Kinetic Resolution of Pipecolic Acid Using Partially-Purified Lipase from *Aspergillus niger*

M. Christine Ng-Youn-Chen, Alessio N. Serreqi, Qingli Huang, and Romas J. Kazlauskas*

Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montréal, Québec H3A 2K6, Canada

Received March 5, 1993 (Revised Manuscript Received December 14, 1993•)

Synthesis of biologically active peptides, alkaloids, and immunosuppresants such as FK506 requires enantiomerically-pure pipecolic acid (2-piperidinecarboxylic acid). We report an efficient kinetic resolution of pipecolic acid esters by enzyme-catalyzed hydrolysis. We screened commercially available hydrolases and identified crude lipase from *Aspergillus niger* (ANL) as the most enantioselective catalyst for the hydrolysis of (±)-methyl pipecolate, $E = 20 \pm 4$ in favor of the (*S*)-enantiomer at pH 7. Changing of the ester group to *n*-pentyl or *n*-octyl did not increase the enantioselectivity, while addition of an *N*-acetyl group decreased the enantioselectivity. Partial purification of ANL by fractional precipitation with ammonium sulfate (25–45% saturation) increased the enantioselectivity to >100. A synthetic scale resolution of (±)-*n*-octyl pipecolate using this partially purified ANL gave (*S*)-(−)-pipecolic acid (93% ee, 0.89 g) and (*R*)-(+)-pipecolic acid (97% ee, 1.20 g). Further purification of ANL confirmed that the lipase (apparent molecular weight of 32 kDa), and not an impurity, was responsible for the enantioselective hydrolysis of octyl pipecolate.

Pipecolic acid, 2-piperidinecarboxylic acid, is a precursor of numerous bioactive compounds. For example, alkaloids such as swainsonine (an α-mannosidase inhibitor),[1] thioridazine (an antipsychotic),[2] pipradol (an anticonvulsant),[3] local anesthetics,[4] a K-opioid analgesic,[5] and a protein kinase C inhibitor,[6] have all been prepared from pipecolic acid.[7] The immunosuppressants FK506 and rapamycin[8] as well as simple analogs of FK506[9] contain a pipecolic acid moiety, as do efrapeptins, a class of peptide insecticides isolated from fungus,[10] and nonapeptide inhibitors of HIV protease.[11] Cyclic hexapeptides containing pipecolic acid inhibit uterine contractions and may prevent premature birth.[12] To maximize biological potency and to minimize side effects, synthesis of these compounds requires enantiomerically-pure pipecolic acid.

Pipecolic acid[13] is not a proteinogenic amino acid, but small amounts of the L-enantiomer (*S*) occur naturally, for example, 0.1 wt % in dried clover leaves. (*S*)-Pipecolic acid can also be prepared by oxidation of (*S*)-lysine.[14] Racemic pipecolic acid is readily available, so the best current route to the (*R*)-enantiomers is classical resolution of the racemate by fractional crystallization as the tartrate salt.[15] A less convenient alternative is destruction of one enantiomer by fermentation[16] or by oxidation with amino acid oxidase.[13,17]

Although hydrolase-catalyzed kinetic resolution is a convenient route to many enantiomerically-pure amino acids,[18] it has not yet been successful for pipecolic acid. Amino acid acylase from porcine kidney[19] or bacterial aminopeptidase[20] can resolve many acyclic amino acids, but these enzymes do not accept cyclic amino acids. Two (*S*)-enantioselective proline acylases have been reported,[21] but no acylase for pipecolic acid has been reported. Porcine liver esterase (PLE) catalyzed slow hydrolysis of derivatives of pipecolic acid, but with low enantioselectivity.[22]

In this paper we report an efficient kinetic resolution of octyl pipecolate catalyzed by lipase from *Aspergillus*

• Abstract published in *Advance ACS Abstracts*, March 1, 1994.

(1) Harris, C.; Harris, T. *Tetrahedron Lett.* **1987**, *28*, 2559–2562.

(2) Patrick, K. S.; Singletary, J. L. *Chirality* **1991**, *3*, 208–211.

(3) Portoghese, P. S.; Pazdernik, T. L.; Kuhn, W. L.; Hite, G.; Shafi'ee, A. *J. Med. Chem.* **1968**, *11*, 12–15.

(4) Af Ekenstam, B. T.; Bovin, C. US Patent 4695576, 1987; *Chem. Abstr.* **1988**, *108*, 75228; Aberg, G.; Af Ekenstam, B.; Smith, E. *Arzneim.-Forsch.* **1983**, *33*, 706–710.

(5) Vecchietti, V.; Giordani, A.; Giardina, G.; Colle, R.; Clarke, D. *J. Med. Chem.* **1991**, *34*, 397–403.

(6) Perumattam, J.; Shearer, B. G.; Confer, W. L.; Mathew, R. M. *Tetrahedron Lett.* **1991**, *32*, 7183–7186.

(7) Two groups have patented mind-restorative alkaloids derived from pipecolic acid: Soeda, M.; Aozuka, T.; Ogura, K.; Toritsuka, M.; Tanaka, Y.; Kato, H.; Nakada, N.; Miura, N. Jpn Patent 02207070, 1990; *Chem. Abstr.* **1992**, *114*, 6303. Chung, J. Y. I.; Garvey, D. S. WO Patent Appl. 91 00,724; *Chem. Abstr.* **1992**, *115*, 114360.

(8) Wasserman, H. H.; Rotello, V. M. *J. Org. Chem.* **1989**, *54*, 2785–2786. Danishefsky, S. J.; Egbertson, M. *J. Org. Chem.* **1989**, *54*, 12–15. Chen, S. H.; Horvath, R. F.; Joglar, J.; Fisher, M. J.; Danishefsky, S. J. *J. Org. Chem.* **1991**, *56*, 5834–5845.

(9) Hauske, J. R.; Dorff, P.; Julin, S.; DiBrino, J.; Spencer, R.; Williams, R. *J. Med. Chem.* **1992**, *35*, 4284–4296. Tung, R. D.; Meyers, H. V. WO Patent Appl. 92 04,370; *Chem. Abstr.* **1992**, *117*, 90804b. Armistead, D. M. WO Patent Appl. 92 19,593; *Chem. Abstr.* **1993**, *119*, 95338d. Duffy, J. P. WO Patent Appl. 92 21,313; *Chem. Abstr.* **1993**, *118*, 212898d.

(10) Gupta, S.; Krasnoff, S. B.; Roberts, D. W.; Renwick, J. A. A.; Brinen, L. S.; Clardy, J. *J. Am. Chem. Soc.* **1991**, *113*, 707–709.

(11) Copeland, T. D.; Wondrak, E. M.; Tozser, J.; Roberts, M. M.; Oroszlan, S. *Biochem. Biophys. Res. Commun.* **1990**, *169*, 310–304.

(12) Perlow, D. S.; Erb, J. M.; Gould, N. P.; Tung, R. D.; Freidinger, R. M.; Williams, P. D.; Veber, D. F. *J. Org. Chem.* **1992**, *57*, 4394–4400 and references cited therein.

(13) Review: Rodwell, V. W. *Methods Enzymol.* **1971**, *17B*, 174–188. Greenstein, J. P. In *Chemistry of the Amino Acids*; Greenstein, J. P., Milton, W., Eds.; John Wiley & Sons: New York, 1961; Vol. 3, pp 2532–2539.

(14) Kisfaludy, L.; Korenczki, F.; Katho, A. *Synthesis* **1982**, 163. Aketa, K.; Terashima, S.; Yamada, S. *Chem. Pharm. Bull.* **1976**, *24*, 621–631. Fujii, T.; Miyoshi, M. *Bull. Chem. Soc. Jpn.* **1975**, *48*, 1341–1342.

(15) Yamada, S.; Hohgo, C.; Chibata, I. *Agric. Biol. Chem.* **1977**, *41*, 2413–2416. Shiraiwa, T.; Shinjo, K.; Kurokawa, H. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 3251–3255.

(16) Mochizuki, K.; Yamazaki, Y.; Maeda, H. *Agric. Biol. Chem.* **1988**, *52*, 1113–1116.

(17) Huh, J. W.; Yokoigawa, K.; Esaki, N.; Soda, K. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 2081–2082. An amino acid oxidase specific for L-pipecolic acid has been isolated from monkey's liver: Mihalik, S. J.; McGuinness, M.; Watkins, P. A. *J. Biol. Chem.* **1991**, *266*, 4822–4830.

(18) Review: Williams, R. M. *Synthesis of Optically Active α-Amino Acids*; Pergamon: Oxford; 1989, Chapter 7.

(19) Chenault, H. K.; Dahmer, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 6354–6364.

(20) Schoemaker, H. E.; Boesten, W. H. J.; Kaptein, B.; Hermes, H. F. M.; Sonke, T.; Broxterman, Q. B.; van den Tweel, W. J. J.; Kamphuis, J. *Pure Appl. Chem.* **1992**, *64*, 1171–1175.

(21) Groeger, U.; Drauz, K.; Klenk, H. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 417–419. Kikuchi, M.; Koshiyama, I.; Fukushima, D. *Biochim. Biophys. Acta* **1983**, *744*, 180–188.

0022-3263/94/1959-2075$04.50/0

Table 1. Hydrolysis of Pipecolate Esters by Crude Lipase from *A. niger*

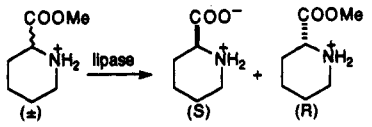
	methyl ester			*n*-pentyl ester			*n*-octyl ester			
pH[a]	activity,[b] units/g	*c*,[c] %	E_{app}[d]	activity,[b] units/g	*c*,[c] %	E_{app}[d]	activity,[b] units/g	*c*,[c] %	E_{app}[d]	$t_{1/2}$,[e] h
4	0.2	26	16 ± 4	nd	nd	nd	0.6[f]	15	20 ± 7	nd
5	10	nd	nd	nd	nd	nd	3	28	23 ± 5	>2000
6	13	34	27 ± 4	1.4	29	12 ± 4	2	59	17 ± 8[g]	200
7	14	46	20 ± 4[h]	0.8	28	20 ± 8	2	38	10 ± 4[h]	20
8	14	42	4[h]	nd	nd	nd	nd		nd	9

[a] pH 4–7: phosphate buffer, 10 or 100 mM; pH 8; sodium pyrophosphate buffer, 100 mM; at pH 6 and 8 similar results were observed in triethanolamine buffer. The initial concentration of substrate was 50 mM for the methyl and *n*-pentyl esters; 10 mM for the *n*-octyl ester. [b] Unit = μmol of ester hydrolyzed per minute per gram of solid. The solid contained ~2 wt % protein. [c] Usually three separate reactions were used to measure the enantiomeric ratio. The conversion, *c*, listed is a typical value. [d] E_{app} represents the enantiomeric ratio as defined by Sih (ref 24); nd = not determined. [e] Stability of the octyl ester to hydrolysis in the absence of enzyme. [f] 50 mM substrate. [g] The enantiomeric purities used to calculate this value were checked independently by NMR. [h] The lower enantiomeric ratio was probably due to nonspecific hydrolysis of the ester. No correction was made for the competing chemical hydrolysis at pH 7 and 8.

niger (ANL). The commercially available preparation showed moderate enantioselectivity (E ~20). Partial purification of this enzyme by a simple ammonium sulfate precipitation enhanced the enantioselectivity to >100. A synthetic-scale resolution using partially purified lipase yielded both (*R*)- and (*S*)-pipecolic acid in ≥93% ee.

Results

Resolution of Pipecolic Acid Esters. We screened commercially available hydrolases and identified lipase from *A. niger* (ANL, lipase AP-6 from Amano Enzyme Co.) as the most enantioselective catalyst for the hydrolysis of methyl pipecolate, eq 1. The enantiomeric purity of



(1)

the product pipecolic acid was measured by HPLC using ligand exchange chromatography,[23] and the enantioselectivities of the hydrolysis reactions were compared using the apparent enantiomeric ratio, E_{app}, as defined by Sih.[24] While ANL favored the (*S*)-enantiomer at pH 7 by a factor of 20 ± 4, lipases from *Candida rugosa*, *Rhizomucor miehei*, and *Pseudomonas cepacia* had enantioselectivities of only 1.4–2.4, favoring the (*S*)-enantiomer, and were not further investigated.

Modification of the substrate has often increased the enantioselectivity of lipase catalyzed resolutions;[25] therefore, in an attempt to increase the enantioselectivity of the ANL-catalyzed reaction, we first modified the ester group, Table 1. Unfortunately, the enantioselectivities of the hydrolysis of the methyl and octyl esters were similar (E ~20) at pH 6, while the hydrolysis of the pentyl ester was slightly less enantioselective. The rate of ANL-catalyzed hydrolysis was 6–10 times slower for the octyl and pentyl esters than for the methyl ester. In spite of this slower rate, we chose the octyl ester for further work because the hydrophobic octyl group simplified separation of the ester and acid in the synthetic-scale reactions described below.

In another attempt to increase the enantioselectivity by modification of the substrate, we added a larger group on the nitrogen. Increasing the size of a substituent has often increased the enantioselectivity of enzyme-catalyzed resolutions, especially when two of the original substituents at the stereocenter were similar in size[25] (cf. CH_2 and NH_2^+). Addition of an acetyl group gave *N*-acetyl methyl pipecolate. Hydrolysis of this new substrate was four times slower and not enantioselective (E_{app} = 1). This decrease in enantioselectivity suggested that the lipase distinguished between the two enantiomers of pipecolic acid based on the charge of the substituents, and not by their size.

Another way to increase the enantioselectivity of an enzyme-catalyzed resolution is to change the reaction conditions.[26] Adding salts (1 M NaCl, 100 mM $CoCl_2$, or 10 mM $HgCl_2$) did not change the enantioselectivity, but adding an organic phase (17 vol % 1-octanol) decreased the enantioselectivity to ~12 (data not shown). Increasing the pH to 7 also decreased the observed enantioselectivity for hydrolysis of octyl pipecolate, Table 1, but this decrease was probably due to spontaneous hydrolysis of octyl pipecolate. Pipecolate esters were stable in aqueous solution below pH 6 but hydrolyzed spontaneously above pH 6 ($t_{1/2}$ = 20 h for octyl pipecolate at pH 7). We did not correct for this nonspecific hydrolysis; therefore, the lower observed enantioselectivities at pH 7 and 8 are likely due to this competing hydrolysis. At pH 4, the specific activity of ANL dropped approximately 5-fold. Thus, the pH range between 5 and 6 was optimal because it maximized the rate of the enzymic reaction and minimized the chemical hydrolysis of the starting ester.

The final way to increase the enantioselectivity was to purify the enzyme. Five different groups have purified

(22) Toone, E.; Jones, J. B. *Can. J. Chem.* **1987**, *65*, 2722–2726. Subtilisin catalyzes the enantioselective hydrolysis of the methyl ester in methyl *N*-acetylpipecolate (E > 100), but the acetyl group is difficult to remove: Sih, C. J.; Gu, R.-L. In *Microbial Reagents in Organic Synthesis*; Servi, S., Ed.; Kluwer Academic: Amsterdam, 1992; pp 79–92.

(23) Cilon, C.; Leshem, R.; Tapuni, Y.; Grushka, E. *J. Am. Chem. Soc.* **1979**, *101*, 7612–7613. Lam, S.; Azumaya, H.; Karmen, A. *J. Chromatogr.* **1984**, *302*, 21–29.

(24) Chen, C. S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294–7299. The enantiomeric ratio of a reaction measures its enantioselectivity and is useful to compare different reactions because the enantiomeric ratio is independent of conversion and substrate concentration. However, when the catalyst is a mixture of enzymes, the measured enantiomeric ratio is only an apparent value, E_{app}, because it depends on the amount of each enzyme and their enantioselectivities. More important, this apparent enantiomeric ratio may vary with substrate concentration if the K_M values of the enzymes differ widely. To minimize these effects we used similar initial substrate concentrations (usually 5–10 mg/mL) for all experiments.

(25) Kazlauskas, R. J.; Weissfloch, A. N. E.; Rappaport, A. T.; Cuccia, L. A. *J. Org. Chem.* **1991**, *56*, 2656–2665. Kim, M. J.; Choi, Y. K. *J. Org. Chem.* **1992**, *57*, 1605–1607. Goergens, U.; Schneider, M. P. *J. Chem. Soc., Chem. Commun.* **1991**, 1066–1068.

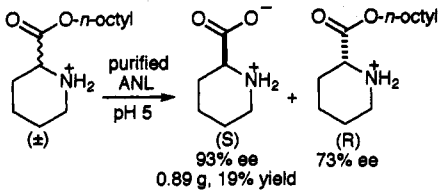
(26) Lam, L. K.; Raymond, A.; Hui, H. G.; Jones, J. B. *J. Org. Chem.* **1986**, *51*, 2047–2050.

Table 2. Partial Purification of Lipase from *A. niger*

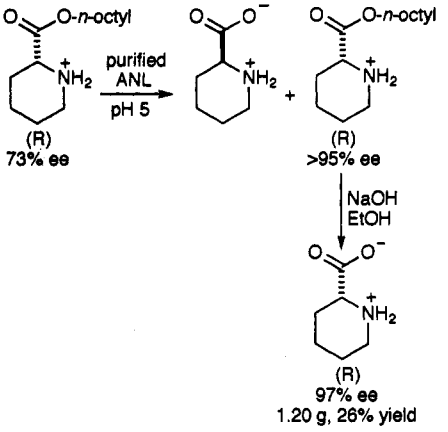
	wt of solid, mg	amt of protein, mg	activity,[a] units/mg protein	total units	fold purification	c,[b] %	E_{app}[c]
method 1							
crude	10 000	200	0.059	12	1	34	26 ● 8
DEAE–trisacryl M		32	0.15	4.8	2.5	42	>100
method 2							
crude	5000	102	0.056	5.7	1	26	27 ● 10
$(NH_4)_2SO_4$[d]	130	2.3	0.61	1.4	11	41	>100
Mono Q		0.13	2.1	0.26	37	50	>100

[a] Octyl pipecolate at pH 5. [b] Conversion. [c] E_{app} represents the enantiomeric ratio as defined by Sih (ref 24) for the hydrolysis of octyl pipecolate at pH 5. [d] Proteins precipitated between 25 and 45% saturation.

Scheme 1



Scheme 2



lipase from *A. niger*.[27] Höfelmann *et al.*[27c] showed that the crude material is only 2 wt % protein and contains, in addition to lipase, several proteases, numerous glycosidases, and an esterase. We hypothesized that nonspecific hydrolase or a hydrolase with reverse enantioselectivity lowered the overall enantioselectivity. To test this hypothesis, we partially purified ANL as described by Höfelmann *et al.*[27c] The anion exchange chromatography (DEAE–Trisacryl M) increased the specific activity 1.8-fold with *p*-nitrophenyl acetate as the substrate and 2.5-fold with octyl pipecolate as the substrate, Table 2. This degree of purification was similar to the 4.3-fold increase with tributyrin as the substrate that Höfelmann *et al.* reported. More significantly, this purification step raised the enantiomeric ratio to >100 for octyl pipecolate. This increase suggests that the crude enzyme did contain other hydrolases that lowered the overall enantioselectivity.

We examined two precipitation methods for partial purification of ANL as simpler alternatives to anion exchange. Fractional precipitation of crude ANL with 2-propanol (30–66 vol %)[27e] did not change the enantioselectivity, but fractional precipitation with ammonium sulfate (25–45% saturation)[27a,b] raised the enantioselectivity to >100. This precipitation with ammonium sulfate was not only simpler than the DEAE–Trisacryl M column, but was also a more effective purification, Table 2. The specific activity of the crude lipase increased 11-fold with octyl pipecolate as the substrate, a factor of 4.4 more than with anion exchange.

To demonstrate the synthetic usefulness of partially purified ANL, we resolved 10 g of (±)-octyl pipecolate using lipase partially purified by ammonium sulfate precipitation, Schemes 1 and 2. Starting with 200 g of crude ANL, approximately 19 000 units (*p*-nitrophenyl acetate assay), we purified it by precipitation with ammonium sulfate 25–48% saturation and desalted the enzyme on a size-exclusion column yielding 1950 units of activity (10% yield). A small scale reaction (100 mg of octyl pipecolate in 20 mL, 16 units of ANL, 3 h) gave product (*S*)-pipecolic acid in 98% ee and recovered (*R*)-octyl pipecolate in 29% ee, corresponding to an enantiomeric ratio of >100. A larger scale resolution using the same enzyme preparation (10 g of octyl pipecolate in 1.5 L, 1400 units of ANL, 24 h) gave product (*S*)-pipecolic acid with 93% ee (0.89 g, >95% chemical purity, 19% yield; 50% is the maximum yield possible). The recovered octyl pipcolate had 73% ee corresponding to an enantiomeric ratio of 60 for this resolution. Further hydrolysis of the octyl pipecolate (4 d) increased its enantiomeric purity to >95% ee. Upon hydrolysis we isolated (*R*)-pipecolic acid with 97% ee (1.20 g, >95% chemical purity, 26% yield). We cannot explain why the same enzyme preparation was less enantioselective in the large-scale than in the small-scale reaction. We also observed a similar difference in another sample of purified enzyme.

Biochemical characterization of our catalyst by electrophoresis showed that this partially purified material still contained many proteins, including at least two hydrolases. Electrophoresis under denaturing conditions (SDS–PAGE) followed by Coomassie Blue dye to stain all proteins, Figure 1a, showed numerous bands for both the crude and the partially purified enzyme. One difference between the two preparations was a protein with an apparent molecular weight of 32 kDa. This protein was visible in the partially purified enzyme, but not in the crude material. Previous workers showed that pure ANL isolated from the crude Amano preparation had an apparent molecular weight of 35–37 kDa.[24d,e] Thus, the increased amount of the 32-kDa protein in the partially purified enzyme is consistent with the notion that ANL was the enzyme that catalyzes the enantioselective hydrolysis of octyl pipecolate.

(27) (a) Fukumoto, J.; Iwai, M.; Tsujisaka, Y. *J. Gen. Appl. Microbiol.* **1963**, *9*, 353–361. (b) Chang, S.-M. *Bull. Int. Chem. Acad. Sin.* **1973**, *22*, 17–24. (c) Höfelmann, M.; Hartmann, J.; Zink, A.; Schreier, P. *J. Food Sci.* **1985**, *50*, 1721–1725. (d) Sugihara, A.; Shimada, Y.; Tominaga, Y. *Agric. Biol. Chem.* **1988**, *52*, 1591–1592. (e) Torossian, K.; Bell, A. W. *Biotechnol. Appl. Biochem.* **1991**, *13*, 205–211.

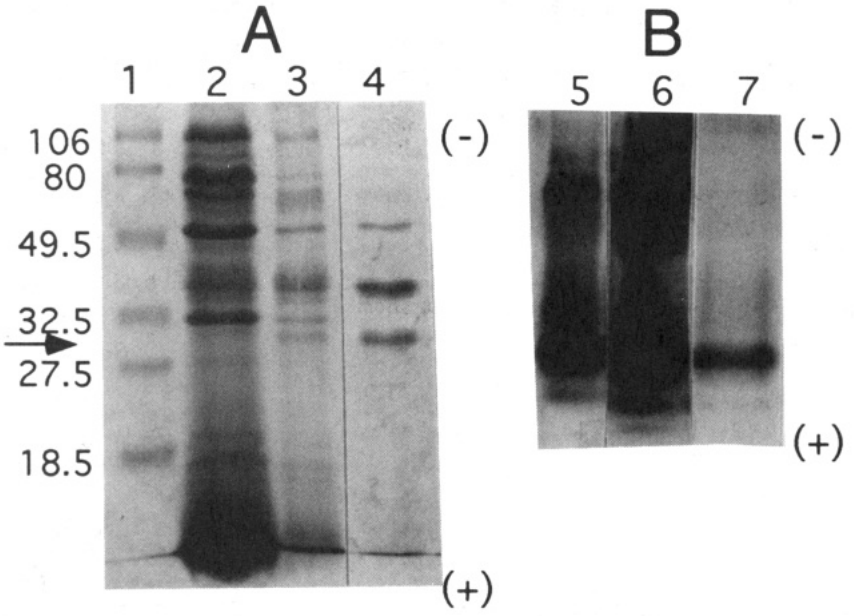


Figure 1. Gel electrophoresis of lipase from *A. niger*. (A) Denaturing gel (SDS–PAGE) stained with Coomassie Blue to show all proteins. The band marked with an arrow corresponds to an apparent molecular weight of 32 kDa. Lane 1: molecular weight standards. Lane 2: crude lipase as received from Amano. Lane 3: lipase partially purified by precipation with 25–45% saturation of ammonium sulfate. Lane 4: after additional purification by anion-exchange chromatography (Mono Q). (B) Nondenaturing gel stained with 1-naphthyl acetate/Fast Blue RR to show the hydrolases. Lane 5: crude lipase as received from Amano. Lane 6: lipase partially purified by precipation with 25–45% saturation of ammonium sulfate. Lane 7: after additional purification by anion exchange chromatography (Mono Q).

To identify the hydrolases in this mixture, we separated the proteins by electrophoresis under nondenaturing conditions and stained with 1-naphthyl acetate/Fast Blue RR. The 1-naphthol released by hydrolysis reacted with the diazo compound Fast Blue RR to give a dark brown color. This technique has been used previously to stain ANL[24e] as well as lipase from *C. rugosa*.[28] The hydrolase stain showed that both the crude and the partially purified enzyme contained two major hydrolases, Figure 1b. Because the partially purified material was highly enantioselective, we concluded that one or both hydrolases catalyzed the hydrolysis of (*S*)-octyl pipecolate, while neither hydrolase catalyzed the hydrolysis of (*R*)-octyl pipecolate.

Another fraction from the ammonium sulfate precipitation (60–80% saturation) contained small amounts of other hydrolases as shown by activity staining on nondenaturing gels. Resolution of octyl pipecolate with this fraction showed lower enantioselectivity, $E = 16 \pm 3$. We believe that these were the hydrolases responsible for the lower enantioselectivity of the crude material.

To identify ANL as the enzyme responsible for the enantioselective hydrolysis of octyl pipecolate, we further purified the enzyme by anion exchange chromatography (Mono Q column). This additional step gave material containing three major proteins with apparent molecular weights of 58, 40, and 32 kDa, Figure 1A. Activity staining of a nondenaturing gel showed a single hydrolase, Figure 1B. Kinetic resolution of octyl pipecolate with this material showed an enantioselectivity of >100, indicating that one of these three proteins was the enantioselective hydrolase. Further purification by gel filtration confirmed that the hydrolase was the 32 kDa protein. Thus, the enzyme responsible for the enantioselective hydrolysis of octyl pipecolate was a hydrolase with an apparent molecular weight of 32 kDa.

Previous workers isolated the lipase from the crude Amano enzyme by a similar procedure[24d] and found an apparent molecular weight of 35–37 kDa. The slight difference in apparent molecular weight between our work and that of previous workers is probably due to the new formulation for the molecular weight standards after 1990. Using the old molecular weight standards, we measured an apparent molecular weight of 35 kDa for our enzyme.

Discussion

Screening identified crude lipase from *A. niger* as an enantioselective catalyst for the hydrolysis of pipecolic acid esters. The enantioselectivity of the hydrolysis was not high enough for efficient synthetic use so we examined several approaches to increase the enantioselectivity. Neither substrate modification, nor modification of reaction conditions increased the enantioselectivity of the crude enzyme. However, a partial purification increased the enantioselectivity by at least a factor of five to >100. This result suggested that the crude enzyme contained nonspecific hydrolases or hydrolases of opposite enantioselectivity. Complete purification was not necessary since the partially purified enzyme was highly enantioselective even though it still contained at least two

(28) Baillargeon, M. W.; Sonnet, P. E. *JAOCS* **1988**, *65*, 1812–1815.

(29) (a) Kitazume, T.; Sato, T.; Ishikawa, N. *Chem. Lett.* **1984**, 1811–1814. (b) Gu, Q. M.; Reddy, D. R.; Sih, C. J. *Tetrahedron Lett.* **1986**, *27*, 5203–5206. (c) Miyazawa, T.; Takitani, T.; Ueji, S.; Yamada, T.; Kuwata, S. *J. Chem. Soc., Chem. Commun.* **1988**, 1214–1215. (d) Sonnet, P. E.; Baillargeon, M. W. *Lipids* **1989**, *24*, 434–437. (e) Ngooi, T. K.; Guo, Z.-W.; Sih, C. J. *Biocatalysis* **1990**, *3*, 119–128. (f) Gu, R. L.; Lee, I. S.; Sih, C. J. *Tetrahedron Lett.* **1992**, *33*, 1953–1956. (g) Chiou, A.-J.; Wu, S.-H.; Wang, K.-T. *Biotechnol. Lett.* **1992**, *14*, 461–464.

hydrolases. The enantioselectivity of previously reported resolutions of carboxylic acids with crude ANL[29] might also increase with partially purified ANL.

Using the same enzyme, Itoh *et al.* reached a different conclusion for the resolution of secondary alcohols.[30] They found that purification of ANL did not increase the enantioselectivity and concluded that all hydrolases in the crude enzyme had a similar enantioselectivity for secondary alcohols. Both this increase in enantioselectivity toward carboxylic acids upon purification and the lack of any change in enantioselectivity toward secondary alcohols upon purification is consistent with our proposed binding sites for these stereocenters in lipases.[31] On the basis of X-ray crystal structures of phosphonate inhibitors bound to *C. rugosa* lipase, we proposed that the alcohol binding site is similar in all lipases because it is formed by conserved structural elements; thus, purification has little effect on enantioselectivity. On the other hand, the acid binding site is formed by nonessential amino acid residues and likely differs among lipases. Purification can therefore strongly influence enantioselectivity toward carboxylic acids.

The kinetic resolution of octyl pipecolate is a simple and efficient route to both enantiomers of pipecolic acid. The partial purification of ANL by precipitation with ammonium sulfate is simple and accessible to synthetic organic chemists. It may be possible to extend this method to other cyclic amino acids, as well as derivatives of proline and pipecolic acid that are used as chiral auxiliaries[32] and for the construction of glycoside inhibitors. A pipecolic acid specific enzyme may also be useful for the synthesis of peptides containing pipecolic acid. Unpurified ANL also catalyzes the enantioselective hydrolysis of related substrates—piperidine-containing esters of primary alcohols.[33]

Further purification confirmed that the lipase from *A. niger* (apparent molecular weight of 32 kDa) was the enzyme responsible for the enantioselective hydrolysis of octyl pipecolate. Even though this lipase has been previously purified, little is known about the pure lipase's substrate specificity or enantioselectivity. The mechanism of discrimination between enantiomers appears to involve the charge on the substituents (CH_2 vs NH_2^+), while other lipases often discriminate between enantiomers based on the size of the substituents.

Experimental Section

General. Lipase from *C. rugosa* (L-1754, 0.16 units/mg solid using olive oil, also known as *C. cylindracea*[34]) was purchased from Sigma Chemical Co., St. Louis, MO. Lipase from *Pseudomonas cepacia* (lipase P-30, 0.05 units/mg solid using olive oil), lipase from *Rhizomucor miehei* (MAP-10, 0.05 units/mg solid using olive oil, synonym *Mucor m.*[34]), and lipase from *A. niger* (AP-6, 2 units/g solid using ethyl butyrate) were purchased from Amano International Enzyme Co., Troy, VI. Enzyme purification was done at 4 °C. Protein concentrations were measured by binding of Coomassie Blue dye (Bio-Rad Laboratories, Mississauga, ON). Denaturing (SDS–PAGE) electrophoresis was done on a 12% polyacrylamide gel using Mini-Protean II apparatus (Bio-Rad) following the Laemmli protocol.[35] Prestained proteins (Bio-Rad) were used as apparent molecular weight standards: phosphorylase B (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa), and lysozyme (18.5 kDa). Chemicals were purchased from Aldrich Chemical Co. unless otherwise noted. Tris–HCl (tris(hydroxymethyl)-aminomethane hydrochloride) was purchased from Sigma Chemical Co. The size exclusion gel, Bio-Gel P6 (BioRad) was used to desalt protein solutions. Ultrafiltration membranes (YM10 and PM10, cutoff at 10 kD) were purchased from Amicon Canada (Oakville, ON). (±)-Methyl pipecolate hydrochloride,[22] (±)-*n*-octyl 2-pipecolate hydrochloride,[36] and (±)-methyl *N*-acetyl-2-pipecolate[22] were prepared by acid-catalyzed esterification according to literature procedures.

(±)-Pentyl-2-pipecolate hydrochloride was prepared by acid catalyzed esterification as for the octyl ester.[36] mp 88–89 °C (methanol/ethyl acetate); R_f = 0.81 (3:2 ethyl acetate/methanol/1% NH_4OH); ^{1}H-NMR (D_2O, 200 MHz) δ 0.65-0.76 (m, 3), 1.1–1.7 (m, 11), 2.14 (m, 1, H3), 2.87 (ddd, 1, $^2J_{H6eq}$ = 13 Hz, $^3J_{H6ax-H5ax}$ = 13 Hz, $^3J_{H6ax-H5eq}$ = 3 Hz), 3.29 (m, 1), 3.85 (dd, 1, $^3J_{H2ax-H3ax}$ = 13 Hz, $^3J_{H2ax-H3eq}$ = 3.5 Hz), 4.06–4.12 (m, 2); ^{13}C-NMR (CD_3OD, 75.4 MHz) δ 14.3, 22.76, 22.82, 23.3, 27.2, 29.0, 29.2, 45.2, 57.9, 67.6, 170.

Spontaneous Hydrolysis of Octyl Pipecolate. The ester (28 mg) was suspended in buffer (100 mM, phosphate for pH 6, 7, or 8; acetate for pH 5). The solution was stirred at room temperature, and the initial rate of hydrolysis (5–10% conversion) was monitored with a pH stat which controlled the addition of 0.1 N NaOH.

Enzyme Assay. Activity is given in units/wt where 1 unit equals 1 μmol of ester hydrolyzed per minute. Typical activity of crude ANL was 3 units/g, measured by pH stat at pH 5 with octyl pipecolate as the substrate. Activity was also assayed using *p*-nitrophenyl acetate (PNPA) as a substrate. An aliquot (5 μL) of enzyme solution followed by an aliquot of PNPA (5 μL, 50 mM solution in acetonitrile) were added to phosphate buffer (1.0 mL, pH 7.5, 10 mM) at 25 °C. The initial rate of formation of *p*-nitrophenolate was monitored at 404 nm for 30 s. Activity was calculated using an extinction coefficient of 11 600 M^{-1} cm^{-1}. This value accounts for the incomplete ionization of *p*-nitrophenolate at pH 7.5. Typical activity of crude ANL was 95 ± 15 units/g with this assay.

Enantiomeric Purity. The enantiomeric purity of pipecolic acid was determined directly using ligand-exchange chromatography[23] on either a Whatman Partisil 5 ODS-3 reversed-phase column eluted with water containing aspartame (7.4 mg/L) and $CuSO_4$ (103 mg/L) with the detector set at 270 nm or a ChiralPak WH (Daicel Chemical Industries, Fort Lee, NJ) column eluted at 50 °C with water containing $CuSO_4$ (62 mg/L) with the detector set at 254 nm. Samples were dissolved in the eluting buffer and adjusted to pH 7 (pH paper) for chromatography. The measured values of enantiomeric excess for repeated injections typically varied by ±2%. The enantiomeric purity of esters of pipecolic acid was determined by ^{1}H-NMR of either the *N*-acetyl derivative[22] in the presence of chiral shift reagent (1.5 equiv of Eu(hfc)$_3$

(30) Itoh, T.; Kuroda, K.; Tomosada, M.; Tagaki, Y. *J. Org. Chem.* **1991**, *56*, 797–804. Other resolutions of secondary alcohols with crude ANL: Miura, S.; Kurozumi, S.; Toru, T.; Tanaka, T.; Kobayashi, M.; Matsubara, S.; Ishimoto, S. *Tetrahedron* **1976**, *32*, 1893–1898. Akita, H.; Matsukura, H.; Oishi, T. *Tetrahedron Lett.* **1986**, *27*, 5241–5244. Sonnet, P. E.; Moore, G. G. *Lipids* **1988**, *23*, 955–960. Glaenzer, B. I.; Faber, K.; Griengl, H. *Enzyme Microb. Technol.* **1988**, *10*, 689–690. Naemura, K.; Takahashi, N.; Tanaka, S.; Ida, H. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2337–2343. Li, Y. F.; Hammerschmidt, F. *Tetrahedron: Asymmetry* **1993**, *4*, 109–120.

(31) Ahmed, S. N.; Kazlauskas, R. J.; Morinville, A. H.; Grochulski, P.; Schrag, J. D.; Cygler, M. *Biocatalysis*, in press; Cygler, M.; Grochulski, P.; Kazlauskas, R. J.; Schrag, J. D.; Bouthillier, F.; Rubin, B.; Serreqi, A. N.; Gupta, A. J. *J. Am. Chem. Soc.*, in press.

(32) Coppola, G. M.; Schuster, H. F. *Asymmetric Synthesis. Construction of Chiral Molecules Using Amino Acids*; Wiley: New York, 1987; Chapter 8.

(33) Chênevert, R.; Dickman, M. *Tetrahedron: Asymmetry* **1992**, *3*, 1021–1024.

(34) *Catalogue of Fungi/Yeasts*, 17th ed.; American Type Culture Collection: Rockville, MD, 1987.

(35) Laemmli, U. K. *Nature* **1970**, *227*, 680–685.

(36) Crider, M. A.; Tita, T. T.; Wood, J. D.; Hinko, C. N. *J. Pharm. Sci.* **1982**, *71*, 1214–1219.

in $CDCl_3$) or the (*R*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid amide (Mosher's acid amide). The Mosher's acid amides were synthesized using Ward and Rhee's procedure,[37] with additional washings in the workup to obtain purer product. Once the reaction (10–20 mg of octyl pipecolate) was complete (TLC on silica gel, 1:1 chloroform/methanol; ninhydrin stain) the mixture was diluted with chloroform (10 mL), washed with saturated $NaHCO_3$ solution (5 mL), 10% HCl solution (5 mL), and saturated NaCl solution (5 mL), dried over $MgSO_4$, and concentrated by rotary evaporation followed by a vacuum pump: R_f = 0.75 (silica gel eluted with 4:1 hexanes/ethyl acetate). Integration of the ^{1}H-NMR resonances for the proton α to the carbonyl gave the relative amount of the two diastereomers: δ 5.55 (d, J = 4 Hz), 5.38 (d, J = 4 Hz). To measure the enantiomeric purity of pipecolic acid with the Mosher's amide, pipecolic acid was first converted to the ethyl ester. Pipecolic acid (16 mg, 0.12 mmol) was dissolved in dry ethanol (10 mL) saturated with HCl gas. The reaction mixture was stirred for 24 h, and then the solvent was removed by rotary evaporation. Excess HCl was removed by dissolving the residue in chloroform (5 mL) and concentrating by rotary evaporation three times. The amide was prepared as described above. The completion of the reaction was determined also as described above.

Small-Scale Resolutions. Ester (50 mg) was added to a rapidly stirred solution of enzyme (typically 50 mg of crude powder) in buffer (5 mL). The course of the reaction was followed with a pH stat which regulated the addition of 0.1 N NaOH. At a convenient percent conversion, an aliquot (1 mL) was extracted with chloroform (5 × 5 mL) to remove the unreacted ester, and the combined extracts were dried over magnesium sulfate and concentrated by rotary evaporation. Small amounts of organic solvent were removed from the aqueous phase by rotary evaporation followed by ultrafiltration (PM-10 membrane) to remove enzyme. The enantiomeric purity of the pipecolic acid in the aqueous phase was determined by ligand-exchange chromatography. The enantiomeric purity of the ester was determined by ^{1}H-NMR as the Mosher amide or by ligand-exchange chromatography after hydrolysis to pipecolic acid. Hydrolysis procedure: aqueous NaOH (2.5 N, 100 μL) was added to a sample of ester (~10 mg) dissolved in ethanol (~5 mL). After 1.5 h, a TLC analysis (1:1 chloroform: methanol; ninhydrin stain) indicated that hydrolysis was complete. The reaction mixture was concentrated by rotary evaporation, and the residue was analyzed by ligand exchange chromatography. The apparent enantiomeric ratio[24] for the resolution was calculated from the measured ee of the starting material (ee_s) and of the product (ee_p) using $c = ee_s/(ee_s + ee_p)$ and $E_{app} = \{\ln[(1-c)(1-ee_s)]\}/\{\ln(1-c)(1+ee_s)]\}$ or $E_{app} = \{\ln[1-c(1+ee_p)]\}/\{\ln[1-c(1-ee_p)]\}$. Alternately, E_{app} was calculated from the measured percent conversion and either ee_s or ee_p using the above equations. Both methods agreed with each other. The error limits represent the standard deviation of nine measurements (three ee measurements for pipecolic acid isolated from three separate reactions). For some reactions, the pipecolic acid was further purified by cation-exchange chromatography. Crude pipecolic acid was dissolved in aqueous H_2SO_4 (0.25 N, 5 mL). Cation exchange resin (Dowex 50W H^+, ~1 g) was added, and the mixture was stirred for 1 h. The resin was transferred to a column (0.5-cm diameter) and washed with water until the eluent contained no more chloride ($AgNO_3$ test) and then with 5% NH_4OH (~20 mL) to elute pipecolic acid. The fractions containing pipecolic acid were concentrated by rotary evaporation and analyzed by ligand-exchange chromatography.

Partial Purification of ANL by Anion-Exchange Chromatography. Crude lipase from *A. niger* (Amano AP-6, 10 g) was stirred in Tris–HCl buffer (0.06 M, pH 7, 85 mL) for 60 min, and the mixture was centrifuged at 12000*g* for 15 min. The supernatant (82 mL) was loaded on a DEAE–Trisacryl M Ion exchange column (2.6 × 55 cm) equilibrated in Tris–HCl buffer (0.06 M, pH 7). After the column was washed with the above buffer (1300 mL) at a flow rate of 85 mL/h, proteins were eluted with a linear gradient from 0.06 to 0.23 M Tris–HCl buffer (1600 mL). Fractions (10 mL) were collected at a flow rate of 70 mL/h. After the gradient, the column was washed with Tris–HCl (0.5 M, pH 7, 600 mL). The active fractions (45–94) were pooled and concentrated to 8 mL using a YM-10 membrane.

Partial Purification of ANL by Precipitation with Ammonium Sulfate (Small Scale). Crude lipase from *A. niger* (Amano AP-6, 5.0 g, 5.7 units with octyl pipecolate) was dissolved in Tris–HCl buffer (75 mL, 25 mM, pH 7.5) and centrifuged (10 000 rpm, 30 min) to remove a small amount of insoluble material. Solid ammonium sulfate (10.8 g) was added to the supernatant in small portions over 4 h to give 25% saturation. (Slow addition was important.) The suspension was centrifuged (10 000 rpm, 30 min), the pellet discarded, and additional ammonium sulfate (8.6 g) added to the supernatant in small portions over 4 h to give 45% saturation. The suspension was centrifuged (10 000 rpm, 30 min), and the pellet was dissolved in sodium acetate buffer (3 mL, 100 mM, pH 5), dialyzed twice against acetate buffer (2 L, 100 mM, pH 5), and lyophilized to give a brown solid (130 mg, 1.4 units, 25% yield).

Partial Purification of ANL by Precipitation with Ammonium Sulfate (Large Scale). Crude lipase from *A. niger* (Amano AP-6, 200 g, 20 000 units by PNPA assay) was added all at once to Tris–HCl buffer (1 L, 25 mM, pH 7.5) and stirred at 4 °C until all the powder dissolved (~8 h). More buffer was added to give a total volume of 1.5 L followed by ammonium sulfate (216 g) added in small portions over 4 h to give 25% saturation. The solution was stirred overnight at 4 °C then centrifuged (20 min, 10 000 rpm, 4 °C) to remove a small amount of precipitate. More $(NH_4)_2SO_4$ (218 g) was added in small portions to the supernatant over 4 h to give 48% saturation. The suspension was stirred overnight at 4 °C and centrifuged (20 min, 10 000 rpm, 4 °C). The pellet was collected and dissolved in phosphate buffer (350 mL, 10 mM, pH 5.0). This solution contained 2000 units of activity (PNPA assay). The solution was concentrated by ultrafiltration (Amicon PM10 membrane, 9-cm diameter) to 245 mL and desalted on a BioGel-P6 column (500 mL) yielding 500 mL containing 1950 units by PNPA assay (10% yield). In other purifications, the enantioselectivity toward octyl pipecolate was >100 using a 25–50% saturation of ammonium sulfate (4400 units, 22% yield). However, a 25–55% saturation showed a lower enantioselectivity of 71 (5000 units, 25% yield).

(*S*)-(−)-Pipecolic Acid. Racemic *n*-octyl pipecolate hydrochloride (10.0 g, 36.1 mmol) was dissolved in NaOAc buffer (1 L, pH 5.0, 100 mM) at ~40 °C and the solution was cooled to room temperature. Partially-purified lipase from *A. niger* (1400 units, PNPA assay) dissolved in NaOAc buffer (500 mL, pH 5.0, 10 mM) was added, and the mixture was stirred at room temperature for 24 h. The reaction mixture was extracted with chloroform (6 × 500 mL), and the chloroform extracts were pooled, concentrated to 400 mL by rotary evaporation, and saved for further enzymic hydrolysis described below. The aqueous phase was filtered through Celite, concentrated by rotary evaporation to 500 mL without heating the solution over 40 °C, and ultrafiltered (Amicon PM10 membrane, 9 cm) to separate the pipecolic acid from the protein. The recovered ANL (1250 units by PNPA assay, 89% recovery) was stored in phosphate buffer (130 mL, 10 mM, pH 5.0, 0.02% NaN_3). The filtrate was concentrated by rotary evaporation to a brown residue (15 g) which was purified by cation exchange chromatography. The residue was dissolved in H_2SO_4 (0.25 N, 400 mL), and cation-exchange resin (Dowex 50W H^+, 250 g) was added to the solution and stirred overnight. The resin was transferred to a column and eluted first with water until the eluent contained no more chloride ($AgNO_3$ test) and then with 5% NH_4OH. The fractions containing pipecolic acid were pooled, concentrated by rotary evaporation, and freeze-dried to give a tan powder: 2.2 g, 86% pure by ^{1}H-NMR using a weighed amount of dioxane as an internal standard. Another cation-exchange column on 20 g of the same resin gave a tan powder (1.0 g) which was recrystallized from methanol/ethyl ether to give a powder (0.89 g, 6.9 mmol, 19% yield (50% is the maximum possible yield), 93% ee by Mosher amide derivative, >95% chemical purity by ^{1}H-NMR (D_2O, 200 MHz) δ 1.3–1.8 (m, 5), 2.03 (m, 1), 2.80 (ddd, 1, $^2J_{H6ax-H6eq}$ = 13 Hz, $^3J_{H6ax-H5ax}$ = 13 Hz, $^3J_{H6ax-H5eq}$ = 3 Hz), 3.20 (m, 1), 3.40 (dd, 1, $^3J_{H2ax-H3ax}$ = 13 Hz, $^3J_{H2ax-H3eq}$ = 3.5 Hz). A ^{23}Na NMR spectrum showed <1 wt % sodium as NaCl.

(*R*)-(−)-Pipecolic Acid. The recovered octyl pipecolate from

(37) Ward, D. E.; Rhee, C. K. *Tetrahedron Lett.* **1991**, 32, 7165–7166.

the reaction above (73% ee) was concentrated by rotary evaporation and then dissolved in NaOAc buffer (750 mL, 100 mM, pH 5.0), the recovered *A. niger* lipase (1250 units by PNPA assay) was added, and the reaction mixture was stirred at room temperature. The enantiomeric purity of the remaining octyl pipecolate was measured by the Mosher amide method every 24 h. After 4 d, the enantiomeric purity of the remaining octyl pipecolate was >95% so the reaction was stopped by extraction with $CHCl_3$ (5 × 500 mL). The combined organic extracts were dried over $MgSO_4$ and concentrated by rotary evaporation to an oil, 4.7 g. This oil was dissolved in methanol (100 mL) containing aqueous NaOH (1.0 N, 20 mL) and stirred for 1.5 h when TLC analysis indicated that the ester was completely hydrolyzed. The solution was neutralized by the addition of aqueous H_2SO_4 (0.25 N) and concentrated by rotary evaporation. The residue was dissolved in water (50 mL) and extracted with $CHCl_3$ (2 × 50 mL) to remove octanol. The combined organic extracts were reextracted with water (7 × 25 mL). All aqueous phases were pooled and concentrated by rotary evaporation to a tan residue, 6.0 g, which was purified by cation exchange chromatography as described above (100 g resin). Recrystallization from methanol/ethyl ether afforded a white powder (1.20 g, 9.30 mmol, 26% (50% is the maximum possible yield), 97% ee by Mosher amide, >95% chemical purity by ^{1}H-NMR. A ^{23}Na NMR spectrum showed <1 wt % sodium as NaCl.

Identification of ANL as the Enzyme Responsible for Resolution. Lipase partially purified by precipitation with ammonium sulfate (26 mg of protein) was desalted with a Bio-Gel P-6 column, dissolved in Tris–HCl buffer (2 mL, 25 mM, pH 7.4), and injected onto an anion-exchange column (Mono Q HR 5/5 column, Pharmacia, Baie d'Urfé, Québec) equilibrated in the same buffer using a Pharmacia FPLC system. The column was eluted at a flow rate of 0.50 mL/min with the same buffer (15 mL) followed by a linear gradient of 0–0.50 M NaCl over 55 mL. Fractions (2 mL) were collected and assayed with *p*-nitrophenyl acetate. Fractions 17 and 18 contained hydrolase activity. Figure 1 shows the SDS–PAGE stained with Coomassie Blue and nondenaturing gel stained with 1-naphthyl acetate/Fast Blue RR for this sample. To identify the 32 kDa protein as the active hydrolase, the active fractions from an anion exchange column were concentrated to by filtration (PM-10 membrane) to 1 mL and applied to a gel filtration column (Superdex 75 HiLoad 16/60, Pharmacia) equilibrated with Tris buffer (25 mM, pH 7.3 containing 0.15 M NaCl). The column was eluted with the same buffer at a flow rate of 1 mL/min (30 cm/h) while fractions (3 mL) were collected. Fraction 23 contained the 32-kDa protein (>80% pure by SDS–PAGE) and showed the highest hydrolase activity.

Acknowledgment. We thank Prof. Michael Parniak and Ms. Emily Amandoron (Department of Medicine) for help with lipase purification and NSERC and FCAR for financial support.