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Empirical rules for the enantiopreference of lipase from *Aspergillus niger* toward secondary alcohols and carboxylic acids, especially α-amino acids

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Abstract: Lipase from *Aspergillus niger* (ANL, Amano lipase AP) catalyzes enantioselective hydrolysis and acylation reactions. To aid in the design of new applications of this lipase, we propose two empirical rules that predict which enantiomer reacts faster. For secondary alcohols, a rule proposed previously for other lipases also works for ANL, but with lower reliability (77%, 37 of 48 examples). For carboxylic acids, we examined both crude and partially-purified ANL because commercial ANL contains contaminating hydrolases. Partial purification removed a contaminating amidase and increased the enantioselectivity of ANL toward many α-amino acids, including cyclic amino acids. Unlike other lipases, ANL readily accepts positively-charged substrates and shows the highest enantioselectivity toward α-amino acids. Although a rule based on the sizes of the substituents could not predict the fast-reacting enantiomer, a rule limited to α-amino acids did predict the fast-reacting enantiomer. We estimate that the charged α-amino group contributes a factor of 40–100 ($\Delta\Delta G^{\ddagger}$=2.2–2.7 kcal/mol) to the enantioselectivity of ANL towards carboxylic acids. © 1997 Elsevier Science Ltd

Introduction

Lipase-catalyzed reactions are one of the best methods to produce enantiomerically pure compounds.[1] Lipase from the fungus *Aspergillus niger* (ANL) is a useful enantioselective catalyst, especially for the resolution of secondary alcohols and carboxylic acids. These reactions were discovered by trial and error. To identify other applications of this lipase, it would be useful to have generalizations or rules that identify which structures in a substrate are required for high enantioselectivity. The only previous generalization for ANL was limited to secondary alcohols of 2-(methylthio)-3-acetoxy esters.[2]

The simplest rules for hydrolase-catalyzed reactions do not attempt to predict the degree of enantioselectivity, but only which enantiomer reacts faster. For example, a rule based on the size of the substituents at the stereocenter predicts which enantiomer of a secondary alcohol reacts faster[3] (Figure 1).

This rule suggests that the lipases distinguish between enantiomers of secondary alcohols primarily based on the relative sizes of the substituents. For the synthetic chemist, this means that lipases resolve secondary alcohols with different-sized substituents better than alcohols with similarly sized substituents. In this paper, we will show that ANL also follows the rule in Figure 1, but with lower reliability than for other lipases.

Predicting the fast-reacting enantiomer of a carboxylic acid has been more difficult for lipases. For purified lipase from *Candida rugosa*, a rule based on the size of the substituents was reliable,[4] but we show in this paper that this rule is not reliable for ANL. For ANL, the most efficiently resolved carboxylic acids are α-amino acids and the fast-reacting enantiomer has the L-configuration

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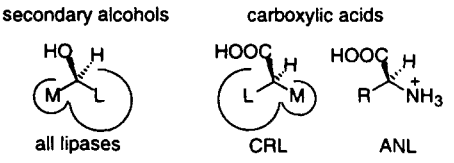

Figure 1. Empirical rules that predict the fast-reacting enantiomer in lipase-catalyzed reactions. For secondary alcohols, all lipases, including ANL, follow a rule that compares the size of the substituents at the stereocenter. For carboxylic acids, a rule based upon the size of the substituents at the stereocenter works for CRL, but not for ANL. A rule for ANL includes only α-amino acids (including cyclic amino acids) and suggests that a charged α-amino group is essential for high enantioselectivity. ANL always hydrolyzes the natural L-enantiomer. M represents a medium sized substituent, e.g., CH_3; L represents a large substituent, e.g., Ph; R represents any alkyl or aryl group.

(Figure 1). This model suggests that charge, not size, of the substituents at the stereocenter sets the enantioselectivity of ANL for carboxylic acids.

Results

Literature survey of the enantiopreference of ANL toward secondary alcohols

To test if the secondary alcohol rule also applies to lipase from *Aspergillus niger*, we summarized all reported ANL-catalyzed reactions of secondary alcohols (Table 1). Figure 2 shows the structures of the fast-reacting enantiomers. The examples include both hydrolyses of esters and acylations of alcohols, but for consistency the structures in Figure 2 are all alcohols. We excluded patents and examples where the enantioselectivity was <2. For eight examples, the rule is equivocal because the size of both substituents is similar according to CPK models. We marked these examples 'sim. size' in Table 1 and excluded them from the tally below. Note, however, that two of these examples showed good enantioselectivity, E>40.

For the remaining 48 substrates, the rule in Figure 1 predicts the absolute configuration of the favored enantiomer in 37 cases, but fails in 11 cases, 77% accuracy. (Guessing alone gives 50% accuracy because there are only two choices.) Six of the 48 substrates showed enantioselectivities >50 and the rule predicted the favored enantiomer for all six of these. Of the 11 exceptions to the rule, eight showed low enantioselectivities, E<10, but three showed moderate enantioselectivity (E=20–41). Five of the six bicyclic alcohols (compounds **23–27**) did not obey the size rule. Thus, the size rule usually predicts the favored enantiomer, but the reliability is less than for other lipases where the reliability is often >90%.[3,5] This lower reliability may be an inherent characteristic of ANL or it may be due to contaminating hydrolases, see below.

A literature survey of enantioselectivity of ANL toward carboxylic acids

Table 2 summarizes all reported ANL-catalyzed hydrolyses of esters of carboxylic acids in the literature and Figure 3 shows the structures of the faster-reacting enantiomers.[6] ANL favors the naturally occurring L-enantiomer of pipecolic acid, **31**, homophenylalanine, **33** (R=CH_2CH_2Ph), and the 12 *N*-Cbz-protected amino acids, **38**. ANL showed no enantioselectivity toward *N*-acetyl pipecolic acid, **39**. For the non-amino acid substrates, ANL showed moderate enantioselectivity toward 2-phenoxypropionic acid, **40**, and low enantioselectivity toward acids **41** and **42**. From such a limited range of substrates (seven structures in total), it is difficult to deduce which classes of carboxylic acids ANL resolves efficiently. For this reason, we resolved a wider range of carboxylic acids with both crude and partially-purified ANL (Table 3).

Enantioselectivity of ANL toward carboxylic acids does not depend on the size of the substituents

We tested the methyl esters of nine α-amino acids, **33**, the octyl ester of **32**, three β-amino acids, **34–36**, and one α-disubstituted amino acid, **37**. In addition, we tested esters of an aryloxypropionic acid, **43**, mandelic acid, **44**, two arylpropionic acids, **45** and **46**, two furoic acids, **47** and **48** and 2-

Table 1. Literature survey of the enantioselectivity of ANL toward secondary alcohols[a]

Struct.	R	E[b]	Follows rule?[c]	Ref	Struct.	R	E[b]	Follows rule?[c]	Ref
1		5	yes	*d*	**12**	CMe=CHEt	12	sim. size	*n*
2		2	yes	*e*	"	CMe=CHMe	10	sim. size	*n*
3		7	yes	*f*	"	$CMe=CH_2$	18	sim. size	*n*
4		6	yes	*g*	"	CH=CHPh	46	sim. size	*n*
5	(4'-OMe-C_6H_4)-O	8	yes	*h*	**13**	2-Furanyl	20	yes	*n*
"	3',4'-*di*OMe-C_6H_3	21	yes	*h*	"	2-Thienyl	46	yes	*n*
"	2',4',6'-*tri*OMe-C_6H_2	25[i]	yes	*h*	"	CMe=CHMe	43	sim. size	*n*
6		74	yes	*j*	**14**		15	yes	*j*
7		11	yes	*j*	**15**	Ph	30	yes	*j*
8	Me	31	yes	*e*	"	Me	3	yes	*j*
"	Et	13	yes	*k*	"	Bu	5	yes	*j*
"	*n*-Propyl	22	yes	*k*	"	CH=CHPh	13	sim. size	*j*
"	*n*-Pentyl	35, 40[i], 150[m]	yes	*k*	**16**		5	no	*j*
"	*n*-Heptyl	17[i]	yes	*k*	**17**		10	yes	*j*
"	Ph	10[i], 138	yes	*e*	**18**	Me	82	yes	*j*
"	Bn	4.8-6[i]	yes	*l*	"	Et	20	yes	*j*
"	4'-OMe-C_6H_4	17, 11[i]	sim. size	*l*	**19**		3	no	*j*
9	Bn	41	no	*l*	**20**		4	sim. size	*j*
"	*i*-Pr	3	no	*k*	**21**		4	yes	*o*
"	*t*-Butyl	2[i]	no	*k*	**22**		3	no	*p*
10	*n*-Pentyl	9	yes	*k*	**23**		~5	no	*q*
"	*n*-Heptyl	8[i]	yes	*k*	**24**		~10	no	*q*
"	*n*-Nonyl	7[i]	yes	*k*	**25**		~7	no	*q*
11	Me	2	yes	*j*	**26**		~30	no	*q*
"	Et	3	yes	*j*	**27**		20	no	*q*
"	Bu	2	yes	*j*	**28**		2	yes	*r*
12	2-Furanyl	52	yes	*n*	**29**		27	yes	*s*
"	2-Thienyl	8	yes	*n*	**30**		>50	yes	*t*

[a]Hydrolysis of the acetate ester using lipase AP-6 from Amano Enzyme Co. unless noted otherwise. Figure 2 shows the absolute configurations of the favored enantiomer. [b]Enantiomeric ratio, E, measures the preference of the enzyme for one enantiomer over the other: Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299. [c]The rule in Fig. 1 is ambiguous when both substituents are similar in size; these examples are marked 'sim. size'. [d]ANL from Röhm; esterification with dodecanoic acid: Gerlach, D.; Missel, C.; Schreier, P. *Z. Lebensm.-Unters. Forsch.* **1988**, *186*, 315-18. [e]Li, Y.-F.; Hammerschmidt, F. *Tetrahedron: Asymmetry* **1993**, *4*, 109-120. [f]Lipase AP; esterification with vinyl acetate: Hirai, Y.; Nagatsu, M. *Chem. Lett.* **1994**, 21-22. [g]Lipase A; esterification with vinyl acetate in carbon tetrachloride: Kaminska, J.; Gornicka, I.; Sikora, M.; Gora, J. *Tetrahedron: Asymmetry* **1996**, *7*, 907-910. [h]Akita, H.; Umezawa, I.; Takano, M.; Ohyama, C.; Matsukura, H.; Oishi, T. *Chem. Pharm. Bull.* **1993**, *41*, 55-63. [i]Hydrolysis of the chloroacetate ester. [j]Itoh, T.; Kuroda, K.; Tomosada, M.; Takagi, Y. *J. Org. Chem.* **1991**, *56*, 797-804. [k]Drescher, M.; Hammerschmidt, F.; Kahlig, H. *Synthesis* **1995**, *10*, 1267-1272. [l]Drescher, M.; Li, Y.-F.; Hammerschmidt, F. *Tetrahedron* **1995**, *51*, 4933-4946. [m]Reaction carried out at 40°C. [n]Akita, H.; Matsukura, H.; Oishi, T. *Tetrahedron Lett.* **1986**, *27*, 5241-5244. [o]Lipase A-6 immobilized on Celite: Akita, H.; Enoki, Y.; Yamada, H.; Oishi, T. *Chem. Pharm. Bull.* **1989**, *37*, 2876-2878. [p]Miura, S.; Kurozumi, S.; Toru, T.; Tanaka, T.; Kobayashi, M.; Matsubara, S.; Ishimoto, S. *Tetrahedron* **1976**, *32*, 1893-1898. [q]Lipase A: Naemura, K.; Takahashi, N.; Tanaka, S.; Ida, H. *J. Chem. Soc., Perkin Trans 1*, **1992**, 2337-2343. Estimated E values based on the reported enantiomeric purities. [r]Lipase from Fluka Chemical Co.: Zaravucka, M.; Zuzana, Z.; Rejzek, M., Streinz, L.; Wimmer, Z.; Mackova, M.; Demnerova, K. *Enz. Microb. Tech.* **1995**, *17*, 866-869. E = 6 for esterification with vinyl acetate in benzene. [s]Lipase APF: Patel, R. N.; Banerjee, A.; Kp, R. Y.; Howell, J. M.; Li, W.-S.; Comezoglu, F. T.; Partyka, R. A.; Szarka, L. *Biotechnol. Appl. Biochem.* **1994**, *20*, 23-33. [t]Hoenke, C.; Kluwer, P.; Hugger, U.; Krieger, R.; Prinzbach, H. *Tetrahedron Lett.* **1993**, *34*, 4761-4764.

chloropropionic acid, **49**. For pipecolic acid, **31**, we previously showed that the enantioselectivity of ANL increased significantly (from E~20 to E>100) upon partial purification of the lipase to remove a contaminating hydrolase.[7] For this reason, we measured the enantioselectivity of both crude and partially-purified ANL.

ANL resolved only the α-amino acids with an enantioselectivity >10. For proline, phenylalanine, and phenylglycine, the enantioselectivity of crude ANL was moderate to good (E=20–70) and increased to >90 upon purification. For alanine the enantioselectivity also increased from 3 to 41 upon purification. The specific activity of ANL towards these substrates increased up to two-fold upon purification (0.21–0.49 U/mg for crude, 0.55–0.96 U/mg for partially-purified). On the other hand, the specific

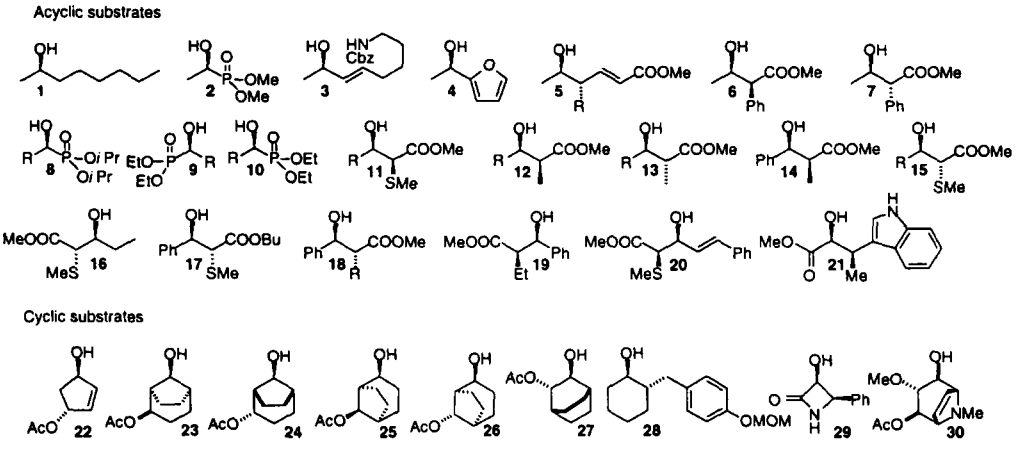

Figure 2. Examples from the literature of secondary alcohols resolved or desymmetrized by ANL. Structures show the fast reacting alcohol enantiomer. Most examples follow the rule in Figure 1. Details in Table 1.

Table 2. Literature survey of the enantioselectivity of ANL toward carboxylic acids[a]

Structure	R[b]	E[c]	Follows size rule[d]?	Reference
31		20, >100	sim. size	*e*
33	CH_2CH_2Ph	13	yes	*f*
38	Me [Ala]	28, 14	no	*g, h*
"	Et	95	no	*g*
"	*i*-Pr [Val]	28, >100	no	*g, h*
"	*n*-Pr	18	no	*g*
"	*n*-Bu	18	no	*g*
"	*n*-Pentyl	87	sim. size	*g*
"	CH_2=$CHCH_2$	21	no	*g*
"	*n*-Hexyl	49	sim. size	*g*
"	(4-Thiazolyl)methyl	56	no	*g*
"	Bn [Phe]	50, 22	no	*g, h*
"	$CH_3SCH_2CH_2$ [Met]	86	no	*h*
"	$(CH_3)_2CHCH_2$ [Leu]	15	no	*h*
39		1	na	*e*
40		19	no	*i*
41		3	yes	*j*
42		6	no	*k*

[a]Hydrolysis of the methyl ester using lipase AP-6 from Amano Enzyme Co. unless noted otherwise. Figure 3 shows the absolute configuration of the favored enantiomer. [b]When the structure is a common amino acid, its three letter abbreviation is given in brackets. [c]Enantiomeric ratio, E, measures the preference of the enzyme for one enantiomer over the other: Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299. [d]The rule in Fig. 1 is ambiguous when both substituents are similar in size; these examples are marked 'sim. size'; 'na' indicates not applicable because E = 1. [e]Crude ANL showed an E of 20, but partially-purified ANL showed an E of > 100: Ng-Youn-Chen, M. C.; Serreqi, A. N.; Huang, Q.; Kazlauskas, R. J. *J. Org. Chem.* **1994**, *59*, 2075-2081. [f]Hydrolysis of the ethyl ester: Houng, J.-Y.; Hsieh, C.-L.; Chen, S.-T. *Biotechnol. Techn.* **1996**, *10*, 353-358. [g]Hydrolysis of the 2-chloroethyl ester: Miyazawa, T.; Takitani, T.; Ueji, S.; Yamada, T.; Kuwata, S. *J. Chem. Soc., Chem. Commun.* **1988**, 1214-1215. [h]Chiou, A.- J.; Wu, S.-H.; Wang, K.- T. *Biotechnol. Lett.* **1992**, *14*, 461-464. [i]Transesterification of the vinyl ester with methanol in heptane: Miyazawa, T.; Kurita, S.; Ueji, S.; Yamada, T.; Kawata, S. *Biotechnol. Lett.* **1992**, *14*, 941-946. [j]Chenevert, R.; Lavoie, M.; Courchesne, G.; Martin, R. *Chem. Lett.* **1994**, 93-96. [k]Ngooi, T. K.; Guo, Z. W.; Sih, C. J. *Biocatalysis* **1990**, *3*, 119-128.

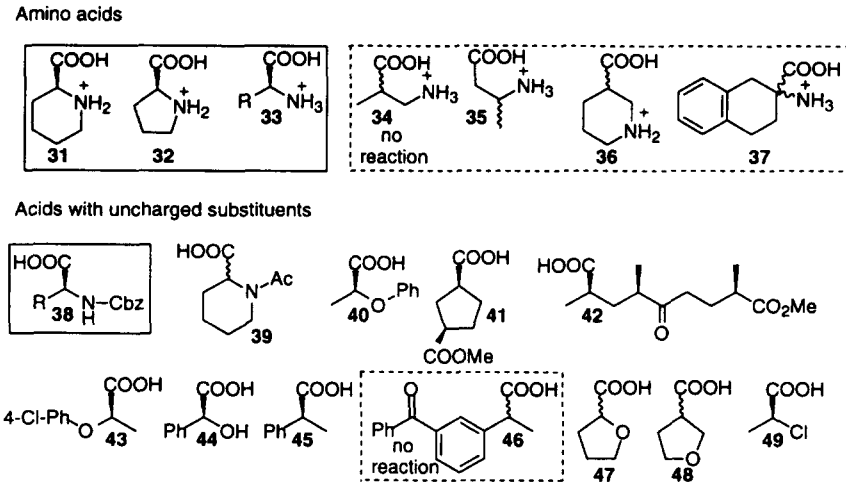

Figure 3. Carboxylic acids tested in enantioselective reactions with ANL. Enantioselectivities are moderate or better (E>10) only for the examples in the solid-line boxes. Examples in the dotted-line boxes did not react with ANL. Structures show the fast reacting enantiomer where the absolute configuration is known. Table 2 provides details for the examples from the literature and Table 3 lists experimental results for examples reported in this paper.

activity of ANL towards *N*-Cbz-phenylalanine, **38** (where R=Bn), decreased by a factor of nine upon purification and the enantioselectivity decreased from 20 to 13. These decreases suggest that a contaminating hydrolase in crude ANL contributes to the hydrolysis of *N*-Cbz-phenylalanine-methyl ester. For the non-charged substrates, **43**, **45**, **47–49**, the specific activity increased as much as 20-fold (0.051–1.4 U/mg for crude, 0.072–3.0 U/mg for partially-purified), but the enantioselectivity remained low. Thus, purification increased the specific activity toward many substrates, but increased the enantioselectivity only toward substrates with a charged α-amino group.

The specific activity of crude ANL toward α-amino acids with larger side chains — tryptophan, tyrosine, and *O*-methyl tyrosine — were 5–50 times lower than for the those with smaller side chains. The enantioselectivity was low to moderate (E=5–25). There was little change upon purification. The specific activity increased two-fold towards tyrosine and *O*-methyl tyrosine, but decreased two-fold towards tryptophan, however the enantioselectivies remained approximately the same (E=2.5–21). Three sterically hindered, unnatural α-amino acids, *tert*-leucine (**33**, R=*t*-Bu), 2,6-dimethyltyrosine (**33**, R=4′-OH-2′,6′-Me-C_6H_2) and the α-disubstituted amino acid, **37**, did not react. The slow hydrolysis and lower enantioselectivity towards large substrates suggest that there is a limit to the size of the side chain that ANL can accommodate.

ANL did not catalyze the hydrolysis of any of the three β-amino acids, **34–36**, even though these are not sterically hindered. Both crude and partially-purified ANL showed low enantioselectivity toward the aryloxypropionic acid, **43**, mandelic acid, **44**, the two arylpropionic acids, **45** and **46**, the two furoic acids, **47** and **48**, and chloropropanoic acid, **49**.

A rule comparing the size of the substituents at the stereocenter predicted the enantiopreference of purified lipase from *Candida rugosa* towards carboxylic acids,[4] but this rule does not work for ANL (Figure 1). For the α-amino acids, **33**, the larger group, R, lies to the left, but for the *N*-Cbz-α-amino acids, **38**, the larger group, *N*-Cbz, lies to the right. Yet ANL favors the L-enantiomer for both. For substrates **41** and **43**, the larger group lies to the left, but for **42** the larger group lies to the right. We also compared the overall accuracy of the size rule. Among the 40 carboxylic acids tested in either this work or literature reports, eight did not react. We excluded another seven because both substituents had similar sizes (**31**, **32**, **33** (R=CH_3 [Ala]), **38** (R=*n*-pentyl, *n*-hexyl) and **47**, **48**). We also excluded

Table 3. Enantioselectivity of ANL in the hydrolysis of carboxylic acid esters[a]

Struct	R[c]	Crude ANL[b] Rate (U/mg)[d]	ee_s (%)	ee_p (%)	E[e]	Partially-Purified ANL[b] Rate (U/mg)[d]	ee_s (%)	ee_p (%)	E[e]	Follows size rule?
32		0.49	nd	86	20[f,g]	0.62	90	94	100[g]	sim. size
33	Me [Ala]	0.40	59	26	3	0.55	99	78	41	sim. size
"	$CH_3SCH_2CH_2$ [Met]	0.58	78	85	30	0.74	97	99	>100	yes
"	Ph	0.45	92	91	70[h]	0.91	93	93	94[h]	yes
"	Bn [Phe]	0.21	93	86	45	0.96	>99	91	>100	yes
"	Indoyl-CH_2[Trp]	0.057	42	58	5.6	0.026	38	29	2.5	yes
"	4'-OH-C_6H_4-CH_2 [Tyr]	0.014	97	33	7	0.028	53	61	7	yes
"	4'-OMe-C_6H_4-CH_2	0.12	73	84	25	0.21	86	76	20	yes
"	4'-OH-2',6'-Me_2-C_6H_2-CH_2	<0.00001	-	-	-	<0.00001	-	-	-	-
"	*t*-Bu	<0.00001	-	-	-	<0.006	-	-	-	-
34		<0.004	-	-	-	<0.0001	-	-	-	-
35		<0.0001	-	-	-	<0.0009	-	-	-	-
36		<0.001[i]	-	-	-	<0.001[i]	-	-	-	-
37		<0.005	-	-	-	<0.004	-	-	-	-
38	Bn [Phe]	1.4	28	88	20	0.16	9	85	13	no
"	Ph	<0.0001	-	-	-	<0.0001	-	-	-	-
43		0.088	64	43	5	0.15	1	54	3	yes
44		1.00	0	0	1	0.82	0.2	8.1	1.2	na
45		0.051	4	nd	1.2[j,k]	0.072	nd	12	1.3[f,j]	na
46		<0.001	-	-	-	<0.00004	-	-	-	-
47		0.047	10	nd	1.6[g,k]	0.25	15	nd	6[g,k]	sim. size
48		0.045	0	nd	1[g,k]	0.82	4	nd	1.8[g,k]	sim. size
49		1.39	42	51	4.5	3.00	50	66	8.0	yes

[a]Hydrolysis of the methyl ester unless noted otherwise. Figure 3 shows the absolute configuration of the favored enantiomer. 'nd' = not determined; '-' = could not be measured because no reaction was detected; 'na' = not applicable because the enantioselectivity is below 2 or because the absolute configuration is unknown. [b]Crude ANL is lipase AP-6 from Amano as received and partially-purified ANL is lipase AP-6 precipitated with 25-55% saturation of ammonium sulfate. [c]When the structure is a common amino acid, its three letter abbreviation is given in brackets. [d]Initial rate of hydrolysis in units/mg protein. Unit = μmol of ester hydrolyzed per minute. Crude ANL contained 2.8 wt% protein by the Bio-Rad protein assay . [e]E represents the enantiomeric ratio which measures the preference of the enzyme for one enantiomer over the other: Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299. Unless otherwise noted, E was calculated from ee_s and ee_p. [f]E was calculated from ee_p and percent conversion. [g]Hydrolysis of the octyl ester. The methyl ester of **32** was unstable at room temperature; the methyl esters of **47** and **48** were very volatile. [h]Phenylglycine methyl ester partially racemized after 24 hours at pH 5. To determine E accurately, we used short reaction times (typically 1 h) and measured ee_p and ee_s immediately after we stopped the reaction. Others also noted the racemization of phenylglycine, for example, Shiraiwa, T.; Sakata, S.; Fujishima, K; Kurokawa, H. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 191-195. [i]Hydrolysis of the ethyl ester. [j]Hydrolysis of the chloroethyl ester. [k]E was calculated from ee_s and percent conversion.

39, **44**, **45** from the tally because the enantioselectivity was <2. For the remaining 22 substrates, only 10 obeyed the size rule corresponding to 45% accuracy. This accuracy is similar to guessing alone.

High enantioselectivity requires a protonated amino group ($-NH_3^+$)

Additional evidence that size of the substituents at the stereocenter is not important comes from good to excellent enantioselectivity toward several carboxylic acids that contain similarly sized substituents. Purified ANL resolved three α-amino acids, pipecolic acid, proline and alanine, with an enantioselectivity of 41 to >100 even though the two substituents differ only in the charge of the substituents at the stereocenter. This difference corresponds to a $\Delta\Delta G^{\ddagger}$ of 2.2–2.7 kcal/mol.

The enantioselectivity of purified ANL toward 2-phenylglycine (**33** where R=phenyl, E=94) was much higher than for analogs without a protonated amino group. The enantioselectivity toward mandelic acid, **44**, and 2-phenylpropionic acid, **45**, was only 1.2–1.3. Similarly, the enantioselectivity of purified ANL toward alanine (**33** where R=methyl, E=41) was much higher than toward 2-chloropropionic acid, **49** (E=8).

To test whether high enantioselectivity requires a protonated amino group, we measured the enantioselectivity of ANL as a function of pH (Table 4). Both enantioselectivity and specific activity

Table 4. Influence of pH on the enantioselectivity of partially-purified ANL toward phenylalanine-methyl ester[a]

pH	rate U/mg[b]	% substrate protonated[c]	ee_s[d]	ee_p[d]	E[e]
4.02	0.047	>99	0.068	0.68	6±1
5.02	0.91	99	0.68	0.95	80±20
6.00	0.16	87	0.53	0.65	8±1
7.07	0.17	47	0.81	0.63	18±1
7.97	0.12	10	0.11	0.70	1.2±0.04
8.90	0.13	1	0.88	0.45	6±2[f]

[a]Reaction conditions: 15 units of lipase (by PNPA assay), room temperature, 25 mM substrate in 10 ml sodium acetate buffer (10 mM) for pH 4-6 and Tris-HCl buffer (10 mM) for pH 6-9. E values at pH 6 were similar in both buffers. Reactions were monitored by pH stat. [b]Unit = μmol of ester hydrolysed per minute. [c]The fraction of protonated Phe-methyl ester at the given pH was calculated using a pK_a = 7.00: Jencks, W.P.; Regenstein, J. *CRC Handbook of Biochemistry. Selected Data for Molecular Biology* CRC: Boca Raton, FL, **1986**, pp J187-J226. [d]Determined by HPLC with a Crownpak CR(+) column. The preferred enantiomer was L as established by the order of elution on the CR(+) column and compared to authentic samples; the D-enantiomer elutes first for both acid and methyl ester. [e]E was calculated from ee_s and ee_p as defined by Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299. Error limits are estimates. [f]Phenylalanine-methyl ester hydrolyzed spontaneously at pH 8.9 at a rate of 9.2 x 10^{-4} min^{-1} (6.6% hydrolysis during the 1 h reaction).The ee values were corrected for this hydrolysis. The uncorrected values are ee_s = 0.72 and ee_p = 0.40. Spontaneous hydrolysis was not detected at other pH values.

were highest at pH 5 toward phenylalanine-methyl ester. At higher pH both the rate and the enantioselectivity decreased by factors of 5.3–7.6 and 4–66, respectively. These decreases are consistent with the $-NH_3^+$ form of phenylalanine-methyl ester as the most reactive and best resolved form of the substrate. At pH 4 the enantioselectivity also dropped to 6, but we attribute this drop to partial denaturation of ANL. After incubation at pH 4 for 1 h, the lipase activity measured at pH 7.5 with PNPA as the substrate dropped by 50% for crude lipase and 25% for partially-purified ANL. Incubation at pH 5–9 showed no drop in activity by PNPA assay.

To confirm that ANL accepts other positively-charged substrates, we tested *S*-acetylthiocholine iodide, a thioester with an $-NMe_3^+$ group in the thiol portion. Indeed, crude ANL catalyzed the efficient hydrolysis of this substrate with a rate of 0.19 U/mg protein. This rate was 100 times faster than with lipase from *Candida rugosa* and 10,000 times faster than with lipase from *Pseudomonas cepacia*. However, ANL was 10,000 times slower at hydrolyzing this substrate than acetylcholine esterase.[8]

ANL is not an amidase

High enantioselectivity toward α-amino acids suggests that partially-purified ANL may be an amidase, not a lipase. However, we confirmed that partially-purified ANL is not an amidase by testing its ability to catalyze hydrolysis of D,L-phenylalaninamide (Table 5). Partially-purified ANL showed no detectable hydrolysis of the amide. In contrast, crude ANL catalyzed the slow hydrolysis of D,L-phenylalaninamide favoring the natural L-enantiomer with excellent enantioselectivity, suggesting that crude ANL contains a contaminating amidase. The activity of the crude lipase towards phenylalaninamide was 2–6 times faster at pH 4–5 than at pH 7.5 and 9. Researchers have previously isolated proteases with optimal activities at acidic pH from *Aspergillus niger*.[9]

Purified ANL is indeed a lipase because it catalyzed hydrolysis of insoluble substrates 100 times more efficiently than crude ANL: olive oil (0.29–0.62 U/mg protein for partially purified vs 0.005 U/mg protein for crude) and ethyl butyrate (0.44–2.42 for partially purified vs 0.02 U/mg protein for crude).

Discussion

Because all lipases follow the secondary alcohol rule in Figure 1, it is not surprising that ANL also follows the rule. However, the reliablity of the rule for ANL is significantly lower than for other lipases: 77% for ANL, but >90% for most other lipases. Although the lower reliablity may be an inherent characteristic of ANL (no sequence or structure is known for this lipase), it is most likely due to the contaminating amidase. This amidase may favor the opposite enantiomer of secondary alcohols

Table 5. Hydrolysis of D,L-phenylalaninamide by *Aspergillus niger* lipase[a]

	crude ANL				PP ANL
pH	activity[b] (U/mg)	ee_s	ee_p	E[c]	activity[b] (U/mg)
4.1	0.016	29	>99	>100	<0.0002
5.0	0.036	48	>99	>100	<0.0008
7.5	0.0068	63	>99	>100	<0.0003
8.9	0.0058	59	>99	>100	<0.0002

[a]Reaction conditions: 15 units of lipase, room temperature, 25 mM substrate in 10 ml sodium acetate buffer (100 mM) for pH 4.1 and pH 5 and sodium phosphate buffer (100 mM) for pH 7.5 and 8.9. Reactions were monitored by TLC (1:1; $CHCl_3$:MeOH; 1% NH_4OH) and terminated when product and starting material spots appeared approximately equal in intensity. [b]Unit/mg = μmol of amide hydrolysed per minute per mg of protein. [c]Enantiomeric excesses and degree of conversion were determined by HPLC (Crownpak CR(+) column). E was calculated from ee_s and ee_p as defined by Chen, C. S.; Fujimoto Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299. The preferred enantiomer was L according to the order of elution on the CR(+) column compared to authentic samples; the D-enantiomer elutes first for both acid and amide.

and thus account for the exceptions to the rule. A recent survey showed that lipases and the protease subtilisin favor the opposite enantiomers of secondary alcohols,[10] thus it is reasonable to suggest that the amidase in crude ANL may also favor the opposite enantiomer.

Many groups have identified contaminating hydrolases in crude ANL.[7,11,12] For example, Hofelman *et al.* identified esterases, proteases and amylases as well as a lipase. The main commercial use of ANL is in cheese-making to accelerate the ripening of flavors.[13] Both lipases and proteases contribute to this ripening, so there is no need for purer enzymes. For synthetic applications, however, a purer form of ANL would be useful.

Our survey showed ANL, unlike other lipases, readily accepts positively-charged substrates. Carrea *et al.* found that ANL was the best lipase for deprotection of cephalosporin derivatives containing a carboxylate near the reactive site.[12] This result suggests that ANL may also readily accept negatively-charged substrates.

A protonated α-amino substituent was the most important feature for high enantioselectivity of ANL toward carboxylic acids. ANL resolves small to medium sized α-amino acids with very good enantioselectivity, but larger amino acids react slowly with lower enantioselectivity. The L-enantiomer was always favored as shown in Figure 1. Many proteases resolve amino acids esters,[14] but ANL is the only lipase that shows high enantioselectivity toward a range of amino acid esters.

Although crude lipase from *Humicola lanuginosa* resolved α-amino acids and α,α-disubstituted α-amino acids, Liu *et al.* found that the true catalyst was an impurity. Because this impurity did not catalyze hydrolysis of *o*-nitrophenyl butyrate or olive oil, it was probably a protease.[15] Similarly, Houng *et al.* reported enantioselective hydrolysis of amino acid esters catalyzed by crude PPL, but again the true catalyst may be a contaminating protease.[16]

For carboxylic acids, a rule based on the size of the substituents did not work for either crude or purified ANL. This result suggests that electronic effects, specifically charge, control the enantioselectivity of ANL. We estimate that interactions of the lipase with the $-NH_3^+$ or $=NH_2^+$ group contribute a factor of 40 to 100, corresponding to a 2.2–2.7 kcal/mol difference in $\Delta\Delta G^{\ddagger}$. This estimate comes from the enantioselectivity of ANL toward substrates whose substituents at the stereocenter differ only in charge. Similarly, Rotticci *et al.* found that electronic effects influence the enantioselectivity of lipase B from *Candida antarctica* (CAL-B).[17] Replacing a bromo substituent by methyl changed the enantioselectivity of CAL-B by a factor of >30 (>2 kcal/mol in $\Delta\Delta G^{\ddagger}$) and the enantioselectivity either decreased or increased depending of the location of the substituent.

Another unique feature of ANL is that it catalyzes the enantioselective hydrolysis of cyclic amino acids — proline and pipecolic acid. This ability may be useful for synthesis of peptides containing cyclic amino acids. Although some proteases can accept proline, they also catalyze the hydrolysis of peptide bonds.[18]

Experimental section

General

Lipase from *Aspergillus niger* (AP-6) and *Pseudomonas cepacia* (LPL-80) were purchased from Amano International Enzyme Co. (Troy, VI). Lipase from *Candida rugosa* (type VII) and acetylcholine esterase (isolated from electric eel) were purchased from Sigma Chemical Co. (Oakville, Ontario). Chemicals were purchased from Sigma–Aldrich Chemical Co. (Oakville, ON) and used without further purification unless stated otherwise. D,L-β-Aminoisobutyric acid, **34**, and D,L-3-aminobutyric acid, **35**, were purchased from Fluka Chemical Co. (Oakville, ON). D,L-dimethyl tyrosine and D,L-2-aminotetralin-2-carboxylic acid, **37**, were provided by Dr P. Schiller, IRCM, Montréal. (±)-Ketoprofen chloroethyl ester, **46** and (±)-methyl-2-(4-chlorophenoxy)propanoate, **43**, were provided by Dr Ian Colton.

Ultrafiltration was performed with an Amicon ultrafiltration kit (Oakville, ON) under N_2 using a YM-10 filter. Melting points were taken on an Electrothermal melting point apparatus and were corrected. Protein concentrations were measured using a dye-binding assay from Bio-Rad (Missisauga, ON) with bovine serum albumin as the standard. Purified ANL was desalted with Bio-Gel P6 (Bio-Rad), a size exclusion gel. Enzyme assays were carried out on a Hewlett Packard 8452A diode array spectrophotometer equipped with a Neslab RTE-100 water bath temperature control unit. The rate of lipase-catalyzed ester hydrolysis was measured on a Radiometer RTS 822 pH stat. HPLC chiral stationary phase columns were purchased from Daicel Chemical Industries Ltd (Fort Lee, NJ). NMR spectra were recorded on either a Varian Gemini 200 MHz, Jeol 270 MHz or Unity 500 MHz NMR spectrometer.

Enzyme activity

Hydrolase activity was measured using *p*-nitrophenyl acetate (PNPA) as a substrate. An aliquot (5 μL) of enzyme solution was added to phosphate buffer (1.00 mL, pH 7.5, 10 mM), allowed to equilibrate to 25°C, followed by addition of an aliquot (5 μL) of PNPA (50 mM solution in 1:1 acetonitrile/ 10 mM phosphate buffer, pH 7.5). The initial rate of formation of *p*-nitrophenolate was monitored at 25°C, 404 nm for 30 seconds. Activity was calculated using the Beer–Lambert law, with a cell length of 1 cm, and extinction coefficient of 11,600 M^{-1} cm^{-1} which accounts for the incomplete ionization of *p*-nitrophenolate at pH 7.5. Crude ANL showed an activity of 48 U/g solid (1.7 U/mg protein) with this assay. U=μmol of ester hydrolysed per minute.

Activity of the partially-purified lipase towards olive oil (0.6–35 mM) and ethyl butyrate (9–250 mM) were measured at pH 5 (sodium acetate buffer, 10 mM) using a pH stat which maintained the pH at 5 by automatic titration with 0.1 N NaOH. Insoluble substrate remained during the assay to ensure interfacial activation.

Partial purification of ANL by precipitation with ammonium sulphate

The procedure was carried out according to Ng-Youn-Chen *et al.*[7] All steps during enzyme purification were carried out at 4°C. Crude lipase from *Aspergillus niger* (Amano AP-6, 32 g, 1500 U by PNPA assay) was stirred in Tris-HCl buffer (250 mL, 25 mmol, pH 7.5) for 3 h, then centrifuged (10,000 rpm, 20 min) to remove insoluble material. Solid ammonium sulphate (36 g, 25% saturation) was added to the stirring supernatant in small portions every 15 min over 4 h. The solution was stirred overnight. The resulting suspension was centrifuged (10,000 rpm, 20 min), the very small pellet was discarded, and additional ammonium sulphate (51 g, 55% saturation) was added in small portions over 4 h. The solution was stirred overnight then centrifuged (3000 rpm, 45 min). The supernatant was discarded and a brown pellet containing the lipase was dissolved in sodium acetate buffer (25 mL, 10 mM, pH 5). This solution was desalted with a size exclusion gel (BioGel-P6) using 10 mM phosphate buffer, 10 mM NaCl, pH 7 as eluent at a linear flow rate of 12.7 cm/h. Fractions were collected and assayed using the PNPA assay. Those fractions with lipase activity were pooled yielding 70 mL of solution containing 189 units (13% yield). The solution was concentrated by ultrafiltration with

an Amicon YM-10 membrane yielding 13 mL (14.5 U/mL, 4.25 mg protein/mL) of lipase solution. Partially-purified ANL solution showed a specific activity of 3.41 U/mg protein accounting for a two-fold increase in specific activity over the crude preparation, and an enantioselectivity of >100 towards phenylalanine-methyl ester HCl. The dark brown solution retained full activity when stored at 4°C with 0.02 wt/vol% NaN_3 solution as preservative for at least 3 months. With other lots of crude ANL, the best partial-purification used 25–48% saturation with ammonium sulphate.

Partial purification of ANL by anion-exchange chromatography

Several lots of crude ANL gave <10% yield when purified by ammonium sulphate precipitation. These lots were partially-purified by first precipitating ANL at 60% saturation with ammonium sulphate, and second, anion exchange chromatography. The 60% pellet (285 units, 19% yield) was dissolved in sodium acetate buffer (23 mL, 10 mM, pH 5) and desalted with a Bio-Gel P-6 column as described above. The fractions were assayed using the PNPA assay and those with lipase activity were pooled, yielding 27 mL of solution containing 236 units. The phosphate buffer was exchanged for Tris-HCl buffer (25 mM, pH 7.48) by ultrafiltration using an Amicon YM-10 membrane, for a final volume of 5 mL (4.00 U/mL, 2.18 U/mg protein).

The solution (2.5 mL) was then injected onto a MonoQ anion exchange column (MonoQ HR 5/5 column, Pharmacia, Baie d'Urfé, QC) equilibrated with Tris-HCl buffer (25 mM, pH 7.48) using a Pharmacia FPLC system. The column was eluted at a flow rate of 0.5 mL/min with the same buffer for 10 mL, followed by a linear gradient of 0–0.5 M NaCl over 70 mL, then eluted with 0.5 M NaCl for 10 mL. Fractions (2 mL) were collected and assayed by PNPA. Fractions 13–15 contained hydrolase activity. The procedure was repeated and fractions 13–15 from both runs were pooled, yielding 12 mL of solution containing 77 units (27% yield overall from the 60% pellet). ANL partially purified by this technique showed a specific activity of 7.74 U/mg protein accounting for a 4.5-fold increase in specific activity over the crude preparation and enantioselectivity of >100 towards phenylalanine-methyl ester HCl. The dark brown solution retained full activity when stored at 4°C with 0.02 wt/vol% NaN_3 solution as preservative for 1 month.

Synthesis of esters of carboxylic acids

Esters of all carboxylic acids were previously prepared in the literature. ^{1}H-NMR and ^{13}C-NMR data are provided because not all literature references included complete NMR data. Amino acid esters were purified by recrystallization from methanol/diethyl ether; all other esters were purified by flash chromatography.

D,L-*2-Phenylglycine-methyl ester-HCl* was prepared by Fischer esterification:[19] 98% (white solid); mp=129.1–130.1°C; R_f=0.51 (chloroform:methanol=95:5); ^{1}H-NMR (CD_3OD, 200 MHz) δ 3.82 (s, 1H), 4.88 (br s, 3H), 5.30 (s, 3H), 7.49 (m, 5H); ^{13}C-NMR (CD_3OD, 200 MHz) δ 54.9, 58.5, 129.3, 130.6, 131.3, 133.3, 169.6.

D,L-*Proline-octyl ester-HCl*, methyl ester of **32**, was prepared by Fischer esterification:[19] 74% (clear oil); R_f=0.81 (chloroform:methanol=50:50); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.87 (t, 3H), 1.45 (m, 10H), 2.11 (m, 2H), 2.39 (m, 2H), 3.43 (m, 2H), 4.10 (m, 1H).

2,6-Dimethyl-DL-*tyrosine-methyl ester-HCl* was prepared according to Pitzele *et al.*:[20] 70% (light brown solid); mp=139.4–140.5°C; R_f=0.88 (ethyl acetate:methanol=3:2, 1% NH_4OH); ^{1}H-NMR (D_2O, 270 MHz) δ 2.18 (s, 6H), 3.06 (m, 2H), 3.67 (s, 3H), 4.16 (t, 1H, *J*=8.2 Hz), 6.58 (s, 2H); ^{13}C-NMR (CD_3OD, 200 MHz) δ 21.6, 21.8, 32.5, 54.4, 54.6, 116.6, 123.3, 139.5, 157.1, 170.6.

O-*Me*-DL-*Tyrosine methyl ester-HCl* was prepared according to Moersch *et al.*:[21] 85% (white solid); mp=167–168°C; R_f=0.78 (ethyl acetate:methanol=3:2, 1% NH_4OH); ^{1}H-NMR (d_6-DMSO, 200 MHz) δ 3.10 (m, 2H), 3.68 (s, 3H), 3.75 (s, 3H), 4.21 (t, 1H, *J*=6.8 Hz), 6.90 (d, 2H, *J*=8.6 Hz), 7.17 (d, 2H, *J*=8.6 Hz); ^{13}C-NMR (CD_3OD, 200 MHz) δ 37.7, 54.2, 54.5, 56.2, 56.6, 115.7, 127.0, 131.5, 160.5, 170.0.

D,L-tert-*Leucine-methyl ester-HCl* was prepared according to Brenner *et al.*:[22] 91% (white solid);

mp=228.4–234.7°C (sublimes without melting); R_f=0.80 (ethyl acetate:methanol=3:2, 1% NH_4OH); ^{1}H-NMR (D_2O, 500 MHz) δ 1.07 (s, 9H), 3.29 (s, 3H), 3.72 (s, 1H). ^{13}C-NMR (CD_3OD, 500 MHz) δ 28.1, 34.8, 63.7, 182.9.

(±)-Methyl β-aminoisobutyrate-HCl, methyl ester of **34**, was prepared according to Brenner *et al.*:[22] 99% (white solid); mp=109.9–110.6°C; R_f=0.76 (chloroform:methanol=1:1); ^{1}H-NMR (D_2O, 200 MHz) δ 1.16 (d, 3H, *J*=7.68 Hz), 2.85–2.93 (m, 1H), 3.01–3.27 (m, 2H), 3.68 (s, 3H); ^{13}C-NMR (D_2O, 200 MHz) δ 17.9, 40.8, 44.8, 56.3, 178.0.

(±)-Methyl 3-aminobutyrate-HCl, methyl ester of **35**, was prepared according to Brenner *et al.*:[22] 96% (yellow oil); R_f=0.79 (methanol:chloroform=1:1); ^{1}H-NMR (D_2O, 200 MHz) δ 1.29 (d, 3H, *J*=6.9 Hz), 2.69 (d, 2H, *J*=6.1 Hz), 3.63–3.74 (m, 1H), 3.69 (s, 3H); ^{13}C-NMR (D_2O, 200 MHz) δ 21.6, 41.5, 48.0, 56.1, 180.1.

(±)-Methyl 2-aminotetralin-2-carboxylate, methyl ester of **37**, was prepared by the method of Obrecht *et al.*:[23] 48% (white solid); mp=131–132°C; R_f=0.68 (hexanes:ethyl acetate=3:1); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 1.92 (m, 1H), 2.16 (m, 1H), 2.76 (d, 2H, *J*=16 hz), 2.84 (m, 1H), 2.99 (m, 1H), 3.30 (d, 1H, *J*=16 hz), 7.06–7.13 (m, 4H). ^{13}C-NMR (CD_3OD, 200 MHz) δ 14.2, 25.3, 31.7, 39.7, 56.6, 61.2, 126.1, 128.8, 129.7, 132.8, 134.5, 176.2.

The *N*-Cbz derivatives **38** (R=Bn, Ph) were prepared from their respective methyl esters following a procedure by Bodansky:[24]

D,L-N-*Cbz-Phenylalanine-methyl ester:* 62% (yellow oil); R_f=0.82 (chloroform:methanol=95:5); ^{1}H-NMR ($CDCl_3$, 270 MHz) δ 3.10 (m, 2H), 3.71 (s, 3H), 4.65 (m, 1H), 5.08 (s, 2H), 5.21 (br d, 2H, *J*=7.9 Hz), 7.09–7.33 (m, 10H); ^{13}C-NMR ($CDCl_3$, 200 MHz) δ 39.6, 53.5, 55.9, 127.4, 128.3, 128.4, 128.8, 128.9, 129.5, 135.8, 136.4, 155.5, 171.7.

D,L-N-*Cbz-Phenyglycine-methyl ester*: 54% (white solid); mp=79.8–82.1°C; R_f=0.37 (hexanes:ethyl acetate=3:1); ^{1}H-NMR ($CDCl_3$, 270 MHz) δ 3.72 (s, 3H), 5.09 (s, 2H), 5.84 (d, 1H, *J*=7.2 Hz), 5.85 (br d, 2H, *J*=5.67 Hz), 7.17–7.35 (m, 10H); ^{13}C-NMR ($CDCl_3$, 200 MHz) δ 53.9, 59.0, 68.1, 127.4, 128.3, 128.4, 128.6, 128.8, 128.8, 129.1, 129.2, 136.3, 136.8, 155.2, 171.0.

We prepared the octyl esters of **47** and **48** and the 2-chloroethyl ester of **45** using a modified DCC coupling employing a water soluble coupling reagent (*N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide):[25]

(±)-2-Chloroethyl 2-phenylpropanoate, chloroethyl ester of **45**: 74% (yellow oil); R_f=0.60 (hexanes:ethyl acetate=3:1); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 1.54 (d, 3H, *J*=7.4 Hz), 3.64 (t, 2H, *J*=6 Hz), 3.77 (q, 1H, *J*=7 Hz), 4.3 (m, 2H), 7.2–7.4 (m, 5H); ^{13}C-NMR (CD_3OD, 200 MHz) δ 20.0, 42.7, 46.6, 65.2, 127.5, 127.8, 128.9, 140.2, 173.9.

(±)-Octyl tetrahydro-2-furoate, octyl ester of **47**: 69% (clear oil); R_f=0.59 (hexanes:ethyl acetate=3:1); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.86 (br t, 3H, *J*=1.13), 1.25 (br s, 12H), 1.62 (m, 2H), 1.94 (m, 3H), 2.28 (m, 1H), 4.08 (m, 2H), 4.12 (t, 2H, *J*=6.8 Hz), 4.39 (m, 1H); ^{13}C-NMR (CD_3OD, 200 MHz) δ 15.7, 24.1, 26.7, 27.3, 30.0, 31.6, 33.1, 66.0, 70.3, 173.2.

(±)-Octyl tetrahydro-3-furoate, octyl ester of **48**: 72% (clear oil); R_f=0.69 (hexanes:ethyl acetate=3:1); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.86 (br t, 3H), 1.27 (m, 12H), 1.60 (m, 2H), 2.1 (m, 2H), 3.07 (q, 1H, *J*=8 Hz), 3.85–4.04 (m, 4H), 4.11 (t, 2H, *J*=6.6 Hz); ^{13}C-NMR ($CDCl_3$, 500 MHz) δ 13.9, 22.4, 25.0, 25.6, 28.4, 28.9, 30.0, 30.6, 31.5, 64.8, 69.1, 72.4, 77.7, 103.0, 161.9.

(±)-Methyl-2-chloropropanoate, methyl ester of **49**. (±)-2-Chloropropionyl chloride was added dropwise to a stirring solution of methanol in an ice bath, under an atmosphere of N_2. The reaction was allowed to warm to room temperature and was stirred for 1 h. The reaction was then neutralized with saturated sodium bicarbonate, extracted with diethyl ether (3×15 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*: 89% (yellow oil); R_f=0.51 (hexanes:ethyl acetate=2:1); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 1.69 (d, 3H, *J*=6.9 Hz), 3.79 (s, 3H), 4.41 (q, 1H, *J*=6.9 Hz); ^{13}C-NMR ($CDCl_3$, 200 MHz) δ 23.0, 53.5, 54.1, 170.3.

General procedure for ANL-catalyzed hydrolyses of amino acid esters, esters of ***32–37***

Aspergillus niger lipase (25 units) was added to sodium acetate buffer (8 mL, 10 mM, pH 5) and stirred for 1 h to ensure complete dissolution. D,L-Amino acid esters (100 mg) dissolved in sodium acetate buffer (1 mL, 10 mM, pH 5) were added and the rate of hydrolysis was monitored by pH stat which maintained the pH at 5 by automatic titration with 0.1 N NaOH. Reactions were usually terminated at approximately 40% conversion, as noted on the pH stat, by removing the enzyme by ultrafiltration with a YM-10 Amicon membrane. The aqueous extract containing both the starting ester and product acid was concentrated *in vacuo*. The enantiomeric purities of the acid and ester were measured as described below and the enantiomeric ratio, E, was calculated according to Sih.[26] When the enantiomeric excess of both starting ester and product acid could not be determined in a single HPLC run, the starting ester and acid were separated by adjusting the solution to pH 8 with saturated $NaHCO_3$ and extracting the ester with ethyl acetate (3×15 mL). Ethanol was added dropwise during the workup to break up emulsions when necessary. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated *in vacuo*, producing the ester. The aqueous phase was concentrated *in vacuo*, yielding the product acid. There was no detectable chemical hydrolysis of the amino acid esters at pH 5.

General procedure for ANL-catalyzed hydrolyses of carboxylic acid esters, esters of ***38, 39, 43–49***

The procedure was similar to above, with the following changes. (±)-Carboxylic acid esters (100 mg) were dissolved in 2 mL diethyl ether and added to the stirring enzyme solutions. The stirring reactions were sealed with Parafilm to prevent appreciable evaporation of the ether layer during the reactions. Reactions were terminated at a convenient conversion by adjusting the solution to pH 1 using 1 N HCl and the product acids and starting esters were extracted with diethyl ether (3×20 mL). The starting ester and acid were separated by adjusting the solution to pH 8 with saturated $NaHCO_3$ and extracting the ester with ethyl acetate (3×15 mL). The aqueous layer was adjusted to pH 2 and the acid extracted with ethyl acetate (3×15 mL). Ethanol was added dropwise during workup to break up the emulsion and inactivate the enzyme. Both extracts were dried with Na_2SO_4, filtered, and concentrated *in vacuo*. The enantiomeric excesses of the acids and esters were measured as described below and the enantiomeric ratio, E, was calculated according to Sih.[26] There was no detectable chemical hydrolysis of the above substrates at pH 5.

Determination of enantiomeric purity by HPLC using a chiral stationary phase

High performance liquid chromatography (HPLC) was performed on a Spectra Physics liquid chromatograph, model 8800 equipped with a Spectra FOCUS forward optical scanning detector, SP8880 autosampler and Spectra Physcis software. The analytical chiral columns used for enantiomeric resolution were a Chiralpak AD amylose derivatized column, a Chiralcel OD column, a Chiralpak WH column and a Crownpak CR(+) crown ether derivatized column (Daicel Chemical Industries Ltd, Fort Lee, NJ). Enantiomeric excesses were measured as described below and the enantiomeric ratio, E, was calculated. The area of the peaks was measured either by electronic integration or cut and weigh when electronic integration gave unequal areas for the racemate.

Enantiomers of the proline, **32**, were analysed with a Chiralpak WH column using 0.25 mM $CuSO_4$ as eluting buffer with the detector set at 254 nm: 50°C, 1.5 mL/min, k_L'=2.23; k_D'=3.90; α=1.75; R_s=1.77. Proline was dissolved in eluting buffer (1 mg/mL), and neutralized to pH 7 with dilute NaOH prior to injection. Proline esters were hydrolysed with aqueous NaOH for analysis. The absolute configuration was established by comparison with an authentic sample of L-proline.

Enantiomers of α- and β-amino acids, **33–35**, their methyl esters, and phenylalaninamide were separated with a Crownpak CR(+) modified crown ether column, using aq. $HClO_4$ solution as eluent, with the detector set at 200 nm. Concentrations of 0.1 mg compound/mL mobile phase were injected for analysis except for alanine, 1.0 mg/mL. Absolute configurations were inferred from the order of elution on a CR(+) column for which the D-enantiomer always elutes first and by the reported orders of

elution in literature for the compounds below.[27] CAUTION: Aqueous solutions containing perchloric acid should not be evaporated and can explode if heated.

Alanine: 0°C, aq. $HClO_4$ pH 1.5, 0.4 mL/min, k_D'=0.75; k_L'=2.30; α=3.07; R_s=6.00.

Alanine-methyl ester: 25°C, aq. $HClO_4$ pH 2, 0.3 mL/min, k_D'=0.38; k_L'=0.70; α=1.84; R_s=1.8.

Methionine: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=0.82; k_L'=1.6; α=1.95; R_s=2.12.

Methionine-methyl ester: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=1.79; k_L'=4.04; α=2.26; R_s=3.00.

Phenylalanine: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=3.93; k_L'=5.20; α=1.32; R_s=3.86.

Phenylalanine-methyl ester: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=8.29; k_L'=10.09; α=1.22; R_s=2.76; and at 5°C, aq. $HClO_4$ pH 1.5, 0.8 mL/min, k_D'=11.7; k_L'=20.9; α=1.78; R_s=3.87.

Phenylalaninamide: 5°C, aq. $HClO_4$ pH 1.5, 0.8 mL/min, k_D'=7.66; k_L'=14.8; α=1.93; R_s=5.05.

2-Phenylglycine: 40°C, aq. $HClO_4$ pH 2, 1.0 mL/min, k_D'=1.37; k_L'=4.46; α=3.25; R_s=10.1.

2-Phenylglycine-methyl ester: 40°C, aq. $HClO_4$ pH 2, 1.0 mL/min, k_D'=3.89; k_L'=10.18; α=2.62; R_s=10.0.

Tyrosine: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=2.11; k_L'=2.84; α=1.34; R_s=1.09.

Tyrosine-methyl ester: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=4.27; k_L'=6.24; α=1.46; R_s=1.62.

4-O-Me-Tyrosine: 25°C, 1.2 mL/min, aq. $HClO_4$ pH 2 k_D'=6.62; k_L'=8.73; α=1.31; R_s=2.80.

4-O-Me-Tyrosine-methyl ester: 25°C, aq. $HClO_4$ pH 2, 1.2 mL/min, k_D'=14.72; k_L'=19.88; α=1.35; R_s=1.89.

2,6-dimethyltyrosine: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=10.19; k_L'=11.29; α=1.11; R_s=0.84.

2,6-dimethyltyrosine-methyl ester: 25°C, aq. $HClO_4$ pH 2, 0.8 mL/min, k_D'=10.89; k_L'=12.11; α=1.11; R_s=1.08.

Tryptophan: 25°C, aq. $HClO_4$ pH 2, 1.2 mL/min, k_D'=11.61; k_L'=14.32; α=1.23; R_s=1.20.

Tryptophan-methyl ester: 25°C, aq. $HClO_4$ pH 2, 1.2 mL/min, k_D'=24.61; k_L'=32.47; α=1.32; R_s=1.40.

β-Aminoisobutyic acid, **34**: 0°C, aq. $HClO_4$ pH 1, 0.4 mL/min, k_D'=3.48; k_L'=4.15; α=1.19; R_s=1.04.

Methyl β-aminoisobutyrate: 0°C, aq. $HClO_4$ pH 1.5, 0.4 mL/min, k_D'=3.03; k_L'=4.00; α=1.32; R_s=1.63.

3-Aminobutyric acid, **35**: 0°C, aq. $HClO_4$ pH 1.5, 0.4 mL/min, k_D'=2.05, k_L'=2.64, α=1.29; R_s=1.29.

Methyl 3-aminobutyrate: 0°C, aq. $HClO_4$ pH 1.5, 0.4 mL/min, k_D'=4.19; k_L'=5.17; α=1.23; R_s=1.73

Enantiomers of **43**, 2-(4-chlorophenoxy)propionic acid, were separated using a Chiralpak AD column as described previously by Colton *et al.*:[28] 254 nm, 25°C, hexanes:isopropanol:trifluoroacetic acid=95:5:1, 0.5 mL/min, k_S'=2.13; k_R'=2.80; α=1.31; R_s=3.78. The absolute configuration was established by comparison with the reported order of elution by the column manufacturer. The ester of **43** was hydrolyzed to **43** with 1.5 equivalents of aqueous NaOH prior to analysis.

Enantiomers of **38** (R=Bn, Ph) and **45** were separated using a Chiralcel OD column. The ester of **45** was hydrolyzed to **45** with 1.5 equivalents of aqueous NaOH prior to analysis. The absolute configurations were established by comparison with the reported order of elution by the column manufacturer.

2-Phenylpropionic acid, **45**: 254 nm, 25°C, hexanes:isopropanol:formic acid=98:2:1, 0.5 mL/min, k_R'=3.35; k_S'=4.05; α=1.21; R_s=2.13.

N-*Cbz-Phenylalanine*: 254 nm, 25°C, hexanes:isopropanol:trifluoroacetic acid=79:20:1, 0.5 mL/min, k_D'=1.84; k_L'=2.13; α=1.16; R_s=1.52.

N-*Cbz-Phenylalanine-methyl ester*: 254 nm, 25°C, hexanes:isopropanol=80:20, 0.5 mL/min, k_L'=4.74; k_D'=5.34; α=1.13; R_s=1.47.

N-*Cbz-Phenylglycine-methyl ester*: 254 nm, 25°C, hexanes:isopropanol=80:20, 0.5 mL/min, k_S'=2.87; k_R'=3.37; α=1.17; R_s=2.00.

Determination of enantiomeric purity by ^{1}H-NMR

The racemic esters of **47** and **48** were dissolved in $CDCl_3$ and the ^{1}H-NMR spectra were obtained using a 200 MHz spectrometer. Solid tris[(3-heptafluoropropylhydroxymethylene)-(+)-camphorato]

europium(III), $Eu(hfc)_3$, was added portion-wise until separate signals for the $COOCH_2$ methylene triplet of the racemic octyl esters were obtained. The triplets of **47** were separated by 2.4 Hz and those of **48** were separated by 3.7 Hz. The number of equivalents of shift reagent necessary to obtain the separations was then added to the esters for which the enantiomeric purity was to be determined. The absolute configurations were not determined.

Determination of enantiomeric purity by gas chromatography using a chiral stationary phase

Gas chromatography was performed on a Varian 589-Series II gas chromatograph equipped with chiral stationary phases.

Enantiomers of methyl mandelate (methyl ester of **44**) were separated on a Chiraldex G-TA30 column (Astec Inc., Whippany, NJ) which contained 2,6-di-*O*-pentyl-3-*O*-trifluoroacetyl derivative of γ-cyclodextrin as the stationary phase: 110°C, split ratio 115:1, k_S'=17.8; k_R'=18.4; α=1.03; R_s=0.91. Product acid was converted to the ester derivative with ethereal CH_2N_2 for analysis. The absolute configuration was established by comparison with the reported order of elution by the column manufacturer.

Enantiomers of the methyl ester of **49** were separated using a Chirasil-DEX CB column (Chrompack Inc., Raritan, NJ) which contained a polydimethylsiloxane derivative of β-cyclodextrin as the stationary phase: 60°C, k_S'=3.8; k_R'=4.47; α=1.17; R_s=5.00. Product acid **49** was converted to the methyl ester for analysis by dissolution in methanol containing a catalytic amount of sulfuric acid. The absolute configuration was established by comparing the order of elution with an authentic sample of (*S*)-methyl-2-chloropropanoate.

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