Enantioselective Inhibition: A Strategy for Improving the Enantioselectivity of Biocatalytic Systems

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Abstract: Dextromethorphan (DM) and levomethorphan (LM) were found to be effective enantioselective inhibitors of *Candida cylindracea* lipase-catalyzed hydrolysis of a variety of (±)-arylpropionic and (±)-(aryloxy)propionic esters. The enantioselectivity of the biocatalytic resolution of (±)-methyl 2-(2,4-dichlorophenoxy)propionate (DCPP) was enhanced 20-fold in the presence of either DM or LM. A general model for enantioselective inhibition has been developed, and a quantitative expression has been derived to show the underlying parameters that govern enantioselective inhibition. To define the mechanism of action of DM, a series of kinetic inhibition experiments was conducted with enantiomerically pure (*R*)-(+)-DCPP and (*S*)-(−)-DCPP. The observed inhibition pattern was that of partial noncompetitive inhibition for (*R*)-(+)-DCPP and that of pure noncompetitive inhibition for (*S*)-(−)-DCPP.

Enantioselective biocatalysis in aqueous[1] and nonaqueous[2] media is becoming increasingly important as a possible alternative method for the resolution of enantiomers. In recent years, hydrolytic enzymes such as pig liver esterase,[3] porcine pancreatic lipase,[1c] and microbial lipases[1c] (EC 3.1.1.3) have been widely used for the kinetic resolution of racemic acids and alcohols, because these enzymes have broad substrate specificities and frequently catalyze reactions with high degrees of enantioselectivity. However, despite the many successful published examples achieved with only a few enzymes, biocatalytic procedures have not yet attained the status of a standard procedure. Most biocatalytic kinetic resolutions are only partially enantioselective with E^*[4] values of ≤10, and there is a lack of an arsenal of enzymes (as in the case of antibodies) with well-defined stereochemical specificities which would allow the synthetic chemist to select the one that is highly enantioselective toward a given racemate. This shortcoming is likely to be overcome by the continual but gradual discovery to new enzymes with the desired stereochemical features. However, if alternative strategies could be developed for improving enantioselectivity, the usefulness of biocatalysts could be extended, and the number of enzymes required for synthetic applications could be reduced considerably.

In 1930, Bamann and Laeverenz[5] first observed that the enantioselectivity of the human liver esterase-catalyzed hydrolysis of (±)-methyl mandelate was prominently enhanced when the alkaloid strychnine was added to the reaction mixture. In the following year, Ammon and Fischgold[6] in a series of noncompetitive experiments using L- and D-methyl mandelate showed that while strychnine had no effect on the kinetic parameters K_m and V_{max} with D-methyl mandelate as substrate, it markedly enhanced the V_{max} without affecting the K_m of the human liver esterase-catalyzed hydrolysis of L-methyl mandelate. This observation strongly suggests that this enhancement of enantioselectivity is the result of an apparent allosteric activation of the enzyme resulting in the potentiation of the catalytic turnover rate for the L enantiomer. To determine whether this allosteric potentiating effect is a general occurrence, we examined the effect of a number of chiral amines on the enantioselectivity of several biocatalytic processes. In the course of these exploratory studies, we encountered the phenomenon of enantioselective inhibition. In this paper, we describe the theoretical and experimental development of this concept as a general method for enhancing the enantioselectivity of biocatalytic reactions.

Table I. Effect of Various Chiral Amines on the Lipase-Catalyzed Enantioselective Hydrolysis of (±)-DCPP:

(±)-DCPP → (*R*)-(+)-DCPPA + (*S*)-(−)-DCPP

amine	reaction time (h)	ee_S	ee_P	c	E^*
none	10	0	0	0.34[a]	1
dextromethorphan	42	0.38	0.87	0.30	20
quinidine	10	0.15	0.61	0.20	5
quinine	18	0.15	0.52	0.22	4
cinchonidine	18	0.16	0.34	0.32	2
brucine	18	0.14	0.36	0.28	2
morphine	22	0.25	0.27	0.48	2
codeine	22	0.28	0.22	0.56	2
strychnine	18	0.13	0.12	0.52	1
(*R*)-(+)-α-methylbenzylamine	18	0.08	0.10	0.44	1
(*S*)-(−)-α-methylbenzylamine	18	0	0	0.5[b]	1
cinchonine	18	0	0	0.5[b]	1

[a] Determined by HPLC. [b] Estimated by TLC.

Results

Careful consideration was given to the experimental design of the biocatalytic system. *Candida cylindracea* lipase was chosen for these studies, because it is widely used for biocatalytic resolutions. Moreover, the commercial Sigma preparation is devoid of contaminating enzyme(s) of opposite stereochemical preference.[7] (±)-Methyl 2-(2,4-dichlorophenoxy)propionate (DCPP) was used as the model substrate for most of the studies, because the *Candida* lipase exhibits very low enantioselectivity toward this compound with an E^* value of 1–1.5. This provides us with a sensitive system to detect small changes in enantioselectivity.

The effect of various chiral amines on the enantioselectivity of lipase-catalyzed hydrolysis of (±)-DCPP is shown in Table I. Of the 12 amines examined, only dextromethorphan (DM) markedly enhanced the enantioselectivity of this reaction (E^* = 20). Moderate improvement was noted with quinidine and quinine with E^* values of 4–5. In the presence of these amines, the *R* enantiomer became overwhelmingly the faster reacting enantiomer. From the extent of conversion (c) and reaction time, it is apparent that the enhancement of enantioselectivity is the consequence of a differential inhibitory phenomenon. Both DM and levomethorphan (LM) are active as enantioselective inhibitors toward the enzymatic hydrolysis of a series of halogenated phenoxypropionate esters (Table II). Also, the enantioselectivity of lipase-catalyzed hydrolyses of arylpropionates was markedly improved in the presence of DM (Table III). In the latter, the *S* enantiomer is preferentially hydrolyzed (sequence-rule change).

(1) (a) Jones, J. B. *Tetrahedron* **1986**, *42*, 3351. (b) Butt, S.; Roberts, S. M. *Chem. Br.* **1987**, 127. (c) Sih, C. J.; Wu, S. H. *Top. Seterochem.*, in press.
(2) (a) Klibanov, A. M. *Chem. Tech.* **1986**, 354. (b) *Biocatalysis in Organic Media*; Laane, C.; Tramper, J.; Lilly, M. D., Eds.; Elsevier: Amsterdam, 1987. (c) Chen, C. S.; Wu, S. H.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1987**, *109*, 2812.
(3) Ohno, M. *Org. React.*, in press.
(4) Chen, C. S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294. The enantiomeric ratio (abbreviated as E^* to prevent confusion with the free enzyme, abbreviated as E, in this paper) is calculated from $E^* = \ln[(1-c)(1-ee_S)]/\ln[(1-c)(1+ee_S)]$, where $c = ee_S/(ee_S + ee_P)$.
(5) Bamann, E.; Laeverenz, P. *Ber. Dtsch. Chem. Ges.* **1930**, *63*, 394.
(6) Ammon, R.; Fischgold, H. *Biochem. Z.* **1931**, *234*, 54.
(7) Wu, S. H. Ph.D. Thesis, University of Wisconsin, December 1987.

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Table II. Effect of DM and LM on the Lipase-Catalyzed Enantioselective Hydrolysis of Halogenated (±)-(Aryloxy)propionic Esters:
(±)-$R_1OCHCH_3CO_2CH \rightarrow$ (*R*)-(+)-$R_1OCHCH_3CO_2H$ + (*S*)-(−)-$R_1OCHCH_3CO_2CH_3$

R_1	inhibitor (0.03 M)	reaction time (h)	ee_S	ee_P	*c*	*E**
2-chlorophenyl	none	7	0.88	0.28	0.76	4
	DM	7	0.68	0.85	0.44	25
	LM	2	0.15	0.88	0.15	18
3-chlorophenyl	none	7	0.51	0.17	0.75	2
	DM	7	0.95	0.58	0.62	13
	LM	2	0.31	0.85	0.27	17
4-chlorophenyl	none	2	0.78	0.77	0.50	17
	DM	2	0.69	>0.98	0.41	>100
	LM	1.5	0.45	>0.98	0.31	>100
2,4-dichlorophenyl	none	10	0	0	0.34[a]	1
	DM	42	0.38	0.87	0.30	20
	LM	10	0.14	0.89	0.14	20
2-methyl-4-chlorophenyl	none	22	0	0	0.50[a]	1
	DM	30	0.31	0.93	0.25	37
	LM	23	0.22	0.97	0.18	81

[a] Determined by HPLC.

Table III. Effect of DM on the Lipase-Catalyzed Enantioselective Hydrolysis of (±)-Arylpropionic Chloroethyl Esters:
(±)-$R_1CHCH_3CO_2C_2H_5Cl \rightarrow$ (*S*)-(+)-$R_1CHCH_3CO_2H$ + (*R*)-(−)-$R_1CHCH_3C_2H_5Cl$

R_1	inhibitor (0.03 M)	reaction time (h)	ee_S	ee_P	*c*	*E**
phenyl	none	120	0.80	0.62	0.56	10
	DM	120	0.39	0.98	0.28	>100
2-fluoro-4-biphenyl	none	72	0.13	0.80	0.14	10
	DM	144	0.11	0.96	0.10	55
3-benzoylphenyl	none	120	0.25	0.52	0.32	4
	DM	192	0.06	0.95	0.06	42

Table IV. Effect of Various Concentrations of DM on the Enantioselective Hydrolysis of (±)-DCPP Catalyzed by *Candida* Lipase[a]

dextromethorphan concn (M)	reaction time (h)	ee_S	ee_P	*c*	*E**
none	1	~0	0.13	0.25[b]	1.4
0.006	3	0.17	0.32	0.35	2.3
0.012	3.5	0.30	0.72	0.29	8
0.018	4	0.41	0.81	0.34	14
0.024	4.5	0.44	0.85	0.34	19
0.03	5	0.43	0.87	0.33	22
0.06	5.5	0.37	0.87	0.30	21

[a] Substrate concentration was 0.05 M, and 225 units of LW lipase in 10 mL of 0.2 M phosphate buffer, pH 7.0, was used. [b] Estimated by TLC.

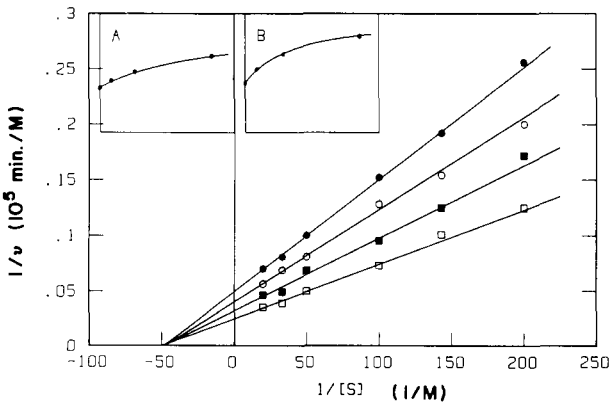


Figure 1. Parital noncompetitive inhibition by dextromethorphan (DM) with (*R*)-(+)-DCPP as the variable substrate. Each solid line was drawn from the data fit to eq D1. The concentrations of DM were 0 (□), 3 (■), 10 (○), and 30 mM (●). Insets: (A) Replots of slopes versus [DM]. The solid line represents the fit of the apparent K_m/V_{max} values of (*R*)-(+)-DCPP to eq D4. (B) Intercept replots versus [DM]. The solid line represents the fit of the apparent $1/V_{max}$ values of (*R*)-(+)-DCPP to eq D5.

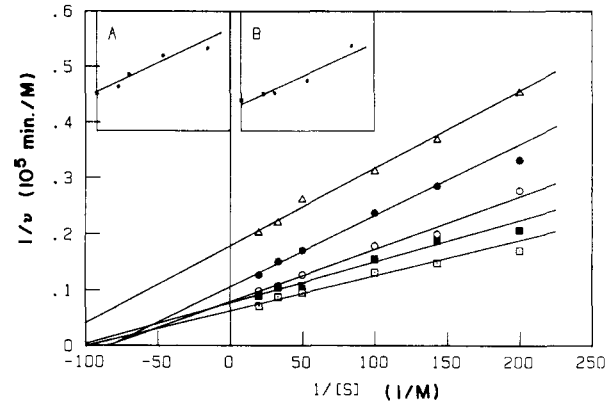


Figure 2. Linear noncompetitive inhibition by dextromethorphan (DM) with (*S*)-(−)-DCPP as the variable substrate. Each solid line was drawn from the data fit to eq D1. The concentrations of DM were 0 (⊡), 2 (■), 3 (○), 6 (●), and 10 mM (△). Insets: (A) Slope replots versus [DM]. The solid line represents the fit of the apparent K_m/V_{max} values of (*S*)-(−)-DCPP to eq D2. (B) Intercept replots versus [DM]. The solid line represents the fit of the apparent $1/V_{max}$ values of (*S*)-(−)-DCPP to eq D3.

To be an effective inhibitor, DM must exist in the free base form, and its apparent optimum concentration is in the range of 0.01–0.03 M (Table IV).

To gain an insight into the mechanism of inhibition of the *Candida* lipase by DM, we prepared (*R*)-(+)-DCPP and (*S*)-(−)-DCPP in their enantiomerically pure forms. This then allowed us to study the degree and the type of inhibition caused by DM against each enantiomer separately. The inhibition pattern of *Candida* lipase activity produced by DM was determined by varying the concentration of (*R*)-(+)-DCPP in the presence of several fixed concentrations of DM. Double-reciprocal plots afforded an intersecting pattern (Figure 1), but replots of the slopes and intercepts versus inhibitor concentration are hyperbolic (insets of Figure 1), which is indicative of partial noncompetitive inhibition. In contrast, when (*S*)-(−)-DCPP was used as the varying

Table V. Kinetic Parameters of *C. cylindracea* Lipase: I = DM

	R-(+)	*S*-(−)
k_{cat} (s^{-1})	13.3 ± 0.4	5.3 ± 0.2
K_m (M)	0.02 ± 0.002	0.01 ± 0.001
k_{cat}/K_m (M^{-1} s^{-1})	665 ± 33	530 ± 42
K_i (M)	0.004 ● 0.001	0.007 ± 0.002
α	1.07 ± 0.18	1.05 ± 0.29
β	0.44 ± 0.08	$1.2 \times 10^{-8} \pm 0.2 \times 10^{-8}$

substrate, an intersecting pattern is again obtained, but replots of these slopes and intercepts versus inhibitor concentration were both linear (insets in Figure 2). This inhibition pattern is consistent with that of pure noncompetitive inhibition. The apparent kinetic constants, obtained by fitting the kinetic data to the general equation for inhibition (eq 3), are listed in Table V.

Discussion

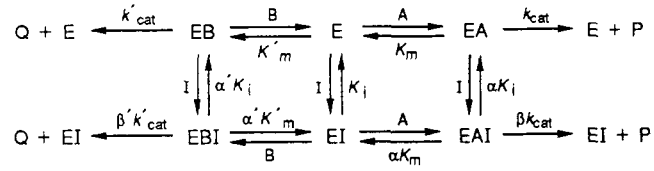
Although phenethylamines are known inhibitors[8] of rat and human pancreatic lipases, the phenomenon of enantioselective inhibition has not been observed until now. Both DM and LM as their free bases markedly enhanced the enantioselectivity (20-fold) of the *Candida* lipase-catalyzed hydrolysis of (±)-DCPP; some enhancement of enantioselectivity was also noted with the chiral amines quinidine and quinine, but strychnine had no apparent effect in this biocatalytic system. The optimum concentration of DM or LM appears to be in the range of 0.03 M, which is approaching their solubility limit in the buffer system. The binding of DM and DCPP to the lipase appears to occur independently of one another as indicated by the values of α and $\alpha' \simeq 1$. This supposition is also consistent with the observation that DM or LM enhanced the enantioselectivity of lipase-catalyzed hydrolysis of many (±)-(aryloxy)propionic and (±)-arylpropionic esters. Although our investigation has been restricted to the *C. cylindracea* lipase and a limited number of substrates, this finding could be quite general, for DM improved the enantioselectivity of the lipase-catalyzed hydrolysis of all of the chiral esters (α-methyl substituted) that we have thus far examined. However, at this stage, it is not known whether this concept of enantioselective inhibition could be extended to the enzymatic hydrolysis of acyloxy esters for the kinetic resolution of chiral alcohols or to the enantiotopically selective hydrolysis of prochiral compounds. Nevertheless, the observed phenomenon extends the usefulness of the *C. cylindracea* lipase and provides the chemist with an additional strategy for improving the enantioselectivity of biocatalytic systems.

To gain an understanding of the mechanistic aspects of enantioselective inhibition, it is necessary to consider the general mechanism as depicted in Scheme I. For the sake of convenience, the nomenclature of Segel[9] will be used for all of our subsequent discussions. Because the k_{cat}/K_m values for both the *R* and *S* enantiomers of DCPP are $<10^3$ M^{-1} s^{-1} (Table V), well below the value of 10^8–10^9 M^{-1} s^{-1} expected for diffusion-controlled reactions,[10] it is reasonable to assume rapid equilibrium conditions for this system.

In this system, A and B are the competing enantiomers, and under rapid equilibrium conditions, the various equilibrium constants are

$$K_m = [E][A]/[EA] \qquad \alpha K_m = [EI][A]/[EAI]$$
$$K'_m = [E][B]/[EB] \qquad \alpha' K'_m = [EI][B]/[EBI]$$
$$K_i = [E][I]/[EI] \qquad \alpha K_i = [EA][I]/[EAI]$$
$$\alpha' K_i = [EB][I]/[EBI]$$

Scheme I. Equilibria Describing General Enantioselective Inhibition



k_{cat} and βk_{cat} are the rate constants for the breakdown of [EA] and [EAI], respectively, to P, and k'_{cat} and $\beta k'_{cat}$ are the rate constants for the breakdown of [EB] and [EBI], respectively, to Q.

It is to be noted that α is the factor by which K_m changes when I is bound to the enzyme and K_i therefore must change to αK_i when A is bound to the enzyme. This is because the overall equilibrium constant for the formation of EAI must be the same regardless of the path (E → EA → EAI ← EI ← E). Similar reasoning may be made for the factor α' for the competing enantiomer, B. At any [I], the velocity at which P is formed is $k_{cat}[EA] + \beta k_{cat}[EAI]$; similarly, the rate of formation of Q is $k'_{cat}[EB] + \beta' k'_{cat}[EBI]$.

From these relationships, a general expression for enantioselective inhibition (eq 1) may be readily obtained (see Appendix).

$$\frac{\ln \dfrac{[A]}{[A_0]}}{\ln \dfrac{[B]}{[B_0]}} = E^* \frac{1 + \dfrac{\beta}{\alpha}\dfrac{[I]}{K_i}}{1 + \dfrac{\beta'}{\alpha'}\dfrac{[I]}{K_i}} \qquad (1)$$

$$E^* = k_{cat}K'_m/k'_{cat}K_m$$

Various types of enantioselective inhibition systems, which are summarized in Table VI, are governed by eq 1. It is evident that enantioselective enhancement by the inhibitor is dependent on the values of β, β', α, and α' as well as [I] and K_i. To obtain enantioselective enhancement, it is imperative that the inhibition pattern for one of the enantiomers (A or B) be either partial competitive, partial noncompetitive, or mixed type II inhibitors (Table VI). No enhancement in enantioselectivity will be observed in other systems where $\beta = \beta' = 0$ or 1 and where $\alpha = \alpha' = 1$. Under these conditions, eq 1 is reduced to the conventional homocompetitive equation[4] (eq 2). In the extreme case, one may envisage

$$\ln ([A]/[A_0])/\ln ([B]/[B_0]) = E^* \qquad (2)$$

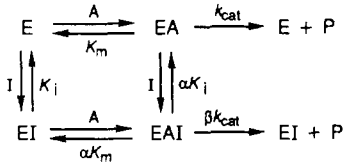
an enhancement in enantioselectivity by an inhibitor when $\alpha = 1$, $0 < \beta < 1$ and $\alpha' = 1$, $\beta' = 0$. At infinitely high [I], all the enzyme will exist as [EI], [EAI], and [EBI], but only EAI is functional to give product, albeit at a reduced rate. When $\beta > \beta'$ and $0 < \beta < 1$, improvement of enantioselectivity by the inhibitor is observed. When $\beta > 1$, the inhibitor becomes an activator.

To deduce the mechanism of enantioselective inhibition produced by DM, a series of kinetic experiments were conducted with each enantiomer of DCPP separately. At this juncture, it is worthy of note that many authors have raised the difficulty associated with the determination of the kinetic parameters V_{max} and K_m[11] for lipase-catalyzed reactions. At best, these parameters are only apparent quantities and must be interpreted accordingly. This is because insoluble substrates form a lipid–water interface and lipolytic enzymes such as lipase bind reversibly to the interface; this so-called penetrated enzyme then reacts with the substrate.

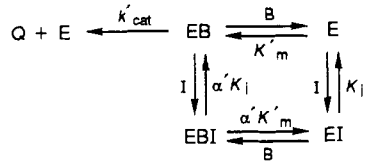
Table VI. Enantioselective Inhibition Systems: When $\alpha < 1$ or $\beta > 1$, Activation May Occur

inhibition pattern		
A	B	$\ln ([A]/[A_0])/\ln ([B]/[B_0])$
partial competitive ($\alpha \neq 1$ and $\beta = 1$)	pure competitive ($\alpha' = \infty$)	$E^*(1 + [I]/\alpha K_i)$
partial competitive	partial competitive	$E^*(1 + [I]/\alpha K_i)/(1 + [I]/\alpha' K_i)$
partial noncompetitive ($\alpha = 1$ and $\beta \neq 0$)	pure noncompetitive ($\alpha' = 1$ and $\beta' = 0$)	$E^*(1 + \beta[I]/K_i)$
partial noncompetitive	partial noncompetitive	$E^*(1 + \beta[I]/K_i)/(1 + \beta'[I]/K_i)$
mixed type II ($\alpha \neq 1$ and $\beta \neq 0$)	mixed type I ($\alpha' \neq 1$ and $\beta' = 0$)	$E^*(1 + \beta[I]/\alpha K_i)$
mixed type II	mixed type II	$E^*(1 + \beta[I]/\alpha K_i)/(1 + \beta'[I]/\alpha' K_i)$

Scheme II. Equilibria for Partial Noncompetitive Inhibition



Scheme III. Equilibria Describing Pure Noncompetitive Inhibition



Hence, the experimentally determined K_m may represent the dissociation constant of the enzyme–interface complex.[12] Likewise, the V_{max} measured at saturating amounts of insoluble substrate may represent the real V_{max} multiplied by the factor $S/(K_m^* + S)$, where K_m^* is the interfacial K_m. Some authors[13] have claimed to have circumvented this difficulty by increasing the area of interface with vigorous stirring. Under these experimental conditions, all the enzyme is adsorbed to the interface, and the concentration of enzyme in solution may be considered to be that at the interface. Despite these potential problems, we reasoned that at the very least the kinetic inhibition pattern for each enantiomer of DCPP could provide us with useful information relative to the mechanism of inhibition.

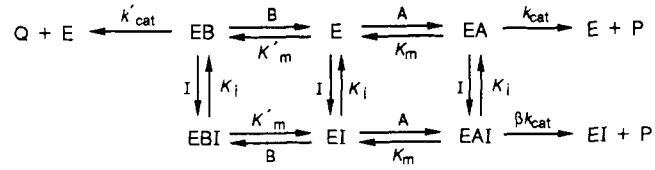
Lineweaver–Burk[14] plots of the inhibition kinetic data (individually line fitted, Figure 1) using (*R*)-DCPP (A) as the varying substrate in the presence of fixed concentrations of the inhibitor, DM, gave a pattern that is consistent with that of partial noncompetitive inhibition.[9] This is manifested by the curves intersecting on the 1/[S] axis and the hyperbolic effects on both slopes and intercepts (inset of Figure 1). In this system, the inhibitor (I) and substrate (A) combine independently and reversibly to the enzyme at different sites to produce the complexes EA, EI, and EAI. The EAI complex can also produce product (P), but not as effectively as EA. It is this latter feature that distinguishes it from pure noncompetitive inhibition. The equilibria describing partial noncompetitive inhibition may be envisaged as shown in Scheme II. As the family of reciprocal lines intersect on the 1/[S] axis, the value of α must equal 1. That is, I has equal affinity for E and EA, and A binds equally well to E and EI.

The inhibition kinetic data for the interaction of DM and the enzyme during hydrolysis of (*S*)-DCPP (B) are more scattered (Figure 2). However, the overall pattern appears to fit that of pure noncompetitive inhibition because replots of slopes and intercepts versus [I] were both linear (inset of Figure 2). The equilibria for this system is represented in Scheme III.

To further verify these inhibition patterns, the kinetic data for each enantiomer were fitted to general-inhibition eq 3 to obtain

$$v = \frac{V_{max}\frac{[S]}{K_m} + \beta V_{max}\frac{[S][I]}{\alpha K_m K_i}}{1 + \frac{[S]}{K_m} + \frac{[I]}{K_i} + \frac{[S][I]}{\alpha K_m K_i}} \quad (3)$$

Scheme IV. Equilibria Describing Enantioselective Inhibition by Dextromethorphan



the apparent kinetic constants shown in Table V. The values of $\alpha = 1$ and $0 < \beta < 1$ for the *R* enantiomer (A) system are characteristic for partial noncompetitive inhibition, whereas $\alpha' = 1$ and $\beta' = 0$ for the *S* enantiomer are reminiscent of pure noncompetitive inhibition. We are now able to rationalize the enhancement of enantioselectivity by DM under competitive conditions. This observed phenomenon is accommodated by Scheme IV. One can readily envisage that as [I] is increased more and more of the enzyme exists as the complexes EBI, EI, and EAI; however, only EAI is converted into P, but at a reduced rate.

The significance of enantioselective inhibition is not confined to the enhancement of enzymatic enantioselectivity in preparative chemistry, but rather, it also has important implications in the in vivo metabolism of racemic compounds. Many drugs are administered as racemates and are metabolized stereoselectively by enzymes in the body. Our findings raise the awareness that enantioselective inhibition may also occur in vivo. This in turn could complicate the pharmacokinetic investigations of racemic drugs and the problem of drug incompatibility. It is hoped that the general model and the quantitative expressions herein presented will serve as a conceptual platform from which investigators will launch further experimentation to determine the full dimension of this phenomenon among different enzyme systems as well as among nonenzymic enantioselective systems.

Experimental Section

^{1}H NMR spectra were recorded on a Bruker WM-200 spectrometer in deuteriochloroform with tetramethylsilane as the internal standard. Optical rotations were measured with a Perkin-Elmer Model 241C polarimeter in the indicated solvents. A Model M-6000 pump equipped with a U6K injector and a Model 77 double-beam UV (254 nm) detector (Waters Associates) were used for high-pressure liquid chromatography (HPLC). Thin-layer chromatography (TLC) was performed on plastic sheets coated with a 0.25-mm thickness of silica gel 60F-254 (Macherey-Nagel, West Germany). Flash column chromatography was performed with Baker silica gel (40 μm). All solvents were glass distilled prior to use. Fluorbiprofen was obtained from Boots Pharmaceuticals Inc.; DM was a product of Vicks; LM was obtained from Hoffman-La Roche. All other chemicals and solvents of the highest quality grade available were purchased from Aldrich Chemical Co. or Sigma Chemical Co. Chloroethyl esters of arylpropionic acids were prepared via the acid chloride method.[15]

Lipase Preparation. *C. cylindracea* lipase (EC 3.1.1.3) powder (L1754 type VII) was purchased from the Sigma Chemical Co. This preparation was purified to homogeneity following a five-step published procedure[16] to obtain the purified lipase form A (LA). The steps of the purification procedure include (1) water extraction, (2) ammonium sulfate precipitation, (3) sodium deoxycholate–organic solvent treatment, (4) SP-Sephadex C-50 chromatography (this was used instead of SE-Sephadex), and (5) Sephadex G-100 chromatography. Lipase LA had a protein concentration of 0.12 mg/mL and a specific activity of 45 units/mL or 375 units/mg of protein; the molecular weight of enzyme LA was 1.2 ($\pm$0.1) $\times 10^5$ as determined by SDS–polyacrylamide gel electrophoresis.[7] Protein concentration was estimated according to the dye binding method.[17] Lipase activity was assayed according to the published procedures with a poly(vinyl alcohol)-emulsified system.[16] One unit is defined as the amount of enzyme that liberates 1 μequiv of acid from olive oil (Sigma) in 1 min at pH 7.0 and 37 °C. The lipase preparation derived from step 1 (LW) of the purification procedure exhibited a single band on polyacrylamide gel electrophoresis; it is only contaminated with low molecular weight proteins at the origin.[7] Because the enantioselective properties of this lipase are markedly altered upon treatment with de-

(8) Comai, K.; Sullivan, A. C. *J. Pharm. Sci.* **1982**, *71*, 418.
(9) Segel, I. H. *Enzyme Kinetics*; Wiley: New York, 1975; pp 125–169.
(10) Hammes, G. G. *Enzyme Catalysis and Regulation*; Academic Press: New York, 1982; p 101.
(11) (a) Verger, R.; De Haas, G. H. *Annu. Rev. Biophys. Bioeng.* **1976**, *5*, 77. (b) Verger, R. *Methods Enzymol.* **1980**, *64*, 340.
(12) Fitzharris, T. J.; Quinn, D. M.; Goh, E. H.; Johnson, J. D.; Kashyap, M. L.; Srivastava, L. S.; Jackson, R. L.; Harmony, J. A. K. *J. Lipid Res.* **1981**, *22*, 921.
(13) Hirohara, H.; Mitsuda, S.; Ando, E.; Komaki, R. In *Biocatalysis in Organic Syntheses*; Tramper, J.; Van der Plas, H. C.; Linko, P., Eds.; Elsevier: Amsterdam, 1985; p 119.
(14) Lineweaver, H.; Burk, D. *J. Am. Chem. Soc.* **1934**, *56*, 658.
(15) Ottenheigm, H. C. J.; DeMan, J. H. M. *Synthesis* **1975**, 163.
(16) Tomizuka, N.; Ota, Y.; Yamada, K. *Agric. Biol. Chem.* **1966**, *30*, 576.
(17) Bradford, M. *Anal. Biochem.* **1976**, *72*, 248.

oxycholate and organic solvent[7] (step 3), preparation LW was used for our kinetic inhibition studies since it is devoid of any other esterase activity; it was diluted to a specific activity of 45 units/mL.

General Procedure for Enzymatic Enantioselective Hydrolyses. Unless otherwise stated, the reaction mixture contained 30 mg of *C. cylindracea* lipase powder in 7 mL of 0.2 M phosphate buffer, pH 8.0. In reactions where an inhibitor was used, a solution of premade 0.2 M phosphate buffer, pH 8.0, containing the inhibitor at the indicated concentrations were used instead. To the above suspension was added 0.9 mmol of a racemic substrate. The reaction mixture was stirred vigorously at 24 °C, and the progress of the reaction was monitored by TLC or HPLC. When the conversion reached 30–50%, the mixture was acidified with aqueous 1 N HCl to pH 2 and then extracted with ethyl acetate (3 × 4 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The remaining substrate and the product were separated by flash chromatography (0.8 × 12 cm) with hexane–ethyl acetate–acetic acid (70:10:1) as the eluting solvent. The remaining substrate ester was directly used for the determination of optical purity (ee_S), and the product acid was methylated by diazomethane treatment and then was used for the determination of ee_P. Optical rotations of the remaining substrates and products were measured when required.

Determination of Enantiomeric Excess (ee). All the ee analyses were made with 200-MHz ^1H NMR analysis of the esters in the presence of the chiral shift reagent Eu(hfc)$_3$. The remaining substrates (esters) were employed directly for ee_S determination. The products (acids) of the enzymatic reactions were first converted to their corresponding methyl esters by treatment with diazomethane, and their ee_P was then determined. For the 200-MHz ^1H NMR analysis, each sample contained 5–15 mg of the ester, 40 mg (for arylpropionic esters) or 80 mg [for (aryloxy)propionic esters] of the chiral shift reagent, and 0.6–0.8 mL of $CDCl_3$. The doublet of the α-CH_3 of these substrates at δ 1.2 was shifted to δ 3–4 and split into two doublets. The signals of (*R*)-(+)-methyl (aryloxy)propionates were more downfield than those of the *S*-(−) enantiomers. In contrast, the signals of the (*S*)-(+)-arylpropionates were more downfield than those of their corresponding *R*-(−) enantiomers. The ee of each sample was calculated from the ratio of the integral areas of the signals for each of the two enantiomers. In the case of methyl 2-(4-chlorophenoxy)propionate, the doublet of the α-CH_3 could not be resolved by the chiral shift reagent, but the singlet at δ 3.7 ($-CO_2CH_3$) was shifted and split into two peaks: the *R*-(+) enantiomer at δ 6.3 and the *S*-(−) enantiomer at δ 6.2. For samples of very high optical purity, the signal corresponding to the minor enantiomer could not be detected. A small quantity of the racemate was then added to the sample and was then again analyzed to confirm the validity. In these samples of very high ee, a value of >0.98 was assigned.

Preparation of (*R*)-(+)- and (*S*)-(−)-DCPP. For the kinetic resolution of (±)-DCPP, lipase LA was employed; this preparation has an *R* stereochemical preference with an E^* value of 14.[7] To a solution of 100 mL of 0.2 M phosphate buffer, pH 7.0, was added 450 units of lipase LA and 10 g of (±)-DCPP. After the reaction mixture was stirred at 24 °C for 48 h, the contents were acidified to pH 2.0, and the general procedure was followed for the isolation of remaining substrate and product. The conversion (c) was determined to be 0.44 by HPLC analysis. With flash column chromatography, 5.5 g of remaining ester, (*S*)-(−)-DCPP, and 4.5 g of product, (*R*)-(+)-(dichlorophenoxy)propionic acid (DCPPA) (ee_P = 0.72), were obtained. The product, DCPPA (4.5 g), was esterified with diazomethane and incubated again with 200 units of lipase LA until the conversion reached approximately 0.7 as estimated by TLC. With the same workup procedure, 3 g of the product was obtained. Recrystallization of the acid from hexane–ethyl acetate (10:1) gave 2 g of (*R*)-(+)-DCPPA, $[\alpha]^{25}_D$ +28.9° (c 1.6, EtOH), which was again esterified with diazomethane and purified to yield 2 g of (*R*)-(+)-DCPP, $[\alpha]^{25}_D$ +37.5° (c 2.5, EtOH); 200-MHz ^1H NMR analysis in the presence of Eu(hfc)$_3$ confirmed the sample to have ee > 0.98.

The remaining substrate fraction (5.5 g) was reincubated with lipase LA to give 3.1 g of (*S*)-(−)-DCPP with ee = 0.95. The ester grouping was hydrolyzed in a solution consisting of 15 mL of aqueous 2 N NaOH and 15 mL of methanol at 24 °C. The resulting (*S*)-(−)-DCPPA was recrystallized twice, methylated with diazomethane, and purified to afford 2 g of pure (*S*)-(−)-DCPP, $[\alpha]^{25}_D$ −36.8° (c 2.8 Et,OH and ee > 0.98.

Determination of Reaction Velocity. The measurement of initial velocity of lipase-catalyzed hydrolysis of artificial substrates is complicated by many factors. First of all, the substrate is insoluble in water, and the reaction rate is slow ($k_{cat}/K_m < 10^3$ M^{-1} s^{-1}). Hence, at low substrate concentrations, it is difficult to obtain accurate initial velocities by measuring the tangent to the v versus [S] curve. For a conventional enzymatic reaction, initial velocities are best measured with a continuous assay. However, in our system, it is imperative to maintain a constant pH, because the solubility of the inhibitor is highly sensitive to changes in pH. For example, the pK_a of the conjugate acid of DM is 8.3.[18] Thus, the percentage of the free base {[B]/([B] + [HB$^+$])} is 50, 33, 5, 3, and 0.5 at pH 8.3, 8.0, 7.0, 6.8, and 6.0, respectively. The maximal solubility of DM in 0.2 M phosphate buffer at pH 7.0 is about 0.03 M at 24 °C. The reported solubility of DM hydrobromide in water is 1.5% (0.043 M) at 25 °C, and the pH of a 1% solution in water is around 5.2–6.5. This eliminates the possible use of pH-stat or indicator color changes to continuously monitor the progress of the reaction. These considerations compelled us to use a discontinuous sampling method.

Experimental reaction velocity was calculated from the substrate concentration, reaction time, and conversion; $v = [S]c/t$. The extent of conversion was determined by HPLC analysis using a 4.6 × 500 mm (Alltech Porasil, 10 μc) column, which was eluted with a solvent system consisting of hexane–ethyl acetate–acetic acid (500:100:6) at a rate of 4 mL/min. The absorbance at 254 nm was monitored with a Waters Model 440 detector and a Hewlett-Packard 3390A integrator. A standard mixture of (±)-DCPP and (±)-DCPPA at varying ratios gave equal molar responses of integral areas. All incubations were conducted under identical reaction conditions. For each sample, the substrate, (*R*)-(+)-DCPP or (*S*)-(−)-DCPP, was dissolved in hexane, and appropriate aliquots were distributed to reaction vials. The hexane was then removed by evaporation using a stream of nitrogen. A premade solution {1 mL of 0.2 M phosphate buffer containing 22.5 units of lipase LW ([E_t] = 5 × 10^{-7} M) and varying amounts of DM as indicated} was preincubated in a 25 °C water bath for 10 min; this solution was then carefully added to the vials containing the substrate. The reaction mixture was stirred vigorously at 25 °C with a Corning PC-351 stirrer. When the conversion reach 0.2–0.3, the reaction was terminated by acidification (1 N HCl) to pH 2.0 and the incubation time recorded. The reaction mixture was then extracted with ethyl acetate (4 × 1 mL), dried over Na_2SO_4, filtered, and evaporated to dryness under reduced pressure. The residue was dissolved in a known aliquot of ethyl acetate and subjected to HPLC analysis to determine the extent of conversion. The experimental velocity was then employed as the initial velocity.

Data Analysis. Kinetic data were analyzed on a Northstar Horizon computer using the BASIC program of Duggleby,[19] prepared by Dr. Dexter B. Northrop. The data were first fitted line by line to eq D1 to generate a double-reciprocal plot. To establish the pattern of inhibition, replots of slopes and intercepts were examined graphically, and the apparent V/K and V values were fitted to eq D2 and D3, respectively, weighted by the standard deviations obtained from the primary regression. Apparent V/K and V values generating hyperbolic replots were fitted to eq D4 and D_5, respectively. To obtain the kinetic constants (Table V), the data were then fitted to eq D6, which is the equivalent form of eq 3.

$$v = \frac{V[S]}{K_m + [S]} \tag{D1}$$

$$\frac{V_i}{K_m} = \frac{V_0}{K_m(1 + [I]/K_{is})} \tag{D2}$$

$$V_i = \frac{V_0}{1 + [I]/K_{ii}} \tag{D3}$$

$$\frac{V_i}{K_m} = \frac{V_0(1 + [I]/K_{id})}{K_m(1 + [I]/K_{is})} \tag{D4}$$

$$V_i = \frac{V_0(1 + [I]/K_{id})}{1 + [I]/K_{ii}} \tag{D5}$$

$$v = \frac{v[S]}{K_m\frac{1 + [I]/K_{is}}{1 + [I]/K_{id}} + [S]\frac{1 + [I]/K_{ii}}{1 + [I]/K_{id}}} \tag{D6}$$

where

$$K_{is} = K_i$$

$$K_{ii} = K_i\alpha$$

$$K_{id} = K_i\alpha/\beta$$

$$V = k_{cat}[E_t]$$

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(18) Newton, D. W.; Kluza, R. In *Principles of Medicinal Chemistry*, 3rd ed.; Foye, W. O., Ed.; Lea & Febiger: Philadelphia, 1989; p 865.
(19) Duggleby, R. G. *Comput. Biol. Med.* **1984**, *14*, 447.

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Appendix

For Scheme I, we may write

$$[E_t] = [E] + [EA] + [EB] + [EI] + [EAI] + [EBI] \quad (A1)$$

$$[EA] = ([A]/K_m)[E] \quad (A2)$$

$$[EB] = ([B]/K_m)[E] \quad (A3)$$

$$[EI] = ([I]/K_i)[E] \quad (A4)$$

$$[EAI] = \frac{[A][I]}{K_m\alpha K_i}[E] \quad (A5)$$

$$[EBI] = \frac{[B][I]}{K'_m\alpha' K_i}[E] \quad (A6)$$

Let

$$X = 1 + \frac{[A]}{K_m} + \frac{[B]}{K'_m} + \frac{[I]}{K_i} + \frac{[A][I]}{K_m\alpha K_i} + \frac{[B][I]}{K'_m\alpha' K_i} \quad (A7)$$

from which

$$[E_t] = [E]X \text{ or } [E] = [E_t]/X \quad (A8)$$

and

$$[EA] = \frac{[A]}{K_m}\frac{[E_t]}{X} \quad (A9)$$

$$[EB] = \frac{[B]}{K'_m}\frac{[E_t]}{X} \quad (A10)$$

$$[EAI] = \frac{[A][I]}{K_m\alpha K_i}\frac{[E_t]}{X} \quad (A11)$$

$$[EBI] = \frac{[B][I]}{K'_m\alpha' K_i}\frac{[E_t]}{X} \quad (A12)$$

The reaction velocity for A is

$$-d[A]/dt = v = k_{cat}[EA] + \beta k_{cat}[EAI] \quad (A13)$$

Substituting eq A9 and A11 into eq A13 and rearranging, we obtain

$$\frac{-d[A]}{dt} = v = \left(1 + \frac{\beta}{\alpha}\frac{[I]}{K_i}\right)\frac{k_{cat}}{K_m}\frac{[E_t]}{X}[A] \quad (A14)$$

Similarly, the reaction velocity for B is

$$\frac{-d[B]}{dt} = v' = \left(1 + \frac{\beta'}{\alpha'}\frac{[I]}{K_i}\right)\frac{k'_{cat}}{K'_m}\frac{[E_t]}{X}[B] \quad (A15)$$

Dividing eq A14 by eq A15, we obtain

$$\frac{d[A]}{d[B]} = \frac{[A]}{[B]}\frac{k_{cat}K'_m}{k'_{cat}K_m}\frac{1 + \frac{\beta}{\alpha}\frac{[I]}{K_i}}{1 + \frac{\beta'}{\alpha'}\frac{[I]}{K_i}} \quad (A16)$$

Integration of eq A16 gives

$$\frac{\ln\frac{[A]}{[A_0]}}{\ln\frac{[B]}{[B_0]}} = E^*\frac{1 + \frac{\beta}{\alpha}\frac{[I]}{K_i}}{1 + \frac{\beta'}{\alpha'}\frac{[I]}{K_i}} \quad (A17)$$

where

$$E^* = k_{cat}K'_m/k'_{cat}K_m \quad (A18)$$

Communications to the Editor

Liquid Xenon: An Effective Inert Solvent for C–H Oxidative Addition Reactions

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The C–H activating intermediate generated by irradiation of $Cp^*(PMe_3)IrH_2$ (**1**; $Cp^* = \eta^5$-C_5Me_5) reacts with all C–H bonds to which it is exposed.[1] This property is useful for inducing chemical transformations in normally unreactive substrates. However, such ubiquitous reactivity is also a problem because it prevents the employment of common organic liquids as inert solvents for C–H activation studies. This restriction has prevented investigation of C–H insertion in organic substrates that are available in limited quantity, prohibitively expensive, or difficult to liquify, such as low molecular weight gases and high-melting solids. Fluorocarbons have been used as inert solvents in some related C–H activation systems, such as those initiated by irradiation of $Cp^*Ir(CO)_2$.[2,3] However, their general applicability has been frustrated by the fact that intermediates generated from $Cp^*(PMe_3)IrH_2$ react even with these materials[4] and by the low solubility of many organic and organometallic compounds in fluorocarbons.

Liquified noble gases have been used as solvents in numerous spectroscopic investigations,[5] including many involving organometallic complexes, most notably by Poliakoff, Turner, and coworkers.[5e-h] However, noble gases have not previously been employed in preparative reactions. We now report that the noble gas xenon, which exists as a liquid under modest pressures in the temperature range from −100 to −60 °C (2–9 atm),[6] can be used successfully as an inert solvent[7] for preparative C–H oxidative

(1) (a) Janowicz, A. H.; Bergman, R. G. *J. Am. Chem. Soc.* **1983**, *105*, 3929–3939. (b) Buchanan, J. M.; Stryker, J. M.; Bergman, R. G. *J. Am. Chem. Soc.* **1986**, *108*, 1537–1550.

(2) Hoyano, J. K.; McMaster, A. D.; Graham, W. A. G. *J. Am. Chem. Soc.* **1983**, *105*, 7190–7191.

(3) Marx, D. E.; Lees, A. J. *Inorg. Chem.* **1988**, *27*, 1121–1122.

(4) Sponsler, M. B.; Bergman, R. G., unpublished results.

(5) See, for example: (a) Bulanin, M. O. *J. Mol. Struct.* **1973**, *19*, 59–79. (b) Beattie, W. H.; Maier, W. B., II; Holland, R. F.; Freund, S. M.; Stewart, B. *Laser Spectros. S.P.I.E.* **1978**, *158*, 113–121. (c) Rentzepis, P. M.; Douglass, D. C. *Nature* **1981**, *293*, 165–166. (d) Andrea, R. R.; Luyten, H.; Vuurman, M. A.; Stufkens, D. J.; Oskam, A. *Appl. Spectrosc.* **1986**, *40*, 1184–1190. (e) Upmacis, R. K.; Poliakoff, M.; Turner, J. J. *J. Am. Chem. Soc.* **1986**, *108*, 3645–3651. (f) Jackson, S. A.; Upmacis, R. K.; Poliakoff, M.; Turner, J. J.; Burdett, J. K.; Grevels, F.-W. *Chem. Commun.* **1987**, 678–680. (g) Gadd, G. E.; Poliakoff, M.; Turner, J. J. *Organometallics* **1987**, *6*, 391–397. (h) Firth, S.; Klotzbücher, W. E.; Poliakoff, M.; Turner, J. J. *Inorg. Chem.* **1987**, *26*, 3370–3375.

(6) Theeuwes, F.; Bearman, R. J. *J. Chem. Thermodynamics* **1970**, *2*, 507–512.

(7) The term "inert solvent" is meant to indicate that the solvent does not give stable products with the intermediates generated by irradiation of $Cp^*(PMe_3)IrH_2$.

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