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

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Abstract

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

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

1. Introduction

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

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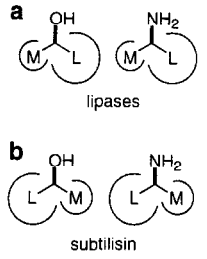


Fig. 1. Empirical rules that predict the enantiopreferences of lipases and subtilisins toward secondary alcohols and primary amines of the type NH_2CHRR'. (a) Lipases favor the enantiomer with the shape shown where L is a large substituent such as phenyl and M is a medium substituent such as methyl.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

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

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

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

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

2. Results

2.1. Enantiopreference of lipases toward primary amines

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

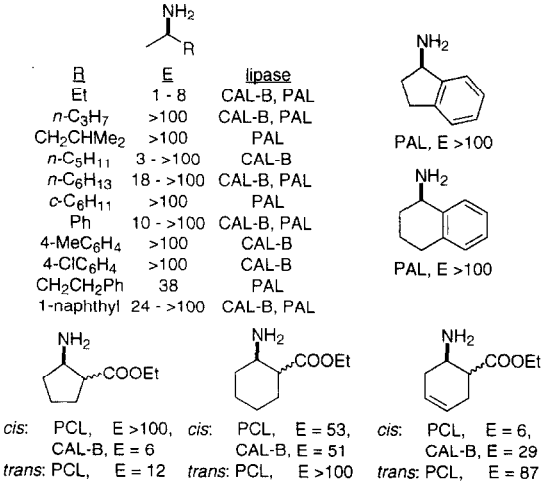


Fig. 2. Enantiopreference of lipases toward primary amines of the type NH_2CHRR'. Lipases favored acylation of the enantiomer shown or hydrolysis of the corresponding amide. CAL-B: acylation or hydrolysis using lipase B from *Candida antarctica*; PCL: acylation using lipase from *Pseudomonas cepacia*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

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

2.2. *Enantiopreference of subtilisin toward secondary alcohols and primary amines*

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

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

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

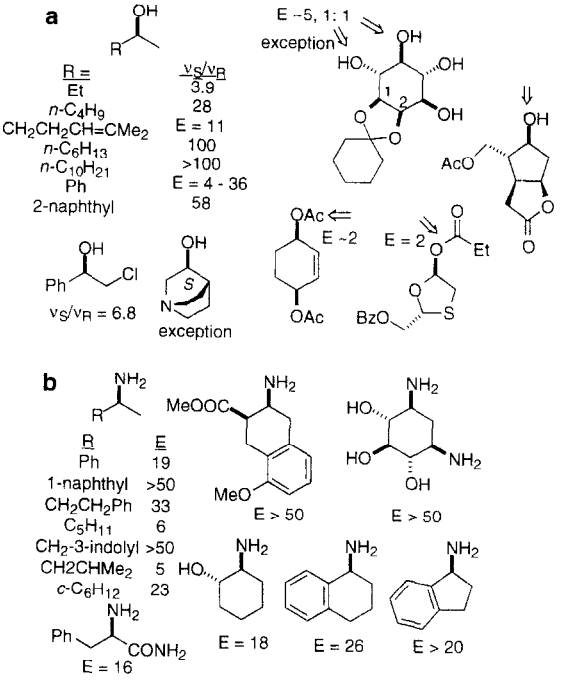


Fig. 3. Enantiopreference of subtilisins (Carlsberg or BPN') toward secondary alcohols and isosteric primary amines. (a) Fast-reacting enantiomer in the acylation of the alcohol or in the hydrolysis of the corresponding ester. Some researchers estimated the enantioselectivity by measuring the initial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

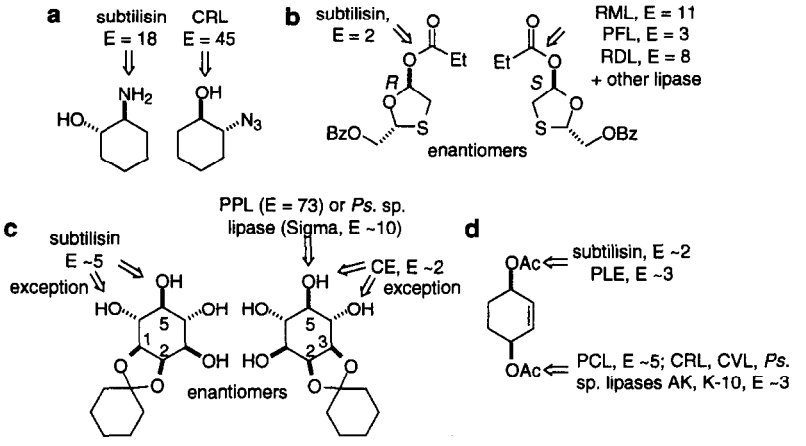


Fig. 4. Four examples of opposite enantioselectivity of lipases and subtilisin in the same or similar molecules. (a) Subtilisin catalyzed the allyloxycarbonylation of the amino group at the (*S*)-stereocenter, while in a similar molecule, CRL catalyzed the enantioselective hydrolysis of the butyrate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

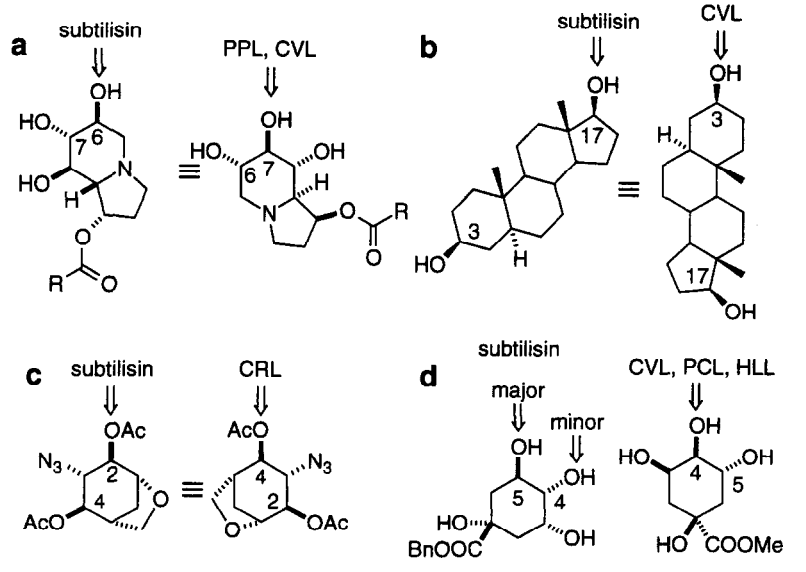


Fig. 5. Four examples of opposite regioselectivity of lipases and subtilisin. (a) Subtilisin favored acylation of the 6-position of 1-*O*-acylcastanospermine by as much as > 20: 1, while lipases porcine pancreatic lipase (PPL) and lipase from *Chromobacterium viscosum* (CVL) favored acylation of the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

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

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

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

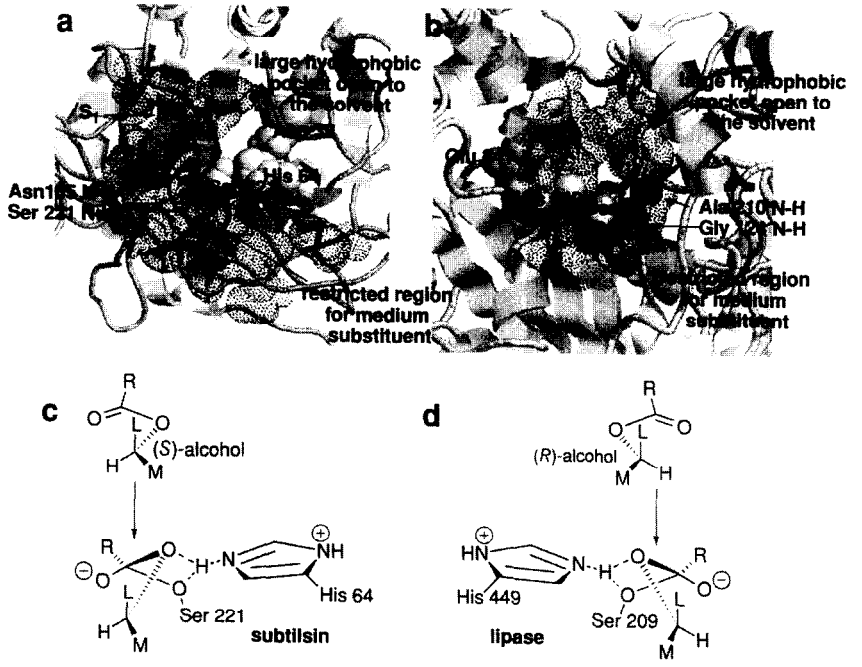


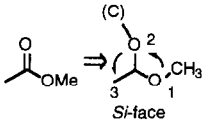
Fig. 6. Structures of subtilisin Calsberg and lipase from *Candida rugosa*. (a) X-ray crystal structure of subtilisin showing the catalytic machinery (Ser 221, His 64, Asp 32, and the N–H's of Asn 155 and Ser 221) and a portion of the proposed substrate binding site. The acyl chain binds in the region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

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

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

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

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



Si-face

3. Discussion

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

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

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

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

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

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

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