Tetrahedron Asymmetry Vol. 4, No. 5, pp. 865-874, 1993
Printed in Great Britain

0957-4166/93 $6.00+.00
Pergamon Press Ltd

A Practical Chemoenzymatic Synthesis Of An LTD_4 Antagonist

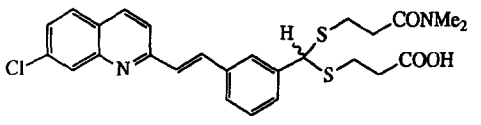
D. L. Hughes*, Z. Song, G. B. Smith, J. J. Bergan, G. C. Dezeny, E. J. J. Grabowski, P. J. Reider

Department of Process Research, Merck Research Laboratories, Box 2000, Rahway, NJ 07065

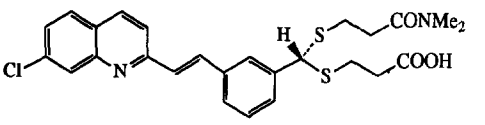
(*Received* 6 *February* 1993; *accepted* 18 *March* 1993)

Abstract. Enzymatic asymmetrization of a prochiral diester having 4 bonds between the ester group and prochiral center is the cornerstone of a short and efficient synthesis of an LTD4 antagonist. The enzymatic hydrolysis occurs in a heterogeneous slurry, but a kinetic analysis shows that the reaction takes place in solution. Product inhibition of the enzyme is severe, requiring that a substantial amount of enzyme be used relative to substrate. To more efficiently use the expensive enzyme, it was immobilized on several supports, the most effective of which was XAD 7 with crosslinked enzyme.

Leukotrienes are naturally occurring molecules in humans and animals that are derived from metabolism of arachidonic acid. No beneficial role of leukotrienes has been discovered, yet they have been implicated in mediating several diseases, such as asthma, inflammatory bowel disease, and psoriasis.[1] Two approaches to diminish the undesirable effects of leukotrienes have been to inhibit the production of leukotrienes by blocking the enzymatic pathway (usually the 5-lipoxygenase enzyme) or by blocking the action of leukotrienes via competitive receptor antagonists. Two compounds, developed in the laboratories of Merck Frosst Canada, that block the leukotriene D_4 (LTD_4) receptor have been shown to be clinically effective in controlling asthma. The first compound developed, MK-0571,[2] is a racemate while the more recent compound is the (R)-enantiomer (MK-0679, verlukast).[3] To support extensive safety assessment studies in animals and large scale clinical trials in humans, a practical synthesis is required, meaning one that is safe, environmentally sound, efficient, and economical. In this paper we describe (1) the practical synthesis of verlukast (MK-0679) using an enzymatic asymmetrization of a prochiral diester as the key step, (2) the kinetics of the enzymatic transformation, and (3) enzyme immobilization techniques to recycle the enzyme.



MK-0571 (Racemate)

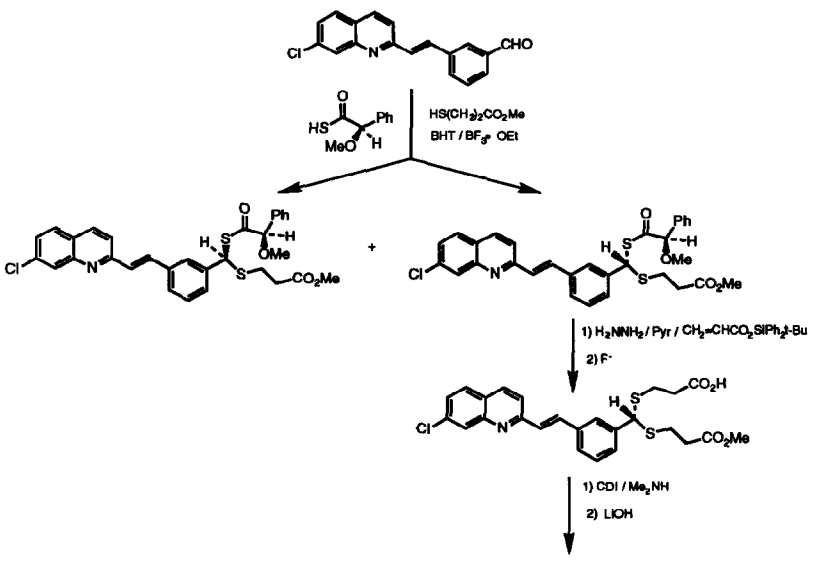


Verlukast (MK-0679) (R-enantiomer)

I. Synthesis of MK-0679 (Verlukast)

Since a reasonable synthesis of the racemic compound MK-0571 was available,[4] the most straightforward approach to the synthesis of the enantiomers would be through a classical resolution. However, due to the similarity of the two thioalkyl side chains, attempts to resolve MK-0571 by methods such as crystallization of diasteromeric salts or chromatographic separation of diastereomeric derivatives, proved difficult. To circumvent these problems, the medicinal chemists at Merck-Frosst prepared and separated diastereomeric dithioacetals early in the synthetic sequence, and then converted each of the individual diastereomers to the enantiomers of MK-0571. The synthesis is shown below in Scheme I.[5]

SCHEME I

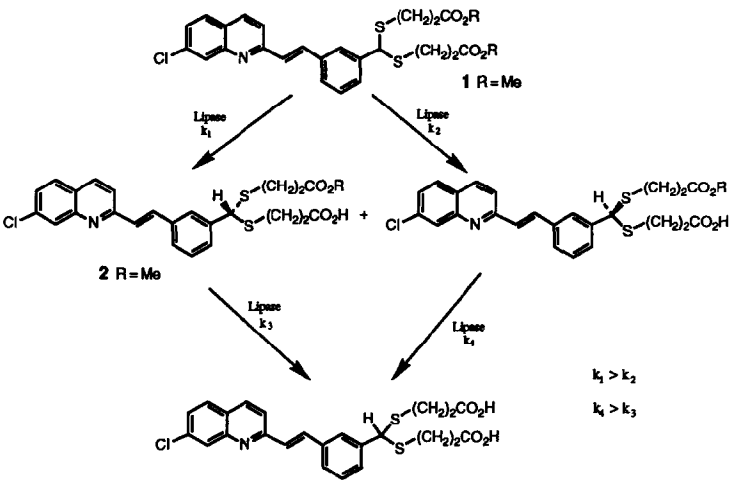


This novel synthesis hinges on two key unexpected transformations: (1) the formation of the mixed dithioacetals in 74% yield, with minimal formation of the two symmetrical dithioacetals; and (2) the *in situ* cleavage of the thioester (hydrazine in pyridine) and trapping of the resulting thiolate anion with the silyl acrylate with no racemization occurring. This synthesis not only provided samples of each enantiomer for testing, but also provided a crystalline intermediate (the mixed (S,R)-dithioacetal diastereomer) suitable for x-ray crystallography, establishing the stereochemistry of each enantiomer prepared.

With the decision to develop the (R)-enantiomer as a drug candidate, a more practical synthesis was required. The increased availability and demonstrated usefullness of enzymes in recent years prompted us to explore their use in the present case. To take full advantage of the capabilities of the enzyme, we wished to use it to asymmetrize a prochiral center, where the theoretical yield of the desired enantiomer would be 100%, rather than use it in the more conventional way of resolving enantiomers, wherein 50% yields of each

enantiomer are the maximum yields. Consequently, the chiral hydrolysis of the prochiral diester **1** became the pivotal transformation (Scheme II).

SCHEME II



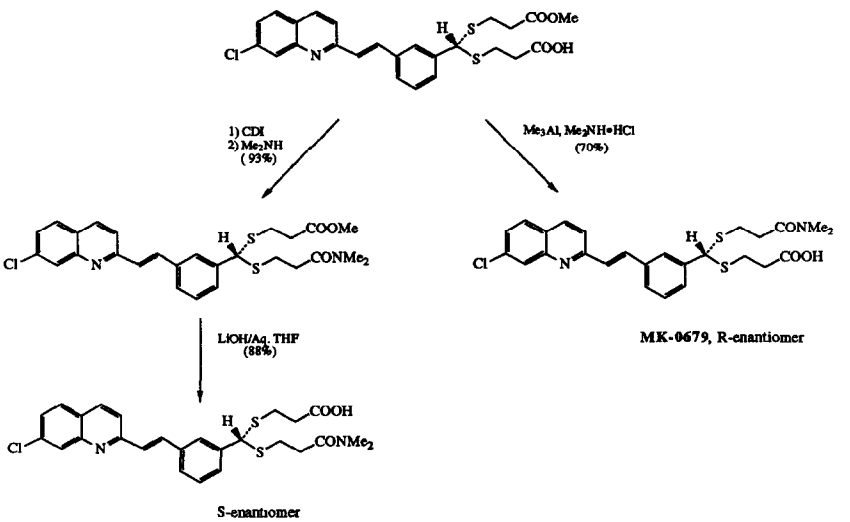
There is little literature precedent for the enantioselective hydrolysis of esters when the chiral or prochiral center is remote from the ester being hydrolyzed.[6] Therefore, it was remarkable to find enzymes that could readily hydrolyze the diester shown in Scheme II, wherein the ester is 4 bonds remote from the prochiral center, with >98% ee. Both the lipase from *Pseudomonas* species and from *Chromobacterium viscosum* gave high yielding hydrolyses with high ee on the methyl esters, while the lipase from *Candida cyclindracea* gave an 86% ee on the activated ester, R = CH_2CONEt_2.[7] In all cases, some hydrolysis to the diacid occurred. With the lipase from *Pseudomonas* species[8] the extent of over hydrolysis was only 2-3%, and it was shown that the minor half ester hydrolyzed faster than the major one ($k_4 > k_3$, Scheme II), producing an increase in ee through a kinetic resolution.

With half ester of high ee in hand, it was straightforward to convert it to final product by performing chemistry on one or the other of the arms of the unsymmetrical dithioacetal (Scheme III). To prepare MK-0679, the ester group of the ester-acid was converted to the amide by reaction with Weinreb's dimethylamine-trialkylaluminum reagent. To prepare the other enantiomer, the acid group of the ester-acid was activated with carbonyl diimidazole and converted to the amide with dimethylamine, then the ester group was hydrolyzed. Thus, although the ester hydrolysis produces only one enantiomer, either enantiomer of the desired final product can be readily obtained.

II. Kinetics of the Enzymatic Hydrolysis

In early experiments in which only diester **1** and Amano lipase P30 were stirred in a pH 7 aqueous buffer, very sluggish hydrolysis occurred, presumably due to the very low solubility of the diester. Adding

SCHEME III



2% of the nonionic surfactant, Triton X-100, increased the hydrolysis rate substantially without denaturing the lipase. Since the diester solubility was still low in this mixture, it was not certain whether the rate increase was due to increased solubility or to a wetting of the insoluble substrate such that an interfacial hydrolysis could take place. Lipases are known to react *in vivo* at interfaces.[9] With 2 % Triton X-100 present in the reaction mixture, the reaction remains heterogeneous throughout. Initially, the diester is virtually all out of solution, then as the reaction proceeds, the free acid of the product ester-acid **2** crystallizes, even at pH 6.5 to 7.5, due to the extremely low solubility of the free acid in the aqeuous solution. From a preparative standpoint, this makes the enzymatic reaction very simple to run: the diester, Triton X-100, and enzyme are stirred in an aqeuous buffer solution for a day at 40°C, then the reaction is cooled to ambient temperature and the product is isolated simply by filtration. The amount of Triton X-100 was optimized at 1.5 wt %. Less of it resulted in a more sluggish reaction while more of it increased the solubility of product, thus reducing the isolated yield.

To determine if the enzymatic hydrolysis was taking place in solution or at an interface, and to further understand and explore this unusual enzymatic reaction, an investigation of the kinetics of the reaction was initiated.[10] Shown below in Figure 1 is a plot of the disappearance of diester **1** during a typical, preparative scale, hydrolysis. The reaction profile can roughly be divided into three sections: the first part in which the initial 10 % reaction occurs rapidly in an inital burst, the middle part in which about 70% of the diester is consumed with nearly zero-order kinetics, and a final part in which the rate slowly decreases as the reaction nears completion. Further kinetic experiments and solubility measurements provided an understanding of this unusual reaction profile. First of all, the initial rapid reaction followed by a clear cut slowing suggested product inhibition. This is not unusual behavior for enzymes, as product inhibition in nature is a feed back mechanism to control enzyme activity. However, proving that inhibition was occurring under the complex,

Figure 1. Reaction Profile for the Lipase-mediated Hydrolysis of Prochiral Diester **1** in Aqueous 1.5% Triton X-100 Solution at 40°C

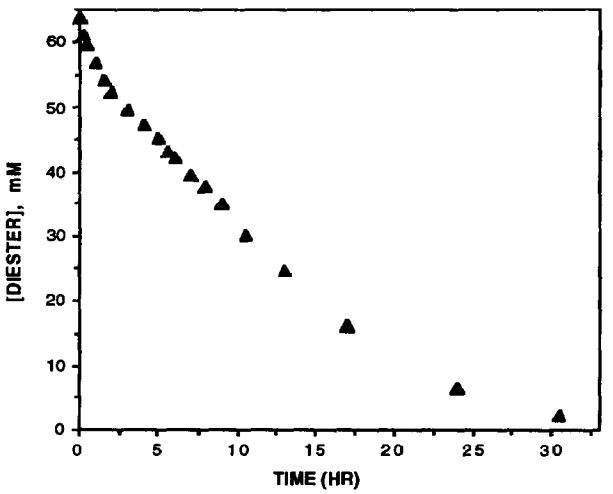


Table I. Product Inhibition in the Lipase-mediated Hydrolysis of Diester **1** in the Presence of Ester-acid **2**

Lipase concentration (mg/mL)	Ester-acid conc. (mM)	Apparent reactivity (hr^{-1})
1.33	0	5.7
0.67	0	2.9
1.33	1.0	4.4
0.67	2.0	1.7
1.33	2.6	2.8
0.67	2.6	1.5
1.33	4.0	1.9
1.33	6.0	1.1
1.33	10.0	0.55
0.67	10.0	0.29

heterogeneous conditions would be difficult. Instead, it was found that the reaction could be run under homogeneous conditions. The solubility of the diester **1** in the 1.5 % aqueous Triton X-100 solution was determined to be 0.8 mg/ mL, or 1.5 mM, at 40°C. Therefore, kinetic experiments were performed at a concentration of 1 mM at 40°C, wherein all diester was in solution initially and the reaction remained homogeneous throughout. Reactions were run with varying amounts of product ester-acid **2** present initially, and, as the data in Table I below indicate, severe product inhibition occurred, with the rate decreasing 10-fold with a product concentration of 10 mM.

An attempt was made to fit the data to a model involving product inhibition as a single equilibrium with the enzyme, but no good fit was obtained. Instead, a model involving two equilibria was used, and the data could be adequately fit, although a fairly large range of equilibrium constants could be used with good fits still being obtained.[10]

$$E + P \rightleftharpoons EP_1$$
$$EP_1 + P \rightleftharpoons EP_2$$

Therefore, the initial slowing of the rate in the heterogeneous reaction can be explained by product inhibition. The reason for the apparent zero-order kinetics during the middle part of the reaction became apparent from the solubility data acquired during the course of the reaction. As mentioned above, the intial diester solubility was 1.5 mM. As the reaction proceeded, this solubility increased to 12 mM, and this increase of diester concentration nearly offsets the inhibitory effect of the product that is being produced during this period, resulting in a reaction profile that appears zero-order. During the final stages of the reaction, the solubility of both the diester and the product decrease and this results in a slowing of the rate as the reaction reaches completion.

The above data suggest that the hydrolysis in the heterogeneous mixture is occurring in the solution phase, although probably within micelles at the micelle/solution interface. Three additional pieces of data further support this. (1) The observed heterogeneous hydrolysis rates agreed with those calculated from the homogeneous rate expression, k = [diester] x [lipase], using reactivities based on the product inhibition curve. (2) The rates of reaction were independent of the mode of agitation, whether it be mechanical stirring with a paddle, magnetic stirring with a stir bar, or mixing via use of an ultrasonic probe. (3) The rates of reaction were independent of the amount of diester initially present, indicating that the rate was controlled only by the amount of diester dissolved, not by the total amount present in the mixture.

III. Lipase Immobilization

From a practical standpoint, the consequence of the severe lipase inhibition by product is that a relatively large amount of the expensive enzyme must be used for reasonable rates, productivity, and throughput. Batch mode operation without enzyme recycle would make the cost of the enzyme prohibitive on a commercial scale. To address this issue, means of recycling the lipase via immobilization was studied.

The primary ways of immobilization are physical adsorption or covalent bonding of the enzyme to a solid support, or physical adsorption on a support followed by enzyme crosslinking. Because our solvent system was an organic-aqueuos mixture, simple physical adsorption on a support was not feasible since desorption was considerable in this solvent mixture. Therefore, we concentrated our efforts on covalent

bonding of the enzyme, and to crosslinking adsorbed enzyme on the support. In all cases examined, the enantioselectivity of the hydrolysis was unaffected by having the lipase immobilized.

Immobilization via Covalent Bonding. Agarose having tethers of varying natures have been widely used for enzyme immobilization,[11] and several of these are available commercially in small quantities. Use of the hydrophobic agarose gave the best results in the present case. The lipase (Amano PS-800) was immobilized to 12-aminododecyl agarose with glutaraldehyde. The solvent system used for the hydrolysis reaction was 20% Triton X-100/ 80% 0.1M phosphate buffer at pH 7.5. In this solvent mixture the reaction remained homogeneous throughout with substrate concentrations up to 0.2 M. In addition, the hydrolysis rate was about 3-fold faster than that in the heterogeneous conditions, in which only 1.5% Triton X-100 was used. With the immobilized enyzme, the rate of reaction on the first cycle was 47% as fast as that of the free enzyme, measured in the same solvent and at the same temperature (40°C). The slower rate of the immobilized lipase is expected, as access of the substrate to the enzyme is restricted in the immobilized preparation. Four more cycles were run and the loss of lipase activity per cycle averaged 26%. Some of the enzyme was leached in the first two cycles, as indicated by enzymatic activity in the filtrate, but none was observed after that. The loss in acitivity appeared primarily to be due to enzyme denaturation. Given the high cost of the activated agarose and the limited initial success of this approach, this route was abandoned.

Immobilization via Physical Adsorption and Crosslinking. Amberlite XAD-7 and XAD-8, and Amberchrome 71 are macroporous, neutral polyacrylate resins that have been used extensively for lipase immobilization via adsorption.[12] About 2 wt% lipase (based on dry wt of resin) was adsorbed on XAD-8, then the enzyme was crosslinked twice using dimethyl diadipimidate.[13] Since the other resins have higher surface areas, about 10 wt% lipase (Amano PS 800) was adsorbed on XAD-7 and 15% on Amberchrome 71. The XAD resins showed similar behavour in the hydrolysis reactions, while the Amberchrome resin gave a slower rate.

Three solvent systems were used in the hydrolysis reactions of the immobilized enzymes, 0.25 M potassium phosphate buffer (pH 7.5) saturated t-BuOH, 20% Triton X-100/ 80% 0.1 M phosphate buffer (pH 7.5), and 50% tetraglyme/ 50% 0.15 M N-methylmorpholine buffer (pH 8). The hydrolysis rates using the XAD-8 immobilized lipase were fastest in the aq. Triton X-100 solvent, with the tetraglyme and t-BuOH solvent systems being 3-fold and 15-fold slower, respectively. The aq. Triton X-100 and aq. tetraglyme solvent systems were used for further work. With both of these solvent systems and the crosslinked lipase on the XAD-8 resin, the initial rate was about 50% of that of the free enzyme. Ten cycles were run, and in the aq. Triton X-100 system, the rate loss per cycle was 15% and in the aq. tetraglyme system, the rate loss was 20% per cycle. In both cases, filtrates from the first two cycles showed some lipase activity, indicating some leaching occurred, but after the third cycle no leaching was occurring, so loss of lipase activity was due to denaturation. Given the faster rates in the aq. 20% Triton X-100 solvent and the greater amounts of enzyme that could be adsorbed on XAD 7, these are the preferred conditions for greatest productivity.

Immobilization on glutaraldehyde-activated polyacrylate resins by covalent bonding. An alternative to simple adsorption of the enzyme on the XAD resins is to first polymerize glutaraldehyde on the resin surface, then covalently bond the lipase to the polymer.[14,15] Two preparations were made. In the first, the polymerization of glutaraldehyde was carried out at pH 10.5 in unbuffered solution for 15 h. The pH dropped to 9 by the end of the polymerization. Lipase binding was then done at pH 7.5 for 16 h. This material was used in 10

hydrolysis cycles in 20% aq. Triton X-100. The initial rate was 30% of the free enzyme rate, and the loss per cycle averaged 12% per cycle. In the second preparation, polymerization was done at pH 10.5 in buffered solution, then the excess glutaraldehyde was washed out by an overnight slurry in aq. Triton X-100. Then, the lipase was bound to the resin for 48 h at pH 7.5. This preparation was used in 5 cycles and again lost an average of 12 % per cycle. The simplicity of this method and the use of inexpensive raw materials makes this approach attractive for practical lipase immobilization.

Summary of Immobilization Techniques for *Psuedomonas* Lipase. To summarize, three techniques were examined for immobilization of the *Pseudomonas* lipase. The first problem that had to be addressed was the solubility of the substrate and product. The diester had virtually no solubility in water, so a large amount of a cosolvent was required. Several alcohols and polar solvents were examined, but most caused severe lipase denaturation. The best cosolvent turned out to be the polyether surfactant, Triton X-100, which gave excellent solubility when used as a 20% solution in water. However, using a surfactant as cosolvent meant that all enzyme that was not covalently bound to the resin would be rapidly desorbed, limiting the options available for immobilization. Of the methods investigated, direct covalent bonding to a suitably activated resin gave poor results, and this combined with the expense and labor involved in preparing the immobilized enzyme, made this route the least attractive. The method involving physical adsorption followed by enzyme crosslinking gave improved results and uses the XAD resins that are readily available and relatively inexpensive. The best results were obtained with XAD 8; however, since this resin has a low surface area, only 2 wt% enzyme could be adsorbed, which limits the productivity of this system. The method wherein glutaldehyde is polymerized on XAD 7 resin, followed by lipase immobilization via Shiff base formation, gave the best results for a practical immobilization that could be used on a large scale. As mentioned above, the resin is inexpensive and readily available, the glutaraldehyde pretreatment and enzyme immobilization were simple and straightforward processes, and up to 10 weight percent enzyme could be immobilized on the resins. Furthermore, this method had enzyme activity losses of only 12 % per cycle, the best of the three methods examined.

EXPERIMENTAL

Preparative Enzymatic Hydrolysis. The dimethyl diester 1 (10.32 g, 0.02 mol) was suspended in 300 mL of 0.1 M potassium phosphate buffer (pH 7.5), and 5 mL of Triton X-100 and 300 mg of LPL-80 (previously renamed as PS-800, Amano Enzyme Co.) were added. The heterogeneous mixture was stirred mechanically at 40°C for 40 h. The mixture was then cooled to ambient temperature, filtered, and washed with 1 x 50 mL of buffer solution (pH 7) and 1 x 50 mL of water to provide 9.05 g of ester-acid of 96% purity (86% yield).

Covalent Immobilization of Lipase on Agarose. The 12-aminododecyl agarose resin (Sigma) was washed with pH 7 buffer and filtered. The wet gel (2.5 g) was suspended in 10 mL of pH 7 buffer, and 2.5 mL of 50% glutaraldehyde was added. The mixture was stirred overnight at 5°C, then filtered and washed extensively with buffer solution. The gel was resuspended in 10 mL buffer which contained 50 mg of the PS-800 lipase and stirred overnight at 5°C. The resin was isolated by filtration. No lipase activity was detected in the filtrate.

Immobilization of Lipase on XAD-8 Followed by Crosslinking. Wet XAD-8 (10 g containing 50% water) was washed with 0.1 M potassium phosphate buffer and then slurried with 177 mg of PS-800 lipase in 25

mL of pH 7 phosphate buffer for 24 h at 5°C. The slurry was then filtered to recover the beads. An estimated 95 mg of lipase was adsorbed to the beads based on the UV absorption at 280 nm of the filtrate and the original solution of lipase. The beads were then resuspended in 20 mL of a pH 7 buffered solution containing 50 mg of dimethyl diadipimidate. The solution pH was adjusted to 7.5 and the mixture was stirred at 5°C for 48 h. The beads were isolated by filtration, and then treated again by the same procedure with more dimethyl diadipimidate. Based on subsequent hydrolysis reactions, the second crosslinking made no difference to the performance or stability of the lipase.

Immobilization of Lipase on Glutaraldehyde-activated XAD-7 by Shiff Base Formation. XAD-7 (10.1 g of resin containing 62% water) was suspended in 10 mL of 0.1 M sodium carbonate at pH 10.6. Glutaraldehyde (4.35 g of a 50% aq. solution) was added and the mixture was gently shaken for 22 h at ambient temperature. The suspension was filtered and washed with 3 x 20 mL water. To wash out any unpolymerized glutaraldehyde, the resin was suspended in 15 mL of 0.1 M phosphate buffer (pH 7.5) and 3 g of Triton X-100 and gently shaken for 2 h at ambient temperature. The suspension was filtered and washed with 4 x 20 mL water. A portion of the activiated resin (3.5 g wet) was suspended in 15 mL of 0.1 M phosphate buffer and Amano PS-800 lipase (81 mg) was added. The suspension was shaken for 45 h at ambient temperature. The resin was then filtered and washed with 4 x 20 mL buffer solution. After drying for 2 h by suction filtration, 2.0 g of resin was recovered.

Enymatic Hydrolyses Using Immobilized Lipase. Dimethyl diester (150 mg) was dissolved in Triton X-100 (2.0 g) and 8 mL of 0.1 phosphate buffer (pH 7.5) and 0.53 g of the immobilized lipase (approx. 20 mg of lipase on the glutaraldehyde-activated XAD-7) were added. The mixture was mechanically stirred at 40 °C for 8 h (90% complete reaction), then the resin was filtered and the cycle was repeated with fresh diester/solvent. By cycle 10 the activity of the resin had dropped to 31% of that measured in the first cycle, an average loss of activity of 12% per cycle. The activity of the enzyme was based on either the initial rate or the time required to reach one half life.

Reaction Monitoring. The reactions involving free or immobilized enzyme were followed by HPLC analysis. The extent of diester hydrolysis was determined based on peak integration at 350 nm. A DuPont Zorbax RX C-8, 25 cm column was used at ambient temperature, with an eluent consisting of 60% acetonitrile and 40% 0.1 % aq. H_3PO_4. At a flow rate of 1.5 mL/min the retention times were 2.6 min for the diacid, 4.3 min for the ester-acid **2**, and 9.0 min for the dimethyl diester **1**.

REFERENCES AND NOTES

1. Rokach, J. (Ed.) *Leukotrienes and Lipoxygenases*; Elsevier: New York, 1989.

2. a) Zamboni, R.; Belley, M.; Champion, E.; Charette, L; DeHaven, R.; Frenette, R.; Gauthier, J. Y.; Jones, T. R.; Leger, S.; Masson, P.; McFarlane, C. S.; Metters, K.; Pong, S. S.; Piechuta, H.; Rokach, J.; Therien, M.; Williams, H. W. R.; Young, R. N. *J. Med. Chem.* **1992**, *35*, 3832-3844. b) Margolskee, D.; Bodman, S.; Dockhorn, R.; Israel, E.; Kemp, J.; Mansmann, H.; Minotti, D. A.; Spector, R.; Stricker, W.; Tinkelman, D.; Townley, R.; Winder, J.; Williams, V. C. *J. Allergy Clin. Immunol.* **1990**, *87*, 309, Abstract 677.

3. Young, R. N.; Gauthier, J. Y.; Therien, M.; Zamboni, R. *Heterocycles* **1989**, *28*, 967-978.
4. McNamara, J. M.; Leazer, J. L.; Bhupathy, M.; Amato, J. S.; Reamer, R. A.; Reider, P. J.; Grabowski, E. J. J. *J. Org. Chem.* **1989**, *54*, 3718-3721.
5. Gauthier, J. Y.; Jones, T. R.; Champion, E.; Charette, L.; DeHaven, R.; Ford-Hutchinson, A. W.; Hoogsteen, K. K.; Lord, A.; Masson, P.; Piechuta, H.; Pong, S. S.; Springer, J. P.; Therien, M.; Zamboni, R.; Young, R. N. *J. Med. Chem.* **1990**, *33*, 2841-2845.
6. Some success has been achieved with compounds having 3 bonds between the ester carbonyl and the chiral center. a) Cambou, B.; Klibanov, A. M. *J. Am. Chem. Soc.* **1984**, *106*, 2687-2692. b) Ladner, W. E.; Whitesides, G. M. *J. Am. Chem. Soc.* **1984**, *106*, 7250-7251.
7. a) Hughes, D. L.; Bergan, J. J.; Amato, J. S.; Reider, P. J.; Grabowski, E. J. J. *J. Org. Chem.* **1989**, *54*, 1787-1788. b) Hughes, D. L.; Bergan, J. J.; Amato, J. S.; Bhupathy, M.; Leazer, J. L.; McNamara, J. M.; Sidler, D. R.; Reider, P. J.; Grabowski, E. J. J. *J. Org. Chem.* **1990**, *55*, 6252-6259.
8. *Pseudomonas sp.* lipase is available from several sources in varying purity. Most of our work has been done with lipase from Amano Enzyme Co. Their crude lipase is designated P30, while the more pure lipase is designated as either LPL-80 or PS-800. Lipase from Sigma also performed well, while lipase from Beohringer-Mannheim gave no reaction with our substrates.
9. a) Semiriva, M.; Desneulle, P. *Advances in Enzymology* , Meister, A., Ed.; John Wiley and Sons, Inc: New York, Vol. 48, 1979, pp. 319-370. b) Borgstrom, B.; Brockman, H. L.Eds. *Lipases* Elsevier: New York, 1984, pp. 443-469. c) Burdette, R. A.; Quinn, D. M. *J. Biol. Chem.* **1986**, *261*, 12016-12021.
10. Smith, G. B.; Bhupathy, M.; Dezeny, G. C.; Douglas, A. W.; Lander, R. J. *J. Org. Chem.* **1992**, *57*, 4544-4546.
11. a) Porath, J.; Axen, R. *Methods in Enzymology*, Mosbach, K., Ed.; Academic Press: New York, Vol. 44, 1976, pp. 19-45. b) Scouten, W. H. *Methods in Enzymology*, Mosbach, K., Ed.; Academic Press: New York, Vol. 135, pp. 30-65.
12. a) Hsu, S.-H.; Wu, S.-S.; Wang, Y.-F.; Wong, C.-H. *Tetrahedron. Lett.* **1990**, *31*, 6403-6406. b) Hamaguchi, S.; Asada, M.; Hasegawa, J.; Watanabe, K. *Agric. Biol. Chem.* **1985**, *49*, 1661-1667. c) Pederson, R. L.; Liu, K. K.-C.; Rutan, J. F.; Chen, L.; Wong, C.-H. *J. Org. Chem.* **1990**, *55*, 4897-4901. d) von der osten, C. H.; Sinskey, A. J.; Barbas, C. F. III; Pederson, R. L.; Wang, Y.-F.; Wong, C.-H. *J. Am. Chem. Soc.* **1989**, *111*, 3924-3927.
13. a) Hunter, M. J.; Ludwig, M. L. *Methods in Enzymology*; Hirs, C. H. W., Timasheff, S. N., Eds.; Academic Press: New York, 1972, pp. 585-596. b) Wold, F. *Ibid.* pp. 623-651. c) Gray, D. N.; Keyes, M. H. *Chemtech* **1977**, *7*, 642-648. d) Keyes, M. H.; Semersky, F. E.; Gray, D. N. *Enzyme Microb. Tech.* **1979**, *1*, 91-94.
14. Carleysmith, S. W.; Dunnill, P.; Lilly, M. D. *Biotech. Bioeng.* **1980**, *22*, 735-756.
15. Structures of glutaraldehyde oligomers: a)Tashima, T.; M.; Imai, M.; Kuroda, Y.; Yagi, S.; Nakagawa, T. *J. Org. Chem.* **1991**, *56*, 694-697. b) Nakagawa, T.; Izawa, K.; Yagi, S.; Shibukawa, A.; Tanaka, H.; Tashima,T.; Imai, M. *Chem. Pharm. Bull.* **1989**, *37*, 2463-2466.