Lipase Selectivities[1]

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One considerable advantage that enzymes potentially have over conventional chemical methods resides with the inherent selectivities of the natural catalysts. The methods for gauging these parameters are sometimes tedious, and often are not without ambiguity. Prompted by projected needs of those engaged in protein engineering to alter lipolytic activity, a number of compounds were evaluated as pseudolipids using reactivity with commercial lipases as a criterion for suitability. These then were employed to assess fatty acid- and stereoselectivities of these lipase preparations. Lipases indeed show small but significant stereobiases with esters of the acetonide of glycerol, compounds whose reactivities with lipases are comparable to, and sometimes exceed, those of the corresponding triglycerides.

The theme of lipase selectivity is very visible today in research journals; it also is a major focus in review articles that deal with the potential for industrial applications of lipase technology (1–5). A recent, useful review that gives leading reference to methodology for determining fatty acid and position selectivities for the lipases is provided by MacCrae (6). Frequently, one compares the slower reacting methyl esters to obtain information about fatty acid preferences, or employs triglycerides with the attendant complexities of a polyfunctional substrate that additionally can undergo position isomerization. A few examples of positional and fatty acid selectivities of some common extracellular microbial lipases are shown in Table 1.

Although additional non-selective triglyceride lipases have been reported (6), the majority of the microbial lipases are considered 1,3-"specific." Fatty acid preferences, however, seem to be modest (7). The lipase from *Geotrichum candidum* apparently is an exception, although its selectivity shows considerable strain variation (8). Moreover, literature is sparse on the subject of triglyceride lipase stereoselectivity: porcine pancreatic lipase is reportedly non-selective (9) as is *Rhizopus arrhizus* lipase (10), although a mammalian (human) gastric lipase reportedly exhibits limited selectivity for the *sn*-3 position of synthetic triglycerides (11). On the other hand, a rather large body of literature dealing with the preparation of chiral esters and alcohols employing lipase-mediated kinetic resolution of racemic (non-triglyceride) substrates exists. Actually, given the nature of enzymes as chiral catalysts with sophisticated molecular architecture, one might expect selectivity of each type to be the norm, and non-selectivity to be an exception. We probably are dealing in all cases with degrees of selection rather than absolutes, and perhaps one of the most dramatic accomplishments of protein engineering would be to enhance the existing substrate preferences to industrial utility.

STEREOSELECTION OF *MUCOR MIEHEI* LIPASE: ALCOHOLS

In an initial screening of commercial lipases to affect resolution of methyl *n*-alkylcarbinols, we learned that Novo's preparations from the fungus *Mucor miehei* were particularly useful (12). We employed equations that had been derived to characterize biochemical kinetic resolutions (Fig. 1) (13,14) as an aid to the screening process. In addition, we required a convenient procedure to document the configurational bias of the alcohols in question. Diastereomeric carbamates were formed using (S)-α-methylbenzylisocyanate that were resolvable by gas chromatography; i.e., a wide variety of alcohols can be analyzed for configuration, as well as for configurational purity in this manner. In the screening procedure, racemic 2-octanol was allowed to react with octanoic acid in hexane in the presence of a lipase. The conversions were determined by free fatty

TABLE 1

Positional and Fatty Acid Selectivities of Some Extracellular Microbial Lipases

Organisim	Mfr. code	P selectivity	FA selectivity
Candida rugosa	Enzeco	Non-	18 (*cis*-Δ9)
Aspergillus niger	Amano-K	Non-	10,12
Geotrichum candidum	Amano-GC4	Non-	18 (*cis*-Δ9)
Aspergillus niger	Amano-AP	1,3-	18 (*cis*-Δ9)
Mucor miehei	Novozyme 225	1,3-	12
Rhizopus arrhizus	Gist Brocades	1,3-	8,10

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RATE RATIOS (K_R/K_S) WERE CALCULATED FROM THE EXPRESSION:

$$\frac{K_R}{K_S} = \frac{Ln(1-C)\ (1-ee)}{Ln(1-C)\ (1+ee)}$$

C = FRACTION OF RACEMIC STARTING MATERIAL CONVERTED.

ee = + ENANTIOMERIC EXCESS OF RESIDUAL STARTING MATERIAL.

FOR HYDROLYSIS, THE "ee" WAS DETERMINED IN THE PRODUCT AND THEN RELATED TO THE STARTING MATERIAL:

$$ee_A = \frac{C\ (ee_B)}{1-C}$$

A REFERS TO STARTING MATERIAL; B TO PRODUCT.

FIG. 1. Equations used in lipase selectivity screening process.

TABLE 2

Esterification of (±)-2-Octanol with Octanoic Acid (Hexane, 30 C)

Lipase	K_R/K_S
Aspergillus niger (Amano-AP)	Too slow
Aspergillus niger (Amano-K)	6.1
Candida Rugosa (Enzeco)	1.1
Mucor miehei (Amano-MAP)	Too slow
Mucor miehei (G.B. S)	2.8
Mucor miehei (Novo)	>50
Rhizopus arrhizus (Sigma)	5.5
Pancreatic (Sigma)	5.4

acid titration, and the ee (enantiomeric excess) of the alcohol was determined to allow calculation of the enantiomeric ration. Several lipases showed a stereobias (Table 2); interestingly the bias exhibited by the *M. miehei* lipase was quite dependent upon the supplier of the enzyme. Only the Novo product had sufficient stereopreference to warrant pursuit of the application we had in mind.

Insect sex attractants, although they are a small commercial market, use chemicals that are in many cases derived from the fats and oils-related industries. Moreover, the technique employed to generate chiral centers has broad application and can tap the enzyme technology that now is developing while itself providing useful feedback. Several pheromones are secondary alcohols or their esters; the configuration of which are critical to biological activity. A case in point is the pheromone of the Western corn rootworm, a beetle in which the larval form feeds on the roots of corn. The structure of the pheromone, 8-methyl-2-decanol propanoate (Fig. 2) has two centers of asymmetry, and the 2R,8R-isomer is the most biologically active isomer (15). At least six related beetles, of which several also are economically important pests, apparently use the same parent alcohol structure. Some respond to the 2R,8R- and some to the 2S,8R-diastereomer. Because only the 8R-configuration is biologically active, contamination with 8S-isomers results only in dilution and thus materials racemic at that center may be suitable for many practical applications. The configuration at the 2-position, on the other hand, is critical, and, particularly because both isomers are desired, this alcohol seems ideally suited for kinetic resolution. Having located a useful enzyme preparation for such a resolution using 2-octanol as the model, racemic 8-methyl-2-decanol then was resolved by esterification with (optimally) octanoic acid in hexane, or by hydrolysis of the octanoate ester. The esterification procedure was a more convenient lab procedure; the enzyme formulation could be filtered and reused when samples of the reacting mixture gave indication that the desired degree of resolution/conversion had been achieved.

An interesting aspect of stereoselective hydrolysis is the sensitivity to chain length of the acid residue (Fig. 3). Occasional mention of this phenomenon ap-

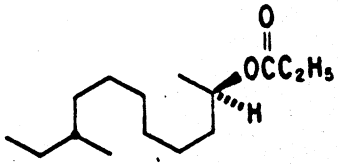


FIG. 2. Sex pheromone of the western corn rootworm: (R)-8-methyl-(S)-2-decanol propanoate.

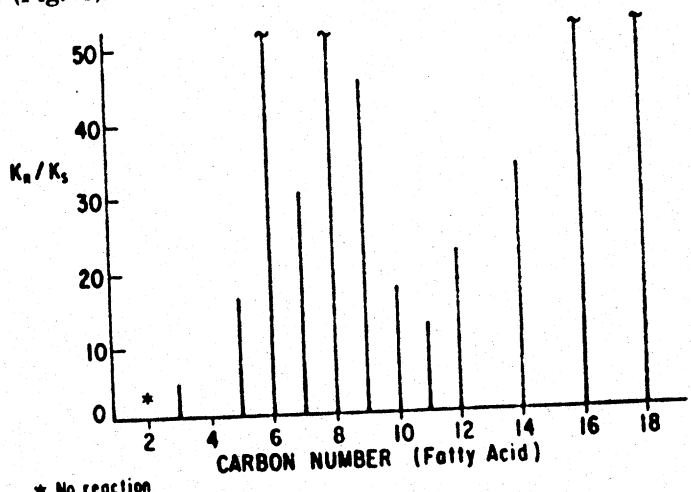


FIG. 3. Rate ratio vs fatty acid chain length for esterification of racemic 2-octanol in hexane in the presence for *Mucor Miehei* lipase.

TABLE 3

Esterification of Selected Alcohols with Octanoic Acid

Alcohol	E_R
Cyclohexyl methylcarbinol	>50
Phenyl methylcarbinol	42
3-Dodecyn-1-ol	>50
3-Octanol	1.2[a]
2-Methyl-1-decanol	1.2
2-Methyl-1-dodecanol	1.3
2-Phenyl-1-propanol	0.29[b]
1,2-Isopropylidene glycerol	1
Citronellol	1.1[c]
4-methyl-2-pentanol	23.5
3-methyl-2-hexanol	—[d]

Reactions were conducted at 30 C in hexane.

[a]The enantiomeric ratio was 1 with propanoic acid and 4.6 with lauric acid.

[b]$K_S/K_R = 3.4$.

[c]Diastereomer elution order not determined.

[d]Two stereoisomers react significantly faster.

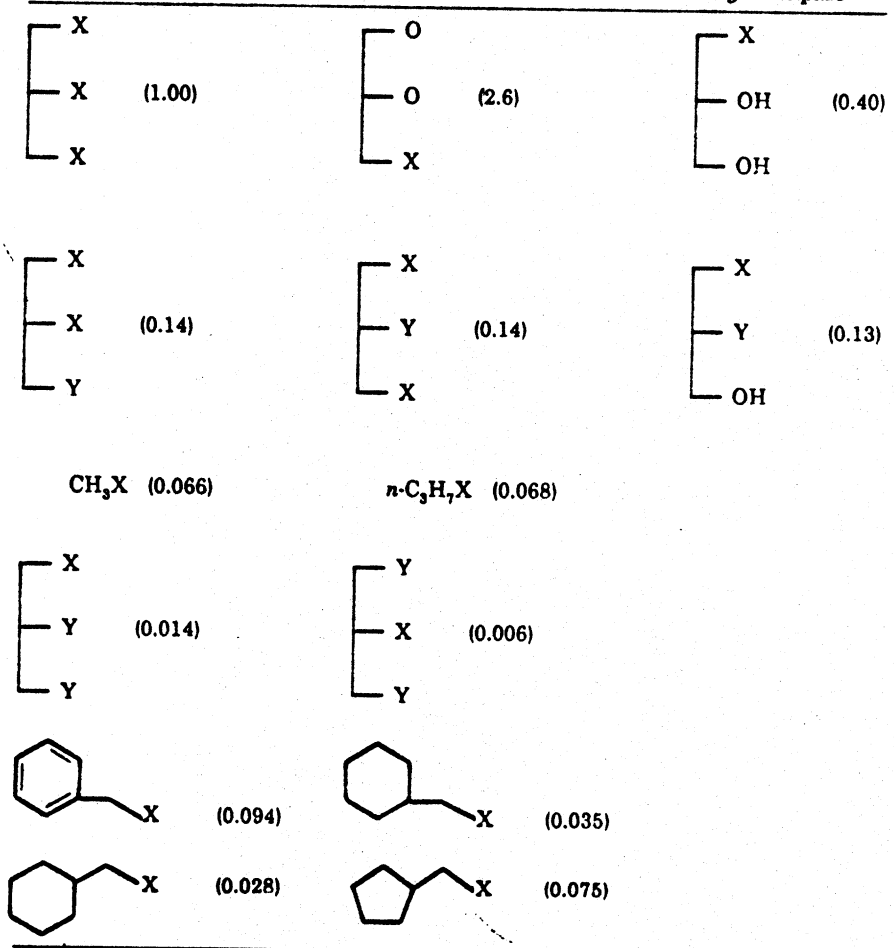
pears in the literature; it seems that one may consider that each possible acylated lipase may have some unique character that can express itself in competitive reactions that measure stereo- or positional-bias (secondary vs primary alcohols). In any case, the scope of the *M. Miehei* stereoselection (octanoic acid, hexane) is shown in Table 3. Stereobias remains high for cyclohexyl- and phenylmethanols, although reaction rates are lower. Shifting the hydroxyl group to the 3-position as in 3-octanol, or employing a primary alcohol with a shifted center of asymmetry as in items 5-9, markedly reduces stereoselection (16).

USE OF PSEUDOLIPIDS TO PROBE LIPASE SELECTIVITIES

We became interested in examining some additional substrates that could be useful in characterizing lipase selectivities and, perhaps, thereby circumventing the problems associated with using triglycerides. In fact, alkylglycerol ethers have in the past been used as "pseudolipids" in biological studies (17). The ethers are very stable, the alkyl chain lengths can be made to imitate any fatty acid residue, the compound can be mono- or

TABLE 4

Relative Reactivity of Octanoate Esters in the Presence of *Candida rugosa* Lipase[a]



[a]X = *n*-octanoyloxy (octanoate ester); Y = *n*-octyloxy (*n*-octyl ether).

diesters, and one could potentially determine any of the types of selectivity by using them as substrates. A number of glycerol derivatives were prepared and hydrolyzed lipolytically; the initial reaction velocities were obtained by a selected protocol (18). Although the compounds varied in their ability to hold an emulsion, qualitative comparisons of reactivity could be made reliably. Reaction velocities relative to trioctanoin normalized for numbers of acid residues in reactions involving *Candida rugosa* lipase are shown in Table 4. The octanoate ester of the acetonide of glycerol is more than twice as reactive as trioctanoin. By comparison, monooctanoin is slower, and methyl octanoate is slower yet. Analogs in which one of the OH groups is etherified with *n*-octyl react at about the same low rate (0.13–0.14); the diether analogs react even more slowly. The cyclopentylmethyl octanoate is essentially isosteric with the acetonide ester, yet it also is much less reactive.

In Table 5, the rate of hydrolysis of various fatty acid esters of glycerol, methanol, and the acetonide of glycerol with *C. rugosa* lipase are listed. Section B gives reactivities relative to the corresponding triglyceride. In all cases, the acetonide ester reacts as fast, or faster, than the triglyceride. Section C gives fatty acid selectivities relative to octanoic acid residues as 1.0; the data suggest that the acetonide ester may provide a better comparison than methyl esters do. In Table 6, the reactivities of octanoic and oleic esters relative to trioctanoin and triolein with several lipases

TABLE 5

Reactives of Selected Esters in the Presence of *Candida rugosa* Lipase

Calculated pseudo first order rates × 10^3 (25 C)

Alcohol	Fatty acid carbon number 4	6	8	9	12	18:1
Glycerol[a]	1.36	0.39	8.81	3.9	—[b]	2.50
Methanol	0.12	0.07	0.58	0.90	0.14	0.59
Acetonide of glycerol	1.90	0.38	23.0	5.05	2.89	2.59

Reactivity relative to corresponding triglyceride

Alcohol	Fatty acid carbon number 4	6	8	9	12	18:1
Glycerol[a]	1.0	1.0	1.0	1.0	—[b]	1.0
Methanol	0.09	0.18	0.07	0.23	—	0.24
Acetonide of glycerol	1.4	1.0	2.6	1.3	—	1.0

Reactivity relative to octanoate ester

Alcohol	Fatty acid carbon number 4	6	8	9	12	18:1
Glycerol[a]	0.15	0.04	1.0	0.44	—[b]	0.28
Methanol	0.21	0.12	1.0	1.55	0.24	1.0
Acetonide of glycerol	0.08	0.02	1.0	0.22	0.13	0.11

[a]The triglyceride was employed.

[b]Solid at reaction temp.

TABLE 6

Relative Reactivities of Selected Esters in the Presence of Various Commercial Lipases

Ester	A	B	C	D	E	F	G
Trioctanoin	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Methyl octanoate	0.10	0.07	0.05	0.21	0.50	0.06	0.04
Acetonide-octanoate	1.50	2.60	0.07	0.18	1.23	0.39	0.15
Triolein	1.00	1.00	1.00		1.00		
Methyl oleate	0.37	0.60	0.12		1.28		
Acetonide-oleate	1.53	2.81	0.12		2.15		

A, *A. niger* (non-selective); B, *C. rugosa*; C, *R. arrhizus*; D, Porcine pancreas; E, *M. miehei* (Novo); F, *M. miehei* (Amano); G, *M. miehei* (Gist-Brocades).

TABLE 7

Enantiomeric Ratios obtained in Hydrolysis of Acetonide Esters of Glycerol

Enzyme	Ester	C	ee	E
A. niger	butyrate	0.500	0.55	4.4
	octanoate	0.500	0.36	3.0
C. rugosa	butyrate	0.500	0.14	1.5
	octanoate	0.500	0.23	2.0
	dodecanoate	0.408	0.04	1.2
M. miehei	butyrate	0.500	0.16	1.6
	octanoate	0.500	0.09	1.3
Porcine pancreas	butyrate	0.500	0.38	3.1
	octanoate	0.500	0.33	2.7

Reactions were conducted at pH 7.00 and 25 C. C, % conversion; ee, enantiomeric excess (mol fraction enantiomer A-mole fraction enantiomer B); E, enantiomeric ratio. Reactions of the butyrate were conducted at various pH; pH (E): 5.2 (ca. 1.0), 6.0 (2.13), 7.0 (4.38), 8.0 (2.36), 9.0 (2.26).

are shown. Again, the acetonide esters generally are more reactive than the methyl esters, and frequently are more reactive than the triglycerides.

STEREOSELECTION OF LIPASES WITH A PSEUDOLIPID

Because the acetonide esters appear to be specific substrates for the lipases, and the asymmetric center of that molecule is a good handle on the stereochemistry of lipolysis, a method was devised to assess the configuration of the acetonide of glycerol. The alcohol was oxidized to a carboxylic acid that then was converted via the acid chloride to an amide with (S)-α-methylbenzylamine. The reactions could be carried out on 50 mg of sample to give diastereomeric amides that were analyzed by gas chromatography. The hydrolysis of butyrate and octanoate esters was conducted under pH stat conditions to 50% conversion using several lipases, and the enantiomeric ratios were calculated (Table 7). The lipases examined exhibited low, but significant, biases; the *sn*-3 glycerol position was shown to be the faster reacting site in all cases.

Finally, Figure 4 illustrates some of the classes of compounds reported in the literature to have undergone stereoselective lipase reactions and indicates the faster reacting enantiomer. The studies cover a variety of substrates, namely, straight-chain secondary alcohols (12,19), acetonide esters (16,20), oxazolidones (21), glycidyl ethers (20), cyclohexanols (22) and 2-benzylglycerol ether (23). The information accumulated to this point albeit with impure proteins, nevertheless suggests that the steric bulk distribution of the nucleophilic component of a reaction for a favored nucleophile are quite similar. Current work is directed to examining related ether-esters in the hope that they may prove useful as probes by which to monitor efforts at protein engineering of lipases, and that they may shed more light on the mechanism by which these enzymes operate.

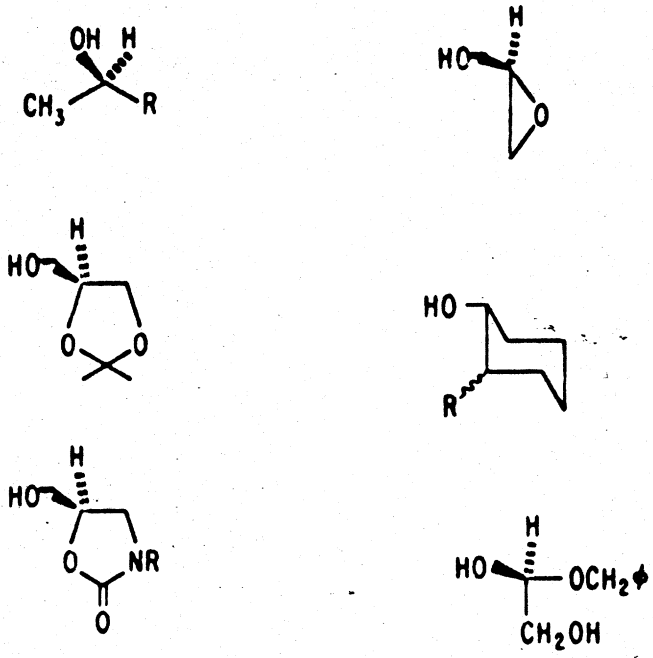


FIG. 4. A selection of chiral alcohols showing the faster reacting enantiometer in lipase-mediated reactions.

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