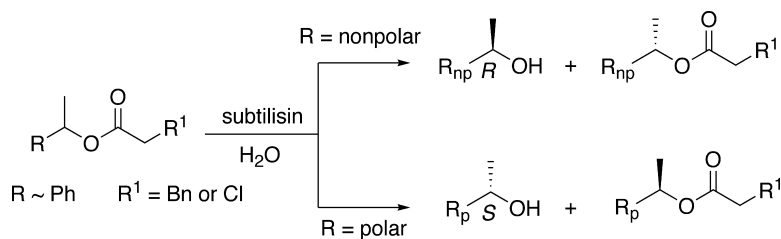


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## How Substrate Solvation Contributes to the Enantioselectivity of Subtilisin toward Secondary Alcohols

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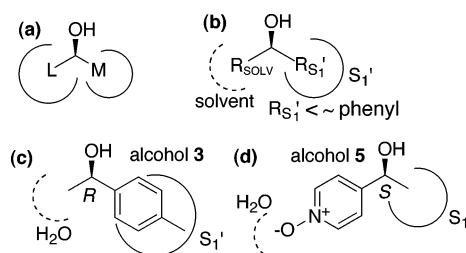
Enantioselective enzymes, especially hydrolases, are useful catalysts to make enantiomerically pure pharmaceuticals, agrochemicals, and fine chemicals.<sup>1</sup> Several empirical rules predict which substrate/hydrolase combinations work best. For example, a rule to predict the enantiopreference of subtilisin toward secondary alcohols is based on the size of the substituents at the stereocenter (Figure 1a).<sup>2,3</sup> This model implies that subtilisin has two differently sized pockets for these substituents, but several experiments are inconsistent with this rule. First, the X-ray crystal structure shows only one pocket (the S<sub>1</sub>' pocket) to bind secondary alcohols.<sup>4</sup> Second, the rule often predicts the incorrect enantiomer for reactions in water. In this communication, we resolve this contradiction with a more general rule that shows subtilisin binds only one substituent of a secondary alcohol and leaves the other in solvent. This refined rule allows quantitative design of enantioselective reactions and rationalizes why solvent alters the enantioselectivity.

X-ray crystal structures of subtilisin reveal one pocket (the S<sub>1</sub>' pocket) that binds the alcohol portion of an ester. Molecular modeling of **1a** reveals that (*S*)-**1a** places the methyl group in the S<sub>1</sub>' pocket, while (*R*)-**1a** places the phenyl group in this pocket (see SI Figure S2). In both cases, the other substituent remains in the solvent. The S<sub>1</sub>' pocket is a shallow crevice large enough to accommodate para-substituted aryl groups, but too small for multisubstituted aryl groups.

Although the rule in Figure 1a is reliable for reactions in organic solvents,<sup>2,3</sup> it is not reliable in water. In organic solvent, the subtilisin-catalyzed transesterification of secondary alcohols **1–13** with dihydrocinnamic acid vinyl ester favored the predicted (*S*)-enantiomer for 26 out of 29 reactions with varying enantioselectivity (*E* = 1.5 to 66; see SI Tables S2–S4). In water, however, subtilisin favored hydrolysis of the opposite (*R*)-enantiomer in most cases: 20 out of 33 reactions (Table 1).

To resolve these contradictions, we propose a revised rule for the enantiopreference of subtilisins with secondary alcohols (Figure 1b). This rule places one substituent in solvent and limits the size of the other substituent to approximately the size of a phenyl group. This rule predicts that solvation of one substituent contributes to the enantiopreference of subtilisin. In particular, placing a nonpolar substituent in water is unfavorable. Reactions in water involving methyl and nonpolar aryl substituents will favor the nonpolar aryl substituent in the S<sub>1</sub>' pocket, opposite to that predicted based on size alone. Thus, the revised rule predicts that subtilisin favors the (*R*)-enantiomer of **3a** in water, but the (*S*)-enantiomer in organic solvents. On the other hand, with a polar aryl group such as that in **5a** (4-pyridine *N*-oxide), the (*S*)-enantiomer is favored both in water, where solvation of the pyridine *N*-oxide is favorable, and in organic solvent, where placing the pyridine *N*-oxide in the solvent avoids steric interactions in the S<sub>1</sub>' pocket.

The revised rule in Figure 1b correctly predicted the (*R*)-enantiomer for reactions in water for substrates with hydrophobic



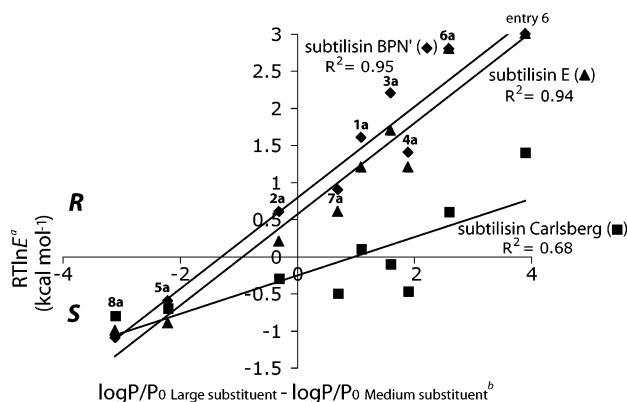
**Figure 1.** Empirical rules that predict the enantiopreference of subtilisins toward secondary alcohols. (a) A rule based on relative substituent size, where L is the large substituent and M is the medium substituent, is reliable in organic solvent. (b) A revised rule that is reliable in water as well. One substituent (R<sub>SOLV</sub>) remains in solvent, while the other (R<sub>S1'</sub>) binds in a hydrophobic pocket. (c) In water, the nonpolar aryl group of alcohol **3** favors binding in the S<sub>1</sub>' pocket, thus favoring the (*R*)-enantiomer. (d) An isosteric substrate alcohol **5** contains a polar aryl group that favors the water-solvated orientation, thus favoring the (*S*)-enantiomer.

**Table 1.** Enantioselectivity of Subtilisin BPN<sup>−</sup>, Carlsberg-, and E-catalyzed Hydrolysis of **1a–13a**<sup>a</sup>

entry	substrate	log <i>P/P</i> <sub>0</sub> diff. <sup>c</sup>	enantioselectivity, <i>E</i> <sup>b</sup>		
			subtilisin E	subtilisin Carlsberg	subtilisin BPN'
1	<b>1a</b>	+1.1	7.0 ( <i>R</i> )	1.2 ( <i>R</i> )	15 ( <i>R</i> )
2	<b>2a</b>	−0.3	1.5 ( <i>R</i> )	1.7 ( <i>S</i> )	2.6 ( <i>R</i> )
3	<b>3a</b>	+1.6	16 ( <i>R</i> )	1.1 ( <i>S</i> )	37 ( <i>R</i> )
4	<b>4a</b>	+1.9	7.7 ( <i>R</i> )	2.2 ( <i>S</i> )	9.9 ( <i>R</i> )
5	<b>5a</b>	−2.2	4.5 ( <i>S</i> )	3.1 ( <i>S</i> )	2.5 ( <i>S</i> )
6	<i>N</i> -HCinn- <i>p</i> -TS <sup>d</sup>	+3.9	> 150 ( <i>R</i> ) <sup>e</sup>	11 ( <i>R</i> ) <sup>f</sup>	50 ( <i>R</i> ) <sup>f</sup>
7	<b>6a</b>	+2.6	110 ( <i>R</i> )	2.5 ( <i>R</i> )	109 ( <i>R</i> )
8	<b>7a</b>	+0.7	2.8 ( <i>R</i> )	2.3 ( <i>S</i> )	4.2 ( <i>R</i> )
9	<b>8a</b>	−3.1	5.5 ( <i>S</i> )	3.6 ( <i>S</i> )	6.2 ( <i>S</i> )
10	<b>9a</b> <sup>g</sup>		20 ( <i>R</i> )	2.0 ( <i>R</i> )	18 ( <i>R</i> )
11	<b>10b</b> <sup>g</sup>		17 ( <i>R</i> )	1.7 ( <i>S</i> )	4.9 ( <i>R</i> )
12	<b>11a</b> <sup>g</sup>		1.8 ( <i>R</i> )	3.1 ( <i>S</i> )	3.1 ( <i>S</i> )
13	<b>12b</b>		n.r. <sup>h</sup>	n.r.	n.r.
14	<b>13a</b>		n.r.	n.r.	n.r.

<sup>a</sup> See SI Tables S2–S5 for complete details. <sup>b</sup> Relative rate of the fast vs slow enantiomer.<sup>5</sup> <sup>c</sup> Substituent hydrophobicity difference (log *P/P*<sub>0</sub>*R*<sub>Large substituent</sub> − log *P/P*<sub>0</sub>*R*<sub>Medium substituent</sub>). <sup>d</sup> *N*-Dihydrocinnamoyl-*p*-toluenesulfonamide. This is a secondary alcohol ester isostere with the methine replaced with sulfur and the methyl replaced with oxygen. <sup>e</sup> Reference 6. <sup>f</sup> Reference 7. <sup>g</sup> Not included in Figure 2 because one substituent is much larger than phenyl. <sup>h</sup> No reaction.

aryl groups (**1a**, **3a**, **6a**, **9a**, **10a**, and **11b**) for 14 out of 18 reactions and the (*S*)-enantiomer for substrates with hydrophilic aryl groups (**2a**, **5a** and **8a**) for eight of nine reactions. It is difficult to predict the favored enantiomer for moderately hydrophilic aryl groups (**4a** and **7a**), and indeed the enantioselectivity in these cases is low to



**Figure 2.** Differences in substituent hydrophobicity affect the enantioselectivity of subtilisins toward secondary alcohols. All reactions are in water. This plot does not include substrates **9a–11a** because their substituted aryl groups are too large to fit in the  $S_1'$  pocket of subtilisins. (a) Enantioselectivity data from Table 1 is given in energy using  $\Delta\Delta G^\ddagger = -RT \ln E$ . (b) Hydrophobicity partition coefficient ( $\log P/P_0$ ).

moderate ( $E = 2.2$  to  $9.9$ ). With nonpolar substituents and nonpolar solvents, the rule simplifies to the previous rule in Figure 1a.

The revised rule also suggests a quantitative link between enantioselectivity and solvation of the substituents. For example, reaction of dihydrocinnamoyl esters **1a–13a** with subtilisin E showed that the enantioselectivity toward secondary alcohol esters in water varied linearly with the difference in hydrophobicity ( $\log P/P_0$ )<sup>8</sup> between the large aryl substituent and the methyl group (Figure 2). This hydrophobicity difference accounts for the solvation of one substituent in water and the other in the hydrophobic  $S_1'$  pocket. Increases in hydrophobicity of the aryl group favored the (*R*)-enantiomer, while decreases favored the (*S*)-enantiomer. For example, subtilisin E-catalyzed hydrolysis of **6a** containing the nonpolar 4-isopropylphenyl group gave (*R*)-**6** with  $E = 110$ , while **7a** containing the more polar, but similar sized 4-nitrophenyl group gave (*R*)-**7** with lower enantioselectivity ( $E = 2.8$ ), and **8a** containing the hydrophilic carboxylate group gave the opposite enantiomer (*S*)-**8** with  $E = 5.5$ . Subtilisin BPN' showed similar enantioselectivity toward substrates **1a–13a** consistent with the similar  $S_1'$  pocket in both cases. The enantioselectivity of subtilisin Carlsberg was lower, and the change in enantioselectivity (slope of the line in Figure 2) varied less with changes in substituent hydrophobicity, presumably due to weaker interaction between substrate and  $S_1'$  pocket.

This revised model also predicts that increasing the polarity difference between the substituents will increase the enantioselectivity of subtilisins. Consistent with this prediction, subtilisin shows high enantioselectivity toward arylsulfonamides (entry 6).<sup>6</sup> This toluenesulfonamide is a polar isostere of **3a**, where a polar oxygen replaces the methyl group and thereby increases the difference in polarity between the two substituents ( $\log P$  difference =  $+1.6$  for **3a** and  $+3.9$  for the sulfonamide). The enantioselectivity of the subtilisin-E-catalyzed hydrolysis increases from  $E = 16$  for **3a** to  $E = >150$  for the sulfonamide.

Increasing the hydrophobicity difference by adding nonpolar substituents to the aryl group is not a good strategy to increase enantioselectivity because it creates a substituent too large for the  $S_1'$  pocket. For example, compounds **9a–11a** contain very large aryl groups. The poor fit of this aryl group in the  $S_1'$  pocket destabilizes reaction of the (*R*)-enantiomer. Subtilisins favor the (*S*)-enantiomer in these cases, but the enantioselectivity is usually low.

This model also rationalizes how changing the organic solvent can increase the enantioselectivity of subtilisins. The enantioselectivity of subtilisin Carlsberg toward 1-phenethyl alcohol (**1**) increases

from  $E = 3$  (*S*) in acetonitrile to  $E = 54$  (*S*) in benzene, likely due to better solvation of the solvent-exposed phenyl substituent in benzene as compared to acetonitrile.<sup>2</sup> Researchers previously explained changes in enantioselectivity of subtilisins toward chiral acids using a similar rationale for solvation of the solvent-exposed groups,<sup>9,10</sup> but our model is the first to use this approach for chiral alcohols.

Unlike subtilisins, which bind substrates in an extended conformation,<sup>11</sup> lipases bind substrates in a folded conformation.<sup>12</sup> This folding and the deeper hydrophobic pockets in lipases place both substituents of typical secondary alcohols in hydrophobic pockets that substantially shield the substituents from the solvent.<sup>13</sup> For this reason, the enantioselectivity of lipase-catalyzed resolutions of secondary alcohols shows less variation with changes in substituent polarity<sup>14</sup> or solvent.<sup>15</sup> The SI shows that lipase from *Burkholderia cepacia* (PCL) favors the (*R*)-enantiomer for all compounds in Table 1 and shows no reversal in enantiopreference upon changing from water to organic solvent.

In conclusion, this revised model of the enantioselectivity of subtilisins toward secondary alcohols is consistent with the structure of subtilisin, rationalizes why enantioselectivity changes and even reverses with changes in solvent, and provides a strategy to increase enantioselectivity by modifying the substrate.

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**Supporting Information Available:** Synthesis of compounds **1a–13a**, determination of absolute configuration, preparation of subtilisin E and BPN', molecular modeling details for **1a**, and enantioselectivity data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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