methane was added slowly until a yellow solution persisted. The excess diazomethane was quenched with acetic acid, and the solvent was removed at reduced pressure. The pale yellow oil obtained was crystallized from methanol/water to give 180 mg (90%) of white crystals, mp 88-90 °C. The spectral data were the following: $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 1.06 (s, 3 H), 1.12 (d, 1 H, $J$ = 14.4 Hz), 1.17 (d, 2 H, $J$ = 12.5 Hz), 1.20 (s, 3 H), 1.26 (s, 3 H), 1.53 (d, 1 H, $J$ = 13.1 Hz), 2.03 (s, 3 H), 2.75 (d, 1 H, $J$ = 12.9 Hz), 2.80 (d, 1 H, $J$ = 13.2 Hz), 2.95 (dd, 1 H, $J_1$ = 5.9 Hz, $J_2$ = 8.6 Hz), 3.44 (dd, 1 H, $J_1$ = 8.10 Hz, $J_2$ = 6.4 Hz), 3.60 (s, 3 H), 5.25 (dd, 1 H, $J_1$ = 5.9 Hz, $J_2$ = 2.10 Hz), 7.22 (m, 5 H); IR ($CH_2Cl_2$) 2966, 2932, 1728, 1680, 1496, 1462, 1429, 1383, 1319, 1207, 1169, 1093, 1020, 736, 702 cm$^{-1}$; MS $m/e$ 399 ($M^+$), 356, 340, 296, 268, 212, 194, 181 amu; HRMS for $C_{23}H_{29}NO_5$ calcd 399.2046, found 399. 2046.

**Imide Methyl Ester 5b.** This compound was synthesized and purified by use of the same conditions as those used for **5a**. The yield was 75%; mp 98-100 °C. The spectral data were the following: $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 0.75 (d, 3 H, $J$ = 6.8 Hz), 0.93 (d, 3 H, $J$ = 6.7 Hz), 1.2 (d, 2 H, $J$ = 14.8 Hz), 1.22 (s, 3 H), 1.27 (s, 3 H), 1.31 (s, 3 H), 1.37 (d, 1 H, $J$ = 13.2 Hz), 1.98 (d, 1 H, $J$ = 13.23 Hz), 2.12 (s, 3 H), 2.49 (m, 1 H), 2.79 (d, 1 H, $J$ = 14.5 Hz), 2.86 (d, 1 H, $J$ = 14.31 Hz), 3.62 (s, 3 H); IR ($CH_2Cl_2$) 3026-2947, 1734, 1718, 1684, 1489, 1458, 1431, 1363, 1317, 1259, 1172 cm$^{-1}$. MS $m/e$ 351 ($M^+$), 336, 308, 348, 220, 194, 121 amu; HRMS for $C_{19}H_{29}O_5N$ calcd 351.2046, found 351.2046.

**Keto Acid 9.** The synthesis of this compound was carried out with the procedure described for **3**, starting with the acid chloride[16] of **7** and the hydrochloride of aminoacetone. The compound **8** was obtained as a crystalline solid and was used without further purification. A sample (49 mg) of **8** was dissolved in 8 mL of methanol. To this was added 2 mL of 1 N NaOH, and the solution was heated at reflux for 3 h. Methanol was removed under reduced pressure, and the crude mixture was acidified with 1 N HCl at 15 °C. The aqueous solution was extracted with methylene chloride (3 × 15 mL), and the combined organic layers were dried over sodium sulfate, filtered, and then concentrated to dryness. The compound **9** was obtained in 70% yield. The spectral data were the following: $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 0.7 (m, 6 H), 1.35 (m, 12 H), 1.7 (m, 10 H), 2.29 (s, 3 H), 4.38 (d, 2 H, $J$ = 7.5 Hz), 7.50 (d, 1 H, $J$ = 3.0 Hz), 7.66 (d, 1 H, $J$ = 3.0 Hz), 7.98 (d, 1 H, $J$ = 3.0 Hz), 8.05 (d, 1 H, $J$ = 3.0 Hz), 9.2 (broad, 1 H); $^{13}$C ($CD_3OD$) $\delta$ 9.5, 27.5, 29.0, 31.5, 33.3, 33.35, 38.5, 40.0, 52.0, 129.3, 130.0, 130.3, 130.6, 145.0, 146; IR ($CH_2Cl_2$) 3314, 2965-2876, 1717, 1706, 1684, 1521, 1447, 1191, 1103 cm$^{-1}$.

**Ketone 10.** A sample (120 mg, 0.855 mmol) of benzoyl chloride and 0.34 mL (2.5 mmol) of freshly distilled triethylamine were dissolved in 40.0 mL of $CH_2Cl_2$. A 168.9-mg (0.94-mmol) portion of $\alpha$-aminoacetone hydrochloride was added to the reaction mixture followed by stirring overnight. The crude mixture was washed with a saturated solution of sodium carbonate followed by 10% HCl and then dried over sodium sulfate. The solvent was removed by rotary evaporation at room temperature. Purification by flash column chromatography on silica gel with 75:25 hexanes/ethyl acetate as the solvent system afforded **10** in 68% yield. The spectral data were the following: $^1$H NMR ($CDCl_3$, 270 MHz) $\delta$ 2.35 (s, 3 H), 4.35 (d, 2 H, $J$ = 8 Hz), 6.9 (broad, 1 H), 7.45 (m, 3 H), 7.85 (m, 2 H); IR ($CH_2Cl_2$) 3437, 3332-3300, 1720, 1639, 1540, 1405, 1019 cm$^{-1}$.

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**Registry No. 2**, 79410-29-0; **3a**, 120881-32-5; **3b**, 120881-33-6; **4a**, 120881-34-7; **4b**, 120881-35-8; **5a**, 120881-36-9; **5b**, 120904-96-3; **6**, 130525-40-5; **7**, 132103-56-1; **8**, 132103-57-2; **9**, 132103-58-3; **10**, 132103-59-4; $D_2$, 7782-39-0; $\alpha$-aminoacetone hydrochloride, 7737-17-9.

# Substrate Structure and Solvent Hydrophobicity Control Lipase Catalysis and Enantioselectivity in Organic Media

**Sanghamitra Parida and Jonathan S. Dordick***

*Contribution from the Department of Chemical and Biochemical Engineering and Center for Biocatalysis and Bioprocessing, University of Iowa, Iowa City, Iowa 52242.*
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**Abstract:** The lipase from *Candida cylindracea* catalyzes the enantioselective esterification of 2-hydroxy acids in nearly anhydrous organic solvents with primary alcohols as nucleophiles. The nature of the 2-hydroxy acid and organic reaction medium affects the efficiency of catalysis and the enantioselectivity. Straight-chain 2-hydroxy acids are highly reactive and give nearly 100% enantioselectivities in esterification reactions with 1-butanol. Slight branching with a methyl group adjacent to the 2-hydroxy moiety in toluene causes a substantial loss (up to 200-fold) in the lipase's catalytic efficiency with a concomitant loss in enantioselectivity. Losses in catalytic efficiency and enantioselectivity are also observed when the lipase is employed in hydrophilic organic media such as dioxane or tetrahydrofuran as compared to hydrophobic solvents such as toluene. With straight-chain substrates, the lipase is over 100-fold more active in toluene than in tetrahydrofuran or dioxane, while optimal enantioselectivity is observed in toluene. The loss in enantioselectivity in hydrophilic solvents is mainly due to a drop in the catalytic efficiencies of the *S* isomers, as the *R* isomers' catalytic efficiencies remain largely unchanged. In highly apolar solvents, such as cyclohexane, enantioselective relaxation occurs due to an increase in the reactivity of the *R* isomers relative to that of their *S* counterparts. These findings enabled a rational selection of substrates and solvents for a two-step, chemoenzymatic synthesis of optically active 1,2-diols to be carried out, the first step being the aforementioned enantioselective esterification of 2-hydroxy acids followed by reduction with $LiAl(OCH_3)_3H$ to give the optically active 1,2-diol. Diols such as (*S*)-(+)-1,2-propanediol, (*S*)-(-)-1,2-butanediol, (*S*)-(-)-1,2-hexanediol, and (*S*)-(-)-4-methyl-1,2-pentanediol were produced in high optical purities (at least 98% enantiomeric excess (ee)).

## Introduction

The substrate specificity and enantioselectivity of enzymatic catalysis in aqueous solutions have been well-studied.[1] Both characteristics owe their existence to the remarkable capability of enzymes to discern structural deviations in related molecules.[2] These factors can be quantitatively described by kinetic analyses through differences in the catalytic efficiency constant or specificity constant ($k_{cat}/K_m$ or $V_{max}/K_m$) between two or more substrates or between a given pair of stereoisomers. Alterations in substrate specificity or enantioselectivity can be induced by changes in either the enzyme structure or the reaction medium. The former requires protein engineering,[3] is not applicable to all enzymes, is tedious,

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**Scheme I**

(A) $(R,S)\text{-}R_1CH(OH)COOH + R'OH \xrightarrow{\text{Enzyme}} (S)\text{-}R_1CH(OH)COOR' + (R)\text{-}R_1CH(OH)COOH + H_2O$

(B) $(S)\text{-}R_1CH(OH)COOR' \xrightarrow{LiAl(OCH_3)_3H} (S)\text{-}R_1CH(OH)CH_2OH + R'OH$

| $R_1$ | Compd-Acid | Compd-Ester | Compd-Diol |
|---|---|---|---|
| $CH_3$ | 1 | 7 | 13 |
| $CH_3\,CH_2$ | 2 | 8 | 14 |
| $(CH3)_2\,CH$ | 3 | 9 | 15 |
| $CH_3\,(CH_2)_3$ | 4 | 10 | 16 |
| $(CH_3)_2\,CH\,CH_2$ | 5 | 11 | 17 |
| $Ph\,CH_2$ | 6 | 12 | 18 |

and can be expensive. The advent of nonaqueous enzymology, however, has provided enzymologists and organic chemists with the capability to alter the reaction medium for enzymatic catalysis at will.[4]

There are numerous cases wherein substrate specificity and enantioselectivity have been altered by catalysis in organic solvents and differences in solvent properties have been shown to cause predictable effects on enzyme specificity.[5-7] For example, the substrate specificities of subtilisin Carlsberg, chymotrypsin, and pig liver carboxylesterase are reversed in octane from those in aqueous solution. Hence, in octane, polar amino acid esters are favored over nonpolar substrates, just the opposite of that obtained in water.[5] A mechanistically unrelated enzyme (horseradish peroxidase) also shows solvent-induced specificity alterations.[6] In hydrophobic solvents, the partitioning of hydrophobic phenolic substrates into the enzyme's active site is poor, thereby leading to a low absolute value of $V_{max}/K_m$. Catalytic efficiencies deteriorate as both substrate and solvent hydrophobicities increase. In the peroxidase and protease/esterase cases, the hydrophobicity of the reaction medium appears to dictate the observed specificity shifts.

In addition to substrate specificity, enantioselectivity is also affected by the organic solvent.[7] A relaxation in the enantioselectivity of subtilisin Carlsberg, chymotrypsin, and $\alpha$-lytic protease occurs as the solvent hydrophobicity increases. Thus, D-amino acids are nearly as effective substrates as their L counterparts, a finding of great importance to the synthesis of unnatural peptides.[8]

One of the more useful classes of enzymes in organic synthesis in nonaqueous solvents is the lipases. Lipases are well-known to catalyze a number of potential commercially relevant reactions in nonaqueous solvents, for example, esterifications, transesterifications, interesterifications, lactone synthesis, peptide synthesis, and others.[9] However, no studies have been carried out to elucidate the effects of substrate structure and organic solvent on both catalytic specificity and enantioselectivity of lipase catalysis in nonaqueous media. Such a study would further the potential preparative uses of lipases in organic synthesis.

**Table I.** Substrate Specificity and Enantioselectivity of CCL Catalysis in Toluene[a]

| acid | $10^3(V_{max}/K_m)_{R,S}$,[b] $h^{-1}$ | ee, % |
|---|---|---|
| 1 | 240 | 95 |
| 2 | 200 | 99 |
| 3 | 1.0 | 17 |
| 4 | 71 | 99 |
| 5 | 16 | 98 |
| 6 | 12 | 73 |
| 2-hydroxycaprylic | 330 | 99[c] |
| 2-hydroxypalmitic | 0 | |

[a] Conditions are as described in the Experimental Section. [b] Catalytic efficiencies were measured with the racemic acids. [c] Optical purity obtained by reacting 0.1 M acid with 0.3 M 1-butanol in 0.2 L of toluene catalyzed by 25 g of CCL. The esterification reaction slowed considerably above 45% conversion. Introduction of an additional 25 g of CCL did not increase conversion beyond 50%, thus indicating that CCL obeys nearly complete enantioselectivity. The resulting butyl ester was isolated as per the other 2-hydroxy acid butyl esters. The product had $[\alpha]^{25}_D$ −3.3 ($c$, 1, $CHCl_3$).

In the present work, we have investigated the substrate and enantioselectivity of lipase catalysis in organic solvents using the esterification of 2-hydroxy acids with primary alcohols as a model reaction. In addition to this reaction being used to elucidate solvent effects on lipase catalysis, such a reaction, coupled to subsequent chemical reduction of the hydroxy esters, can also be used in the novel chemoenzymatic synthesis of optically active 1,2-diols as depicted in Scheme I. These compounds are useful intermediates in the synthesis of antibiotics,[10,11] optically active polymers,[12] and chiral solvents[13] and are potentially useful chiral auxiliaries and ligands in hydrogenation catalysts[14] and chiral crown ethers.[15] Chemical methods of synthesis include the osmylation of olefins via slow feeding of the olefin to the metal catalyst,[16] ring opening of epoxides,[17] tin-mediated coupling of aldehydes,[18] and reduction of optically active hydroxy acids or esters.[19] While these reactions can be carried out in high conversions, enantioselectivities vary considerably with typical values ranging from 50 to 95% enantiomeric excess (ee).

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Biological alternatives to chiral 1,2-diol synthesis do exist (e.g., fermentation of xylose or glucose by *Clostridium thermosaccharolyticum* produces (*R*)-(−)-1,2-propanediol,[20] fermentative baker's yeast or glycerol dehydrogenase catalyzes the reduction of α-hydroxy ketones to chiral vicinal diols,[21,22] and microsomal epoxide hydrolase is capable of enantioselectively hydrolyzing epoxides to the corresponding chiral 1,2-diols[23]); however, dilute aqueous productivities, limited substrate specificities, or limited enzyme availability restricts the practicality of the biological routes.

## Results and Discussion

The esterification of 2-hydroxycaproic acid (**4**, see Scheme I) was chosen as our model reaction. This hydroxy acid is commercially available and soluble in a wide number of organic solvents. Lipases, in general and in the presence of alcohols, are well-known to catalyze esterification of organic acids in nearly anhydrous environments, often with high degrees of enantioselectivity.[24] As such, 12 commercially available lipases were tested for esterification activity of **4** in toluene with 1-butanol as the nucleophile.[25] The enzymes were suspended in a toluene solution containing a 3-fold molar excess of 1-butanol.[26] The suspensions were shaken at 250 rpm at 25 °C. The lipase from the yeast *Candida cylindracea* (CCL from Sigma and AY from Amano) gave the highest activities and was deemed as the most appropriate biocatalyst for this study.

Encouraged by the high activities afforded by the yeast lipase, gram-scale ester synthesis was commenced as follows: Powdered CCL was suspended (0.1 g/mL) in 0.5 L of toluene containing 0.1 M **4** and 0.3 M 1-butanol. The reaction was stirred at 250 rpm at 30 °C. The reaction proceeded to nearly 50% conversion after 15 h. No lactone formation was evident (as determined by GC). Introduction of 0.2 g/mL of additional lipase resulted in very slow further esterification (20% additional conversion in 30 h), suggesting that the reaction was highly enantioselective. In the absence of enzyme, no reaction was observed.

CCL is well-known as a highly useful catalyst for enantioselective esterifications of organic acids in organic solvents.[9,24,27] Hence, it was of direct interest for us in this work to elucidate the substrate specificity and enantioselectivity of CCL on 2-hydroxy acids in nonaqueous media. For straight-chain acyl groups, the length of the carbon chain (from $C_3$ to $C_8$) did not significantly affect lipase catalysis in toluene, as reflected in the values for catalytic efficiency ($V_{max}/K_m$) (Table I). However, a very long acyl group such as 2-hydroxypalmitic acid ($C_{16}$) was not a substrate in toluene. It should be noted that the natural reaction with CCL in water is the hydrolysis of triglycerides such as those that contain palmitic acid.[28] As such, it was expected

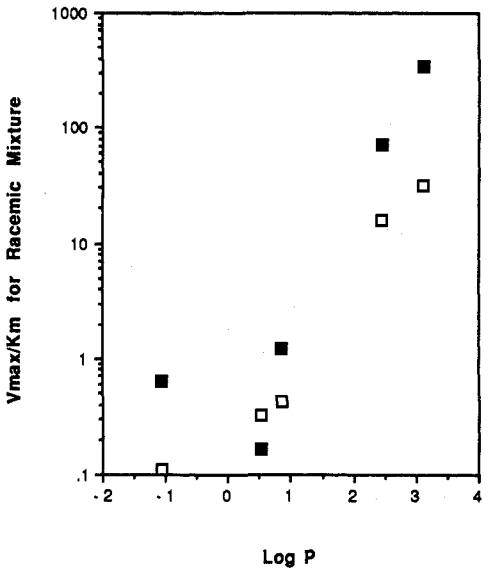


**Figure 1.** Influence of solvent hydrophobicity[36] on the catalytic efficiency of CCL-catalyzed esterification of (*R*,*S*)-2-hydroxycaproic acid (■) and (*R*,*S*)-2-hydroxyisocaproic acid (□). The conditions are as described in the Experimental Section. The unit of $V_{max}/K_m$ is reciprocal hours.

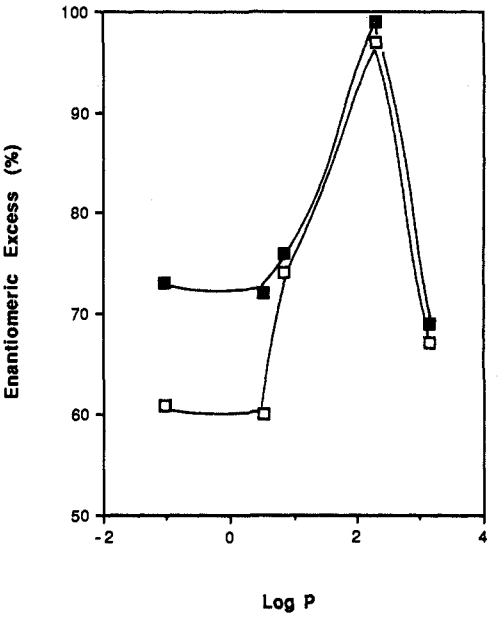


**Figure 2.** Influence of solvent hydrophobicity on the enantioselectivity of CCL catalysis for (*R*,*S*)-2-hydroxycaproic acid (■) and (*R*,*S*)-2-hydroxyisocaproic acid (□).

that the $C_{16}$ 2-hydroxy fatty acid would be a substrate in aqueous solutions.

The presence of bulky groups or chain branching in the vicinity of the 2-hydroxy group did lower the catalytic efficiency of CCL in toluene. For example, the value of $V_{max}/K_m$ for the esterification of the bulky **6** is over 20-fold lower than that for **1**. The location of chain branching also is rate controlling. As the chain branch occurs closer to the hydroxyl group, the reaction rate decreases; **3** reacts 16-fold slower than **5** wherein the methyl branch is β to the hydroxy moiety. These findings suggest that the acyl binding pocket of the active site of CCL best accommodates linear hydroxy acids. Branched hydroxy acids fit less effectively into this binding site and, therefore, are less reactive. The sterically hindered binding pocket of CCL may be further exacerbated by the use of the lipase in organic solvents. It is well-known that enzymes in nearly anhydrous organic solvents are highly rigid.[29] This

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(25) Both toluene and 1-butanol have been used for CCL-catalyzed esterification of organic acids.[4]
(26) The concentrations of **4** and 1-butanol were 0.1 and 0.3 M, respectively. All enzymes tested were completely insoluble in toluene and other organic solvents used in this work. The relatively large amount of enzyme used in a given experiment is a result of the commercial nature of the enzyme preparations. Such enzymes are highly impure and contain inert protein and nonprotein materials. The lipases used in the screen (0.5 g of enzyme in 2 mL of toluene) and the conversion (%) of **4** to the butyl ester **10** after 24 h were as follows: AY, 47; CCL, 45; *M. miehei*, 39; P, 34; MAP-10, 17; CE-10, 15; G, 8; AP, 3; APF, 3; GC-20, 20; FAP-15, 2; PPL, 0.
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structural inflexibility may prevent the lipase's active site from accepting branched-, bulky-, or long-chain 2-hydroxy acids. Therefore, in addition to the relatively low catalytic activity observed with **3** or **6**, 2-hydroxypalmitic acid may be unreactive due to the structural inflexibility of the CCL's active site in nonaqueous media.

The enantioselectivity of CCL-catalyzed ester synthesis in toluene is highly dependent on the catalytic efficiency of the reaction. As depicted in Table I, the enantioselectivity of CCL catalysis in toluene is nearly 100% for straight-chain substrates. Branching close to the 2-hydroxy moiety results in poor enantioselectivities as evidenced by the enantiomeric excess of **9** being only 17%.

The catalytic efficiency of CCL also was significantly affected by the nature of the organic solvent. Two substrates, **4** and **5**, were examined in a number of solvents with widely different hydrophobicities ranging from the hydrophilic dioxane to the hydrophobic cyclohexane. In both cases, the catalytic efficiency of CCL increased as the solvent hydrophobicity increased (Figure 1). This effect was dramatic. The lipase was over 3 orders of magnitude more active in cyclohexane than in tetrahydrofuran. The slower enzymatic reactions in hydrophilic solvents resulted in a relaxation of enantioselectivity for both substrates (Figure 2). It should be note that, in each solvent, CCL catalysis was allowed to proceed to 25% conversion in order to maximize the enantioselectivity of ester formation.[27] Surprisingly, the enantioselectivity does appear to become maximal in toluene as the higher catalytic efficiency in cyclohexane did not translate into nearly complete enantiospecificity.

A relationship between catalytic efficiency and enantioselectivity was expected. The enantioselectivity for lipase catalysis can be represented by the ratio of $(V_{max}/K_m)_S/(V_{max}/K_m)_R$. In order to determine whether the observed specificity shifts were due to changes in the catalytic efficiency of one or both stereoisomers, the catalytic efficiencies of the individual isomers must be known. For **1**, **5**, and **6**, *S* isomers are commercially available, while for **4** the *R* isomer was obtained as described in the Experimental Section. In each case, it is possible to calculate the catalytic efficiency of the opposite isomer given the enantioselectivity of the reaction as follows. Consider the reaction between the two competing isomers *S* and *R*. The specificity of the enzyme is a function of the $V_{max}/K_m$ ratio for each isomer and the concentration of the isomer as shown in eq 1.[2] At initial rate conditions,

$$v_S/v_R = (V_{max}/K_m)_S[S]/(V_{max}/K_m)_R[R] \quad (1)$$

$[S] = [R]$ and the ratio of $v_S/v_R$ (where $v_i$ is the initial rate of reaction with isomer i at a given isomer concentration [i]) is given by the enantiomeric ratio (*E*) and is equal to the ratio of catalytic efficiencies of the *S* and *R* isomers (eq 2).[30] This enantiomeric

$$E = (V_{max}/K_m)_S/(V_{max}/K_m)_R = \ln [1 - C(1 + ee_p)]/\ln [1 - C(1 - ee_p)] \quad (2)$$

ratio can then be related directly to the enantiomeric excess of the ester product ($ee_p$) by eq 2.[30] For initial rate studies, conversions of no greater than 10% were performed; hence, $C = 0.1$. This analysis allows one to calculate the value of catalytic efficiency of the *S* or *R* isomer of a given hydroxy acid once the opposite isomer's catalytic efficiency and the enantiomeric excess of the reaction are known. The calculated values for **1** and **4–6** in a number of organic solvents are given in Table II.

For highly reactive 2-hydroxy acids, the *S* isomer is reactive; for **1** or **4**, the ratio of $(V_{max}/K_m)_S/(V_{max}/K_m)_R$ is calculated to be 220 in each case in toluene. As depicted in Table II, the drop in enantioselectivities in toluene for less reactive substrates is mainly due to a reduction in the value of $V_{max}/K_m$ for the *S* isomers. For example, the catalytic efficiency of the *S* isomer of **6** is 20-fold lower than that of the *S* isomer of **1**, whereas the catalytic efficiencies of the *R* isomers are comparable. More striking is the difference in catalytic efficiency between **1** and **3**; the ratio of efficiencies for the *S* isomers is 490-fold, whereas the ratio of *R* isomers is comparable.

**Table II.** Catalytic Efficiency of CCL Catalysis in Various Organic Solvents[a]

| acid | solvent | $10^3(V_{max}/K_m)_{R,S}$, $h^{-1}$ | $10^3(V_{max}/K_m)_S$, $h^{-1}$ | $10^3(V_{max}/K_m)_R$, $h^{-1}$ |
|---|---|---|---|---|
| **1** | toluene | 240 | 490 | 2.2[b] |
| **3** | toluene | 1.0 | 1.0[c] | 1.0[c] |
| **4** | toluene | 72 | 140[d] | 0.64 |
| **5** | toluene | 16 | 32 | 0.29[b] |
| **6** | toluene | 8.7 | 24 | 3.4[b] |
| **4** | cyclohexane | 350 | 1230[d] | 200 |
| **5** | cyclohexane | 31 | 79 | 15[b] |
| **4** | diethyl ether | 1.2 | 6.9[d] | 0.82 |
| **5** | diethyl ether | 0.42 | 2.3 | 0.32[b] |
| **4** | dioxane | 0.63 | 1.7[d] | 0.24 |
| **5** | dioxane | 0.11 | 0.23 | 0.052[b] |
| **4** | THF | 0.17 | NC | $<10^{-3}$ |
| **5** | THF | 0.32 | 1.4 | 0.33[b] |

[a] Conditions are as described in the text and Experimental Section. [b] Calculated from the value of $(V_{max}/K_m)_S$ and the ee with eq 2. [c] These are assumed values given that the ee is low; hence, the catalytic efficiencies for the individual isomers are similar to that for the racemic mixture. [d] Calculated from the value of $(V_{max}/K_m)_R$ and the ee with eq 2.

With respect to the reaction medium, as the solvent becomes more hydrophilic, the enzyme becomes a less efficient catalyst for butyl esterification. This may be speculated to be due to a number of factors including the stripping of enzyme-bound water away from the catalyst, thereby reducing the catalytic power of the enzyme,[31] or the partitioning of the 2-hydroxy acid substrate away from the lipase's active site and into the bulk reaction medium. This latter effect would reduce the catalytic efficiency of CCL in solvents that can readily dissolve 2-hydroxy acids due to an increase in the apparent $K_m$ of the substrates. It should be noted that the solubility of all 2-hydroxy acids tested was higher in the hydrophilic solvents such as dioxane or THF than in toluene. A mechanistically similar phenomenon has been obtained with peroxidase catalysis in organic media with phenolic substrates.[6] As with less reactive substrates in toluene, the loss in the enantioselectivity of 2-hydroxy acid esterification in hydrophilic solvents is due mainly to a reduction in the value of $(V_{max}/K_m)_S$ (Table II). For example, the catalytic efficiency for the *S* isomer of **4** is 60-fold lower in diethyl ether than in toluene, whereas the *R* isomer's catalytic efficiency is not significantly affected.

It is interesting that the lower catalytic efficiencies of the *S* isomers in hydrophilic solvents are phenomenologically similar to those found with branched substrates in toluene (i.e., only the *S* isomers show reduced catalytic efficiencies). One may envision that, for branched substrates in toluene, the *S* isomers cannot fit properly into the active site and, hence, both reactivity and enantioselectivity drop. Assuming the active site of CCL is stereoselective, then in order for the *R* isomers to be reactive, they must bind to the enzyme incorrectly.[32] This incorrect binding is much less affected by the branched nature of the substrate, as the branched groups of the *R* isomers would not occupy the same spatial position in the active site as the branched groups of the *S* isomers. Similarly, the hydrophilic solvents must impair the ability of the *S* isomers to bind well to the active site of CCL, although the exact mechanism of this effect is still undetermined. Once again, the *R* isomers, which do not bind well to the active site, are less affected.

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The lower enantioselectivities for **4** and **5** in cyclohexane cannot be explained by a drop in activities for the *S* isomers, as the enzyme is significantly more active in cyclohexane than in toluene (Table II), but rather the *R* isomers have also become more reactive. In cyclohexane, the *R* isomers were roughly one-sixth as reactive as the *S* isomers. This explains the relatively low ee's for **10** and **11** obtained in cyclohexane. The change in the enantioselectivity of CCL together with the increase in catalytic efficiency of CCL in cyclohexane, as compared with more polar solvents tested in this work, suggests that CCL can be used to effectively esterify *both R* and *S* isomers of 2-hydroxy acids in very hydrophobic solvents. A similar effect has been observed for serine proteases in highly apolar solvents in which the reactivity of the *R* isomer of amino acid esters increases as the solvent hydrophobicity increases.[7] While the mechanism for enantioselective relaxation for serine proteases has been ascribed to an inhibition in the release of active site water into a highly apolar solvent, such an event cannot be reliably predicted with CCL due to a lack of understanding of the structure of the active site. Clearly, however, the enantioselectivity of CCL catalysis in organic media is controlled by both the organic solvent and the substrate structure. This finding should be of great interest to both enzymologists and organic chemists, as rational approaches to solvent and substrate choice can be made for selective organic synthesis with enzymes.

**Synthesis of (*S*)-(−)-1,2-Hexanediol (16). A Model System.** The ability to control the enantioselective esterification of 2-hydroxy acids catalyzed by CCL in organic media prompted us to investigate an example of where such ester syntheses can be employed. CCL is capable of catalyzing the enantioselective esterification of 2-halo carboxylic acids[27] in hexane. Optically active butyl esters were produced in greater than 95% ee. In principle, a similar technique could be employed in the synthesis of optically active 2-hydroxy carboxylic esters. The resulting esters can be reduced with lithium aluminum hydride (LAH) or its derivatives to yield optically active 1,2-diols.[33] The chemoenzymatic approach is depicted in Scheme I.

The model reaction employed for structure–function studies (2-hydroxycaproic acid (**4**)) was also used in the enzymic step for 1,2-diol synthesis. For synthetic purposes, we initiated a reaction identical with that described earlier (e.g., powdered CCL was suspended (0.1 g/mL) in 0.5 L of toluene containing 0.1 M **4** and 0.3 M 1-butanol). The reaction was stopped after 8 h (38% conversion) and the product isolated (see Experimental Section), yielding 2.2 g (64% isolated yield) of (*S*)-(−)-butyl 2-hydroxycaproate (**10**) in 99% ee.[34] Reduction of 1.4 g of **10** with $LiAl(OCH_3)_3H$ yielded (*S*)-(−)-1,2-hexanediol (**16**) (0.83 g, 96% isolated yield) in 99% ee. Therefore, the chemoenzymatic two-step reaction sequence depicted in Scheme I provides a high degree of optically pure (*S*)-(−)-**16**.

In addition to 1-butanol, a number of other primary alcohols were capable of acting as acyl acceptors in the CCL-catalyzed esterification of **4** in toluene, including ethanol, 1-propanol, and 1-hexanol (Figure 3). In each case, the corresponding ester was isolated in greater than 95% ee. No reaction was observed with secondary alcohols such as 2-propanol or 2-butanol.

The reaction rate with 1-hexanol was virtually identical with that with 1-butanol; hence, we proceeded to investigate this alcohol as a cosubstrate for diol synthesis. After 8 h, 38% conversion of **4** to the hexyl ester was obtained. Following workup, 1.20 g (72% isolated yield) of the hexyl ester was isolated. The hexyl ester (0.80 g) was reduced exactly as with the butyl ester to give (*S*)-(−)-**16** (0.41 g, 96% isolated yield) in 99% ee. Hence, substitution of butanol with a larger acyl acceptor did not effect the conversion of **4** to (*S*)-(−)-**16**. Toluene was by no means the only solvent capable of sustaining CCL catalysis. Effective diol synthesis was achieved with use of cyclohexane, diethyl ether, THF, and dioxane as solvents for the enzymic step, albeit with differing

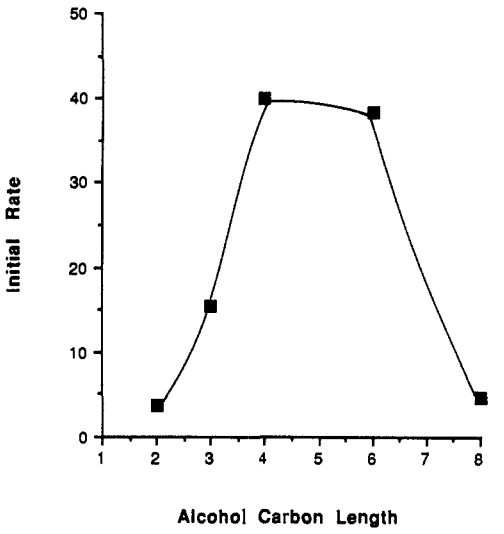


**Figure 3.** Effect of alcohol chain length on CCL-catalyzed esterification of (*R,S*)-2-hydroxycaproic acid. Conditions: 0.1 M acid and 0.3 M *n*-alkyl alcohol dissolved in 10 mL of toluene containing 0.2 g of CCL. The suspension was shaken at 250 rpm at 30 °C. The unit of initial rate is micromoles per hour.

degrees of enantioselectivity as predicted from the structure–function studies.

A significant advantage of enzymatic catalysis in organic as opposed to aqueous media is the ability to directly couple the enzymic step to a chemical synthesis that must be performed in a nonaqueous environment.[4] For example, LAH and its derivatives are highly unstable in water yet are effective reductants in dry organic solvents such as diethyl ether. It occurred to us that the lipase-catalyzed esterification of **4** and the necessary reduction could be carried out sequentially in the same solution of diethyl ether without purification of the intermediate hydroxy ester.[35] The CCL-catalyzed esterification proceeded slowly with less than 10% conversion after 5 days. In contrast to CCL, lipase AY showed higher activity in diethyl ether; in 5 days, 45% of **4** was esterified to **10**. The resulting ester was worked up as described in the Experimental Section and, while remaining in the original ether solution, was reduced with $LiAl(OCH_3)_3H$, resulting in (*S*)-(−)-**16** in 95% ee. Thus, chemoenzymatic synthesis of optically active 1,2-diols can be performed in a single reaction vessel without evaporation of the organic solvent.

**Chemoenzymatic Synthesis of Optically Active 1,2-Diols.** The two-step enzyme/chemical synthetic scheme for optically active 1,2-diols could be extended to a number of related alkyl and aryl 2-hydroxy acids (Table III). All butyl esters were produced in the *S* form (with varying optical purities) and with high GC purities (>98%). Reduction of the butyl esters with $LiAl(OCH_3)_3H$ (Table IV) resulted in the formation of *S*-1,2-diols (with varying optical purities). Complete retention of optical activity was obtained upon reduction of the esters. Straight-chain alkyl hydroxy acids were esterified with nearly complete stereoselectivity. However, chain branching or the presence of bulky groups in the vicinity of the 2-hydroxyl moiety reduced the enantioselectivity of lipase-catalyzed esterification. In addition to the reduction in CCL's enantioselectivity, the rates of esterification with such compounds were lower than with straight-chain substrates.

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(34) The isolated yield is based on a maximum of 50% conversion of the (*R,S*)-**4**.

(35) $LiAl(OCH_3)_3H$ has virtually no solubility in toluene; hence, diethyl ether was used for the chemoenzymatic reaction sequence.

(36) log *P* is defined as the logarithm of the partition coefficient of a given solvent between 1-octanol and water (Laane, C.; Boeren, S.; Vos, K.; Veeger, C. *Biotechnol. Bioeng.* **1987**, *30*, 81–87). Values of log *P* for 1-butanol–solvent mixtures were calculated with use of the following correlation: $\log P = (1 - x) \log P_{\text{solvent}} + x \log P_{\text{1-butanol}}$, where $x$ is the mole fraction of 1-butanol in the mixture (Reslow, M.; Aldercreutz, P.; Mattiasson, B. *Appl. Microbiol. Biotechnol.* **1987**, *26*, 1–8).

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**Table III.** Synthesis of Optically Active Esters from Racemic Acids via Scheme I with CCL in Toluene[a]

| acid | reaction time, h | degree of conv, % | yield, g (%)[b] | bp, °C (mmHg) | purity,[c] % | $[\alpha]^{25}_D$,[d] deg | isomer | ee, % |
|---|---|---|---|---|---|---|---|---|
| **1** | 3.2 | 50 | 4.1 (47) | 183–185 (760) | 98 | −12.3 (*c* 1) | *S* | 95[e] |
| **2** | 2.5 | 38 | 1.5 (42) | 66–68 (2) | 98 | −3.4 (*c* 1) | *S* | 99[f] |
| **3** | 168 | 13 | 0.4 (12) | 204–205 (760) | 98 | 3.2 (*c* 2) | *S* | 17[f] |
| **4** | 8 | 38 | 2.2 (32) | 43–44 (3) | 98 | −6.8 (*c* 1) | *S* | 99[f] |
| **5** | 48 | 40 | 1.4 (37) | 84–85 (7) | 99 | −6.7 (*c* 1) | *S* | 98[g] |
| **6** | 192 | 40 | 2.1 (31) | 138–140 (4) | 98 | −16.8 (*c* 1) | *S* | 73[g] |

[a] Conditions: The concentrations of racemic acids and 1-butanol were 0.1 and 0.3 M, respectively. The amounts of racemic acid and CCL and volumes of toluene were 9.0 g of **1**, 50 g of CCL, 0.50 L; 3.0 g of **2**, 14 g of CCL, 0.30 L; 5.0 g of **3**, 60 g of CCL, 0.30 L; 4.8 g of **4**, 75 g of CCL, 0.36 L; 2.6 g of **5**, 30 g of CCL, 0.20 L; and 2.4 g of **6**, 50 g of CCL, 0.2 L. The reactions were magnetically stirred at 250 rpm at 30 °C. [b] Given as the molar ratio of isolated ester/starting acid; hence, for enantiospecific syntheses the yields are twice that as listed. [c] Determined by gas chromatography. [d] All optical rotations were measured in $CHCl_3$. [e] Compared with authentic (*S*)-**7**. [f] These values are from corresponding ee's of the respective diols (see Table IV). [g] Compared with enzymically prepared *S* butyl esters from the commercially available *S* acids. The synthesis of these *S* esters was similar to that described for the respective racemic starting materials.

**Table IV.** Synthesis of Optically Active 1,2-Diols from Enzymically Prepared 2-Hydroxy Esters[a]

| ester | yield of diol, g (%) | purity,[b] % | $[\alpha]^{25}_D$, deg | isomer | ee, % | resulting diol product |
|---|---|---|---|---|---|---|
| **7** | 1.2 (31) | 99 | 16.2 (*c* 1, EtOH) | *S* | 97 | **13**[c] |
| **8** | 0.6 (76) | 96 | −13.2 (*c* 1, EtOH) | *S* | 99 | **14**[d] |
| **9** | 0.2 (84) | 96 | 1.8 (*c* 3, EtOH) | *S* | 17 | **15**[e] |
| **10** | 0.8 (96) | 99 | −15.9 (*c* 1, EtOH) | *S* | 99 | **16**[f] |
| **11** | 0.6 (97) | 99 | −22.8 (*c* 1, EtOH) | *S* | 89 | **17**[g] |
| **12** | 0.9 (97) | 95 | −21.0 (*c*, 1, EtOH) | *S* | 75 | **18**[h] |

[a] Conditions: The respective amounts of butyl esters and $LiAl(OCH_3)_3H$ and volumes of diethyl ether were 7.5 g of **7**, 18 g, 0.50 L; 1.1 g of **8**, 5.3 g, 0.15 L; 0.4 g of **9**, 2.2 g, 0.10 L; 1.4 g of **10**, 7.0 g, 0.20 L; 1.0 g of **11**, 4.0 g, 0.10 L; 1.3 g of **12**, 7.0 g, 0.20 L. The reaction time was 1 h for each reduction. The diol products **13–18** were purified as described in the Experimental Section. [b] Determined by gas chromatography. [c] Compared with authentic (*S*)-(+)-**13**. [d] Lit.[37] $[\alpha]^{25}_D$ −12.6 (*c* 11, EtOH). [e] Lit.[38] $[\alpha]^{25}_D$ −10.9 (*c* 0.9, $CHCl_3$). [f] Lit.[39] $[\alpha]^{25}_D$ −15.7 (*c* 13, EtOH). [g] From (*S*)-**5** starting material and chemoenzymatic reaction carried through to the diol product. [h] From (*S*)-**6** starting material and chemoenzymatic reaction carried through to the diol product.

## Conclusions

The yeast enzyme from *C. cylindracea* (CCL or AY) is an effective biocatalyst for the enantioselective esterification of 2-hydroxy acids in a variety of organic solvents and in the presence of a number of alcohols. Because the reactivity of the lipase was heavily influenced by the substrate and organic solvent, the substrate specificity and enantioselectivity of CCL in nonaqueous media have been examined in this work. Straight-chain alkyl 2-hydroxy acids are highly reactive and give nearly 100% enantioselectivities in the esterification reactions with 1-butanol. Slight branching with a methyl group adjacent to the 2-hydroxy moiety causes a substantial loss in CCL's catalytic efficiency (up to nearly 200-fold, compare **3** to **2** in toluene). A significant drop in catalytic power is also observed with a bulky aryl group adjacent to the hydroxyl moiety. Similar losses in catalytic efficiency are observed when CCL reacts in hydrophilic media such as dioxane and THF. In general, the lower the reactivity of CCL toward a substrate in a given solvent, the poorer the enantioselectivity. This relaxation in enantioselectivity is due to primarily to a decrease in the catalytic efficiencies of the *S* isomers. These findings provide us with a method to quantitatively describe CCL catalysis in nonaqueous media and allow us to rationally choose both substrate and solvent for enantioselective catalysis.

In addition to the fundamental studies on CCL catalysis, we have discovered that the lipase-catalyzed enantioselective esterification of 2-hydroxy acids could be used advantageously in the single-pot chemoenzymatic synthesis of optically active 1,2-diols. Optically active vicinal diols with enantioselectivities in excess of 95% can be prepared with use of this approach. Application of enzymes in nonaqueous media can be significantly extended if chemoenzymatic syntheses are considered. Enantioselective synthesis of 1,2-diols is one example of this approach.

## Experimental Section

**Materials.** Porcine pancreatic (PPL) and *C. cylindracea* (CCL) lipases were purchased from Sigma Chemical Co. Lipase from *Mucor miehei* was obtained from Novo-Nordisk. All other enzymes used in this work were obtained from Amano International Enzyme Co. These included lipases from *C. cylindracea* (AY), *Pseudomonas fluorescens* (P), *Rhizopus* sp. (FAP-15), *Mucor* sp. (MAP-10), *Geotrichum candidum* (GC-20), *Aspergillus niger* (AP and APF), *Humicola langinosa* (CE-10), and *Pseudomonas* sp. (G). In all experiments, the enzymes were used directly from the bottle without any prior pretreatment.

All racemic 2-hydroxy acids (as well as individual optical isomers when available) and alcohols used in this work were obtained commercially. All organic solvents employed in this work were of analytical grade and, prior to use, were dehydrated by shaking with 4-Å molecular sieves (Linde) (ca. 1 g of sieves/10 mL of solvent) for 24 h at room temperature. The water content of the solvents did not exceed 0.01% (w/w), as determined by the optimized Fisher method with a Karl-Fisher Coulometric titrimeter (Fisher-Scientific).

**Synthesis of $LiAl(OCH_3)_3H$.** This derivative of LAH was prepared fresh each time in diethyl ether solution.[33] In a typical reaction (e.g., for the preparation of **10** reduction), 2 g (0.05 mol) of LAH suspended in 100 mL of diethyl ether was mixed with 6.4 mL (0.166 mol) of methanol at 4 °C. The mixture was magnetically stirred for 2 h prior to use.

**Assays.** All acids, esters, and diols in this work were quantified by gas chromatography with a 25-m capillary column with 530-μm fused-silica gum (Hewlett-Packard), a $N_2$ carrier gas (30 mL/min), and injector and detector port temperatures of 250 °C. The free acids **1–6** were not easily measured by the GC and are, therefore, not reported. However, the esters and diols were measured: (i) In the case of **9–11**, the temperature of the column was 175 °C and the retention times were 3.89 min for **9**, 4.79 min for **10**, and 4.35 min for **11**. (ii) For **7** and **8**, the temperature of the column was 150 °C and the retention times were 3.60 min for **7** and 4.05 min for **8**. (iii) For **12**, the temperature of the column and retention time were 250 °C and 4.30 min, respectively. The hexyl ester of **4** was measured with a column temperature of 175 °C and a retention time of 7.60 min.

The diols were measured at the same column temperature as that for their corresponding butyl esters with the exception of **13**, which was measured at 100 °C. The diols were quantified with authentic standards where commercially available. Retention times were as follows: **13**, 3.21 min; **14**, 2.94 min; **15**, 3.01 min; **16**, 3.45 min; **17**, 3.13 min; and **18**, 3.57 min.

All optical rotations were performed at 589 nm (sodium line) on a Jasco Model DIP-360 optical polarimeter at 25 °C. $^1H$ NMR spectra were recorded on a Brüker 360-MHz spectrometer in $CDCl_3$. All chemical shifts were reported (ppm) with tetramethylsilane as external standard.

**Enzyme-Catalyzed Esterifications.** In a typical reaction, a solution of a racemic 2-hydroxy acid and 1-butanol in toluene was prepared and CCL was added (for details, see footnote *a* to Table III). The resulting suspension was magnetically stirred at 250 rpm at 25 °C to reach a

certain degree of conversion (Table III). The reactions were terminated by filtering the enzyme, and the liquid phase was washed with three 150-mL portions of 0.3 M aqueous $NaHCO_3$. The organic phase obtained was dried with $MgSO_4$ and the solvent evaporated in a rotary evaporator. The pure esters were isolated free from the 1-butanol by distillation. Optical rotations and ee values are given in Table III.

**Synthesis of (*R*)-(+)-2-Hydroxycaproic Acid ((*R*)-(+)-4).** A solution of racemic **4** was prepared in toluene as follows: 2.4 g of (*R,S*)-**4** (0.1 M) was dissolved in 0.18 L of toluene containing 0.3 M 1-butanol and the reaction initiated upon the addition of 9.0 g of CCL. The reaction was stirred at 250 rpm at 30 °C. The reaction was terminated after 30 h by filtering off the CCL. The conversion at this time was 70%. The unreacted acid was extracted into 0.3 M $NaHCO_3$ solution, acidified with HCl, and reextracted into $CH_2Cl_2$. The solvent was then dried with $MgSO_4$ and then evaporated, leaving behind the pure acid. The (*R*)-(+)-**4** showed $[\alpha]^{25}_D$ + 27.5° (*c* 1, 1 N NaOH).

**Chemical Reduction. Preparation of (*S*)-(-)-1,2-Hexanediol ((*S*)-(-)-16).** A solution of 1.4 g of (*S*)-(-)-butyl 2-hydroxycaproate dissolved in 100 mL of diethyl ether was slowly added over a period of 1 h with magnetic stirring to 100 mL of diethyl ether solution containing 7.0 g of $LiAl(OCH_3)_3H$. The addition was carried out at 4 °C. When addition was complete, 25 mL of water was added to destroy the unreacted hydride and 100 mL of 4% aqueous sulfuric acid solution was added to extract the salts. The 1,2-hexanediol product remained in the organic phase and was washed three times with 100 mL of sulfuric acid solution. The 1,2-hexanediol was isolated free from 1-butanol by distillation.

The single-pot synthesis of 1,2-hexanediol was carried out in a similar fashion by sequential esterification and reduction steps in diethyl ether. No purification of the butyl ester intermediate was performed; however, the unreacted **4** was removed from the diethyl ether by extraction and the organic phase dried over $MgSO_4$ prior to reduction.

The following data were obtained: bp 91-92 °C (2 mmHg); $^1$H NMR (360 MHz, $CDCl_3$) $\delta$ 4.15-4.08 (1 H, br s, OH), 4.08-3.93 (1 H, br s, OH), 3.70-3.55 (1 H, m, CHOH), 3.42-3.35 (2 H, m, $CH_2OH$), 1.45-1.30 (6 H, m, 3 $CH_2$), 0.94-0.88 (3 H, t, $CH_3$).

Other 1,2-diols were prepared in an analogous manner. For (*S*)-(+)-**13**, the recovery from the reduction step was through the water phase, as the product does not partition into the organic layer. The diol product was then purified by distillation.

**(*S*)-(+)-1,2-Propanediol (13)**: bp 186-187 °C (760 mmHg); $^1$H NMR ($CDCl_3$) $\delta$ 4.70-4.35 (2 H, br s, OH), 3.77-3.69 (1 H, m, CHOH), 3.45-3.20 (2 H, m, $CH_2OH$), 1.01-0.98 (3 H, t, $CH_3$).

**(*S*)-(-)-1,2-Butanediol (14)**: bp 195-197 °C (760 mmHg); $^1$H NMR ($CDCl_3$) $\delta$ 4.25-4.20 (2 H, br s, OH), 3.65-3.52 (1 H, m, CHOH), 3.42-3.30 (2 H, m, $CH_2OH$), 1.45-1.30 (2 H, m, $CH_2$), 0.93-0.85 (3 H, t, $CH_3$).

**(*S*)-(+)-3-Methyl-1,2-butanediol (15)**: bp 62-63 °C (4.1 mmHg); $^1$H NMR ($CDCl_3$) $\delta$ 3.73-3.67 (1 H, m, CHOH), 3.53-3.41 (2 H, m, $CH_2OH$), 1.75-1.66 (1 H, m, CH), 0.98-0.96 (3 H, d, $CH_3$), 0.93-0.91 (3 H, d, $CH_3$). Note: the two OH protons were too broad for accurate measurement by NMR.

**(*S*)-(-)-4-Methyl-1,2-pentanediol (17)**: bp 80-82 °C (6.8 mmHg); $^1$H NMR ($CDCl_3$) $\delta$ 4.60-4.35 (2 H, br s, OH), 3.81-3.75 (1 H, m, CHOH), 3.63-3.57 (1 H, m, $CH_2OH$), 3.42-3.33 (1 H, m, $CH_2OH$), 1.83-1.75 (1 H, m, CH), 1.42-1.32 (2 H, m, $CH_2$), 0.96-0.91 (6 H, t, $CH_3$).

**(*S*)-(-)-3-Phenyl-1,2-propanediol (18)**: bp 135-139 °C (2 mmHg); $^1$H NMR ($CDCl_3$) $\delta$ 7.30-7.15 (5 H, m, $C_6H_5$), 3.90-3.83 (1 H, m, CHOH), 3.64-3.42 (2 H, m, $CH_2OH$), 2.74-2.65 (2 H, m, $CH_2$). Note: the two OH protons were too broad for accurate measurement by NMR.

**Enzyme Kinetics.** Enzyme kinetics were followed by GC for the formation of the butyl esters. The concentrations of the 2-hydroxy esters were varied from 10 to 200 mM, and the concentration of 1-butanol was fixed at 0.3 M. The concentration of CCL was 50 mg/mL, and the reaction volumes were all 10 mL. All analyses were carried out up to 10% conversion of the acid substrate. The water produced during the reaction with 200 mM substrate and 10% conversion amounts to 20 mM. This concentration of water does not affect the linearity of initial rate (ester produced vs time) measurements.

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# Active-Site-Directed Modification of Subtilisin

**Ziyang Zhong, Jeffrey A. Bibbs, Wei Yuan, and Chi-Huey Wong***

*Contribution from the Department of Chemistry, Scripps Research Institute, La Jolla, California 92037.* *Received November 29, 1989. Revised Manuscript Received September 18, 1990*

**Abstract:** This paper describes the development of several competitive inhibitors and mechanism-based inactivators of subtilisin BPN′. Various methyl arylakanesulfonates were prepared and shown to be either competitive inhibitors or selective methylating reagents for the $\epsilon$2-N of the active-site His. Styrene sulfonyl chloride was shown to be a good covalent inactivator and benzyl *N*-(*N*-Boc-L-phenylalanyl)-L-aziridine-2-carboxylate was a strong competitive inhibitor. The second-order rate constant of inactivation and the $K_i$ of the enzyme reacting with the methylating reagents and other inactivators were determined. The methylated enzyme was purified to homogeneity and the kinetic constants for the enzyme-catalyzed ester and amide hydrolyses were determined. It was established that the methylated enzyme lost most of the amidase activity while the esterase activity was still significant and useful for peptide synthesis via aminolysis. A mechanism involving ring flipping of the active-site imidazole, first proposed for methylchymotrypsin activity, was also proposed to explain methylsubtilisin-catalyzed reactions.

Selective modification or inhibition of enzymes at the active site has been a subject of intense investigation. One of our interests in this regard is to develop modified proteases for the aminolytic condensation of peptide segments to form larger peptides without secondary peptide hydrolysis. We have recently developed two strategies to accomplish this goal: one is the use of serine proteases in the presence of water-miscible organic solvents, which selectively inhibit the amide cleavage activities;[1,2] the other is the use of methylchymotrypsin prepared via site-directed methylation.[3] Both approaches provide modified proteases useful for peptide segment coupling. One disadvantage with methylchymotrypsin is that only peptidyl segments that meet the chymotrypsin specificity requirements can be coupled,[2] which presents a limitation of this methodology. We describe here the development of new reagents for selective methylation of subtilisin BPN′, a serine protease with a wide range of substrate specificity,[4] at the $\epsilon$-N of active-site His,

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