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Structure of Solvent Affects Enantioselectivity of Lipase-Catalyzed Transesterification

Kaoru Nakamura,* Masamichi Kinoshita, and Atsuyoshi Ohno

Institute for Chemical Research, Kyoto University
Uji, Kyoto 611, Japan

Abstract: Lipase-catalyzed transesterification of (*rac*)-6-methyl-5-hepten-2-ol (sulcatol) with vinyl acetate has been studied in various solvents and the effect of solvent on the enantioselectivity has been discussed from the viewpoint of molecular shape of the solvent. Alkanes and ethers are selected as solvents. Enantioselectivity of a reaction in a structurally linear solvent is higher than that in the corresponding branched chain solvent. Furthermore, the enantioselectivity decreases specifically with the increase in the ring size of solvent molecule. Thus, lipase recognizes not only the structure of substrate but also that of solvent.

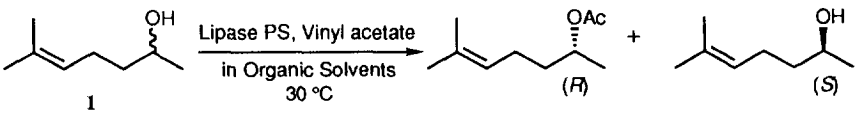
INTRODUCTION

Lipases (EC 3.1.1.3) are hydrolytic enzymes that catalyze hydrolysis of triacylglycerol into the corresponding fatty acids and glycerol. Today, many organic chemists employ lipases as catalysts for synthesizing chiral compounds, because lipases can keep their high activities and stabilities in organic media.[1-3] It has been reported that organic solvents affect enzymatic activity and stereoselectivity of the reaction.[3-18] Most of the effects have been explained from the viewpoint of polarity or hydrophobicity of the solvent, which is a property of bulk solvent.[6,9,12,14] In general, however, only poor correlations have been recognized between enzymatic selectivities and parameters to characterize the solvent. We reported, in the previous paper from our laboratory, that the structure of solvent influences the activity and stereoselectivity of a lipase.[7,16] In this paper, we wish to report that an organic solvent exerts enantioselective inhibition for a lipase-catalyzed transesterification depending upon the shape of solvent molecule.

RESULTS AND DISCUSSION

As an alcoholic substrate, 6-methyl-5-hepten-2-ol (sulcatol; **1**) was employed. Sulcatol is an important chiral building block in synthesizing some bioactive natural products,[19,20] and the *S* isomer is well-known as

male-produced aggregation pheromone of the ambrosia beetles *Gnathotrichus sulcatus* and *Gnathotrichus retusus*.[21] Both enantiomers of sulcatol have been prepared by transesterification reaction catalyzed by pig pancreatic lipase.[22,23] We have measured the initial rates and E-value of transesterification between racemic sulcatol and vinyl acetate catalyzed by a lipase from *Pseudomonas cepacia* (Lipase PS from Amano) in various organic solvents. The reaction is illustrated in Scheme 1.



Scheme 1

For organic chemists, enantioselectivity of the reaction is one of the most important characters of an enzyme along with chemical yield. Therefore, in the present study, we focused our effort on elucidating a mechanism to exert enantioselectivity of the reaction. The selectivity is defined as a ratio of rate constants for (*R*)- and (*S*)-enantiomers (E-value).[24] The ratio was calculated from initial rates of the reaction under the conditions where the concentrations of the substrates are much lower than their K_ms. Results are summarized in Table 1.

Table 1. Enantioselectivity of Lipase PS-Catalyzed Transesterification between Sulcatol and Vinyl Acetate in Various Organic Solvents.

Solvent	log*P*[a]	E-value
Hexane	3.5	28.5
Cyclohexane	3.2	10.9
Toluene	2.5	37.0
Benzene	2.0	38.0
Diisopropyl ether	1.9	21.3
Diethyl ether	0.85	31.1
tert-Butanol	0.80	18.5
Tetrahydrofuran	0.49	24.9

(a) The values are taken from ref. 4.

It is suggested that polarity, or hydrophobicity, of a solvent generally influences enantioselectivity of enzymatic reactions. Although we attempted to correlate E-value with certain parameters of solvents such as dielectric constant, dipole moment, and log*P*, all effort have failed. As an example, an E-log*P* correlation is depicted in Fig. 1.

The result exemplifies the difficulty in correlating the selectivity with one of physical properties of the solvent directly, probably because an enzyme has many sites to be affected by solvent molecules. Although there have been quite a lot of reports describing solvent effect on enzymatic selectivities, they are little correlated with a parameter of solvent except for some scattered examples.[6,9,12,14] Thus, we assume that a solvent influences enzymatic reactions as a molecule rather than as a bulk medium.

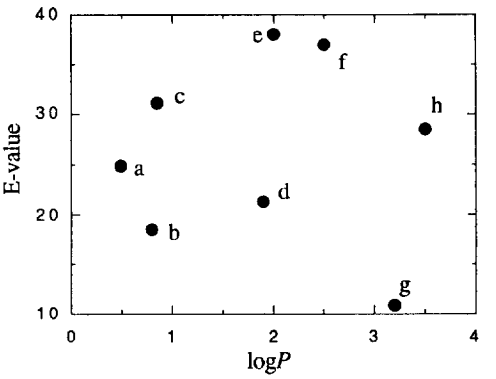


Figure 1. The correlation between the hydrophobicity of solvents and the enantioselectivity of lipase. Solvents: (a) tetrahydrofuran, (b) *tert*-butanol, (c) ether, (d) diisopropyl ether, (e) benzene, (f) toluene, (g) cyclohexane, and (h) hexane.

We discovered previously that stereoselectivity of a lipase depends on the structure of solvent molecule.[7,16] In the present study, therefore, the attention has been paid to the difference in property of geometrical isomers of solvents. Alkanes have been selected as solvents in order to avoid unnecessary complexity from electrostatic interactions between the solvent and an enzyme. The solvents employed are the following couples; hexane and 2,2-dimethylbutane (DMB), heptane and 2,4-dimethylpentane, and octane and 2,2,4-trimethylpentane. Kinetic results are summarized in Table 2.

Table 2. Relation between the Structure of Solvent and the Enantioselectivity of Lipase PS.

Solvent	$10\times k_R$ (h^{-1})[a]	$10^3\times k_S$ (h^{-1})[a]	E-value
Hexane	2.25 ±0.02	7.88 ±0.05	28.5 ±0.1
2,2-Dimethylbutane(DMB)	2.92 ±0.01	30.7 ±0.4	9.5 ±0.2
Heptane	2.2 ±0.1	7.9 ±0.5	28.1 ±0.4
2,4-Dimethylpentane	2.33 ±0.05	12.2 ±0.6	19.2 ±0.6
Octane	2.23 ±0.08	9.5 ±0.5	24 ±2
2,2,4-Trimethylpentane	2.06 ±0.03	11.5 ±0.1	17.8 ±0.3

(a) k_R and k_S: rate constant for (*R*)- and (*S*)-sulcatols, respectively.

The lipase exerts higher enantioselectivity in structurally linear solvents than in solvents with a branched chain(s). For example, E-value measured in hexane is 3 times as large as the value measured in DMB. It is obvious from Table 2 that the difference in enantioselectivity in different solvent is mostly stemed from the difference in reactivity of the (*S*)-enantiomer. The rate constant for the (*R*)-enantiomer in hexane is close to that in DMB, whereas the rate constant for the (*S*)-enantiomer in hexane is reduced to about one fourth of that in DMB. Similar tendency is seen for the other couples of solvents. In addition, the rate constants for the (*S*)-

enantiomer are about one to two orders of magnitude smaller than those for the (*R*)-enantiomer, which seems to suggest that the reaction of the (*S*)-enantiomer is retarded or inhibited in structurally linear solvents.

In order to confirm the generality of the observation, alcohols other than sulcatol have also been subjected to the transesterifications in hexane and DMB. Results are summarized in Table 3.

Table 3. Effect of Solvent Structure on the Lipase PS-Catalyzed Transesterification between Various Alcohols and Vinyl Acetate.

Alcohol	in Hexane			in 2,2-Dimethylbutane		
	k_R (min^{-1})	k_S (min^{-1})	E	k_R (min^{-1})	k_S (min^{-1})	E
2-Pentanol	4.2×10^{-1}	8.3×10^{-2}	5.1	4.2×10^{-1}	1.8×10^{-1}	2.4
2-Hexanol	5.3×10^{-1}	4.1×10^{-2}	13.0	5.4×10^{-1}	1.3×10^{-1}	4.3
2-Heptanol	4.5×10^{-1}	3.0×10^{-2}	14.5	5.5×10^{-1}	1.5×10^{-1}	3.5
Pinacolyl alcohol[a]	5.8×10^{-5}	1.1×10^{-6}	54.0	7.7×10^{-5}	2.3×10^{-6}	32.7
1-Phenylethanol	4.3×10^{-2}	1.1×10^{-4}	375	3.4×10^{-2}	2.6×10^{-4}	132

(a) Pinacolyl alcohol: 3,3-dimethyl-2-butanol.

Similarly to sulcatol, the lipase exerts higher enantioselectivity in hexane than in DMB for all the alcohols studied. Here again, the retardation in hexane is more significant for the reaction of the (*S*)-enantiomer than for that of the (*R*)-enantiomer.

To obtain more detailed insight into the reaction mechanism, apparent Michaelis constant, K_m, and maximum velocity, V_{max}, have been measured for the (*R*)- and (*S*)-sulcatols under the same conditions where kinetic resolution was carried out. Results are listed in Table 4.

Table 4. Apparent Kinetic Parameters for the Lipase PS-Catalyzed Transesterification in Hexane and 2,2-Dimethylbutane.

Solvent	K_m (mM)	V_{max} ($mM\cdot h^{-1}\cdot mg^{-1}$)[a]
(R)-sulcatol		
Hexane	69 ± 6	1.9 ± 0.1
2,2-Dimethylbutane	70 ± 5	2.8 ± 0.1
(S)-sulcatol		
Hexane	122 ± 16	0.134 ± 0.008
2,2-Dimethylbutane	74 ± 3	0.297 ± 0.004

(a) In the units, "mg" refers to the weight of the lipase.

Table 4 reveals that K_m for the (*R*)-enantiomer is almost independent of the shape of solvent molecule. On the other hand, the situation is different for the (*S*)-enantiomer: K_m in hexane is 1.6 times as large as that in

DMB, whereas V_{max} in this solvent is 2.3 times as small as that in DMB, which results in 3.8 times difference in catalytic turnover numbers, V_{max}/K_m, in hexane and DMB.

It is generally believed that hydrolytic enzymes recognize optical isomers of secondary alcohols as follows: the binding site of alcohols is consisted of two pockets, large and small, and catalytic functions of the enzyme are sterically situated to prefer the reaction with one of two enantiomers remaining the other unreacted. In order to subject the unfavorable enantiomer to the reaction, the larger substituent in this substrate has to be incorporated into the small pocket of the enzyme.

Yennawar *et al.* reported the structure of γ-chymotrypsin in hexane.[25] A crystal soaked in hexane contains seven molecules of hexane and two of them are set close to the active site. One of the two hexane molecules at the active site locates itself close to His57 (one of the residues of catalytic triad) and the other is put just outside the specificity pocket. Thus, it is reasonable to assume that a lipase in an organic solvent incorporates the solvent molecule into its pocket.

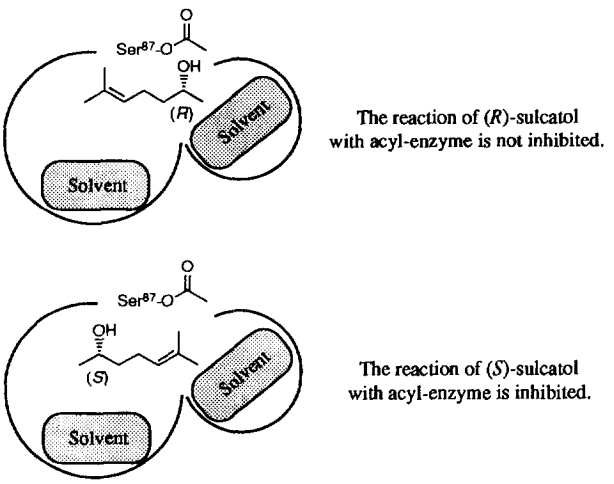


Figure 2. Mechanism of enantioselective inhibition for lipase-catalyzed transesterification by solvent molecules incorporated into the substrate-binding pocket.

Being judged from rate constants listed in Table 2 as well as K_m and V_{max} listed in Table 4 and the X–ray crystallographic result mentioned above, the (*R*)-enantiomer is favorable one in the reaction of sulcatol with lipase PS. In other words, when the small pocket of the lipase is occupied by a solvent molecule(s), apparent size of the pocket becomes smaller and the reaction with (*S*)-sulcatol is retarded with the increase in apparent K_m of (*S*)-sulcatol.

The strength of hydrophobic interaction between a molecule and another depends on the surface area of these molecules.[25] The difference in K_m of (*S*)-sulcatol in hexane and DMB suggests that hexane molecule has larger affinity toward the hydrophobic pocket of lipase than DMB. On the other hand, since the large pocket of

lipase have enough space for (*R*)-sulcatol to bind even in the presence of a solvent molecule(s) in the pocket, the reaction with (*R*)-sulcatol is little affected (see Fig. 2).

A lipase in cyclopentane exerts higher enantioselectivity than that in cyclohexane, cycloheptane or cyclooctane. The results are summarized in Tables 5 and 6, which confirm the dependence of enantioselectivity on the size of solvent molecule, as reported previously.[16] Thus, the assumption of incorporation of a solvent molecule(s) into the hydrophobic pocket of lipase can account for the solvent-dependent enantioselectivity of the reaction.

Table 5. Effect of Cyclic Solvent on the Lipase-Catalyzed Transesterification.

Solvent	$10 \times k_R$ (h^{-1})	$10^3 \times k_S$ (h^{-1})	E-Value
Cyclopentane	2.22 ±0.01	8.9 ±0.2	25.0 ±0.6
Cyclohexane	2.31 ±0.05	21 ±2	10.9 ±0.5
Cycloheptane	1.94 ±0.05	21.4 ±0.9	9.0 ±0.1
Cyclooctane	1.73 ±0.05	15.4 ±0.1	11.3 ±0.1

Table 6. Apparent Kinetic Parameters for the Lipase-Catalyzed Transesterification in Cyclopentane and Cyclohexane.

Solvent	K_m (mM)	V_{max} ($mM \cdot h^{-1} \cdot mg^{-1}$)
(R)-sulcatol		
Cyclopentane	128 ± 8	2.6 ± 0.1
Cyclohexane	222 ± 7	4.7 ± 0.1
(S)-sulcatol		
Cyclopentane	146 ± 12	0.20 ± 0.01
Cyclohexane	140 ± 13	0.35 ± 0.02

K_ms in cycloalkanes are larger than those in normal alkanes, particularly for (*R*)-sulcatol (*e.g.* K_m = 69 mM in hexane and K_m = 222 mM in cyclohexane). Cycloalkanes are expected to be incorperated more easily into the pocket than the corresponding normal alkanes of the same carbon number because of the compactness, or the former has larger affinity toward the hydrophobic pocket of lipase than the latter.[27] The difference between enantioselectivity of the reaction in cyclopentane and that in cyclohexane is due largely to the difference in V_{max} of (*S*)-sulcatol, but not in K_m of (*R*)-sulcatol. It is not surprising that V_{max} is affected by the ring size of solvent molecule, because the incorporated solvent molecules may cause positional distortion at important part of amino acid side-chains[25] such as catalytic triad (Ser87-His286-Asp264)[28] and oxyanion holes, which affects turnover number, k_{cat}.

Interestingly, the effect of solvent on enantioselectivity can also be seen in ethereal solvents as well. For example, as listed in Table 7, the E-value measured in butyl methyl ether is about twice as large as that in *tert*-butyl methyl ether.

Table 7. Effect of Ethereal Solvent on the Lipase-Catalyzed Transesterification.

Solvent	$10\times k_R$ (h^{-1})	$10^3\times k_S$ (h^{-1})	E-Value
Butyl methyl ether	0.84 ±0.02	3.0 ±0.1	27.7 ±0.3
tert-Butyl methyl ether	1.59 ±0.04	13.2 ±0.3	12.0 ±0.5

Although the difference in enantioselectivity between straight and branched chain molecules as solvents is smaller for a series of ethers than that of alkanes, the selectivity is evidently larger in structurally linear ether than that in the corresponding ether with branched chain. The fact that an ether exerts only smaller effect than the corresponding alkane is due to the facts that the former, which is less hydrophobic than the latter, finds difficulty to be incorporated into the hydrophobic pocket and that the former can interact with a lipase electronically at various sites of the enzyme.

Thus, one should take direct interaction between the solvent molecule and the enzyme into consideration instead of treating the solvent as a bulk medium. It is well known that a lipase has a lid covering its active site. In addition, a lipase can assume two stable conformations; opened (active) and closed (nonactive).[29-37] Note that lipases are a group of enzymes that deal with the reaction of fats, the substrates that exist in hydrophobic layers. Since hydrophobic environment stabilizes the opened conformation, the lipase will prefer to take this conformation in alkanes. On the other hand, the closed conformation may increase its importance in ethereal solvents. Thus, the rate constant becomes smaller in the latter than in the former remaining E-value almost unchanged in an alkane and an ether of similar structures (*e.g.* E = 28.5 in hexane and E = 27.7 in butyl methyl ether) despite of large difference in log*P*.

In conclusion, we would like to propose that solvent molecules bind to the substrate-binding pocket and change the enantioselectivity of lipase. A structurally linear solvent can exert higher enantioselectivity than the corresponding solvent with a branched chain(s) in transesterification mediated by a lipase. In addition, a cyclic solvent with small ring is more effective for improving the enantioselectivity than those with large rings.

EXPERIMENTAL

Instruments. Gas chromatograms were recorded on a Shimadzu GC-14A Gas Chromatograph. For the analysis of enantiomeric excess in the product alcohol, a capillary column equipped with Chiraldex G-TA (Tokyo Kasei Kogyo Co., Ltd.), CP-Cyclodextrin-B-236-M-19 (Chrompack) or HR-20M (Shinwa Chemical Industries, Ltd.) was employed.

Materials. Substrates and organic solvents were purchased from Nacalai Tesque Co., Tokyo Kasei Co., and Wako Pure Chemicals Co. Hydrocarbon solvents were dried over molecular sieves 4A before the use. Ethereal solvents were dried over CaH_2 before the use. Lipases PS was provided from Amano Pharmaceutical Co., Ltd.

Lipase PS-Catalyzed Transesterification of Sulcatol with Vinyl Acetate. Lipase PS (10 mg) was placed in a vial and 4 ml of a solvent containing 120 mM of vinyl acetate and 24 mM of an alcohol as a substrate was added to the vial. Then, the resulting suspension was stirred magnetically at 30 °C. Periodically, aliquots were withdrawn from the suspension and analyzed on gas chromatography (Chiraldex G-TA). The concentration of the substrate herein employed is small enough to obtain a first-order kinetics for the present transesterification (*cf.* K_m listed in Table 4). Enantiomeric excesses in the remained substrate (ee_s) and the product acetate (ee_p) were calculated from gas chromatograms of appropriate samples. The ratios, $[R]/[R]_0$ and $[S]/[S]_0$, were calculated on the bases of ee_S and ee_P by Eq. 1, where $[R]_0$ and $[S]_0$ are initial concentrations of (*R*)- and (*S*)-sulcatol, respectively, and [R] and [S] are the concentrations of (*R*)- and (*S*)-sulcatol, respectively, at an appropriate time interval.

$$\frac{[R]}{[R]_0} = \frac{(1 - ee_S)\, ee_P}{ee_S + ee_P}, \qquad \frac{[S]}{[S]_0} = \frac{(1 + ee_S)\, ee_P}{ee_S + ee_P} \tag{1}$$

Kinetics. The reaction was analyzed with the first-order kinetics. Thus, a rate constant was calculated from the slope of a plot of logarithm of $[R]/[R]_0$ or $[S]/[S]_0$ against reaction time, and the E-value was obtained from the slope of a $\ln([R]/[R]_0)$ *vs.* $\ln([S]/[S]_0)$ plot.

The Michaelis-Menten parameters, K_m and V_{max}, for the transesterifications with sulcatol in hexane and DMB were elucidated from the reactions with appropriate initial concentrations of the substrate from 20 mM to 320 mM. Other reaction conditions were kept unchanged from those described above. Initial rates were measured by the aid of gas chromatography (column: HR-20M) as described above. No internal standard for the chromatography was employed in order to avoid its unnecessary interference on the property of the solvent. Therefore, relative amounts of the acetate and the alcohol were obtained by the aid of a calibration curve drawn in advance of the kinetics. The Hanes-Hoolf plot ($[S]/v_o$ *vs.* $[S]$) was employed for elucidating K_m and V_{max}. Results are listed in Table 4.

Optically Pure (R)- and (S)-Sulcatols. A mixture of (*rac*)-sulcatol (1.6 mmol), vinyl acetate (8 mmol), and lipase SP435 (50 mg), provided from Novo Nordisk Bioindustry Ltd.(Chiba, Japan), in 50 ml of hexane was stirred at 30 °C. After 12 h, the reaction was quenched by filtration to remove the lipase and the solvent was evaporated under reduced pressure. The unreacted (*S*)-sulcatol was separated from the acetate of (*R*)-enantiomer by column chromatography eluting with [3 - 100%] ethyl acetate/hexane mixture. Chemical yields of (*S*)-sulcatol and the (*R*)-acetate were 34 and 66 %, respectively (the quantity of racemic substrate is defined as 100 %). Enantiomeric excess in thus obtained (*S*)-sulcatol was determined to be more than 99.9% by gas chromatography (column; Chiraldex G-TA: temp 75 °C) (31 % yield). The (*R*)-acetate (52 % e.e.) was converted into (*R*)-sulcatol by $LiAlH_4$ in dry ether. (*R*)-Sulcatol of more than 99% in enantiomeric excess was obtained after 3 times repetition of the transesterification on enantiomerically concentrated (*R*)-sulcatol (30 % yield).

Absolute configurations of the (*R*)- and (*S*)-sulcatols were determined by comparing their signs of optical

rotations with those reported in the literature.[38]

(*R*)-Sulcatol: $[\alpha]^{24}_D$ = -15.9° (c = 1.00, EtOH); lit[38] $[\alpha]^{23}_D$ = -14.5° (c = 0.74, EtOH).

(*S*)-Sulcatol: $[\alpha]^{24}_D$ = +16.2° (c = 1.00, EtOH); lit[38] $[\alpha]^{23}_D$ = +14.4° (c = 0.998, EtOH).

Lipase PS-Catalyzed Transesterification of Other Secondary Alcohols with Vinyl acetate. Lipase PS (5 mg) was placed in a vial and 4 ml of a solvent containing 120 mM of vinyl acetate and 24 mM of 2-pentanol, 2-hexanol, 2-heptanol, or 1-phenylethanol was added to the vial. The rate constants and E-values were determined in the same way as described for sulcatol. For the reaction in 3,3-dimethyl-2-butanol, 100 mg of lipase PS was placed in a flask and 64 ml of a solvent containing 155 mM of vinyl acetate and 31 mM of the alcohol was added to the flask. In this reaction, only enantiomeric excess of the corresponding acetates, ee_P, was determined by gas chromatography (column: CP-Cyclodextrin-B-236-M-19). Thus, the alcohol separated from the reaction mixture after 147 h was subjected to the determination of the enantiomeric excess (ee_S) after being converted into the corresponding acetate chemically. The rate constants and E-value were calculated from ee_S and ee_P. Configurations of the alcohols studied were assigned on the bases of their retention times on gas chromatography: the enantiomer with smaller retention time is assigned to the (*S*)-enantiomer in analogy with the retention times of (*R*)- and (*S*)-sulcatols.

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