separated using the conditions described above.

Modeling of tetrahedral intermediates bound to subtilisin Carlsberg. All modeling was performed using *Insight II 2000.1 / Discover* (Accelrys, San Diego, USA) on a SGI Octane UNIX workstation using the AMBER force field.[38] We used a nonbonded cutoff distance of 8 Å, a distance-dependent dielectric of 1.0 and scaled the 1-4 van der Waals interactions by 50%. Protein structures in Figure 5 were created using PyMOL (Delano Scientific, San Carlos, CA, USA). The X-ray crystal structure of subtilisin Carlsberg (entry 1CSE)[39] is from the Protein Data Bank. Using the Builder module of *Insight II*, we removed the inhibitor, Eglin C. The 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 (His64) was protonated. The positions of the water hydrogens and then the enzyme hydrogens were optimized using a consecutive series of short (1 ps) molecular dynamic runs and energy minimizations.[40] This optimization was repeated until there was <2 kcal/mol in the energy difference of the minimized structures. Thereafter, an iterative series of geometry optimizations were performed on the water hydrogens, enzyme hydrogens and full water molecules. Finally, the whole system was geometry optimized until the RMS was less than 1 kcal/mol.

The tetrahedral intermediates were built manually and covalently linked to Ser221. Nonstandard partial charges were calculated using a formal charge of –1 for the substrate oxyanion. Energy minimization proceeded in three stages. First, minimization of substrate with only the protein constrained (25 kcal mol^{-1} $Å^{-2}$); second, minimization with only the protein backbone constrained (25 kcal mol^{-1} $Å^{-2}$) and for the final stage the minimization was continued without constraints until the rms value was less than 0.0005 kcal mol^{-1} $Å^{-1}$. A catalytically productive complex required all five hydrogen bonds within the catalytic machinery. The limits for a hydrogen bond were a donor to acceptor atom distance of less than 3.1 Å with a nearly linear arrangement (>120° angle) of donor atom, hydrogen, and acceptor atom. Structures lacking any of the five catalytically relevant hydrogen bonds or encountering severe steric clash with enzyme were deemed nonproductive.

Modeling **3a** with subtilisin Carlsberg gave one productive conformation for each enantiomer. The four other plausible conformations lost catalytically essential hydrogen bonds and/or encountered steric clash with the protein. Similar to previous modeling studies with secondary alcohols with subtilisin Carlsberg,[41] the productive conformation of the (*R*)-**3a** binds with the methyl group in the leaving-group S_1' pocket and the productive conformation of the (*S*)-**3a** has the phenyl group in this pocket.

Productive conformer of (*R*)-**3a.** The acetylene group forms the *syn*-pentane-like interaction with the oxyanion. (*S*)-**3a** fit well in the active site and S_1' residues did not clash with the methyl group.

Non-productive conformers of (*R*)-**3a** When the methyl group forms the *syn*-pentane-like interaction with the oxyanion, the phenyl group of non-productive (*R*)-

3a encounters a steric clash with Met222 (C_{ortho} - S distance = 3.62 Å, C_{meta} - S distance = 3.88 Å and C_{meta} - C_{ε} distance = 3.78 Å).[42] When the phenyl group forms the *syn*-pentane-like interaction with the oxyanion, the phenyl group of non-productive (*R*)-**3a** encounters a steric clash with the oxyanion hole Asn155 (C_{ortho} - $N_{\delta 2}$ distance = 3.29 Å, C_{ortho} - C_{γ} distance = 3.39 Å, C_{meta} - $N_{\delta}2$ distance = 3.73 Å, C_{meta} - C_{γ} distance = 3.40 Å) and G219 (C_{meta} -C_{α}distance = 3.69 Å).[42] An essential hydrogen bond to His64 is lost in this conformer ($N_{\varepsilon 2}$-O_{alc} distance 3.21Å).

Productive conformer of (*S*)-3a. The acetylene group forms the *syn*-pentane-like interaction with the oxyanion. (*S*)-**3a** fit well in the active site, but the phenyl group bumps Met222 (C_{meta} – C_{ε} distance = 3.98 Å) and His64 (C_{meta} - $C_{\delta}2$ distance = 3.72 Å) in the S_1' pocket. This tight fit suggests a favorable hydrophobic interaction between the phenyl group and S_1' pocket residues. Consistent with this hypothesis, higher concentrations of organic co-solvent decreased this hydrophobic interaction and increased the enantioselectivity of subtilisin Carlsberg toward (*R*)-**3a**.

Non-productive conformers of (*S*)-3a When the methyl group forms the *syn*-pentane-like interaction with the oxyanion, the phenyl group of non-productive (*S*)-**3a** encounters a steric clash with catalytic His64 (C_{ortho} - $N_{\delta}2$ distance = 3.64) and the methyl group bumps the oxyanion hole Asn155 (CH_3 - $N_{\delta}2$ distance = 3.74 Å). When the phenyl group formed the *syn*-pentane-like interaction with the oxyanion the phenyl group of non-productive (*S*)-**3a** encountered a steric clash with the oxyanion hole Asn155 (C_{ortho} - $N_{\delta}2$ distance = 3.38 and C_{meta} - $N_{\delta 2}$ distance = 3.45 Å and C_{para} - $N_{\delta 2}$ distance = 3.51 Å, C_{para} - C_{γ} distance = 3.42 Å) and Gly219 (C_{ortho} - C_{α} distance = 3.53 and C_{meta} - C_{α} distance = 3.63 Å).

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Chapter 4

In the previous two chapters we described the reaction of bulky substrates with subtilisin and several other enzymes. In these examples the substrate range was broadened to include esters of a bulky secondary alcohol auxiliary and of tertiary alcohols. This agrees with the hypothesis that subtilisin has a large open active site capable of accepting unnatural sterically hindered molecules.

Beyond substrate specificity is another frontier in biocatalysis - catalytic promiscuity (section 1.3.3). Catalytic promiscuity is the ability of a single active site to catalyze different chemical transformations. Expanding the substrate specificity modifies the substituent, but here the type of reaction is changed so that atypical types of bonds are broken/formed with different mechanisms. Catalytic promiscuity aids in understanding the evolution of new activities, and extends enzymes' usefulness in organic synthesis.

Sulfinamides are commonly used as chiral auxiliaries for the preparation of chiral amines. In this chapter we describe a catalytic promiscuous reaction of subtilisin Carlsberg with *N*-acyl sulfinamides. These substrates contain both an amide (C-N) and sulfinamide (S-N) bond, but subtilisin hydrolyzes the unnatural S-N bond. Closely related subtilisins BPN` and E hydrolyzed the expected amide bond. Evidence is collected to confirm the unusual reaction of subtilisin Carlsberg.

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Chapter 4

Unexpected Subtilisin-Catalyzed Hydrolysis of a Sulfinamide Bond over a Carboxamide Bond in *N*-Acyl Sulfinamides

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Abstract

Subtilisin Carlsberg-catalyzed hydrolysis of *N*-chloroacetyl p-toluenesulfinamide favored cleavage of the sulfinamide (S(O)-N) bond with a minor amount (~25%) of the expected carboxamide (C(O)-N) bond. The sulfinamide hydrolysis was enantioselective (*E* ~17) and yielded remaining starting material enriched in the (*R*)-enantiomer and achiral product – sulfinic acid and chloroacetamide as confirmed by mass spectra and NMR. In contrast, the related subtilisin BPN` and E favored the carboxamide hydrolysis. Hydrolysis of the pseudo-symmetrical *N*-p-toluoyl p-toluenesulfinamide, which contains a sulfinamide and a carboxamide in similar steric and electronic environments, gave only sulfinamide cleavage (>10:1) for subtilisin Carlsberg showing that sulfinamide cleavage is the preferred path even when a similar carboxamide is available.

Introduction

Catalytic promiscuity, the ability of an active site to catalyze more than one chemical transformation,[1] contributes to the natural evolution of new enzymes[2] and can generate new useful catalysts for synthesis.[3] We report a subtilisin-catalyzed hydrolysis of a sulfinamide S-N bond in an *N*-acyl sulfinamide. This reaction is the first example of an enzymatic sulfinamide hydrolysis and also the first example where the substrate

contains both the normal functional group (carboxamide) and the unnatural functional group (sulfinamide) in similar environments, but the enzyme favors the unnatural functional group.

Proteases normally catalyze hydrolysis of the amide C-N bond, but can also catalyze hydrolysis of sulfite[4] or sulfonate esters,[5] phosphodiesters[6] or organophosphate triesters[7] or the condensation of siloxanes.[8] Peptides react via a tetrahedral intermediate while these analogs must involve alternate geometries. Catalytic promiscuity does not include irreversible inactivation of enzymes by phosphorous compounds,[9] sulfonyl fluorides,[10] β–sultams,[11] or similar inhibitors because inactivation does not regenerate the starting enzyme.

Chiral sulfinamides are versatile auxiliaries for the preparation of chiral amines.[12] Previously, we reported the subtilisin E-catalyzed enantioselective hydrolysis of *N*-chloroacetyl *p*-toluenesulfinamide, **1**, by cleavage of the carboxamide bond, path A in Figure 1a.[13] This highly (R_S)-enantioselective cleavage is a good synthetic route to enantiopure *p*-toluenesulfinamide.

Results

During this work, we discovered that a related subtilisin, subtilisin Carlsberg, which favored hydrolysis of the sulfinamide bond, path B, Figure 1. Mass spectrometry combined with ^{1}H-NMR unambiguously identified that hydrolysis of the sulfinamide bond in *N*-chloroacetyl *p*-toluenesulfinamide, **1**, was the major reaction catalyzed by subtilisin Carlsberg. Mass spectrometry identified the sulfinic acid product, while ^{1}H-NMR revealed formation of chloroacetamide from S–N hydrolysis, with a small amount of chloroacetic acid from C–N hydrolysis (1: 0.37 after 6 h), Figure 1b, indicating approximately three times more substrate reacts via the S-N bond hydrolysis than by C-N bond hydrolysis.

HPLC analysis confirmed that subtilisin Carlsberg favored the S–N bond hydrolysis by approximately fourfold over C–N bond hydrolysis and that both reactions were moderately enantioselective, Table 1. The disappearance of the starting compound *N*-chloroacetyl *p*-toluenesulfinamide, **1**, revealed the sum of both reactions. The appearance of the product *p*-toluenesulfinamide revealed the amount of C-N hydrolysis

and the difference revealed the amount of S-N hydrolysis. Control reactions without enzyme revealed ~7% spontaneous hydrolysis, mainly via S-N bond cleavage. For subtilisin Carlsberg (entry 2 in Table 1) approximately four times more substrate reacted via S-N bond hydrolysis than by C-N bond hydrolysis: 39% and 9% conversion, respectively after correcting for chemical hydrolysis. This agrees with the chloroacetamide and small amount of chloroacetic acid in the NMR and a 3-4 fold preference for the S-N reaction by Carlsberg. The S-N cleavage was moderately (R_S)-enantioselective (E = 17) yielding the remaining starting material enriched in the (S_S)-enantiomer. The products – sulfinic acid and chloroacetamide – are achiral. The C-N cleavage was moderately enantioselective (E = 6) and also favored the (R_S)-enantiomer.

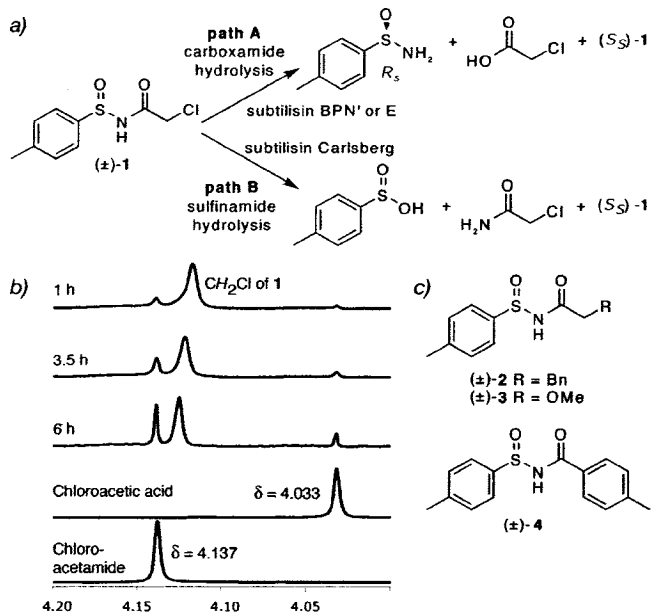

Figure 1: a) Different subtilisins favor hydrolysis of different bonds in *N*-chloroacetyl *p*-toluenesulfinamide, **1**. b) Chemical shift of the methylene protons during subtilisin Carlsberg hydrolysis of **1** showing chloroacetamide as the major product. c) Substrates **2-4**.

Two related subtilisins favored C-N hydrolysis in **1**. Subtilisin E (from *Bacillus subtilis* with 70% sequence identity to subtilisin Carlsberg) showed only a highly enantioselective hydrolysis via the C-N pathway (51% conversion, E >75, entry 3) as reported previously.[13] Subtilisin BPN' (from *Bacillus amyloliquefaciens* with 70% sequence identity to subtilisin Carlsberg and 85% sequence identity to subtilisin E[14]) also

Table 1. Chemoselectivity and enantioselectivity of subtilisin-catalyzed hydrolysis of *N*-acyl-*p*-toluenesulfinamides, **1-4.**

			Total Hydrolysis[a,b]			Sulfinamide Hydrolysis				Carboxamide Hydrolysis			
	Substrate	Subtilisin	Conv. %[b]	ee_S, %[c]	Amount[d]	% S-N[e]	ee_P Cal.[f]	E`[g]	E[h]	% C-N[i]	ee_P, %[j]	E`[g,k]	E[h,k]
1	**1**	None	7	0	0	5	0	N/A[m]	-	2	0	N/A	-
2	**1**	Carlsberg[l]	55	85 (*S*)	6	44 (39)	72 (*R*)	11	17	11 (9)	59 (*R*)	4	6
3	**1**	E	53	98 (*S*)	5	<3	N/A	-	-	53 (51)	91 (*R*)	>75	>75
4	**1**	BPN`	49	85 (*S*)	5	10 (5)	-	-	-	39 (37)	93 (*R*)	54	>75
5	**2**	Carlsberg	57	83 (*S*)	6	30	69 (*R*)	7	7	27	57 (*R*)	N/A	4
6	**2**	BPN`	30	44 (*S*)	5	<3	-	-	-	28	94 (*R*)	N/A	46
7	**3**	None	21	0	0	20	0	N/A	-	1	0	N/A	-
8	**3**	Carlsberg	49	27 (*S*)	6	39 (19)	16 (*R*)	1.5	2.2	10	80 (*R*)	10	18
9	**3**	BPN`	27	13 (*S*)	5	17 (0)	<3	N/A	-	10	90 (*R*)	20	>75
10	**4**	Carlsberg	55	21 (*S*)	20	55		2	2	<3	-	-	-

[a]All reactions performed at 25 °C in BES buffer (50 mM, pH 7.2), Reaction times: **1** = 6 h, **2** = 24h, **3** = 24 h, **4**, = 72 h. [b]Total conversion determined from the disappearance of **1-4** using biphenyl as an internal standard. Error ± ~2% conversion, based on standard deviation. [c]ee_S = enantiomeric excess of remaining starting material measured by HPLC. [d]Amount of enzyme (nmol). [e]Calculated amount of the total conversion attributed to S-N bond hydrolysis, the value with the chemical reaction subtracted is in parenthesis. [f]Calculated enantiomeric excess for the portion of **1-4** that disappeared via the S-N cleavage path. [g]E` = Observed enantioselectivity (uncorrected for spontaneous chemical hydrolysis) was calculated from conversion, c, and ee_P or ee_S according Sih.[15] [h]E = enantioselectivity, corrected for spontaneous chemical hydrolysis using the program Selectivity KreSH[17]. [i]%C-N conversion – Percentage of C-N conversion based on formation of *p*-toluenesulfinamide. [j]Enantiomeric excess of the C-N hydrolysis product, *p*-toluenesulfinamide, measured by HPLC. [k]Small errors in conversion create large errors in E when E is high. For this reason, the E-values are given as lower limits; the E-values calculated from the data are higher in some cases. [l]Three different commercial samples of subtilisin Carlsberg gave similar values. [m]Not applicable.

favored the C-N pathway (37% conversion, E >75, entry 6) with a small amount of S-N cleavage (5%, entry 6).

Two experiments showed that both the C-N and S-N hydrolysis involved the active site. (Details in Supporting Information.) First, inhibition of subtilisin with an active-site-targeted inhibitor – phenylmethyl sulfonyl fluoride – eliminated both reactions. Second, **1** competitively inhibited the hydrolysis of the *N*-suc-AAPF-*p*NA peptide substrate[17] with an inhibition constant (K_i) of 7 ± 2 mM, showing that **1** bound to the active site of subtilisin Carlsberg. The affinity of **1** for other subtilisins was similar (subtilisin BPN`: K_i = 8 ± 2 mM; subtilisin E: 12 ± 3 mM).

Subtilisin Carlsberg also catalyzed sulfinamide hydrolysis in three other *N*-acyl sulfinamides, **2-4**. The *N*-dihydrocinnamoyl acyl group in **2** mimics phenylalanine's side chain. Subtilisin Carlsberg catalyzed approximately equal amounts of sulfinamide and carboxamide hydrolysis: 30% and 27% respectively. The *N*-methoxyacetyl compound **3** was a slow substrate for subtilisin Carlsberg and gave approximately twice as much sulfinamide hydrolysis as carboxamide hydrolysis: 39% S-N hydrolysis (including 20% chemical) and 10% C-N Hydrolysis, entry 8. In contrast, subtilisin BPN' showed only carboxamide hydrolysis with compounds **2** and **3**, entries 6 and 9. The *N*-*p*-toluoyl compound **4** is pseudo-symmetric since both sulfinyl and carbonyl substituents are *p*-tolyl to test the preference for sulfinamide vs. carboxamide hydrolysis in similar steric and electronic environments. Subtilisin Carlsberg catalyzed only sulfinamide cleavage (>10:1, no detected carboxamide hydrolysis) with low enantioselectivity (E = 2), entry 10, while subtilisin BPN' showed no reaction with compound **4**.

Sulf*on*yl compounds are irreversible inhibitors of serine proteases that form stable sulf*on*yl enzyme complexes, which have been characterized by x-ray crystallography.[18] Peptides containing sulf*in*amide functionality were proposed as protease inhibitors, but synthesis was unsuccessful.[19] Sulfinamide hydrolysis by subtilisin Carlsberg may involve a sulf*in*yl serine intermediate, but we currently have no direct evidence for or against such an intermediate. Sulfatases catalyze the hydrolysis of sulfate group via a different mechanism. They contain an α-formylglycine residue, not a serine, at the active site and react via a sulfate

hemiacetal.[20] Similarly, sulfamidase catalyzes the cleavage of the S-N bond in *N*-sulfoglucosamine to give sulfate.[21]

These results clearly demonstrate that subtilisin Carlsberg catalyzes the unnatural sulfinamide cleavage and that this cleavage can be the preferred path even when a carboxamide group is available. A related example involved inactivation of the serine protease elastase with a β–sultam (β–lactam analog) where cleavage favored the strained sulfonamide over the unstrained carboxamide bond.[11] The structurally similar subtilisins BPN` and E do not cleave these sulfinamide groups, suggesting that a few amino acid substitutions may introduce this catalytic promiscuity.

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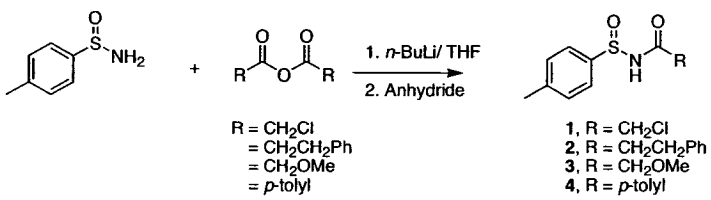
Experimental

Synthesis of *N*-acyl-*p*-toluenesulfinamides. *N*-acyl-*p*-toluenesulfinamides[22] were prepared by acylation of the corresponding *p*-toluenesulfinamide with 2 equivalents of *n*-butyl lithium and addition of the appropriate anhydride[23] to give the *N*-acyl sulfinamide, Scheme 2.



Scheme 2: Synthesis of *N*-acyl-*p*-toluenesulfinamides.

***N*-Chloroacetyl-*p*-toluenesulfinamide, 1.** White solid, 1.22 g (41%), mp 115-117 °C (lit 119-121 °C[24]); ^{1}H NMR (300 MHz, $CDCl_3$ δ): 2.43 (s, 3H), 4.29 (s, 2H), 7.31 (d, J = 8.1, 2H), 7.63 (d, J = 8.1, 2H); ^{13}C NMR ($CDCl_3$, δ): 21.8, 42.5, 124.8, 130.6, 139.9, 143.5, 167.2, HR-EIMS *m/z*: M+Na$^+$ calcd for $C_9H_{10}NO_2SNa$ 254.0023, found 254.0013.

***N*-Dihydrocinnamoyl-*p*-toluenesulfinamide, 2.** White solid, 2.31 g (63%), mp 97-99 °C (lit. 94-96 °C[22]); ^{1}H NMR (300 MHz, $CDCl_3$ δ): 2.39 (s, 3H), 2.65 (m, 2H), 2.96 (t, J = 7.5, 2H), 7.12-7.29 (m, 7H), 7.35 (d, J = 8.4, 2H), 8.39 (s, 1H); ^{13}C NMR ($CDCl_3$, δ): 21.5, 30.9, 37.7, 124.8, 126.5, 128.5, 128.7, 130.1, 140.1, 140.2, 142.6, 173.3, HR-EIMS *m/z*: M+Na$^+$ calcd for $C_{16}H_{17}NO_2SNa$ 310.0883, found 310.0881.

***N*-Methoxyacetyl-*p*-toluenesulfinamide, 3.** White solid, 520 mg (21%), mp 62-64 °C (lit. 57-59 °C[23]); ^{1}H NMR (300 MHz, $CDCl_3$ δ): δ 2.44 (s, 3H), 3.37 (s, 3H), 4.00 (s, 2H), 7.39 (d, J = 8.4, 2H), 7.63 (d, J = 8.4 2H), 8.328 (s, 1 H) ^{13}C NMR ($CDCl_3$, δ): 21.6, 59.3, 71.7, 124.7, 130.2, 140.4, 142.9, 170.6, HR-EIMS *m/z*: M+Na$^+$ calcd for $C_{10}H_{13}NO_2SNa$ 250.0519, found 250.0504.

***N*-*p*-toluoyl -*p*-toluenesulfinamide, 4.** White solid, 1.50 g (43%), mp 124-125 °C, ^{1}H NMR (300 MHz, $CDCl_3$ δ): 2.38 (s, 3H), 2.40 (s, 3H), 7.21 (d, J = 8.4, 2H), 7.29 (m, J = 8.4, 2H), 7.60 (d, J = 8.4, 2H), 7.72 (m, J = 8.4, 2H), 8.93 (s, 1 H) ^{13}C NMR ($CDCl_3$, δ): 21.5, 21.7, 125.0, 128.2, 128.9, 129.5, 130.1, 140.7, 143.6, 144.1, 167.5, HR-EIMS *m/z*: M+Na$^+$ calcd for $C_{15}H_{15}NO_2SNa$ 296.0726, found 296.0734.

Subtilisin Carlsberg. Three commercial samples of subtilisin Carlsberg were used: Fluka 82518 (11.7 U/mg), Sigma P5380 (11.4 U/mg), Sigma P8038 (9.2 U/mg), activity from the manufacturer (Sigma-Aldrich, St. Louis, MO). Electrospray ionization mass spectrometry (Q Star, Applied Biosystems, Foster City, CA) confirmed that the commercial samples were indeed subtilisin Carlsberg. The phenylmethylsulfonyl fluoride-inhibited (PMSF) enzyme revealed a molecular weight of Fluka 82518: 27440 Da, Sigma P8038: 27441 Da, Sigma P5380: 27443 Da. This

agrees with the predicted value of 27,443 Da for subtilisin Carlsberg containing an added phenylmethylsulfonyl group. For comparison the predicted molecular weight for subtilisin BPN` is 246 mass units higher - 27,689 Da. Literature ESI measurements of non-inhibited subtilisin gave molecular weights of Carlsberg: 27289 Da, and BPN`: 27536 Da.[25] The phenylmethylsulfonyl group adds a mass of 155 g/mol, giving a total molecular weight of Carlsberg: 27443 Da, and BPN`: 27690 Da.

**Preparation of Subtilisin BPN` and Subtilisin E.** Protease deficient *B. subtilis* DB104[26] was transformed with vector pS4,[27] which encodes for subtilisin BPN` and chloramphenicol resistance. Transformed cells were grown in 2XSG[28] media (250 mL) containing chloramphenicol (12.5 μg/mL) with shaking overnight at 37 °C. This culture was used to inoculate 5 L bioreactor containing the same medium. After 47 hours the culture was cooled and centrifuged at 7000 rpm/4 °C. Ammonium sulfate was added (473 g/L) and protein was precipitated by allowing the solutions to stand for 1-2 hours at 4 °C. The precipitate removed by filtration through Celite (Fisher C211) and then resuspended in buffer (20 mM Hepes-1 mM calcium chloride, pH 7.0) and filtered. Impurities in the filtrate were removed by adsorption unto Cellex-D anion exchange cellulose (at pH 7 subtilisin is not adsorbed) and re-filtered. The clarified solution placed in a 3.5 kDa cutoff dialysis membrane and dialyzed twice against buffer (20 mM Hepes-1 mM calcium chloride, 6 L, pH 7.0) overnight at 4 °C. The dialyzed protein solution was concentrated by passage through an 8 kDa cutoff ultra filtration cartridge and purified on a BioCad purification system using a Poros 20HP2 (10 mm x 100 mm) column (Applied Biosystems, Foster City, CA). The sample was loaded and washed with 1.8 M NH_4SO_4 in 20 mM HEPES (pH 7.0) and then eluted with a linear gradient of 1.8 M to 0 M NH_4SO_3 in 20 mM HEPES (pH 7.0). SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) gave one band corresponding to the mass of subtilisin. The activity of enzyme was determined using the synthetic peptide *N*-suc-AAPF-*p*NA[29] (0.2 mM, pH = 7.2, 50 mM BES buffer) and the absorbance monitored at 410 nm: BPN` 7.9 U/mg. Subtilisin E was prepared in a similar manner: 21 U/mg.[3] For comparison, commercial samples of subtilisin Carlsberg, which has a 7-fold higher k_{cat}/K_M value for this substrate,[30]

gave the following activities: Fluka 82518: 374 U/mg; Sigma P8038: 374 U/mg; Sigma P5380: 316 U/mg. Protein concentrations were determined by absorbance readings at 280 nm and subtilisin extinction coefficients[31] or using the Bradford assay.[32] Both gave similar values.

General procedure for enzymatic reactions and analysis. Enzymatic reactions were performed under the following conditions: *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer (150 μL, 50 mM, pH 7.2), **1** (10 μL, 63 mM in acetonitrile containing biphenyl (54 mM) as an internal standard) and an enzyme solution (50 μL typically 5-6 nmol subtilisin). After 6 h of stirring at 25 °C, acetonitrile (1.0 mL) was added to precipitate protein and the reaction mixture was centrifuged. The conversion was determined by reverse phase chromatography of this solution on a Zorbax SB-C18 column (4.6 mm × 250 mm, Agilent, Palo Alto, CA) with acetonitrile/water as the mobile phase, at 254 nm. A standard of known composition of *p*-toluenesulfinamide/biphenyl was used to determine the moles of sulfinamide product. At 254 nm, equimolar *p*-toluenesulfinamide/biphenyl gave an area ratio of 1: 1.94. For **1**: 50/50 MeCN/H_2O, flow 0.6 mL/min for 9 min, then gradient to 80/20 over 0.5 min; *p*-toluenesulfinamide: t_R = 5.6 min; **1**: t_R = 7.1 min; biphenyl: t_R = 15.7 min. For **2**: 60/40 MeCN/H_2O, flow 0.6 mL/min for 9 min, then gradient to 80/20 over 0.5 min; *p*-toluenesulfinamide t_R = 5.1 min; 2, *C* = 8.9 min; biphenyl t_R =18.1 min. Dihydrocinnamamide, t_R = 5.0 min. overlaps with *p*-toluenesulfinamide, but the absorbance at 254 nm, bandwidth 50, is negligible. Eluting with 25/75 MeCN/H_2O, flow 0.6 mL/min gives *p*-toluenesulfinamide t_R = 13.1 min and dihydrocinnamamide, t_R = 13.4 min. Only subtilisin Carlsberg shows dihydrocinnamamide product; none was detected for subtilisin BPN`. For **3**: 45/55 MeCN/H_2O, flow 0.6 mL/min for 9 min, then gradient to 80/20 over 0.5 min; *p*-toluenesulfinamide: t_R = 5.8 min; **3**: t_R = 6.3 min; biphenyl: t_R = 20.4 min. For **4**: 65/35 MeCN/H_2O, flow: 0.6 mL/min for 9 min, then gradient to 80/20 over 0.5 min; *p*-toluenesulfinamide and *p*-toluamide: t_R = 4.6 min for both; **4**: t_R = 7.4 min; biphenyl: t_R = 15.8 min. To separate *p*-toluenesulfinamide and *p*-toluamide: 25/75 MeCN/H_2O, flow: 0.65 mL/min; *p*-toluamide: t_R = 11.0 min; *p*-toluenesulfinamide: t_R

= 11.9. There was no *p*-toluenesulfinamide detected for reaction with subtilsisin Carlsberg. To determine the enantiomeric excess the reaction was extracted with EtOAc (3 × 0.75 mL), dried with $MgSO_4$ and evaporated and analyzed using a chiral stationary phase: Chiralpak AD-H or Chiralcel OD columns (4.6 mm × 25 cm, Chiral Technologies, West Chester, PA) at 230 nm. An achiral Discovery Cyano column (4.6 mm × 25 cm, Supelco, Bellefonte, PA) was placed before the chiral column in cases where the sulfinamide and *N*-acyl sulfinamide enantiomers overlapped. For **1**: 80/20 Hexanes/EtOH, flow 0.75 ml/min, Cyano column and Chiralpak AD-H. *p*-toluenesulfinamide, (*S*) t_R = 20.5 min, (*R*) t_R = 16.5 min; **1**: (*R*) t_R = 22.0 min, (*S*) t_R = 29.4 min. For **2**: 90/10 Hexanes/EtOH, flow 1.0 mL/min, Chiralpak AD-H. *p*-toluenesulfinamide, (*S*) t_R = 13.0 min, (*R*) t_R = 19.2 min; **2**: (*R*) t_R = 15.1 min, (*S*) t_R = 30.3 min; dihydrocinnamamide, t_R = 10.9 min. For **3**: 85/15 Hexanes/EtOH, flow 0.75 mL/min, Cyano column and Chiralcel OD. *p*-toluenesulfinamide, (*R*) t_R = 17.0 min, (*S*) t_R = 18.9 min; **3**: (*R*) t_R = 21.3 min, (*S*) t_R = 23.9 min. For **4**: 85/15 Hexanes/EtOH, flow 0.75 mL/min, Cyano column and Chiralpak AD-H. *p*-toluenesulfinamide, (*S*) t_R = 18.5 min, (*R*) t_R = 23.4 min; **4**: (*S*) t_R = 27.5 min, (*R*) t_R = 36.4 min; *p*-toluamide: t_R = 18.1 min. The *p*-toluamide overlaps with (*R*)-*p*-toluenesulfinamide on this chiral column, but there is none present as determined by reversed phase HPLC. The *E* value was determined from the conversion and enantiomeric excess according to:

$$E = \frac{\ln[1 - c(1 + ee_P)]}{\ln[1 - c(1 - ee_P)]} \text{ or } E = \frac{\ln[1 - c(1 - ee_S)]}{\ln[1 - c(1 + ee_S)]}$$

where *E* is the enantioselectivity, c is the conversion, ee_p = enantiomeric excess of product and ee_s = enantiomeric excess of remaining starting material.[33] The enantioselectivity for the C-N cleavage was calculated from the enantiomeric purity of the product *p*-toluenesulfinamide and the conversion that corresponds to the C-N cleavage reaction. The enantioselectivity for the S-N cleavage was calculated from the enantiomeric purity of the remaining starting material, which was corrected for the amount of each enantiomer that reacted to yields *p*-toluenesulfinamide, and from the conversion that corresponds to the S-N cleavage reaction. Both enantiomeric purities were corrected for the small amount of spontaneous hydrolysis. This

calculation is an approximation because both enantioselective steps act simultaneously upon the starting material. The E value is a lower limit of the theoretical value if one path could be suppressed.

Inhibition of reaction with PMSF. Reactions of **1** carried out under the above conditions using enzyme treated with PMSF (phenylmethane sulfinyl fluoride[34]), (5 μL, 120 mM in MeCN) gave reactions identical to those with no enzyme added.

NMR reaction. NMR was used to determine the products of the subtilisin Carlsberg reaction. Reactions conditions were as follows: D_2O (1 mL) containing phosphate buffer (20 mM, pD 7.4, pH+0.4) and subtilisin Carlsberg (7.6 mg, Fluka 82518), d_3-MeCN (100 μL), (±)-**1** (160 mM) and sodium 4,4-dimethyl-4-silapentane-1-sulfonate,[35] (DSS, 1 mM). The reaction was monitored over the course of 6 h using a 500 MHz instrument. Under these conditions the α-CH_2Cl group has the following 500 MHz ^{1}H resonances relative to DSS: Chloroacetic acid δ = 4.033, chloroacetamide δ = 4.137 and **1** δ = 4.110. The major product was chloroacetamide (relative area = 1), while a small amount of chloroacetic acid (relative area = 0.37) was detected. A blank reaction with no enzyme showed no detectable change over 6 h. Over the course of the reaction the pH changes slightly due to the acid produced. This causes a slight shift in the α-CH_2Cl group of **1** as seen in Figure 1b. For the enzymatic reaction at 1 h, δ = 4.117. 3.5 h δ = 4.121 and at 6 h δ = 4.124. The chemical shift of the α-CH_2Cl group of **1** as a function of pH is shown in Figure 2 showing that the change in chemical shift in Figure 1b is entirely due to the decrease in pH.

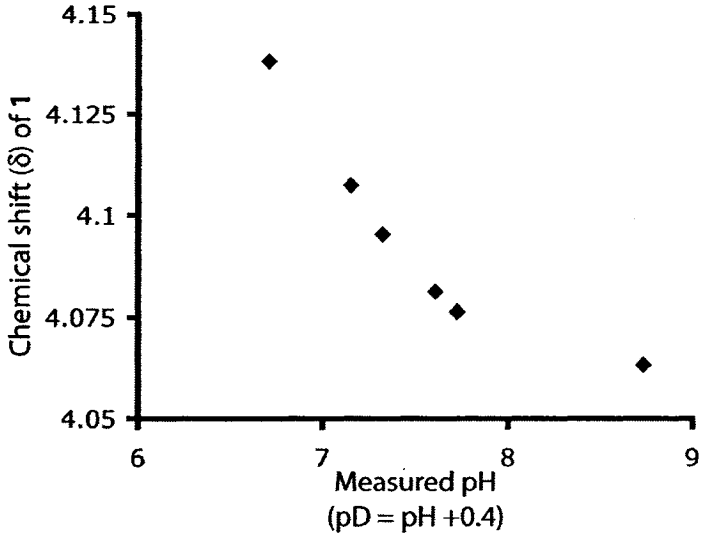


Figure 2: Effect of pH on the chemical shift on the methylene of **1** in D_2O.

Isolation of *p*-toluenesulfinic acid. Compound **1** (10 mg in 0.7 mL MeCN, 0.45 mmol) in BES buffer (9.3 mL, 20 mM, pH 7.2) was stirred with subtilisin Carlsberg. After 6 h the reaction was extracted with methylene chloride followed by acidification of the buffer layer with HCl (0.1 N). The aqueous layer was extracted with ether (2 x 15 mL), dried over $MgSO_4$ and concentrated to yield a white powder (2.1 mg, 60%). TLC (90/10 $CHCl_3$/HCOOH): $R_f = 0.3$; ^1H NMR (400 MHz): δ 2.45 (s, 3 Hz), δ 7.4 (d, $J = 8.2$ Hz), δ 7.6 (d, $J = 8.2$ Hz); HRMS-EI (*m/z*): M+ calcd for $C_7H_8O_2S$: 156.02450, found 156.02450.

Kinetics and inhibition. The inhibition constant (K_I) for the hydrolysis of the synthetic peptide *N*-suc-AAPF-pNA[29] was measured for subtilisins Carlsberg, E, and BPN`.[36] The inhibition constant for the hydrolysis of the peptide *N*-suc-AAPF-pNA by **1** was measured in a 96 well plate. In each well was placed enzyme solution (10 μL) and assay solution containing the peptide and **1** (90 μL). The assay solution consisted of buffer (81 mL, 50 mM BES, pH 7.2), acetonitrile (8.2 μL), and DMSO (0.8 μL). The concentration of the peptide was varied from 0.1 mM to 1.6 mM, from a 200 mM stock in DMSO. The inhibitor, **1**, was varied from 0 mM to 7.2 mM (the solubility limit). Each measurement was preformed in triplicate following the release

of *p*-nitroanaline at 410 nm ($\varepsilon = 8800\ M^{-1}cm^{-1}$) over the first 0-35 s. Non-linear curve fitting was preformed on the rate vs. [S] plots using the OriginPro 7.5 software (OriginLab Corporation, Northampton, MA) to determine V_{max} and K_M (inset of Figures S-2 – S-4). A replot of inhibitor V_{max}/K_M gave the K_I values according to the equation:

$$slope_{(1/s)} = \frac{K_{mapp}}{V_{max}} = \frac{K_m}{V_{max}K_I}[I] + \frac{K_m}{V_{max}}$$

Where K_{Mapp} is the apparent K_M and slope (1/s) is the slope of the reciprocal Lineweaver-Burk plot ($1/v$ vs. 1/[S]) for each inhibitor concentration. This plot gives the x-intercept as $-K_I$.[37]

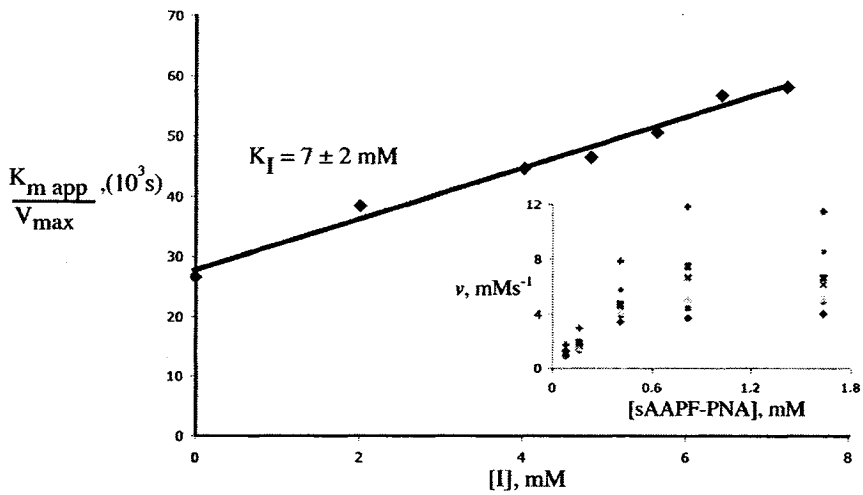


Figure S-2: Replot to determine inhibition constant of subtilisin Carlsberg by **1**. Inset: rate (v) vs. *N*-suc-AAPF-pNA peptide concentration at varying inhibitor concentrations.

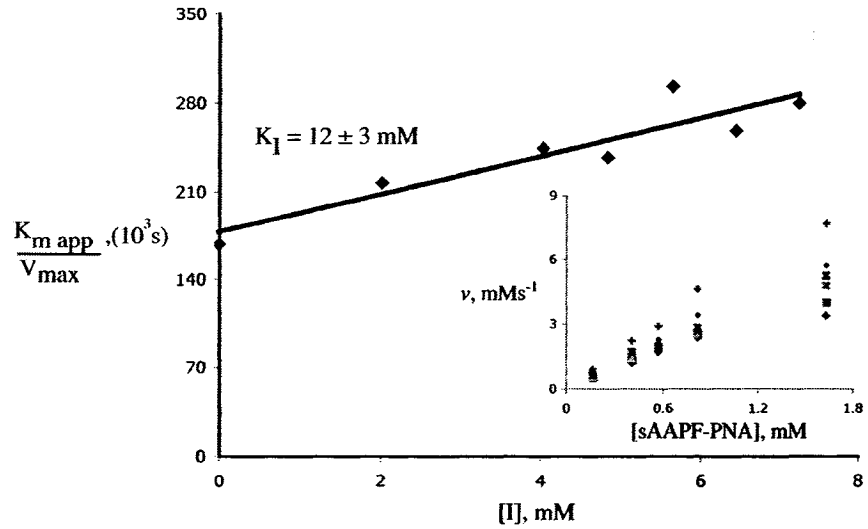


Figure S-3: Replot to determine inhibition constant of subtilisin E by **1**. Inset: rate (v) vs. *N*-suc-AAPF-pNA peptide concentration at varying inhibitor concentrations.

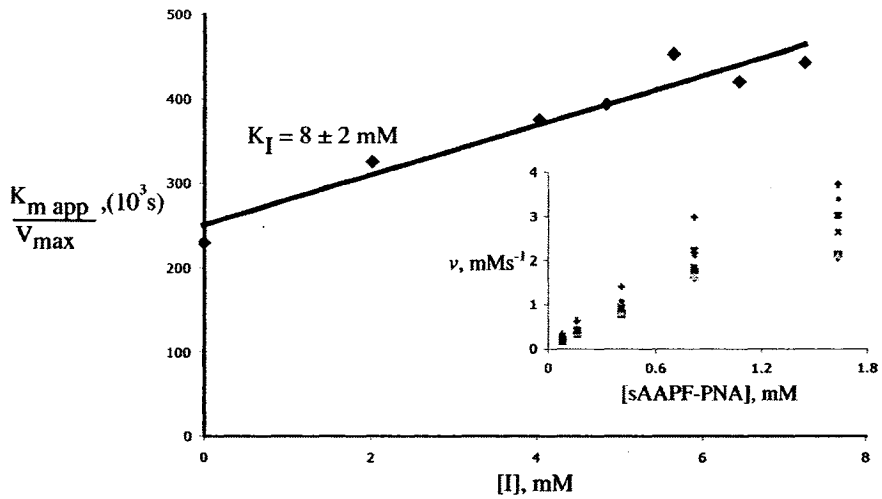


Figure S-4: Replot to determine inhibition constant of subtilisin BPN` by **1**. Inset: rate (v) vs. *N*-suc-AAPF-pNA peptide concentration at varying inhibitor concentrations.

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Chapter 5

In the previous chapter, we examined the catalytic promiscuous reaction of subtilisin Carlsberg with *N*-acyl sulfinamides. Unexpectedly, subtilisin hydrolyzed the unnatural S-N sulfinamide bond rather than the C-N amide bond.

In this chapter we continue this investigation into subtilisin Carlsberg's catalytic promiscuous reaction with *N*-acyl sulfinamides. We identify an intermediate of the reaction and examine the pH dependence for subtilisin BPN` and Carlsberg. Further substrates, including sulfinates are tested, and mutation reveals only a few amino acid substitutions are responsible for the promiscuity.

Chapter 5

Subtilisin-Catalyzed Hydrolysis of *N*-Acyl Sulfinamides and Sulfinates

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Abstract:

N-acyl sulfinamides have two potential sites for hydrolysis – either the C-N amide bond or the S-N sulfinamide bond may be cleaved. Subtilisin, BPN' catalyzes the natural C-N hydrolysis in these substrates. In contrast the protease subtilisin Carlsberg catalyzes an unexpected promiscuous reaction by hydrolyzing the S-N bond in *N*-acyl sulfinamides. Electrospray-mass spectrometry revealed a sulfinyl-enzyme intermediate located at the active site, analogous to an acyl-enzyme. Substrate variation indicated a substrate reversal was responsible for the change in reactivity by binding in the S_1 acyl pocket. The enzymatic hydrolysis of sulfinates supported this hypothesis. Three mutations of subtilisin BPN' towards subtilisin Carlsberg increased the S-N hydrolysis by 14-fold, indicating that only a few mutations were responsible for the catalytic promiscuity. The substrate specificity and mutagenesis were consistent with reversed orientation for sulfinyl reactions.

Introduction

Catalytic promiscuity is the ability of an active site to catalyze more than one chemical transformation.[1] For example, proteases normally catalyze hydrolysis of the amide C-N bond, but can also catalyze hydrolysis of analogs. Pepsin, an aspartate protease, catalyzes hydrolysis of several sulfite esters.[2] α-Chymotrypsin, a serine protease, catalyzes the hydrolysis of cyclic sulfonate esters,[3] the aminopeptidase from *Streptomyces grieus*, a dizinc metalloenzyme, catalyzes the efficient hydrolysis of phosphodiesters.[4] Two proline-specific proteases, manganese dependent enzymes, also hydrolyze organophosphate triesters, giving them flexibility to degrade highly toxic nerve agents and pesticides as a second function.[5] These examples are unusual because peptides react via a tetrahedral intermediate while sulfites, sulfonates, phosphodiesters and phosphotriesters {or sulfur and phosphorous substrates} must have an alternate (trigonal bipyramidal) intermediate. Catalytic promiscuity does not include irreversible inactivation of enzymes by phosphorus compounds,[6] sulfonyl fluorides,[7] β–sultams,[8] and similar inhibitors because inactivation does not regenerate the starting enzyme. Catalytic promiscuity is important to understand the evolution of new enzymes[9] and to generate new useful catalysts for synthesis.[10]

Sometimes an unmodified enzyme exhibits two functions, or a slightly modified enzyme may perform the promiscuous function. Often only a few mutations are necessary to introduce a new function in an enzyme. A typical example is the evolution of melamine hydrolyase to an enzyme that degrades the pesticide atrazine with only 8 amino acid substitutions.[11] Another example is a four amino acid modification of a fatty acid desaturase to produce an efficient hydroxylase.[12] Adding four key catalytic mutations and an additional α–helix modified a transport protein with no activity to a structurally related dehydratase with low activity.[13] Thus catalytic promiscuity is often introduced by a few mutations to a structurally related enzyme.

The chiral sulfinyl group is a versatile chiral director in C-C and C-X bond formations.[14] Chiral sulfinamides are versatile auxiliaries for the preparation of chiral amines.[15] Chiral sulfoxides are common intermediates to control stereochemistry in the totally synthesis of many natural products.[16] The most frequent precursors to sulfoxides and sulfinamides are chiral sulfinates. There are few biocatalytic methods to prepare

chiral sulfinyl compounds. Oxidation of disulfies with cyclohexanone monooxygenase was generally gave low enantiomeric purity.[17] Resolutions of several *N*-acyl sulfinamides by the protease subtilisin E gave sulfinames in high enantiopurity.[18]

Subtilisin (E.C. 3.4.21.62) is a common serine protease for which the structure, the mechanism, and the substrate preference are well understood.[19] Subtilisin is used in detergents and organic synthesis, in particular for organic solvent resolution of alcohols and amines.[20] Protein engineering has modified many properties of subtilisin: thermostability, pH dependence, organic solvent stability and substrate specificity.[21]

Previously we reported the subtilisin catalyzed sulfinamide (S-N) bond hydrolysis of *N*-chloroacetyl-*p*-toluenesulfinamide by subtilisin Carlsberg.[22] In contrast, the related subtilisins E and BPN` hydrolyzed only the carboxamide (C-N) bond with high enantioselectivity ($E > 100$) to give *p*-toluene sulfinamide and enantioenriched starting material.

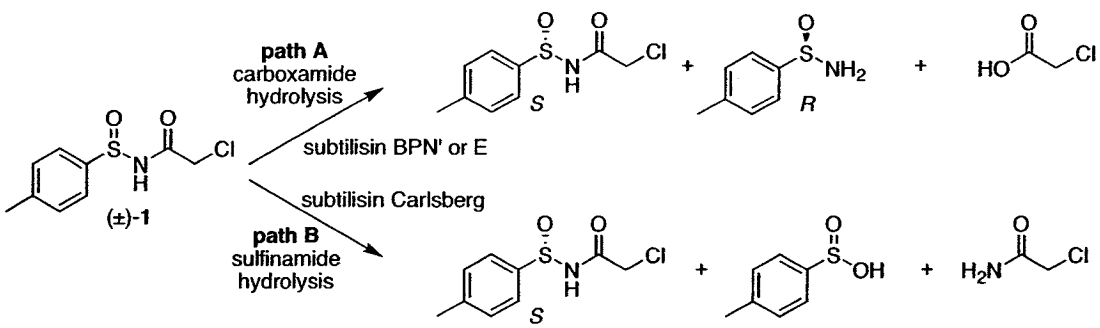

Figure 1: Different subtilisins hydrolyze different bonds in of *N*-chloroacetyl-*p*-toluenesulfinamide

In many cases the molecular basis for the promiscuous function is either not examined, or poorly understood. Sometimes the structure of the enzyme is unknown which prevents interpretation of the promiscuous reaction. This paper focuses on understanding the promiscuous Carlsberg reaction, and explaining the specific amino acid substitutions that cause subtilisins to catalyze different reactions.

Results

Subtilisin-catalyzed hydrolysis of carboxamide bonds involves an acyl enzyme intermediate. The active site serine attacks the carboxamide carbonyl group to form a tetrahedral intermediate, Figure 2. Next, this tetrahedral intermediate collapses as the

active site histidine protonates the leaving group nitrogen. This loss of the amine leaving group yields an acyl enzyme intermediate. Next, water attacks the acyl enzyme to form a second tetrahedral intermediate, which collapses by release of the acyl group as a carboxylic acid and leaves subtilisin ready for the next hydrolysis.

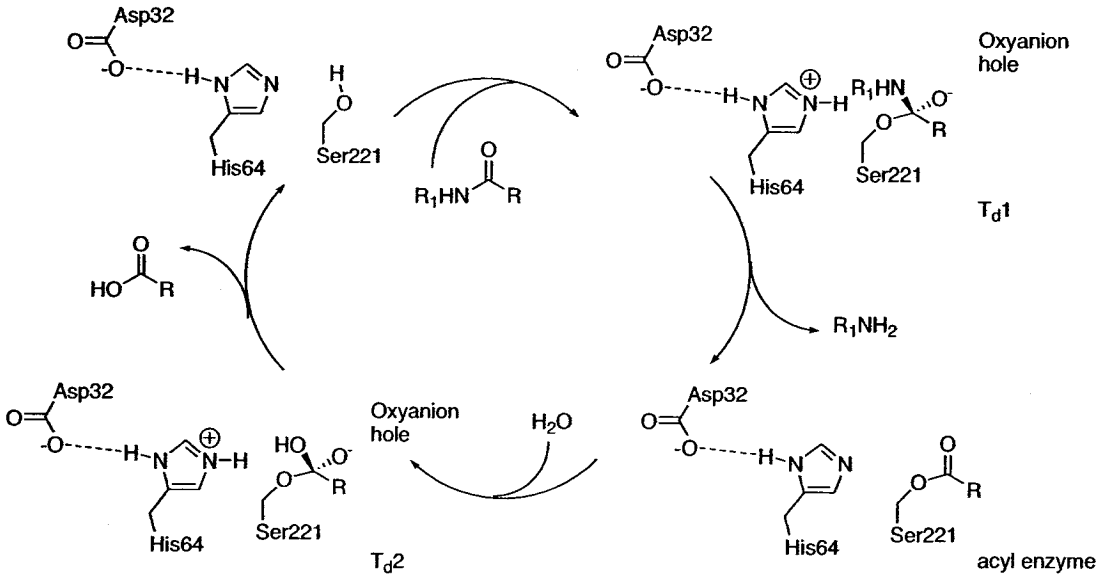

Figure 2. Mechanism of subtilisin-catalyzed hydrolysis of carboxamide bond involves an acyl enzyme intermidate.

The simplest hypothesis for the subtilisin-catalyzed hydrolysis of the sulfinamide link in compound **1** is that it proceeds via a mechanism analogous to that for hydrolysis of the carboxamide link. This hypothesis predicts that the reaction should form a sulfinyl enzyme intermediate analogous to the acyl enzyme intermediate.

Electrospray mass spectrometry can detect an acyl enzyme intermediate in subtilisin catalyzed hydrolysis of carboxamide links. Incubation of acryloyl imidazole with subtilisin at pH to 5 to slow hydrolysis of the cinnamoyl enzyme intermediate showed that ~ 90% of the substilisin was acylated.[23] There are several other examples of acyl enzymes identified by ESI-MS. The serine β-latamase forms an acyl enzyme with four different β–lactam substrates at low pH.[24] Acyl enzymes were directly observed for porcine pancreatic elastase and a heptapeptide susbtrate,[25] α–chymotrypsin and isatoic anhydride.[26]

Electrospray mass spectrometry revealed a similar sulfinyl subtilisin intermediate upon incubation of compound **1** with subtilisin Carlsberg at pH 7 followed by acidification to pH 1. The free enzyme (predicted mass 27,286 amu) appears at 27284 amu, Figure 2a. Several sodium adducts were also present above this peak at 27,305 (+ 21 amu) and 27,323 amu (+ 42 amu).[a] Another major peak occurs at 27,515 amu (M +231 amu), corresponding to subtilisin bound to substrate **1**. This binding is likely an ion pair with the enzyme, see below. Two smaller peaks occur at the predicted mass for a sulfinyl enzyme: a peak appears at 27,422 amu, which is 138 mass units above the free enzyme and as expected for the sulfinyl enzyme intermediate (+139 units for the *p*-tolulenesulfin group, minus 1 unit for the serine proton). Another peak appears at 27653, which is a further 138 units above the non-specific binding peak. This peak corresponds to a sulfinylated enzyme with substrate non-specifically bound to it.

Two control experiments confirm this interpretation. First, subtilisin Carlsberg inhibited with PMSF (phenylmethane sulfonyl fluoride[27]) and then treated with **1** did not give a sulfinylated enzyme peak, Figure 2b. The substrate still bound in a non-specific fashion (M + 231), but **1** did not form sulfinyl-enzyme peaks (M + 138, or M + PMS + 138). This demonstrates that the sulfinylation occurs at the active site; the inhibitor PMSF covalently modifies the catalytic serine in the active site. Secondly, using the phenyl substrate **2** lowered the sulfinyl-subtilisin peak by the expected 14 mass units as compared to the *p*-tolyl substrate **1**. Thus subtilisin Carlsberg treated with **2** gave a phenylsulfinyl peak (M + 124). The mass spectral data provides strong evidence for a sulfinyl-subtilisin intermediate in the active site, but does not prove attachment at the serine or reveal the nature of the intermediate.

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[a] The expected mass of the sodium adduct is M + 22 due to a loss of hydrogen of mass 1 and a gain of sodium ion of mass 23. The observed mass of M + 21 is likely due to errors in mass measurement.

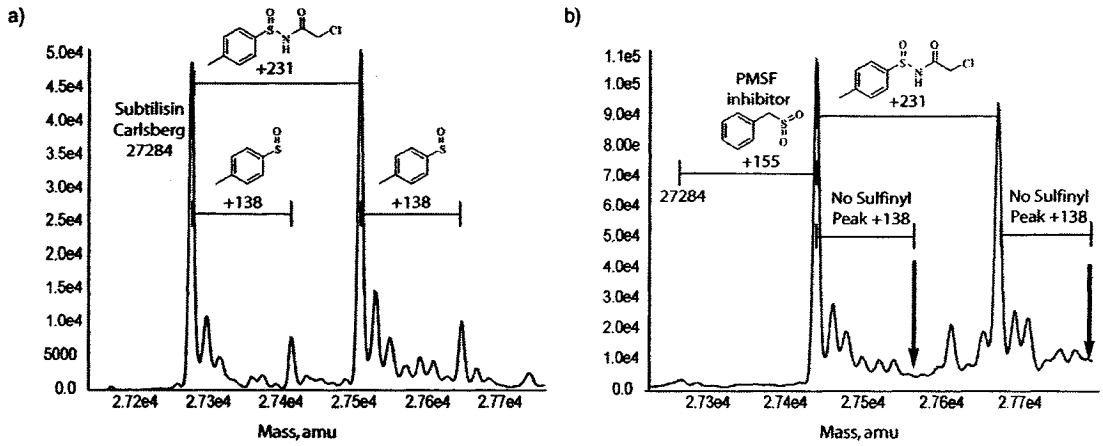


Figure 3: Electrospray mass spectra of subtilisin Carlsberg treated with **1**. (a) The peak at M+138 corresponds to the expected mass for a *p*-tolylsulfinyl-subtilisin intermediate; the peak at M+231 corresponds to the expected mass for an adduct of **1** with subtilisin; the peak at M+231+138 corresponds to an adduct of **1** and the *p*-tolylsulfinyl-subtilisin intermediate. (b) Control experiment with Carlsberg pretreated with the active-site targeted-inhibitor phenylmethane sulfonyl fluoride (PMSF). The peak at M+155 corresponds to phenylmethane sulfonyl subtilisin; the peak at M+155+231 corresponds to an adduct of **1** with phenylmethane sulfonyl subtilisin. No *p*-tolylsulfinyl-subtilisin intermediate forms in this case because the inhibitor blocks the active site.

The pH profiles for hydrolysis of **1** suggest similar mechanisms for hydrolysis of carboxamide and sulfinamide links. Subtilisin Carlsberg- and subtilisin BPN` favor hydrolysis of different links, but show similar bell-shaped pH rate profiles for **1** (Figure 4, traces a and b). The conversion is low at pH below 4.5, rises to a maximum pH 6.5 and decreases to less than 10% conversion above pH 8.5 (Figure 4, a and b). This profile suggests the reaction involves at least two ionizable groups and that they are in the correct protonation state near pH 6.5. We suggest that the decrease in activity of the subtilisins toward **1** above pH 6.5 is due to ionization of the substrate. The N-H proton is acidic (pK_a = 6.5) and deprotonation gives a negatively charged species that is less likely to bind to subtilisin. The similar pH profiles for both the natural (C-N) and unnatural (S-N) N-acyl sulfinamide hydrolyses suggests similar mechanisms for the two reactions. Unlike typical amide or ester hydrolysis, the C-N and S-N hydrolysis still occurs pH 5 -6. Attempts to perform ESI-MS experiments below pH 5 did not give any sulfinyl enzyme intermediate.

In contrast, a typical peptide substrate does not show a bell-shaped pH profile, but a plateau at high pH. For example, the rate acceleration (k_{cat}/K_m) of subtilisin BPN`-

catalyzed hydrolysis of *N*-succ-PFAA-pNP increases near pH 7 and reaches a plateau above pH 8.5 (Figure 4, trace c). The increase in rate near pH 7 is attributed to the deprotonation of the catalytic histidine ($pK_a = 7.17$).[28]

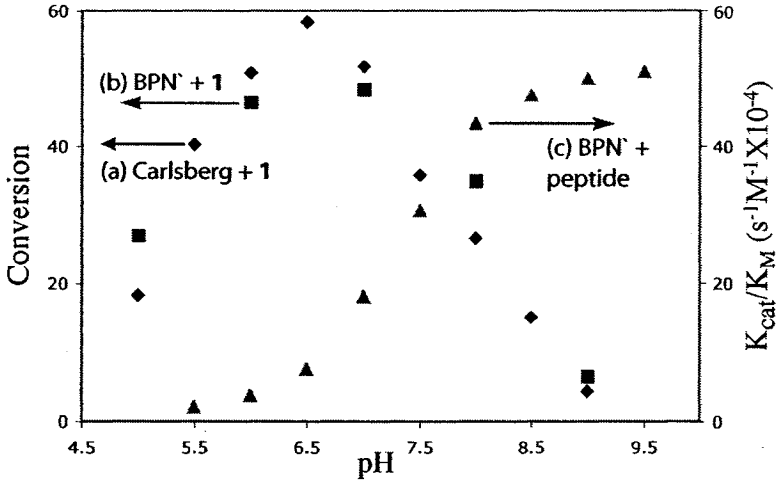


Figure 4: Conversion as a function of pH for the hydrolysis of **1** by (a) ◆subtilisin Carlsberg (mainly S-N hydrolysis) and (b) ■ Subtilisin BPN` (mainly C-N hydrolysis) (c) Subtilisin BPN` pH profile (▲) for a typical peptide substrate *N*-succ-PFAA-pNP included for comparison.[28]

Substrate orientation in the active site

The catalytically productive orientation of carboxamides in the active site of subtilisin places the acyl moiety and the leaving group amine into specific pockets. The acyl moiety binds in the S_1 site according to the protease binding site nomenclature,[29] while the leaving group binds in the S_1' site, Figure 5a. This orientation positions the leaving group nitrogen near the active site histidine so that it can protonate the leaving group. The specificity of proteases for particular peptide links stems primarily from the S_1 site. Subtilisin favors amino acids with large, non-polar side chains such as phenylalanine as the acyl group. This preference is consistent with size and the nonpolar nature of the S_1 site in subtilisin.

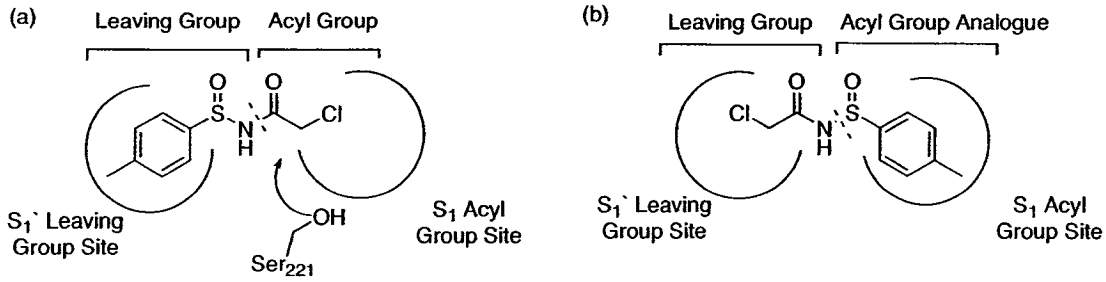


Figure 5. Proposed reversal in substrate orientation to account for the subtlisin-catalyzed hydrolysis of the carboxamide link and the sulfinamide link in compound **1**. (a) Substrate positioned for C-N hydrolysis, with the conventional acyl and leaving group positioning (b) Corresponding orientation for the S-N hydrolysis, in which the substrate reverses to favor the alternate S-N hydrolysis.

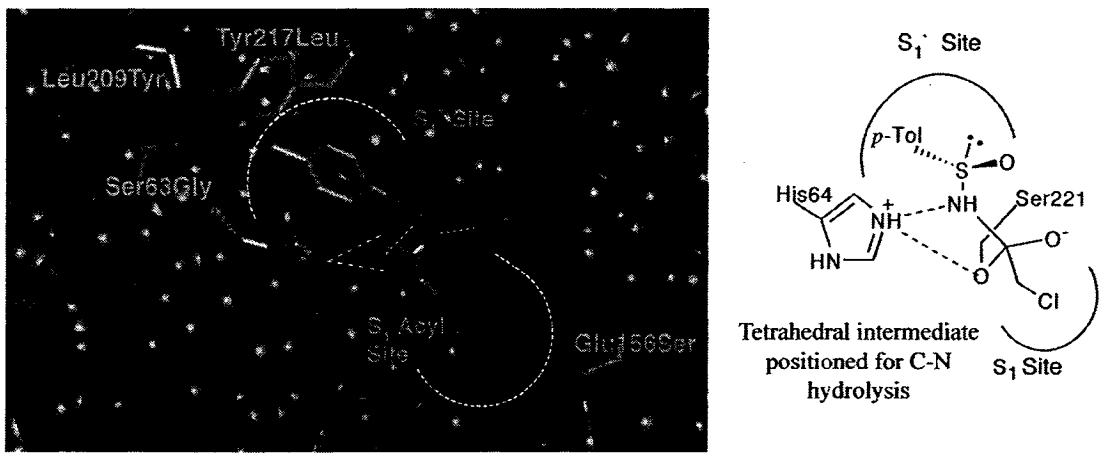


Figure 6: Molecular model of the first tetrahedral intermediate for C-N hydrolysis of *N*-chloroacetyl-*p*-toluene sulfinamide **1** in the active site of subtilisin BPN' (compare with the orientation in Figure 1a). The four positions selected for mutation in BPN' are indicated: Leu209Tyr indicates a mutation from the natural leucine in BPN' to tyrosine. Tyrosine is found at position 209 in subtilisin Carlsberg. Residues 217, 209, and 63 in the S_1' leaving site, as well as residue 156 in the S_1acyl site differ in subtilisins Carlsberg and BPN'.

Formation of the sulfinyl enzyme intermediate from **1** by a mechanism similar to that for formation of an acyl enzyme intermediate requires that the sulfinamide **1** turn around in the active site, Figure 5b. Although simply sliding the substrate to the right in Figure 5a could position the sulfur near the active site serine, it would not position the leaving group nitrogen near the active site histidine to allow it to protonate the leaving group.

We hypothesize that the substrate reverses in the active site. Variation of the substrate structure changed the ratio of sulfinamide to carboxamide hydrolysis in ways that are consistent with a reversal of orientation for two hydrolyses.

We used a 2-phenylethyl substituent to vary the preferred substrate orientation in the active site. Subtilisin favors binding a 2-phenylethyl substituent in the S^1 acyl site because it mimics phenylalanine. We hypothesized that a 2-phenylethyl substituent on the sulfinyl group would favor a substrate orientation for sulfinamide hydrolysis. The ratio of sulfinamide to carboxamide hydrolysis for compounds **3** and **4** support this hypothesis. Compound **3**, which contains a 2-phenylethyl substituent on the sulfinyl group, gave higher ratio of sulfinamide hydrolysis (4: 1) as compared to compound **4** (1:1), which contains a *p*-tolyl substituent, Table 1. Both compounds **3** and **4** reacted at an intermediate rate. Compounds **5** and **6** also differ by a *p*-toluene and 2-phenylsulfinyl but reacted slowly and showed only sulfinamide hydrolysis (>10:1) so any difference in the ratio was outside the detection limit. Compounds **1** and **2** compared a phenyl vs. *p*-tolyl substitutent on the sulfinyl. Both compounds reacted quickly and showed the same 4:1 ratio of sulfinamide to carboxamide hydrolysis.

To further test this hypothesis, we added the 2-phenylethyl substituent to the carbonyl group (corresponding to a dihydrocinnamoyl group) and predicted that it would favor carboxamide hydrolysis. We compared the ratio of sulfinamide to carboxamide hydrolysis in **1, 4 & 6-10**, which all contain *p*-tolyl sulfinamide moiety and differing acyl groups. The *N*-dihydrocinnamoyl compound **4** showed the lowest ratio of sulfinamide to carboxamide hydrolysis (1:1), consistent with the preference of the dihydrocinnamoyl group for the S_1 acyl pocket. Compounds **1** and **7-10** have similar small acyl groups and show similar amounts of sulfinamide hydrolysis (from 4:1 to 2:1). Compounds **1** and **7** contain electron-withdrawing substituents, but these did not change the ratio of sulfinamide to carboxamide hydrolysis as compared to compound **8-10** without electron-withdrawing substituents. Compounds **1** and **7** did react significantly faster than compounds **8-10**. These experiments show that the ratio of sulfinamide to carboxamide hydrolysis does not vary upon adding electron-withdrawing substituents, but does vary upon addition of the 2-phenylethyl substituent.

The enantioselectivity was low for most sulfinamide hydrolyses, despite the fact that the reaction occurs at the sulfur stereocenter. One exception was **5**, which had an enantioselectivity greater than 50 (see Table 1).

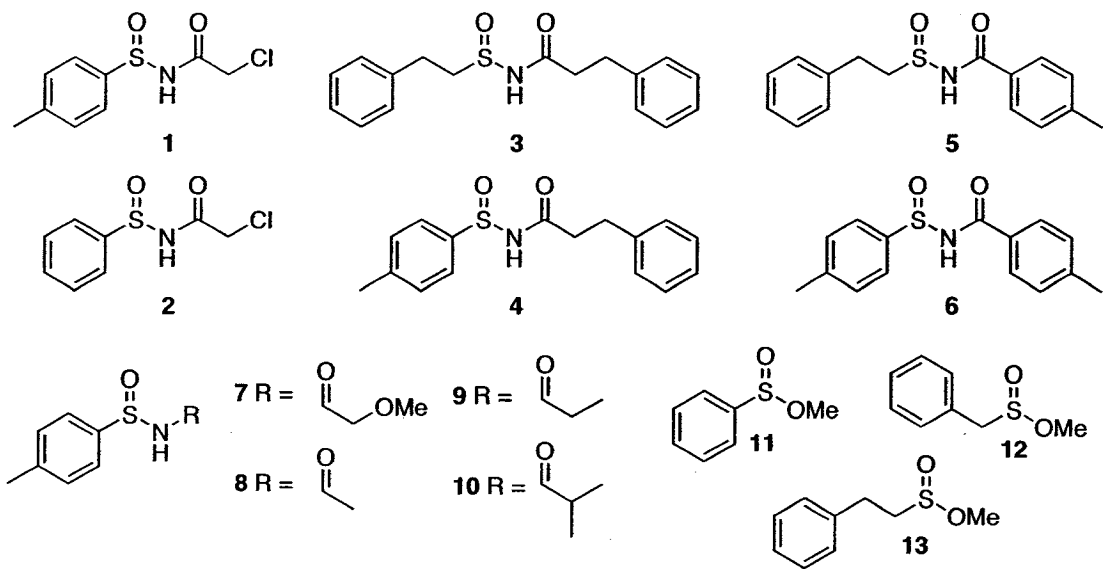


Figure 7: Substrates to test substrate reversal hypothesis with subtilisin Carlsberg by measuring the S-N:C-N hydrolysis ration in N-acyl sulfinamides. Substituents that mimic the phenylalanine side chain (CH_2CH_2Ph) generally bind in the S_1 acyl site. Compounds **1-6** are three pairs in which the acyl group is held constant while the sulfinyl group is varied. Compounds **7-10** expand the acyl group range in the *N*-acyl-*p*-toluenesulfinamides. The sulfinates **11-13** were also tested for enzymatic hydrolysis.

Table 1: *N*-acyl sulfinamides: Total enzymatic hydrolysis and component S-N and C-N values

		Total Hydrolysis[a,b]			Sulfinamide Hydrolysis				Carboxamide Hydrolysis				Ratio
	Subtstrate	Conv. %[b]	Time/ rate[c]	ee_S, %[d]	% S-N[e]	ee_P Cal.[f]	E`[g]	E[h]	% C-N[i]	ee_P, %[j]	E`[g]	E[h]	*S-N:C-N*
1	**1**	55	6, fast	85 (*S*)	44 (39)	72 (*R*)	11	17	11 (9)	59 (*R*)	4	6	4:1
2	**2**	53	6, fast	44 (*S*)	43 (36)	29 (*R*)	2	2	10 (9)	80 (*R*)	10	18	4:1
3	**3**	30	24, int.	37	23 (21)	-	-	-	7 (5)	-	-	-	4:1
4	**4**	57	24, int.	83 (*S*)	30	69 (*R*)	7	7	27	57 (*R*)	N/A[k]	4	1:1
5	**5**	18	72, slow	17	18 (14)	-	9.3	>50	0	-	-	-	>10:1
6	**6**	55	72, slow	21 (*S*)	55	-	2	2	<3	-	-	-	>10:1
7	**7**	49	24, fast	27 (*S*)	39 (19)	16 (*R*)	1.5	2.2	10 (9)	80 (*R*)	10	18	2:1
8	**8**	21	72, slow	8	20 (10)				<3	-	-	-	~4:1
9	**9**	40	72, slow	38	30 (18)	52	4	4	10	71	N/A	6	2:1
10	**10**	23	72, slow	10	19 (13)	25	2	2	4	75	N/A	7	3:1

[a]All reactions performed at 25 °C in BES buffer (50 mM, pH 7.2), [b]Total conversion determined from the disappearance of starting material using biphenyl as an internal standard. Error ± ~2% conversion, based on standard deviation. [c]Approximate rate from conversion and time (h) and grouped into fast, intermediate, and slow categories. [d]ee_S = enantiomeric excess of remaining starting material measured by HPLC. [e]Calculated amount of the total conversion attributed to S-N bond hydrolysis, the value with the chemical reaction subtracted is in parenthesis. [f]Calculated enantiomeric excess from the portion of substrate that disappeared via the S-N cleavage path. [g]E` = Observed enantioselectivity (uncorrected for spontaneous chemical hydrolysis) was calculated from conversion, c, and ee_P or ee_S according Sih.[30] [h]E = enantioselectivity, corrected for spontaneous chemical hydrolysis using the program Selectivity KreSH[31]. [i]%C-N conversion – Percentage of C-N conversion based on formation of sulfinamide. [j]Enantiomeric excess of the C-N hydrolysis sulfinamide product, measured by HPLC. [k]Not applicable.

We tested several sulfinates (ester analogues), but only the aliphatic dihydrocinnamoyl analogue **13** gave enzymatic hydrolysis. Thus choosing a complementary acyl analogue to match the enzyme active site proved critical to enhance the promiscuous reaction. Subtilisin Carlsberg catalyzed hydrolysis of the dihydrocinnamoyl sulfinate, **13**, with high enantioselectivity, E >100, Table 2. The absolute configuration of the favored enantiomer was (*R*)-**13**, which has the same three-dimensional arrangement as the N-acyl sulfinamide above. This is the first example of enzymatic sulfinate hydrolysis by a serine hydrolase, or by any hydrolase. The dihydrocinnamoyl group mimics phenylalanine and binds well to the S_1 acyl pocket. Subtilisin Carlsberg did not catalyze hydrolysis of the corresponding benzyl sulfinate **12** or phenyl sulfinate **11**.

Table 2: Subtilisin Carlsberg catalyzed hydrolysis of sulfinates **11-13**.

Compound	Time (h)	% Chemical[a]	% Enzymatic	ee_S[b]	E[c]
11	72	26%	< 5%	n.r.[d]	-
12	72	6%	None	n.r.	-
13	72	3%	34%	50%	>100

a) chemical (non-enzymatic) hydrolysis b) enantiomeric excess of the substrate determined using chiral HPLC. c) Enantioselectivity determined from conversion and ee_S, and corrected for spontaneous chemical hydrolysis using the program Selectivity KreSH.[30,31] d) No reaction

Site directed mutagenesis identified four key mutations that affect the amount of the sulfinamide vs. carboxamide hydrolysis in subtilisin BPN`. Mutations of subtilisin BPN` to introduce residues found in subtilisin Carlsberg increased the S-N bond hydrolysis. We selected residues close to the active site for mutation because closer mutations usually have a larger effect on the reaction than more distant sites.[32] The S_1` leaving group pocket was mutated at residues 217, 209, and 63, while one position in the S_1 acyl binding pocket was mutated, position 156. The combined mutations increase by 14-fold the amount of sulfinamide vs. carboxamide hydrolysis of **1** (Table 3).

The single mutant Y217L had the single biggest effect on the ratio of C-N vs. S-N reactivity – it increased 4-fold from wild-type BPN` (Table 3, entry 4). The other single mutants in the $S_1$` pocket, L209Y and S63G, did not show a significant change in the selectivity ratio (lines 6 and 7). In the S_1 acyl pocket, the E156S single mutant also had little effect upon the chemoselectivity of the enzyme. This location is unique in that it is located in the acyl-binding pocket, not the leaving group pocket.

Having determined position Y217L as an important position for S-N reactivity, we prepared multiple mutants to combine with this mutation. Positions that previously had little effect now increased the amount of S-N reaction indicating complementary, rather than additive, contributions. We made two double mutants. First, adding the E156S mutant to Y217L increased the S-N ratio 8-fold, to 40 % of the total enzymatic hydrolysis (entry 8, Table 3). Secondly, adding the L209Y mutant to Y217L increased the total S-N reactivity 6-fold (entry 9, Table 3).

We made three triple mutants. The mutant Y217L, L209Y and S63G increased by 15% to 45% over its double mutant (without mutation S63G, compare lines 9 and 10). Adding either a S63G or a L209Y mutation to the Y217L/E156S mutant increase the S-N hydrolysis to 73% and 69%, respectively (compare lines 8, 11, and 12). Either of these mutants increase the amount of S-N reactivity in BPN` 14-fold, comparable to the preference in subtilisin Carlsberg.

We expected the quadruple mutant to combine these successes. However, it only gave 46% S-N hydrolysis of the total enzymatic hydrolysis (9-fold increase). Evidently the many other amino acid differences between the two enzymes are significant; in the case of the quadruple mutant these must affect the selectivity of the reaction.

Table 3: Mutations of Subtilisin BPN` alter the preference for S-N to C-N bond cleavage.

	Subtilisin	Total Hydrolysis[a,b] Conv. %[b]	Total Hydrolysis[a,b] ee_S, %[c]	Sulfinamide Hydrolysis % S-N[d]	Sulfinamide Hydrolysis ee_P Cal.[e]	Sulfinamide Hydrolysis E` [f]	Carboxamide Hydrolysis % C-N[g]	Carboxamide Hydrolysis ee_P, %[h]	Carboxamide Hydrolysis E[f]	Normalized values %S-N:%C-N (enzymatic)	Normalized values Increase in % S-N
1	None (chemical)	7		5			2			-	
2	Carlsberg[i]	55	85	44	72	17	11	59	6	79:21	
3	BPN`	49	85	3			46	93	64	< 5:95	
4	Y217L	32		10			22			20:80	4-fold
5	E156S	51		6			45			< 5:95	
6	S63G	50		8			42			7:93	
7	L209Y	53		6			46			< 5:95	
8	Y217L + E156S	47		21			26			40:60	8-fold
9	Y217L + + L290Y	50		18			32			30:70	6-fold
10	Y217L + + S63G + L209Y	48		23			25			44:56	9-fold
11	Y217L + E156S + S63G	50	81	36	88	25	14	60	4	72:28	14-fold
12	Y217L + E156S + L290Y	39	58	27	90	26	12	65	5	69:31	14-fold
13	Y217L + E156S + S63G + L290Y	51		25			26			46:54	9-fold

[a]All reactions performed at 25 °C in BES buffer (50 mM, pH 7.2), Reaction times 6 h. [b]Total conversion determined from the disappearance of **1** using biphenyl as an internal standard. Error ± ~2% conversion, based on standard deviation. [c]ee_S = enantiomeric excess of remaining starting material measured by HPLC. [d]Calculated amount of the total conversion attributed to S-N bond hydrolysis, the value with the chemical reaction subtracted is in parenthesis. [e]Calculated enantiomeric excess for the portion of **1** that disappeared via the S-N cleavage path. [f]E` = Observed enantioselectivity (uncorrected for spontaneous chemical hydrolysis) was calculated from conversion, c, and ee_P or ee_S according Sih.[30] [g]E = enantioselectivity, corrected for spontaneous chemical hydrolysis using the program Selectivity KreSH31. [h]%C-N conversion – Percentage of C-N conversion based on formation of *p*-toluenesulfinamide. [i]Enantiomeric excess of the C-N hydrolysis product, *p*-toluenesulfinamide, measured by HPLC.

Discussion:

The S-N hydrolysis by subtilisin Carlsberg is a clear example of catalytic promiscuity. Catalytic promiscuity involves a switch in mechanism, or the type of bond broken/formed, or both. The protease hydrolyzes a completely different bond, a S-N sulfinamide bond, rather than C-N carboxyamide bond. It is an unusual example in that *N*-acyl sulfinamides contain both the natural amide and unnatural sulfinamide bond, yet the enzyme prefers the unnatural bond. Other examples of promiscuity do not present a choice between natural and promiscuous function for the enzyme within a substrate.

There are several classes of enzymes that perform sulfate bond cleavage reactions. The S-N bond in N-sulfoglucosamine is cleaved to give sulfate by sulfamidase (E.C. 3.10.1.1).[38] The S-O bond in sulfamates also releases sulfate when cleaved by sulfatases (E.C. 3.1.6.X).[39] However, these reactions occur at a different functional group than the reaction reported here and involve a different intermediate. Sulfatases contain an α-formylglycine residue, not a serine, at the active site and react via a sulfate hemiacetal.

Sulfonates are structurally similar to the sulfinyl compounds reported here. Sulfonates and sulfonyl fluorides inhibit many types of serine proteases because the sulfonyl enzyme does not turn over to regenerate the free enzyme.[40] The sulfonyl inhibitor attaches to the catalytic serine, and X-ray crystal structures of a sulfonylated subtilisin are known.[41] Similar *N*-acyl compounds, β–sultams (analoges to β–lactams), inactivate the serine protease elastase by opening a strained four membered ring to give a sulfonylated enzyme.[42] While there is ample precedent for sulfonylation of serine hydrolases, de-sulfonylation does not occur. With the sulfinyl compounds in this work catalytic turnover does occur. Sulfinyl compounds may react because they are less hindered than sulfonyl compounds with one less oxygen and have an open site for attack of water. Sulfur peptides containing sulfinamide functionality were proposed as protease inhibitors, but synthesis was unsuccessful.[43]

While sulfonyled enzymes are well known from inhibitors, the corresponding sulfinylated enzymes are unknown. The intermediate observed by mass spectrometry indicates that the hydrolysis of *N*-acyl sulfinamides proceeds through a sulfinyl enzyme intermediate. The sulfinyl group is most likely attached to the catalytic serine in an

analogous manner to acyl enzyme intermediates and to sulfonyl inhibitors. However, we do not have direct evidence for the location of the sulfinylated residue. Another possible nucleophile is the imidizole of His64. A crystal structure of the *Candida rugosa* lipase revealed a sulfonylated histidine residue formed with excess sulfonyl chloride,[44] but with smaller amounts of sulfonyl chloride only the catalytic serine reacted.

Subtilisin prefers large non-polar residues such as phenylalanine or tyrosine in the S^1 binding site.[45] Boronic acids are competitive inhibitors of subtilisin Carlsberg and display a similar selectivity profile. The inhibition constant for methylboronic acid is 13 mM, while for 2-phenylethylboronic acid it is 0.27 mM, a 46-fold difference.[46] The dihydrocinnamoyl group also increased the reactivity towards esters[47] and *N*-acyl sulfinamides.[18] Substrates with the dihydrocinnamoyl mimic on the sulfinyl portion provide strong evidence for the position of the substrate binding. Placing this group on the carboxyl portion of the molecule favors the C-N reaction, while placing the group on the sulfinyl portion favors the S-N reaction. This indicates a substrate reversal to place this group in the S_1-acyl binding pocket (see Figure 5). In C-N hydrolysis, the acyl group is positioned in the S_1-acyl binding pocket, while the leaving sulfinamide is located in the leaving group S_1` pocket. For S-N sulfinamide hydrolysis, it is postulated that a reversal occurs to place the sulfinyl group in the $S_1$ acyl pocket, and the leaving amide in the $S_1$` pocket. Binding in this orientation would also be favored if the catalytic histidine performs the analogous role of protonating the leaving group, as occurs in acyl hydrolysis (Figure 2). Merely shifting the substrate, without a reversal, would place it in an unfavorable position to receive a proton from the catalytic histidine.

Further support for binding to enhance reactivity comes from a new class of substrates, sulfinates, made to react by adding the 2-phenylethane group. Subtilisin Carlsberg hydrolyzed only one of the three sulfinates tested. This is likely due to positioning of the substrate by the 2-phenylethyl sulfinyl group in **13**.

Remote binding effects are often cited to enhance reactivity and position the substrate for reaction and favor formation of the transition state.[48] Binding orients the reactive portion of the substrate into the correct distance, orientation and conformation for chemical transformation, similar to a near attack complex.[49] These contributions may supplement the classic enzymatic catalysis by stabilization of the transition state

geometry. However, dynamic effects in proteins make it difficult or impossible to dissect enzyme-substrate interaction into purely ground state binding vs. transition state binding contributions.[50]

Formation of the sulfinyl enzyme intermediate most likely involves nucleophilic substitution. Nucleophilic substitution of sulfinyl compounds proceeds by an associative mechanism from a four-coordinate sulfinyl geometry to a five-coordinate trigonal bipyramidal (sulfurane) geometry around the sulfur atom.[51] The five-coordinate species may be either a transition state, in a concerted S_N2-like process, or an intermediate, in a stepwise addition-elimination process. According to theoretical calculations, an intermediate is formed with an unsymmetrical trigonal bipyramidal structure and the leaving group and nucleophile in apical positions.[52] There are also several examples of stable, isolated, trigonal biypyramidal sulfurane compounds.[53] Reaction to form the sulfinyl-enzyme intermediate may involve sulfurane (5-coordinate) transition state and a pseudorotation.

In addition to reaction requirements, the enzyme also imposes limitations on the reaction. Nucleophilic attack occurs on the (*R*)-enantiomer of the sulfur stereocenter. The attacking nucleophile, most likely Ser221, will occupy an apical position in the trigonal bipyramidal structure, and the sulfinyl oxygen will likely be placed in the oxyanion hole for stabilization of the buildup of charge in the transition state. The dihydrocinnamoyl portion of the molecule will likely bind in the S_1 acyl site. This leaves the position of the leaving group, -NHR, and the lone pair. The stereochemistry requires that the lone pair be in the opposite apical position and the –NHR leaving group occupy the remaining equatorial position. Generally, the leaving group must occupy an apical position before departing. Pseudorotation to place the leaving group in an apical position may further the reaction (Sulfurane A to B, Figure 8), or may not be necessary. Pseudrotation is thought to occur in phosphoryl transfer reactions occurring with retention of configuration.[54] Two diastereomers differing at the phosphorus center of a peptidic substrate gave the same stereochemistry for the resulting phosphorus intermediate.[55] A pseudorotation was suggested to account for the alternate inversion or retention pathways.

Pseudorotation may also account for the low enantioselectivity if both enantiomers can lead to the same intermediate. The stereochemistry of the resulting

sulfinylated enzyme formed is unknown, and the overall retention or inversion is not known because the final product, sulfinic acid, is achiral. The feasibility of this mechanism is currently under investigation using QM/MM methods.

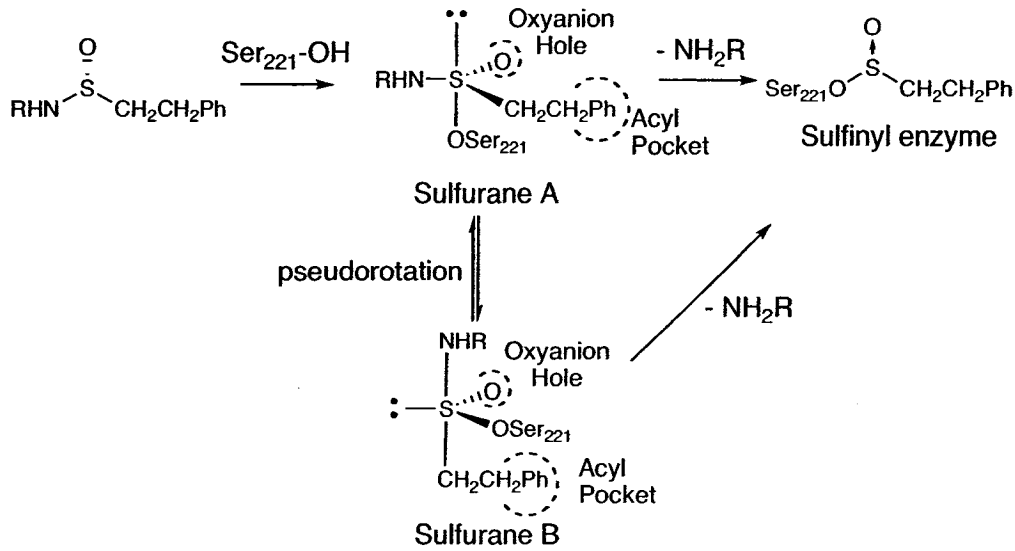


Figure 8: Formation of a possible sulfurane trigonal bipyramidal intermediate. The nucleophilic Ser_{221}-OH and the sulfur lone pair occupy the apical positions in the trigional bipyramidal sulfurane A. Pseudorotation may occur to exchange apical and equatorial positions.

A high enantioselectivity might be anticipated for sulfinamides reactions because hydrolysis occurs at the chiral center. Indeed, reaction with acetylcholinesterase with Soman (2-(3,3-dimethylbutyl)-methylphosphonofluoridate) occurs with high enantioselectivity and reacts exclusively ($E > 10^4$) with the P(*S*) configuration.[56] However, in other examples where reaction occurs at the organophosphorus stereocenter the enantioselectivity is not always high. *Candida antarctica* B lipase is inhibited by ethyl (-)-*p*-nitrophenyl hexylphosphonate only10 times faster than the (+)-enantiomer, a moderate enantioselectivity.[57] The enantiopreference for two diastereomers differing at the phosphorus center was only 2:1 for phosphonate inhibitors of several serine hydrolases, including subtilisin.[58] For the hydrolysis of phosphotriesters by the organophosphorus acid anhydrolase (OPAA), the enantioselectivity was substrate dependant from 3 to 36.[59] Therefore reactions occurring directly at a stereocenter do not always have a high enantioselectivity.

Subtilisin Carlsberg and BPN` differ in 86 of 275 amino acids (69% identical) and have very similar crystal structures (see Figure 9) but hydrolyze peptides at different rates. For the synthetic peptide substrate succinyl-Ala-Ala-Pro-PHE-*p*-nitroanilide, Carlsberg had a 7-fold higher k_{cat}/K_M value of 2500 $sec^{-1}M^{-1}$ compared with the BPN` value of 360 $sec^{-1}M^{-1}$.[60] Three mutations close to the active site of BPN` accounted for these kinetic differences. The E156S single mutant of BPN` increased the k_{cat}/K_M value by 3-fold, attributed mainly to a lower K_M value. The L217Y single mutant increased the k_{cat}/K_M value by 2-fold; both k_{cat} and K_M increased. A combination of these two mutations gave a combined 4-fold increase in the k_{cat}/K_M of the resulting BPN` mutant for this synthetic peptide. A third mutation, G169A, which is not in direct contact with the substrate, gave a 3-fold increase. Combined into a triple mutant with 156/217 the G169A mutation raised the catalytic efficiency to match Carlsberg. The rate enhancement for peptide hydrolysis for residue 217 mutation suggested smaller leucine being able to accommodate larger leaving groups, while other mutations were not rationalized.[61]

In a similar way three mutations account for the increase in the promiscuous S-N reaction vs. the C-N reaction in subtilisins BPN` and Carlsberg. Two mutations sites were the same as the above study; positions 156 ($S_1$ acyl site) and 217 ($S_1$` site) gave a 8-fold increase in the S-N reaction. Two other positions, 63 and 209, differ in BPN` and Carlsberg and were also chosen for mutation. Position 169 was not mutated, as it was further from the active site. The above study examined rate changes resulting from mutation, while here the selectivity, not the rate, changed. The overall reaction rate was approximately the same for each mutant.

All four mutations affected the amount of S-N hydrolysis, indicating that they are key residues near the active site that affect selectivity. The effect of mutations was not additive but complementary. For instance, single mutants of position 63, 156, and 209 showed no change (Table 2, entries 5-7), but in combination each mutant contributed to an increase in S-N hydrolysis (entries 9-13).

These mutations are in both the acyl and leaving group sites, and are consistent with a reversal of the substrate. One mutation (Glu156Ser) is located in the S_1 acyl-binding pocket, while the other three of these mutations are located in the alcohol-binding pocket (S_1`). The changes in the enzyme are not obvious and it would be difficult to

predict or design this result based on simple modeling. In the S_1 acyl pocket, position 156 is mutated from a charged glutamate residue to an uncharged serine residue. This results in a larger, less polar pocket. In the S_1` leaving group pocket, tyrosine 217 is 8.4 Å from the catalytic serine (Cα), and is in direct contact with the substrate during C-N hydrolysis. Leucine is slightly wider than the aromatic tyrosine, but spatially they are quite similar. Another possibility is a hydrogen bond between tyrosine and Ser63 which restricts movement in wild-type BPN`. The hydroxyl group of Tyr217 is 2.8 Å from Ser63, but this interaction is not possible in the mutated enzyme. Position 217 and 209 are complementary in the two enzymes. In subtilisin BPN` these residues are tyrosine and leucine, while in subtilisin Carlsberg they are reversed, leucine and tyrosine.

In addition to the substrate reversal hypothesis, an increased flexibility of the active site to accommodate an alternate reaction mechanism may also contribute to reactivity. Two of these mutations are located next to key residues: position 63 is adjacent to the catalytic His64, while position 156 is next to Asn155, an oxyanion-stabilizing residue. In both cases, the mutations are to smaller residues that may allow more flexibility to accommodate an alternate mechanism. In particular, a pseudorotation of the intermediate may require additional enzyme flexibility.

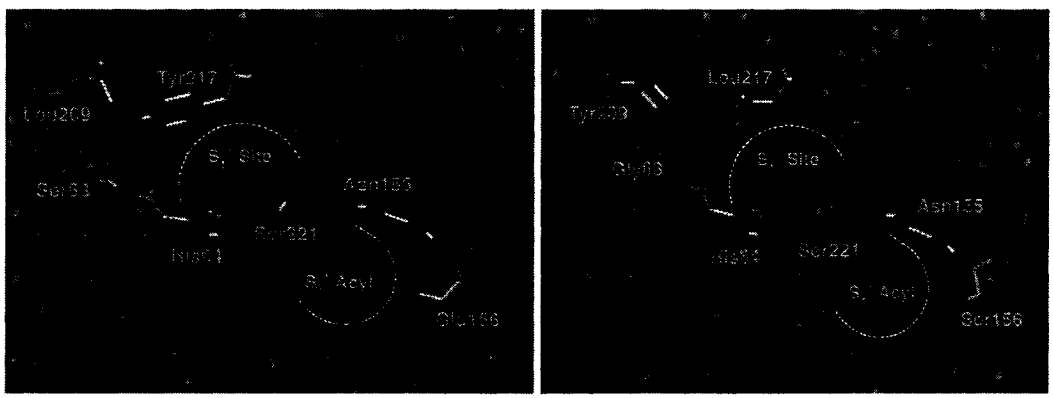


Figure 9: Subtilisins BPN` (left), and Carlsberg (right). Catalytic residues Ser221, His64 and Glu156, the S1acyl site and the S1` leaving group site are shown. Residues differing between the two enzymes are 63, 209 and 217 in the S1` site, and 156 in the acyl site.

There is no reason why these mutations should be the best for S-N hydrolysis. Because this is an example of catalytic plasticity, the natural enzyme (Carlsberg) is not

optimized for the reaction. Saturation mutagenesis on the positions identified, and others, should allow us to improve the reaction even more. The reaction rate was approximately the same for each mutant, and could be improved using mutagenesis and computational results. Catalytic promiscuity is often introduced by a few mutations close to the active site.[32,62] The residues responsible for the chemoselectivity here were all close to the active site.

Enantiopure sulfinamides and sulfinates are valuable synthetic intermediates in the preparation of chiral amides and sulfoxides. The *p*-toluene and *t*-butyl sulfinamides are the most common due to their ease of preparation. Enantiopure *p*-toluene sulfinamide is prepared from the menthol sulfinate crystallization. This is limited to *p*-substituted aromatics, as the aliphatic equivalents are not crystalline and difficult to separate. Thus there are fewer methods to prepare the aliphatic sulfinyl compounds. While in the initial stages, the enzymatic hydrolysis reported here may provide a route to the less accessible aliphatic sulfinates, which are easily transformed to sulfinamides and sulfoxides.

Experimental

General methods. ^{1}H- and ^{13}C-NMR spectra were obtained as $CDCl_3$ solutions at 300 MHz and 75 MHz, respectively. Chemical shifts are expressed in ppm (δ) and are referenced to tetramethylsilane or solvent signal. Coupling constants are reported in Hertz (Hz). Flash chromatography with silica gel (35-75 mesh) was used to purify all intermediates and substrates. All reagents, buffers, starting materials and anhydrous solvents were purchased from Sigma-Aldrich (Milwaukee, USA) and used without purification. All air- and moisture-sensitive reactions were performed under Ar. Subtilisin Carlsberg (E.C. 3.4.21.62) was purchased from Fluka (St. Louis, USA).

Enzymatic Reactions and Analysis

Enzymatic reactions were preformed under the following conditions: BES buffer (250 μL, 50 mM, pH 7.2), substrate (30 μL, 75 mM in MeCN containing biphenyl as an internal standard, and an enzyme solution (50 μL, 10 mg/mL). The reaction was stirred at 25 °C quenched with acetonitrile (1.00 mL) to precipitate the enzyme, centrifuged, and

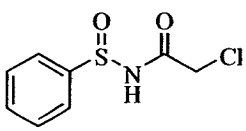
analyzed. Conversion was determined by reverse phase chromatography of this solution on a Zorbax SB-C18 column (4.6 mm x 250 mm, Agilent, Palo Alto, CA) with acetonitrile/water as the mobile phase. Biphenyl was used as an internal standard and sulfinamide standards with biphenyl were used to determine C-N conversion. To determine the enantiomeric excess the reaction was extracted with EtOAc (3 x 0.75 mL), dried with $MgSO_4$ and evaporated and analyzed using a chiral stationary phase: Chiralpak AD-H or Chiralcel OD columns (4.6 mm x 25 cm, Chiral Technologies, West Chester, PA) at 230 nm. An achiral Discovery Cyano column (4.6 mm x 25 cm, Supelco, Bellefonte, PA) was placed before the chiral column in cases where the sulfinamide and *N*-acyl sulfinamide enantiomers overlapped. Retention times are given with compounds analyzed. Reactions for the pH dependence curve were preformed on a Radiometer TitraLab 854 pH stat-titration apparatus, with a buffer of appropriate pKa (2.0 mM), and the pH held constant by titration with NaOH (0.1 mM). The reaction was stopped by addition of acetonitrile and analyzed as above.

Mass Spectra:

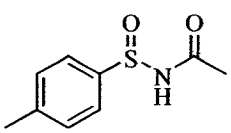
Enzyme mass spectra were obtained under the following conditions. Subtilisin Carlsberg (40 μL, 10 mg/mL in 10 mM BES, pH 7.2) and **1** (3 μL, 100 mM) were reacted for 30 min. and acidified with formic acid (2%, 3 μL). The mixture was desalted using a porous C4 resin, diluted and analyzed by electrospray ionization mass spectrometry (Q Star, Applied Biosystems, Foster City, CA). Inhibited enzyme was prepared by treatment of an enzyme solution with PMSF (3 μL, 100 mM) and the above experiment replicated. A third experiment with *N*-chloroacetyl phenylsulfinamide **2**, was also performed as a control. The molecular weight of the free enzyme shifted by 1-2 mass units depending on calibration, but the adducts were of consistently of expected mass.

Synthesis of *N*-acyl-*p*-toluenesulfinamides.

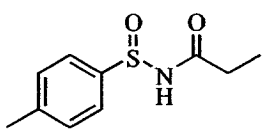
N-acyl-*p*-toluenesulfinamides[63] were prepared by acylation of the corresponding *p*-toluenesulfinamide with 2 equivalents of *n*-butyl lithium and addition of the appropriate anhydride[64] to give the *N*-acyl sulfinamide. *N*-acyl derivatives **1, 4, 6, and 7** were prepared and analyzed as described previously.[22]



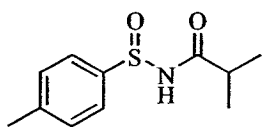
***N*-(chloroacetyl)-benzenesulfinamide, 2,** White solid, (580 mg, 40%), mp 93-95 °C; ^{1}H NMR δ 4.15 (s, 2H), 7.59 (m, 3H), 7.75 (m, 2H), 8.41 (s, 1H); ^{13}C NMR δ 42.4, 124.9, 129.8, 132.7, 143.0, 167.3; MS $[M+H]^+$ $C_8H_9ClNO_2S$ Cal'd = 218.0043, found = 218.0054. Conversion: 50/50 MeCN/H_2O, flow 0.6 mL/min for 9 min, then gradient to 80/20 over 0.5 min; benzenesulfinamide: t_R = 4.8 min; **2**: t_R = 6.1 min; biphenyl: t_R = 15.7 min. **Enantiomeric excess: 90**/10 Hexanes/EtOH, flow 0.5 mL/min, Cyano column and Chiralcel-OD. benzenesulfinamide, (*R*) t_R = 33.4 min, (*S*) t_R = 35.6 min; **2**: (*R*) t_R = 47.3 min, (*S*) t_R = 64.1 min.



***N*-(acetyl)-*p*-toluene sulfinamide, 8,** White solid, (560 mg, 22%), mp 124-126 °C (Lit 125-127 °C[18]); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.19 (s, 3H), 2.44 (s, 2H), 7.35 (d, *J* = 8.1, 2H), 7.60 (d, *J* = 8.1, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.8, 21.9, 124.8, 130.1, 140.2, 142.8, 171.3, HR-EIMS *m/z*: M^+ calcd for $C_9H_{12}NO_2S$ 198.0588, found 198.0585. Conversion: 50/50 MeCN/H_2O, flow 0.6 mL/min for 9 min, then gradient to 80/20 over 0.5 min; *p*-toluenesulfinamide: t_R = 5.3 min; **8**: t_R = 5.5 min; biphenyl: t_R = 15.7 min. Enantiomeric excess: 95/5 Hexanes/EtOH, flow 0.75 ml/min, Cyano column and Chiralcel-OD. *p*-toluenesulfinamide, (*R*) t_R = 42.5 min, (*S*) t_R = 52.0 min; **8**: t_R = 44.3 min, t_R = 58.3 min.

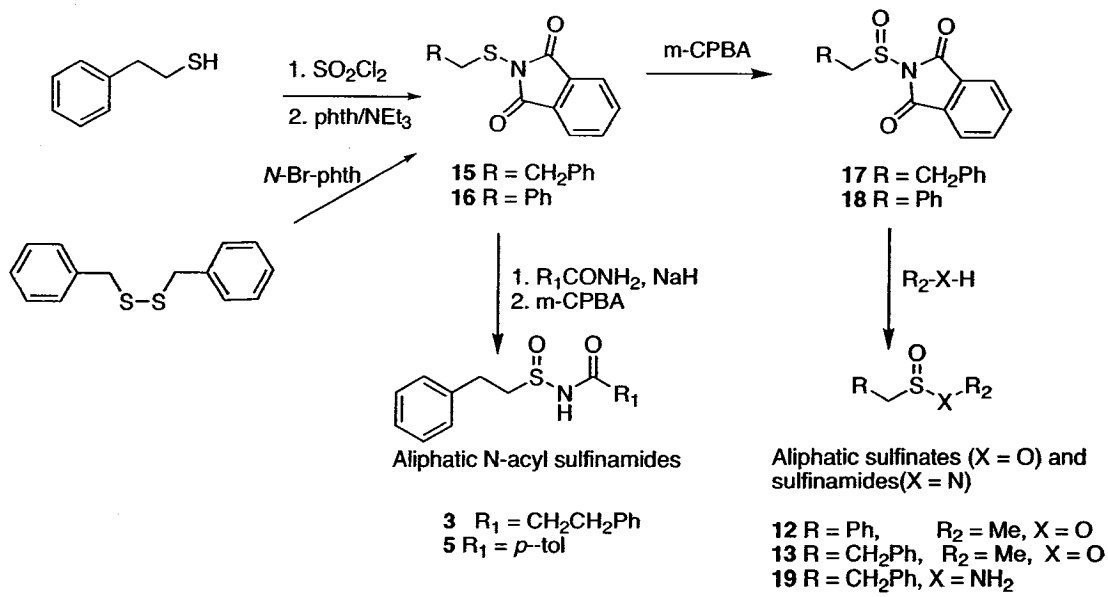


***N*-(propyl)-*p*-toluenesulfinamide, 9,** White solid, (1.3 g, 65%); mp 108-110 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.23 (t, *J* = 7.5, 3H), 2.44 (s (br), 4H), 7.35 (d, *J* = 7.8, 2H), 7.60 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 8.8, 21.5, 124.8, 130.1, 140.3, 142.6, 174.9; HR-EIMS *m/z*: M^+ calcd for $C_{10}H_{14}NO_2S$ 212.0745, found 212.0757. Conversion: 50/50 MeCN/H_2O, flow 0.6 mL/min for 9 min, then gradient to 80/20 over 0.5 min; *p*-toluenesulfinamide: t_R = 5.3 min; **9**: t_R = 6.4 min; biphenyl: t_R = 15.7 min. Enantiomeric excess: 95/5 Hexanes/EtOH, flow 0.75 ml/min, Cyano column and Chiralcel-OD. *p*-toluenesulfinamide, (*R*) t_R = 42.5 min, (*S*) t_R = 52.0 min; **9**: (*R*) t_R = 36.4 min, (*S*) t_R = 48.5 min.



***N*-(Isopropyl)-*p*-toluenesulfinamide, 10,** White solid, (1.4 g ,64%) mp 123-125 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.19 (d, J = 7.0, 6H), 2.44 (s, 4H), 7.32 (d, J = 7.8, 2H), 7.53 (m, J = 7.0, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.9, 19.2, 21.5, 124.7, 130.2, 140.7, 142.8, 177.8, MS $[M+H]^+$ $C_{11}H_{16}NO_2S$ Cal'd = 226.0901, found = 226.0903. Conversion: 50 MeCN/H_2O, flow 0.6 mL/min for 9 min, then gradient to 80/20 over 0.5 min; *p*-toluenesulfinamide: t_R = 5.3 min; **10**: t_R = 7.7 min; biphenyl: t_R = 15.7 min. Enantiomeric excess: 95/5 Hexanes/EtOH, flow 0.60 mL/min, Cyano column and Chiralcel-OD. *p*-toluenesulfinamide, (*R*) t_R = 42.5 min, (*S*) t_R = 52.0 min; **10**: (*R*) t_R = 30.9 min, (*S*) t_R = 39.7 min.

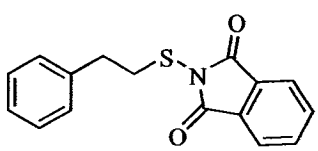
Preparation of aliphatic sulfinamides and sulfinates:



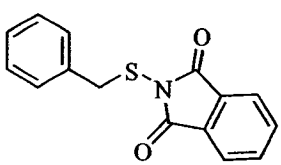
Scheme 1: General method for the preparation of aliphatic sulfinates and *N*-acyl sulfinamides (phth= phthalimide).

Aliphatic sulfinamides were prepared from the corresponding thiols (or disulfides) by first making the *N*-thiophthalimide as a sulfur transfer reagent.[65] Thiophthalimides **15-16** were synthesized by one of two methods: thiols were converted to the sulfinyl chloride followed by treatment with phthalimide in triethylamine.[66] Alternatively, disulfides were refluxed with *N*-bromopthtalimide in benzene.[67] Addition of the corresponding amide in

NaH provided *N*-acyl-sulfenamides, and the resulting product oxidized with *m*-CPBA to give the *N*-acyl-sulfinamide (**3, 5**). This method was preferable to acylation of the sulfinamdie because of acidic alpha protons (oxidation is performed after the displacement). Sulfinates and sulfinamides were from the *N*-sulfenyl phthalimide to the *N*-sulfinylphthalimide with *m*-CPBA (**17-18**), followed by displacement with an alcohol or amine (**12-13, 19**).

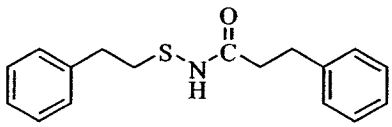


***N*-(2-phenylethanethio)phthalimide, 15.** A solution of benzeneethane thiol (10 g, 72 mmol) in *n*-heptane (75 mL) was cooled to 0 °C and sulfuryl chloride (9.7 g, 72 mmol) was added dropwise followed by stirring for 45 min. This solution was transferred to a second round-bottomed flask containing phthalimide (10.7 g, 72 mmol) and triethyl amine (10 mL) in DMF (75 mL) at 0 °C and stirred for a further 2 hours. After 2 hours, the reaction was added to cold water (1 L) and the solid collected by suction filtration and dried under high vacuum. Beige solid, (16.9 g, 82%), mp 73-74 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.95 (t, J = 5.1, 2H), 3.18 (t, J = 5.1, 2H), 7.20 (m, J = 5H), 7.77 (m, 2H), 7.90 (m, 2H); ^{13}C NMR δ 35.2, 39.6, 124.0, 126.5, 128.5, 128.6, 132.1, 134.7, 139.2, 168.6; HR-EIMS *m/z*: M^+ calcd for $C_{16}H_{14}NO_2S$ 284.0745, found 284.0739.

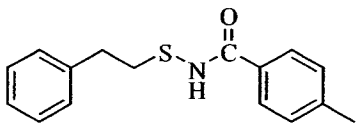


***N*-(benzylthio)phthalimide, 16.**[67] A solution of benzene disulfide (7.0 g, 28 mmol) and *N*-bromophthalimide (12.8 g, 56 mmol) was placed in anhydrous benzene (75 mL) and refluxed for 1 hour. After cooling to room temperature hexanes (75 mL) were added and the white precipitate collected by suction filtration. The crude product was recrystallized from ethanol to give a white solid (6.7 g, 88% yield), mp 163-165 (lit 167-168°C[67]): ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.12 (s, 2H), 7.24 (m, J = 5H), 7.75 (m, 2H), 7.86 (m, 2H); ^{13}C NMR δ 42.5, 123.9, 128.0, 128.7, 129.6, 131.9, 134.6, 135.3, 168.0; HR-EIMS *m/z*: M^+ calcd for $C_{15}H_{12}NO_2S$ 270.0588, found 270.0593.

Formation of *N*-acyl sulfenamides from thiophthalimides

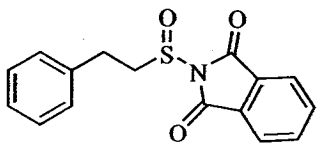


***N*-(dihydrocinnamoyl)-2-phenylethanesulfenamide,** Dihydrocinnamide (1.05 g, 7.0 mmol) in anhydrous THF (50 mL) was cooled to 0 °C followed by addition of NaH (0.34 g, 8.5 mmol, dispersion in mineral oil). Compound **15** (2.0 g, 7.0 mmol) was added and the reaction was stirred at room temperature for 3 hours. The reaction mixture was washed with saturated NH_4Cl (25 mL), water (2X25 mL), brine (25 mL), dried ($MgSO_4$). Purification by column chromatography on basic aluminia activity 1 (50:50 CH_2Cl_2/Hexanes to 50:50 EtOAc/Hexanes) gave a clear yellow oil, (1.33 g, 66%); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.46 (t, J = 7.5, 2H), 2.94 (m, 6H), 5.90 (s, 1H), 7.26 (m, 10H); ^{13}C NMR δ 31.6, 35.1, 38.6, 40.1, 126.5, 128.5, 128.6, 128.7, 140.1, 140.4, 174.2; HR-EIMS *m/z*: M^+ calcd for $C_{17}H_{20}NOS$ 286.1265, found 286.1260.



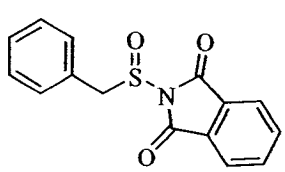
***N*-(*p*-tolyl)-2-phenylethanesulfenamide,** Purified by column chromatography (40% EtOAc/Hexanes) to give a white solid, (1.10 g, 57%), mp 90-94°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.40 (s, 3H), 2.95 (t, J = 7.0, 2H), 3.12 (t, J = 7.0, 2H), 6.62 (s, 1H), 7.26 (m, 7H), 7.55 (m, 2H); ^{13}C NMR δ 21.6, 35.2, 40.2, 126.6, 127.5, 128.7, 129.3, 140.1, 142.9, 169.4; HR-EIMS *m/z*: M^+ calcd for $C_{16}H_{18}NOS$ Cal'd = 272.1109, found = 272.1113.

Oxidation of Thiolphthalimides and *N*-acyl Sulfenamides to Sulfinyl phthalimides and *N*-acyl sulfinamides[65]

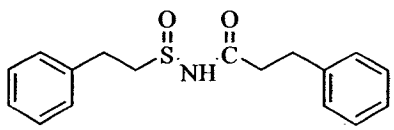


***N*-(2-phenylethanesulfinyl) phthalimide, 17.** A solution of *m*-chloroperbenzoic acid (4.4 g, 18 mmol, 70% purity) in chloroform (50 mL) was added dropwise over 0.5 hours to a solution of **15** (50 g, 18 mmol) in chloroform (75 mL) at 0 °C. The reaction mixture was stirred for 1 hour followed evaporation to give a solid that was triturated with ether (75 mL) and filtered to give a white solid, (4.2 g. 79%), mp 120-122 °C; ^{1}H NMR δ 3.03 (m, 1H), 3.18 (m, 1H), 4.09 (m, 2H), 7.21 (m, 5H), 7.82, (m, 2H), 7.91, .00(m, 2H); ^{13}C NMR

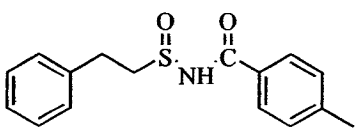
δ 29.6, 53.7, 124.4, 127.0, 128.6, 128.9, 131.6, 135.4, 137.5, 166.2; HR-EIMS *m/z*: $[M+H]^+$ calcd for $C_{16}H_{14}NO_3S$ 300.0694, found 300.0703.



***N*-(benzylsulfinyl) phthalimide, 18,** White solid, (3.0 g, 92%), mp 162-164 °C, Lit[65b] 154-155 °C; ^{1}H NMR δ 4.94 (d, J = 12, 1H), 5.31 (d, J = 12, 1H), 7.35 (m, 5H), 7.81, (m, 2H), 7.92, (m, 2H); ^{13}C NMR δ 59.3, 124.5, 129.0, 129.4, 130.1, 131.7, 135.4, 135.8, 166.3; HR-EIMS *m/z*: $[M+H]^+$ $C_{15}H_{12}NO_3S$ Cal'd = 286.0537, found = 286.0532.



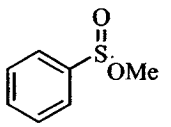
***N*-(dihydrocinnamoyl)-2-phenylethanesulfinamide, 3,** White solid, (640 mg, 85%), mp 116-118 °C; ^{1}H NMR δ 2.60 (s, broad, 2H), 2.94 (m, 4H), 3.14 (m, 2H), 7.26 (m, 10H), 8.57 (s, 1H); ^{13}C NMR δ 28.4, 30.8, 56.2, 126.6, 127.2, 128.5, 128.6, 128.7, 129.0, 137.9, 140.0, 173.6; HR-EIMS *m/z*: M^+ calcd for $C_{17}H_{20}NO_2S$ 302.1214, found 302.1241. Conversion: 40/60 MeCN/H_2O, flow 0.6 mL/min for 9 min, then gradient to 80/20 over 0.5 min; Sulfinamide, t_R = 4.8 min; dihydrocinnamamide, t_R = 4.9 min; compound **3**: t_R = 7.9 min; biphenyl t_R = 17.9 min. To separate the sulfinamide and dihydrocinnamide, 25/75 MeCN/H_2O, flow 0.6 mL/min, dihydrocinnamide, t_R = 12.1 min; sulfinamide, t_R = 14.3 min. **Enantiomeric Excess:** 85/15 Hexanes/EtOH, flow 0.75 mL/min, Chiralcel OD. **3** Enantiomers t_R = 20.7 and t_R = 25.1 min; dihydrocinnamide, t_R = 9.7 min; sulfinamide enantiomers t_R = 16.3 and t_R = 22.2 min.



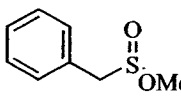
***N*-(*p*-tolyl)-2-phenylethanesulfenamide, 10**. A solution of *m*-chloroperbenzoic acid (910 mg, 3.7 mmol, 70% purity) in chloroform (20 mL) was added dropwise over 0.5 hours to a solution of *N*-(*p*-tolyl)-2-phenylethanesulfenamide (1.0 g, 3.7 mmol) in chloroform (20 mL) at 0 °C. The reaction mixture was stirred for 1 hour followed washing with 5% sodium bicarbonate (3 X 20 mL) and dried ($MgSO_4$). Evaporation of the solvent gave a colorless oil, (980 mg, 92%), mp 132-135 °C; ^{1}H NMR δ 2.37 (s, 3H), 2.99 (m, 2H), 3.37 (m, 2H), 7.21 (m, 7H), 7.67, (m, 2H), 9.63 (s, 1H); ^{13}C NMR δ 21.7, 28.6, 55.6, 127.1, 128.3, 128.6, 129.0, 129.5, 138.2, 144.1, 167.3; HR-EIMS *m/z*: M^+

calcd for $C_{16}H_{18}NO_2S$ 288.1058, found 288.1045. Conversion: 40/60 MeCN/H_2O, flow 0.6 mL/min for 9 min, then gradient to 80/20 over 0.5 min; Sulfinamide and *p*-tolylamide, t_R = 4.8 min; **10**: t_R = 7.9 min; biphenyl t_R = 17.9 min). To separate the sulfinamide and *p*-tolylamide, 25/75 MeCN/H_2O, flow 0.6 mL/min, *p*-tolamide, 12.1 min; sulfinamide, 14.3 min. Enantiomeric excess: 85/15 Hexanes/EtOH, flow 0.75 mL/min, Chiralcel OD. **10** Enantiomers t_R = 16.5 and t_R = 20.8 min; *p*-tolylamide, t_R = 8.7 min.

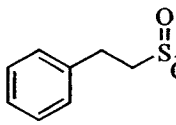
Sulfinate esters[65]



Methyl phenylsulfinate, 11. Conversion: 80/20 MeCN/H_2O, flow 0.6 mL/min; **11**, t_R = 5.3 min; biphenyl t_R = 9.8 min; Enantiomeric Excess: 99:1 Hexanes/Ethanol, flow 0.6 mL/min, Chiralcel OD. **11** Enantiomers t_R = 13.3 and t_R = 14.8 min.



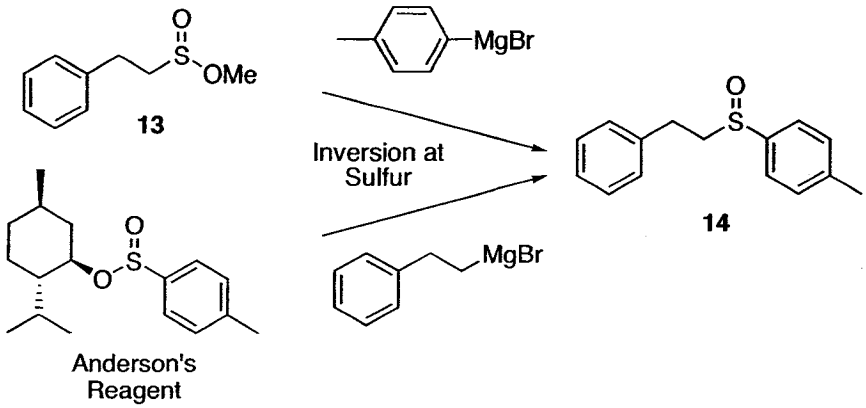
Methyl benzylsulfinate, 12, ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.75 (s, 3H), 3.96 (d, *J* = 12.9, 1H), 4.05 (d, *J* = 12.9, 1H), 7.31 (m, 5H); ^{13}C NMR δ 54.8, 64.1, 128.4, 128.9, 130.5, 134.4; HR-EIMS *m/z*: M^+ calcd for $C_8H_{11}O_2S$ 171.0479, found 171.0477. Conversion: 80/20 MeCN/H_2O, flow 0.6 mL/min; **12**, t_R = 5.0 min; biphenyl t_R = 9.7 min.



Methyl 2-phenylethanesulfinate, 13, Colorless oil, 440 mg (79%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.00 (m, 4H), 3.76 (s, 3H), 7.26 (m, 5H); ^{13}C NMR δ 27.3, 54.7, 58.0, 126.8, 128.6 128.8, 138.8; HR-EIMS *m/z*: M^+ calcd for $C_9H_{13}O_2S$ 185.0636, found 185.0644. Conversion: 80/20 MeCN/H_2O, flow 0.6 mL/min; **13**, t_R = 5.0 min; biphenyl t_R = 9.7 min; Enantiomeric Excess: 85/15 Hexanes/Ethanol, flow 0.75 mL/min, Chiralcel OD. **13** Enantiomers t_R = 8.5 and t_R = 12.0 min.

Absolute configuration of 13

The absolute configuration of the **13** was determined by conversion to a sulfoxide and chemical correlation. Treatment of the remaining enantioenriched starting material (39% ee) with *p*-toluenemangensium bromide proceeds with complete inversion of configuration to give the sulfoxide **14** (37% ee). This sulfoxide was enriched in the (*R*)-enantiomer, as determined by comparison with a standard prepared from Anderson's reagent ((-)-menthyl- (*S*)-*p*-toluenesulfinate) and a gringard to produce **14**.[68] Thus (*R*)-**13** was the fast reacting sulfinate enantiomer, consistent with the previous *N*-acyl sulfinamides.



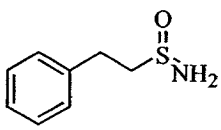
Scheme 2: Absolute configuration of **13** by conversion to a sulfoxide of known configuration.

A – Enzymatic reaction and derivatization

The sulfinate **13** (20 mg, 0.11 mmol) in acetonitrile (2 mL) and BES buffer (20 mL, 2 mM) and subtilisin Carlsberg (50 mg) were stirred at pH 7.2 using a pH stat to control pH for 2 days. The reaction was extracted with dichloromethane (2 X 15 mL), dried, and evaporated. The enantiomeric excess of the remaining starting material was 39% (conditions as above). This compound was then placed in anhydrous ether (10 mL) and *p*-toluene magnesium bromide (0.16 mL of a 0.5 M solution in ether, 0.08 mmol) was added and the reaction stirred at room temperature. The reaction was stopped with the addition of saturated ammonium chloride, washed with water, dried, and evaporated. The resulting sulfoxide was analyzed by chrial HPLC and gave an enantiomieric excess of 37%, 95:5 hexane:ethanol, Chiracel OD, flow 0.75 mL/min, Enantiomers (*S*)-**14**: t_R = 17.3 (S) and (*R*)-**14**: t_R = 18.7 min.

B – Standard of known configuration. (*R*)-(2-phenethyl)-*p*-toluene sulfoxide, 14

To a solution of (1*R*. 2*S*. 5*R*)-(-)-menthyl-(*S*)-*p*-toluenesulfinamide (1.0 g, 3.4 mmol) and (1*S*. 2*R*. 5*S*)-(-)-menthyl- (*R*)-*p*-toluenesulfinamide (100 mg, 0.34 mmol) in ether (50 mL) at 0 °C was added 2- phenylethyl magnesium bromide prepared from 2-bromoethylbenene (2.0 g, 11 mmol) and magnesium (270 mg, 11 mmol) in ether (25 mL). The reaction mixture was stirred for 2 hours and quenched with saturated ammonium chlrode (30 mL), washed with water, dried and Purified by column chromatography (40% Ethyl acetate/hexanes) to give a colorless oil (770 mg, 84%), enantiomieric excess, (*R*)-enriched, of 79%, HPLC as above. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.41 (s, 3H), 2.90 (m, 1H), 3.04 (m, 3H), 7.26 (m, 7H), 7.52 (m, 2H); ^{13}C NMR δ 21.5, 28.3, 58.4, 124.1, 126.8, 128.6, 128.8, 130.1, 138.9, 40.4, 141.6; HR-EIMS *m/z*: $[M+H]^+$ calcd for $C_{15}H_{17}OS$ 245.1000, found 245.1003.



2-phenylethylsulfinamide, 19, white solid (114 mg, 57%), mp 77-78°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.05 (m, *4*H), 4.45 (s, *2*H), 7.26 (m, 5H); ^{13}C NMR δ 29.2, 58.0, 126.8, 128.6 128.8, 138.9; HR-EIMS *m/z*: M^+ calcd for $C_8H_{12}NOS$ 170.0639, found 170.0645.

Mutations of Subtilisin BPN`:

Mutants of the template subtilisin BPN` vector pS4,[69] which encodes for subtilisin BPN` and chloramphenicol resistance were prepared using the QuikChange Site Directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacture's instructions. Primers used were as follows: S63G (5`-CCTTTCCAAGACAACAACGGCCACGGAACTCACGTTGC-3`), cS63 (5`-GCAACGTGAGTTCCGTGGCCGTTGTTGTCTTGGAAAGG-3`); E156S (5`-GCGGCAGCCGGTAACTCTGGCACTTCCGGCAG-3`), cE156S (5`-CTGCCGGAAGTGCCAGAGTTACCGGCTGCCGC-3`); L209Y (5`-CGTATCTATCCAAAGCACGTACCCTGGAAACAAATACGGG-3`), cL209Y (5`-CCCGTATTTGTTTCCAGGGTACGTGCTTTGGATAGATACG-3`); Y217L (5`-GGAAACAAATACGGGGCGCTTAACGGTACGTCAATGG-3`)

cY217L (5`-CCATTGACGTACCGTTAAGCGCCCCGTATTTGTTTCC-3`). After digestion with DPN1 to remove template DNA, the resulting mutants were transformed into *E. coli* DH5α competent cells and plated on LB agar plates containing chloroamphenicol. Colonies were selected and grown in LB media for sequencing. BPN` mutants were expressed in protease deficient *B. subtilis* DB104[70] cells with the mutated DNA, and selected on LB plates containing chloroamphenicol. Subtilisin BPN` mutants were expressed in 2XSG[71] media for 48 hours. The supernatant after centrifugation was filtered through a 0.22 μm membrane filter and then through a bead of Dowex DE-52 anion exchange resin at pH 7.2.[22] The enzyme solution was then concentrated using an ultrafiltration 10 kDa cutoff membrane and used for enzymatic reactions with compound **1,** as described previously. SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) gave one band corresponding to the molecular weight of subtilisin.

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Summary, Conclusions and Future Work

We have expanded the application of subtilisin to hydrolyze unnatural substrates and perform unnatural reactions. The open active site of subtilisin allows it hydrolyze sterically hindered substrates such as a bulky auxiliary and tertiary alcohol esters. A new catalytic promiscuous reaction of subtilisin Carlsberg was discovered where a sulfinamide (S-N) bond is hydrolyzed rather than the expected amide (C-N) bond in *N*-acyl sulfinamides. The active site also catalyzes unnatural reaction using a similar mechanism to the natural hydrolysis.

We identified two enantiocomplementary enzymes, subtilisin and cholesterol esterase, which can resolve a Diels-Alder chiral auxiliary. Both enzymes displayed high enantioselectivity (E >200) and gram quantities were resolved with subtilisin Carlsberg. The molecular basis of subtilisin's high enantioselectivity was determined using molecular modeling. The slow reacting enantiomer disrupted key hydrogen bonds with the enzyme due to steric clashes.

We also performed the first study on the protease catalyzed resolution of tertiary alcohol esters. In general proteases showed good reactivity but low enantioselectivity. Substrate studies determined that internal substrate strain limited the reaction to tertiary alcohols with a methyl or smaller small substituent because this substituent must adopt an unfavorable *syn*-pentane-like orientation with the oxyanion of the first tetrahedral intermediate. This work demonstrates that proteases such a subtilisin can hydrolyze challenging tertiary alcohol esters. Protein engineering may further improve the resolution of tertiary alcohols by increasing the enantioselectivity. Using a complementary acyl group (such as dihydrocinnamoyl for subtilisin and α-chymotrpysin) may enhance the reactivity of challenging substrates even further.

Finally, we discovered a new reaction of subtilisin Carlsberg, the hydrolysis of *N*-acyl sulfinamides by cleavage of the S-N bond. We examined the substrate range of the reaction and proved the reaction proceeds through a sulfinyl enzyme intermediate. This is one of the few examples where the molecular basis for the promiscuity was examined. Only a few amino acid substitutions of subtilisin BPN` changed its selectivity to favour the S-N reaction. Catalytic promiscuity may expand the application of enzymes to organic synthesis by allowing new transformations. Directed evolution may improve the

reaction further by increasing activity and enantioselectivity. QM/MM of the transition state should reveal the exact mechanism in the formation of the sulfinyl enzyme intermediate. The reaction could be applied in the transfer of a sulfinyl group to other amine and alcohol nucleophiles, rather than water.

Contributions to knowledge

1. We established that subtilisin is an excellent catalyst for the resolution of sterically hindered substrates. The bulky secondary alcohol spiro substituent (chapter 2) was resolved with high enantioselctivity. This example should allow further resolutions of sterically hindered substrates with subtilisin.

2. Molecular modeling of the spiro secondary alcohol showed no evidence for the stereoelectronic effect contributing to the enantioselectivity of subtilisin. This was suggested as an important factor in lipase and protease enantioselectivity, but in this example it does not contribute.

3. We performed the first investigation of protease catalyzed hydrolysis of tertiary alcohol esters. This is an important first step in developing efficient methods to produce enantiopure tertiary alcohols using proteases. Currently there are only a few synthetic and biocatalytic methods to prepare enantiopure tertiary alcohols.

4. We examined a new reaction that can be used in the synthesis of important enantiopure sulfinyl compounds, such as sulfinamides and sulfinates. This could eventually be used to transfer sulfinyl groups to other amines and alcohols instead of water.

5. Substrate studies on *N*-acyl sulfinamides and sulfinates indicate that binding in the acyl group portion can switch type of reaction between sulfinamide and carboxyamide hydrolysis. Introducing the dihydrocinnamoyl group turned the substrate around to increase the amount of sulfinamide hydrolysis. Thus the catalytic promiscuity arose from a re-orientation of the substrate rather than an introduction of new catalytic residues.

6. We demonstrated that catalytic promiscuity required only a few mutations to dramatically alter activity. Catalytic promiscuity is an emerging field of biocatalysts,

which should widen the number of functional group transformations available to organic chemists. It will also aid in understanding how enzymes acquire new functions.