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Inhibition of Lipases by Phosphonates

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Abstract—Ethyl hexylchlorophosphonate and analogues thereof were investigated as inhibitors of lipases. Both microbial and mammalian lipases were irreversibly inhibited. The inhibition could be monitored by *p*-nitrophenol release from the corresponding ethyl *p*-nitrophenyl hexylphosphonate inhibitor. Quantitative analysis of the data indicated that a 1:1 lipase–inhibitor complex was formed during inhibition. Enantioselective inhibition was found for the lipases derived from *Candida antarctica* and *Rhizomucor miehei* using pure enantiomers of ethyl *p*-nitrophenyl hexylphosphonate as inhibitors. Using the same inhibitor, reversed enantioselectivity was found for the protease α-chymotrypsin as compared to the two lipases.

Introduction

Organophosphorus compounds, such as diisopropyl fluorophosphate (DFP) (**1**) and diethyl *p*-nitrophenyl phosphate (DPNP) (**2**) are phosphorylating agents known to be powerful inhibitors of serine proteases (Figure 1). They have been shown to specifically and irreversibly react with the active site serine in these enzymes. Thus, these phosphorylating agents have been invaluable tools in the investigation of mechanistic and structural features of proteases.[1–3]

In the case of lipases, several attempts to find efficient inhibitors have been made.[4] The use of classical protease inhibitors such as DPNP (**2**) indicates that lipases belong to the same group of serine hydrolases as proteases even though inhibition occurs only at very high inhibitor concentrations.[5,6]

Recently, we found ethyl hexylchlorophosphonate to be an efficient, irreversible inhibitor of *Rhizomucor miehei* lipase.[7] Furthermore, reactive phosphonates were shown to inhibit human pancreatic and gastric lipases.[8] In the following, the relationship between structure, hydrophobicity (chain length), reactivity (leaving group), and stereochemistry of phosphonate inhibitors and their ability to inhibit lipases are discussed.

Results

Inhibited enzymes

A selection of enzymes was tested for inhibition by ethyl hexylchlorophosphonate (Table 1). Inhibitions were performed in a two phase system where the inhibitor was added in hexane to an aqueous solution of enzyme. Under these conditions, the lipases were efficiently inhibited using 0.01–10 mM, (total concentration in the suspension), of the phosphonate inhibitor. The microbial and mammalian lipases tested were inhibited. Similarly, serine proteases and esterase were efficiently deactivated. A non-serine hydrolase, phospholipase A_2, was not inhibited.

The inhibition of lipases derived from *R. miehei* and *Candida antarctica*, respectively, was studied in more detail. Both enzymes were inhibited at approximately equivalent lipase and inhibitor concentrations.

Chain length of inhibitor

A series of ethyl alkylchlorophosphonates (**3a–e**) (Figure 1, Table 2) with different chain length was prepared in order to study the influence of changes in size and hydrophobicity of the inhibitor on inhibition. The lipases were inhibited with approximately equivalent inhibitor and enzyme concentrations, irrespective of the chain length, using *C. antarctica* B and *R. miehei* lipases.

Inhibitor analogues

A chlorine leaving group was substituted for other possible leaving groups (**3f–i**), in order to find a more stable and convenient inhibitor (Table 2). The inhibition was followed by lipase activity determination in the two phase system described in the experimental part. Only ethyl *p*-nitrophenyl hexylphosphonate (**3f**) inhibited both lipases, but *R. miehei* lipase was also inhibited by ethyl hexylphosphonic acid (**3i**), (10 mM). Diethyl hexylphosphonate (**3g**) and ethyl 2,2,2-trichloroethyl hexylphosphonate (**3h**) in a total concentration of 10 mM in the incubation mixture did not inhibit the lipases significantly within 4–5 h.

Active site titration

Inhibition of *C. antarctica* lipase B by an excess of ethyl *p*-nitrophenyl hexylphosphonate gave a linear relationship between *p*-nitrophenol release and enzyme concentration (Figure 2). A mol *p*-nitrophenol to mol enzyme ratio of 0.7–0.8 was obtained.

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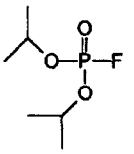

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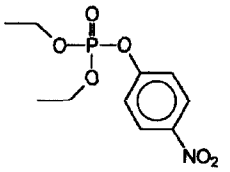

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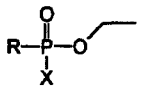

3

3	a	b	c	d	e	f
R =	C_4H_9	C_6H_{13}	C_8H_{17}	$C_{10}H_{21}$	$C_{12}H_{25}$	C_6H_{13}
X =	Cl	Cl	Cl	Cl	Cl	$C_6H_5NO_3$

	g	h	i
R =	C_6H_{13}	C_6H_{13}	C_6H_{13}
X =	C_2H_5O	$C_2H_2Cl_3O$	OH

Figure 1. Phosphorous-containing inhibitors.

Table 1. Enzyme inhibition by ethyl hexylchlorophosphonate. [Enz] and [Inh] is the concentration of enzyme and inhibitor in the incubation mixture. The inhibitor concentrations given represent the concentration needed for full inhibition under the incubation time. Details are given in the experimental section.

Enzyme	Inhibition	[Enz]	Incubation time (min)	[Inh] (mM)
Lipases				
C. cylindracea	+	1 OD_{280}	90	0.3
H. lanuginosa	+	1 OD_{280}	35	0.6
P. cepacia	+	1 OD_{280}	10	1
C. antarctica A	+	0.8 OD_{280}	75	0.06
C. antarctica B	+	0.03 mM[d]	45	0.023
R. miehei	+	0.03 mM[d]	100	0.014
Guinea pig pancreatic	+	1 OD_{280}	90	1
Human pancreatic	+	0.6 mg/mL	15	2.5
Bovine lipoprotein[a]	+		15	10[b]
Proteases				
Trypsin	+	1 mg/mL	15	0.4
Subtilisin	+	1 mg/mL	15	0.4
Phospholipase A_2	-	1 mg/mL	60	1
Hog liver esterase[c]	+	1 mg/mL	13	0.1

+ = Full inhibition, – = no inhibition.
a) The concentration was not determined.
b) Only two incubation mixtures were tested, one without inhibitor and one with inhibitor (10 mM).
c) Inhibitor was added dissolved in DMSO.
d) Extinction coefficients are only known for *C. antarctica* and *R. miehei* lipases.

Table 2. Inhibition of *R. miehei* and *C. antarctica* B lipases by phosphonate inhibitors **3a–i**.

Compound	*R. miehei* lipase Inhibition	Conditions:[a] [Inh] (mM) / Incubation (min)	*C. antarctica* B lipase Inhibition	Conditions:[a] [Inh] (mM) / Incubation (min)
3a	ND		+	0.06 / 5
3b	+	0.06 / 60	+	0.03 / 45
3c	+	0.06 / 125	+	0.03 / 25
3d	+	0.03 / 55	+	0.03 / 35[b]
3e	+	0.06 / 30	+	0.03 / 45[b]
3f	+	10 / 2	+	10 / 2
3g	-	10 / 240	-	10 / 240
3h	-	10 / 240	-	10 / 240
3i	+	10 / 120	-	10 / 240

+ = Full inhibition, – = no inhibition.
ND = not determined.
a) Conditions: enzyme ([Enz] = 1 mg/mL equal to OD_{280} = 1) dissolved in water. [Inh] is the concentration of inhibitor in the incubation mixture. Details are given in the experimental section.
b) The minimum time required for full inhibition. This was only determined for these two samples. For other samples the minimum time may be shorter than the incubation time.

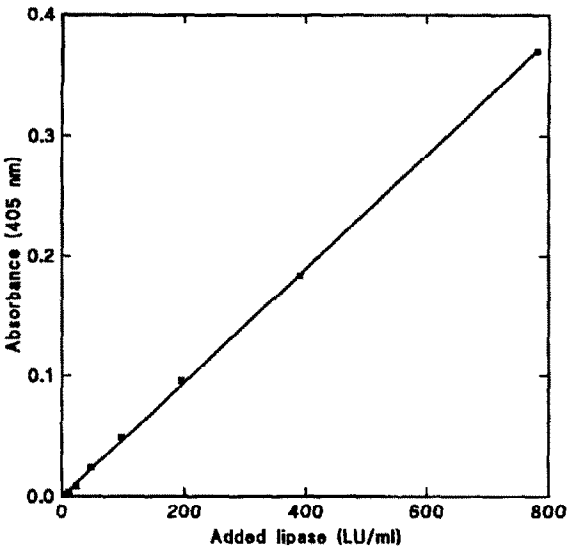

Figure 2. Titration of *C. antarctica* B lipase with ethyl *p*-nitrophenyl hexylphosphonate. Absorbance at 405 nm in a mixture of lipase and inhibitor versus added lipase. An excess of inhibitor was used.

Inhibitor kinetics and stereochemistry

The pure enantiomers of ethyl *p*-nitrophenyl hexylphosphonate (**3f**) were obtained using chiral HPLC. No spontaneous hydrolysis of the pure enantiomers was seen under the conditions used for inhibition.

The inhibition of *C. antarctica* lipase B, *R. miehei* lipase, and α-chymotrypsin by the two enantiomers of (**3f**) was followed in a mixture of enzyme, inhibitor and substrate. The reaction rate was followed spectrophotometrically.

The kinetic calculations are based on the two equations 1 and 2

$$Enz + Subs \rightleftharpoons Enz{*}Subs \rightarrow Enz + Pro \quad (1)$$

$$Enz + InhX \underset{}{\overset{K_d}{\rightleftharpoons}} Enz{*}InhX \overset{k_2}{\rightarrow} Enz - Inh + X \quad (2)$$

where K_d is the dissociation constant for the enzyme–inhibitor complex and k_2 is the rate constant of this complex. Assuming pseudo-first-order kinetics for the reaction between enzyme and substrate and between enzyme and inhibitor, K_d and k_2 can be obtained according to equation 3 by plotting the reciprocal apparent overall pseudo-first-order rate constant for the reaction between enzyme and inhibitor, k_{obs}^{-1}, against the reciprocal inhibitor concentration $[Inh]^{-1}$ (Figure 3A). For the derivation of this equation see appendix and reference.[9]

$$\frac{1}{k_{obs}} = \frac{K_d}{k_2} \times \frac{1}{[Inh]} + \frac{1}{k_2} \quad (3)$$

Enantioselective inhibition was observed for the *C. antarctica* and *R. miehei* lipases when the pure enantiomers of ethyl *p*-nitrophenyl hexylphosphonate (**3f**) were used as inhibitors (Figures 3A, 3B). Only results for *C. antarctica* B lipase could be used quantitatively as only this enzyme gave an absorbance which was linear with time when no inhibitor was present (Figure 3A). The results for *R. miehei* lipase and α-chymotrypsin were used only qualitatively (Figures 3B, 3C).

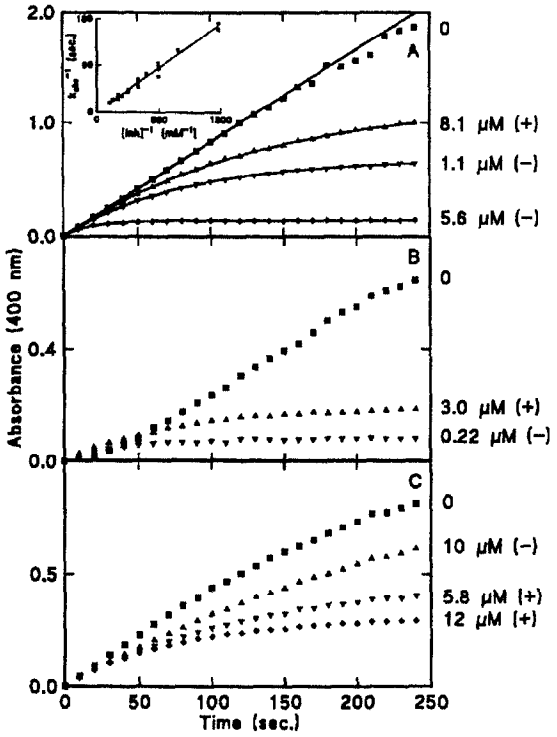

Figure 3. Enantioselective inhibition of *C. antarctica*, *R. miehei*, and α-chymotrypsin with ethyl *p*-nitrophenyl hexylphosphonate. The absorbance is followed in a mixture of enzyme, inhibitor and substrate; the inhibitor is omitted in the control sample (0). Inhibitor concentration together with enantiomer, (+) or (−), is given for each curve. Curve (A) *C. antarctica* lipase B. The inserted curve shows the fit to equation 3. The slope of the inserted curve gives k_2/K_d. Curve (B) *R. miehei* lipase. Curve (C) α-chymotrypsin.

We found a reversed enantioselectivity for the inhibition of α-chymotrypsin as compared with *C. antarctica* and *R. miehei* lipases when using the pure enantiomers of ethyl *p*-nitrophenyl hexylphosphonate (Figure 3 A, B, C).

The inhibition of *C. antarctica* B lipase was followed spectrophotometrically using *p*-nitrophenyl acetate as substrate (Figure 3A). The kinetic determinations confirmed a pseudo-first-order reaction between lipase and inhibitor. Likewise, the hydrolysis of substrate was directly proportional to the enzyme concentration, thus a first-order reaction.

An attempt to determine the Michaelis–Menten constant, K_m, for the reaction between *p*-nitrophenyl acetate and *C. antarctica* B lipase did not succeed because of the low solubility of the substrate. Thus, equation 3 was used to determine K_d, k_2, and k_2/K_d.

A significant difference in the bimolecular reaction constant, k_2/K_d, for the reaction of *C. antarctica* lipase with the two enantiomers of the inhibitor (**3f**) was found. The (−)-enantiomer reacted approximately 10 times faster with the lipase than the (+)-enantiomer (Table 3). The (−)-enantiomer (**3f**) showed the smallest K_d and the largest rate constant k_2 for the irreversible inhibition reaction as compared to the (+)-enantiomer of (**3f**) (Table 3).

Discussion

Recently, the crystal structures of the lipases from *C. antarctica*, *R. miehei*, *Geotrichum candidum* and human pancreas were reported.[10–13] In the crystal structure of the native *R. miehei* lipase, the active site was found to be covered by a surface loop of the protein acting as a 'lid'. The lipases were therefore hypothesized to undergo a conformational change before being catalytically active. This was substantiated by analysis of the crystal structure of a *R. miehei* lipase inhibited by two different inhibitors, in which the covering 'lid' had been moved to accommodate the inhibitors.[7,14]

The lipase inhibitor, ethyl hexylchlorophosphonate (**3b**) used in one of the two studies was found to be more active towards lipases than DFP (**1**) and DPNP (**2**). Furthermore, the chloro-phosphonate inhibitor (**3b**) was found to be covalently bound to the active serine, the hydrophobic side chain being placed in a hydrophobic pocket. The oxygen corresponding to the carbonyl oxygen in natural substrates was stabilized by hydrogen bonding to the protein backbone. It was suggested that the enzyme–inhibitor complex simulates the tetrahedral intermediate formed during lipase-catalyzed hydrolysis of natural substrates.[7]

The prototype of the inhibitors used in this study, ethyl hexylchlorophosphonate, was shown to be a general

Table 3. Kinetic data for inhibition of *C. antarctica* lipase B with the enantiomers of ethyl *p*-nitrophenyl hexylphosphonate.

Enantiomer[a]	K_d (mM)	k_2 (s^{-1})	k_2/K_d
(+)-**3f**	0.048 ± 0.006	0.051 ± 0.006	1.07 ± 0.03
(+)-**3f**	0.046 ± 0.014	0.052 ± 0.016	1.13 ± 0.08
(-)-**3f**	0.007 ± 0.002	0.08 ± 0.02	11.4 ± 0.4
(-)-**3f**	0.013 ± 0.005	0.16 ± 0.06	12.9 ± 0.7

k_2/K_d is the bimolecular reaction constant, K_d is the dissociation constant for the enzyme inhibitor complex and k_2 is the rate constant of this complex. The constants are calculated using equation 3 and are given together with their 70 % confidence limits.
a) Duplicate runs.

inhibitor of serine hydrolases such as proteases, an esterase and both microbial and mammalian lipases (Table 1). Phospholipase A_2 was the only enzyme tested which was not inhibited, which could be anticipated since phospholipase A_2 does not belong to the group of serine hydrolases.[15]

The inhibition of lipases is irreversible and the complex formed is very stable. Only the best leaving groups, chloride and *p*-nitrophenol achieved efficient inhibition of lipases from *R. miehei* and *C. antarctica*. Less reactive alkyl phosphonates, e.g. diethyl hexylphosphonate do not inhibit, or are hydrolyzed by, the lipases (Table 2). The corresponding acid (**3i**) only inhibits *R. miehei* lipase which may be due to a surfactant effect leading to denaturation of the enzyme.

All ethyl alkylchlorophosphonates tested inhibit lipases in nearly equal inhibitor and enzyme concentrations ([Enz] = [Inh]), irrespective of the alkyl chain length (Table 2). This correlates well with findings in the crystal structure[7] where the hydrophobic cleft seems flexible enough to accommodate both short and long chain fatty acids. The effective inhibition at low inhibitor concentrations is probably due to favorable interactions of these hydrophobic phosphonate inhibitors in the active site as well as the possibility of interfacial activation of the lipase. However, a reliable determination of the kinetics for the inhibition of the lipases with the labile chlorophosphonates in this two phase hexane–water system was not practically possible.

The above problems in determining the inhibitor kinetics prompted us to look for an alternative kinetic set-up. The particular aim for this was the determination of the stereoselectivity in the inhibition. We chose a solubilized system, (water–acetonitrile, 96–4 v/v), using a soluble inhibitor, ethyl *p*-nitrophenyl hexylphosphonate, and *p*-nitrophenyl acetate as substrate. This method was found appropriate since it was possible to follow the relatively fast inhibition reaction using only small amounts of enzyme and inhibitor. Unfortunately, we could not use longer chain inhibitors due to low solubility.

The stoichiometry of the reaction between *C. antarctica* lipase B and ethyl *p*-nitrophenyl hexylphosphonate was investigated by measuring the amount of released *p*-nitrophenol spectrophotometrically (Figure 2). The calculated ratio of released *p*-nitrophenol to enzyme was 0.7–0.8, strongly indicating the formation of a 1:1 complex.

Candida antarctica and *R. miehei* lipases were stereoselectively inhibited by the (–)-ethyl *p*-nitrophenyl hexylphosphonate enantiomer. This is also in good agreement with the crystal structure of the lipase–inhibitor complex of *R. miehei* lipase where only the *R*-enantiomer is found covalently bound to the active site serine even though inhibition was performed with a racemic inhibitor in excess.[7]

Our kinetic determinations using *C. antarctica* B lipase, and the pure enantiomers of ethyl *p*-nitrophenyl hexylphosphonate (**3f**) showed a 10 times faster inhibition by the (–)-enantiomer than by the (+)-enantiomer of (**3f**), (k_2/K_d), (Table 3). This may be explained by both a difference in binding and reactivity. Thus, the (–)-enantiomer had both a better affinity (lower K_d) and was reacting faster (higher k_2) than the (+)-enantiomer, which makes the (–)-enantiomer a better inhibitor.

In our attempts to study the kinetics for *R. miehei* lipase we were only able to obtain a qualitative measurement for the selectivity (Figure 3). This may be due to the difference in substrate selectivity of *R. miehei* lipase as compared to *C. antarctica* B lipase, which has a broader selectivity for short chain, water soluble substrates, than *R. miehei* lipase.

Similar investigations of the stereoselectivity of the protease α-chymotrypsin have been performed using *O*-alkyl *p*-nitrophenyl methylphosphonates as inhibitors. In that study an *O*-alkyl chain length dependent enantioselectivity was found.[16]

The inhibition is believed to proceed with inversion of configuration at the phosphorous atom. This would be in good agreement with earlier results where α-chymotrypsin is shown to be phosphorylated by a cyclic phosphate triester via inversion of configuration.[17]

In our investigation, using ethyl *p*-nitrophenyl hexylphosphonate inhibitor, we found a reversed enantioselectivity for the protease α-chymotrypsin versus *C. antarctica* and *R. miehei* lipases. This difference was anticipated from a comparison of the crystal structures of *R. miehei* and *C. antarctica* lipases with the α-chymotrypsin structure.

The active site, comprising the hydrophobic pocket, binding site and catalytic triad has the same orientation in the recently determined crystal structure of *C. antarctica* lipase[9] as in the inhibited form of *R. miehei* lipase.[7] Comparison of this spatial orientation of the active site in the lipases with the well defined active site of α-chymotrypsin[18–21] shows an opposite orientation of the active site in the protease versus the lipases. Thus, when the hydrophobic binding pockets of *R. miehei* lipase and α-chymotrypsin are placed in the same plane and point in the same direction starting from the active site serine, the catalytic triads and oxyanion holes become essentially placed in opposite directions in space for the two enzymes (Figure 4). This means in this case that the *R*-enantiomer of alkyl hexylphosphonate fits best in the lipase and the *S*-enantiomer in α-chymotrypsin.

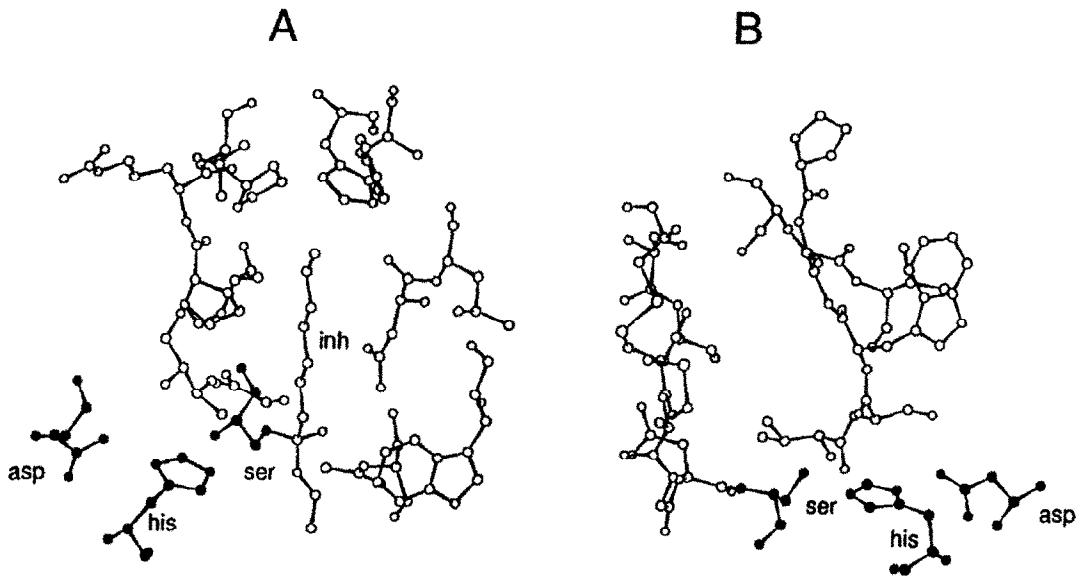

Figure 4. Comparison of the active sites in the inhibited *R. miehei* lipase (A) and α-chymotrypsin (B) as determined by X-ray crystallography. The enzymes are placed looking into the active site with the hydrophobic pockets in the plane of the paper pointing upwards from the active serine. (A) Active site region of *R. miehei* lipase inhibited with ethyl hexylchlorophosphonate.[7] (B) Active site region of α-chymotrypsin. The X-ray structure of α-chymotrypsin was taken from Brookhaven data bank. An X-ray structure of inhibited α-chymotrypsin is reported but the 3D coordinates are not available from Brookhaven data bank.[21]

Experimental

Synthesis of inhibitors

^{1}H NMR Spectra were recorded on a Bruker AC 300 P spectrometer using TMS as internal standard. Separation of the enantiomers of ethyl *p*-nitrophenyl hexylphosphonate was performed by HPLC using a Shimadzu LC-4A equipped with a Chiralpak AS 250 × 4.6 mm column from Daicel Chemical Industries Ltd. Short path (bulb-to-bulb) distillations were performed using a Büchi GKR-50. Optical rotations were measured using a Perkin-Elmer 241 polarimeter.

The chlorophosphonate inhibitors (**3a–e**) were prepared in two steps from triethyl phosphine and alkyl bromide. The diethyl alkylphosphonates thus obtained were subsequently monochlorinated by treatment with phosphorus pentachloride. This mono chloride served as intermediate to inhibitor analogues with different leaving groups.

A typical procedure for the preparation of ethyl alkylchlorophosphonate was as follows:

Diethyl hexylphosphonate (3g):[22,23] Triethyl phosphine (15.6 g, 90.3 mmol) and hexyl bromide (7.5 g, 6.4 mL, 45 mmol) were heated at 165 °C overnight. Ethyl bromide was distilled off during the reaction. Distillation of the crude product gave 6.8 g (68 %) of diethyl hexylphosphonate. $Bp_{1.5}$= 95–101 °C (Lit.[23] bp_2 = 103 °C). ^{1}H NMR ($CDCl_3$) δ: 4.18–3.99 (m, 4H), 1.79–1.66 (m, 2H), 1.65–1.52 (m, 2H), 1.43–1.21 (mm, 12H), 0.89 (t, 3H).

Using the same procedure the following compounds were obtained. The compounds were purified by short path distillation and no precise boiling point determined.

Diethyl butylphosphonate.[23] ^{1}H NMR ($CDCl_3$) δ: 4.18–4.01 (m, 4H), 1.79–1.66 (m, 2H), 1.65–1.52 (m, 2H), 1.47–1.26 (m, 8H), 0.91 (t, 3H).

Diethyl octylphosphonate.[23,24] Yield (85 %). ^{1}H NMR ($CDCl_3$) δ: 4.20–4.00 (m, 4H), 1.82–1.66 (m, 2H), 1.65–1.51 (m, 2H), 1.41–1.20 (m, 16H), 0.89 (t, 3H).

Diethyl decylphosphonate.[25] Yield (90 %). ^{1}H NMR ($CDCl_3$) δ: 4.18–4.01 (m, 4H), 1.79–1.67 (m, 2H), 1.66–1.52 (m, 2H), 1.42–1.21 (m, 20H), 0.89 (t, 3H).

Diethyl dodecylphosphonate.[23,24] Yield (86 %). ^{1}H NMR ($CDCl_3$) δ: 4.18–4.01 (m, 4H), 1.79–1.66 (m, 2H), 1.65–1.53 (m, 2H), 1.43–1.19 (m, 24H), 0.88 (t, 3H).

Additional spectroscopic data for the above intermediates were in accordance with previously published data.

Ethyl hexylchlorophosphonate (3b).[22,23] To diethyl hexylphosphonate (5 g, 22.4 mmol) in CCl_4 (25 mL) was added PCl_5 (5.12 g, 22.6 mmol) and the mixture was heated at reflux overnight followed by bulb-to-bulb distillation to give 3.1 g (69 %) of product. Oven temp. (1 mmHg) = 153 °C ($bp_{9.5}$ = 110–112 °C).[22] ^{1}H NMR ($CDCl_3$) δ: 4.40–4.16 (mm, 2H), 2.21–2.08 (m, 2H), 1.80–1.64 (mm, 2H), 1.49–1.22 (mm, 9H), 0.89 (t, 3H).

Using the same procedure the following compounds were obtained. The compounds were purified by short path distillation and no precise boiling point determined.

Ethyl butylchlorophosphonate (3a)[22] Yield (67 %). ^{1}H NMR ($CDCl_3$) δ: 4.39–4.17 (m, 2H), 2.22–2.00 (m, 2H), 1.79–1.63 (m, 2H), 1.54–1.33 (m, 5H), 0.94 (t, 3H).

Ethyl octylchlorophosphonate (3c). Yield (63 %). ^{1}H NMR ($CDCl_3$) δ: 4.40–4.17 (m, 2H), 2.22–2.18 (m, 2H), 1.81–1.66 (m, 2H), 1.57–1.20 (m, 13H), 0.89 (t, 3H).

Ethyl decylchlorophosphonate (3d). Yield (73 %). ^{1}H NMR ($CDCl_3$) δ: 4.39–4.16 (m, 2H), 2.20–2.07 (m, 2H), 1.79–1.64 (m, 2H), 1.48–1.19 (m, 17H), 0.88 (t, 3H).

Ethyl dodecylchlorophosphonate (3e). Yield (60 %). ^{1}H NMR ($CDCl_3$) δ: 4.39–4.18 (m, 2H), 2.20–2.08 (m, 2H), 1.79–1.66 (m, 2H), 1.47–1.19 (m, 21H), 0.88 (t, 3H).

Ethyl 2,2,2-trichloroethyl hexylphosphonate (3h). To ethyl hexylchlorophosphonate (0.5 g, 2.4 mmol) in toluene (4 mL) and diethyl ether (6 mL) were added 2,2,2-trichloroethanol (0.7 g, 4.8 mmol) and triethylamine (0.5 g, 0.65 mL, 4.7 mmol) in diethyl ether (10 mL). The mixture was refluxed for 2 h followed by filtration, evaporation and chromatography yielding 0.56 g (73 %) of product. ^{1}H NMR ($CDCl_3$) δ: 4.66–4.48 (mm, 2H), 4.28–4.08 (mm, 2H), 1.93–1.78 (mm, 2H), 1.73–1.58 (mm, 2H), 1.46–1.22 (mm, 9H), 0.89 (t, 3H). Anal. calcd for $C_{10}H_{20}Cl_3O_3P$: C, 36.89; H, 6.19; Cl, 32.67. Found: C, 36.21; H, 6.31; Cl, 32.50.

Ethyl p*-nitrophenyl hexylphosphonate (3f).*[22] Sodium (60.3 mg, 2.62 mmol) was added to ethanol (3 mL, 99.9 %). To the resulting solution toluene (40 mL) and *p*-nitrophenol (0.33 g, 2.4 mmol) were added. Ethanol was distilled off and ethyl hexylchlorophosphonate (0.51 g, 2.4 mmol) was added. Reflux overnight followed by evaporation and chromatography gave 0.46 g (61 %) of product. ^{1}H NMR δ: 8.3–8.18 (d, 2H), 7.43–7.34 (d, 2H), 4.31–4.09 (m, 2H), 2.0–1.86 (m, 2H), 1.77–1.60 (m, 2H), 1.50–1.21 (m, 9H), 0.88 (t, 3H).

Separation of enantiomers. Separation of the enantiomers of ethyl *p*-nitrophenyl hexylphosphonate was performed by chiral HPLC using hexane and 2-propanol (9:1) as eluent at a flow of 1 mL/min. Both enantiomers were purified to a stereochemical purity of > 99 % *e.e.* Fraction 1, retention time 14.7 min, $[\alpha]_D^{20} = 13\ °$ ($c = 0.6$, hexane). Fraction 2, retention time 20.0 min, $[\alpha]_D^{20} = -13\ °$ ($c = 0.6$, hexane).

Enzyme assays

Lipase activity assay. Lipase activity was determined by pH-stat using a VIT 90 video titrator equipped with a ABU 91 autoburette from Radiometer, Copenhagen. Lipase activity was measured by hydrolysis of emulsified tributyrin measuring the release of free fatty acid by adding base at constant pH (7).[26] 1 LU (lipase unit) is the amount of lipase liberating 1 μmol of butyric acid/min.

Protease activity assay. Substrates, *N*-succinyl Ala–Ala–Pro–Phe *p*-nitroanilide and benzoyl Arg *p*-nitroanilide were purchased from Sigma. Stock solution: substrate in DMSO (100 mg/mL). Working solutions of substrates were prepared by diluting 1- to 100-fold stock solution in Tris–acetate (50 mM, pH 8.2). Subtilisin and α-chymotrypsin activity were assayed with *N*-succinyl Ala–Ala–Pro–Phe *p*-nitroanilide and benzoyl Arg *p*-nitroanilide was used to assay trypsin activity. Substrate (100 μl) was mixed with diluted enzymes (100 μl) and protease activity was measured spectrophotometrically (405 nm).

Activity assay for hog liver esterase. The activity was measured spectrophotometrically (405 nm) using *p*-nitrophenyl acetate as substrate: a solution of substrate in DMSO (100 mM) was diluted 1- to 100-fold in Tris buffer (50 mM, pH 9.0). Enzyme in buffer (100 μl) was mixed with substrate (100 μl).

Activity assay for phospholipase A_2. Phospholipase activity was determined by a pH-stat method (pH = 8, 40 °C) using a mixture of egg yolk, $CaCl_2$ and deoxycholate.[27] The liberated free fatty acid was titrated at constant pH by adding base. Enzyme activity was given as liberated μmol fatty acids/min.

Enzymes

Lipases. All microbial lipases were purified at Novo Nordisk to a purity showing one band in SDS–PAGE. *Candida antarctica* B component (spec. act. 500 LU/OD_{280}); *C. antarctica* A component (300 LU/OD_{280}); *R. miehei* (8000 LU/OD_{280}); *Humicola lanuginosa* (4500 LU/OD_{280}); *Pseudomonas cepacia* (7000 LU/OD_{280}); *Candida cylindracea* (2000 LU/OD_{280}), Nippon fats and oils; guinea pig pancreatic lipase (1800 LU/OD_{280}); human pancreatic lipase (8000 LU/mg), was a generous gift from Professor B. Borgström, Lund, Sweden; bovine lipoprotein lipase (400 U/mg), phospholipase A_2 from *Naja naja* venom (935 U/mg using soybean 1-α-phosphatidylcholine as substrate), Sigma; hog liver esterase (130 U/mg using butyric acid ethyl ester as substrate), Boehringer Mannheim GmbH.

Proteases. Bovine–trypsin (3500 USP/mg, 1 USP is the amount of enzyme which alters the absorbance by 0.003/min using *N*-benzoyl-L-arginine ethyl ester as substrate, pH 7.6, 25 °C); subtilisin A (30.3 Anson units/g, Anson units are determined using urea-denaturated haemoglobin, the TCA-soluble product is quantified in a colour reaction with Folin–Ciocalteu phenol reagent); α-chymotrypsin (970 USP/mg, 1 USP is the amount of chymotrypsin which alters the absorbance by 0.0075/min using *N*-succinyl Ala–Ala–Pro–Phe *p*-nitroanilide as substrate). All products were from Novo Nordisk A/S.

Inhibition

Inhibition of enzymes with ethyl alkylchlorophosphonates. Performed using the following general procedure: enzymes were dissolved in water. The inhibitor was added in hexane and the mixture shaken. After an incubation period of 15–100 min, all incubation mixtures were assayed for activity using the above described procedures. The results and inhibitory conditions are summarized in Table 1.

Active site titration of C. antarctica *lipase.* Ethyl *p*-nitrophenyl hexylphosphonate was used to titrate the active sites of *C. antarctica* B lipase. The lipase was dissolved in Tris buffer (50 mM, pH 8.0), and a range of concentrations

was prepared. An excess of inhibitor (20 μl, 1 mM) in DMSO was added to the enzyme samples (180 μl). The release of *p*-nitrophenol was followed spectrophotometrically (405 nm) until a constant absorbance was observed. The molar release of *p*-nitrophenol versus enzyme activity added was then determined, using a *p*-nitrophenol standard curve in the same system. The molar concentration of the lipase was calculated using a specific activity of 500 LU/mg and a molecular weight of 33×10^3 g/mol.[28]

Kinetic determinations. Absorbance was measured each second at 400 nm on a Hewlett Packard 8452A, diode array spectrophotometer. The inhibitor concentration was determined measuring released *p*-nitrophenol spectrophotometrically after total hydrolysis in NaOH (1 M).

Assay. *p*-Nitrophenyl acetate was used as substrate for *C. antarctica* B (1 and 2 mM) and *R. miehei* lipases (4 mM) and *N*-succinyl Ala–Ala–Pro–Phe *p*-nitroanilide for α-chymotrypsin (0.1 mM). The release of *p*-nitrophenol or *p*-nitroaniline was followed spectrophotometrically in a mixture of enzyme, substrate and ethyl *p*-nitrophenyl hexylphosphonate (inhibitor) in sodium phosphate buffer (50 mM, pH = 7.0) and acetonitrile (4 % v/v). To determine K_m and to check that the reaction between enzyme and substrate was pseudo-first-order, the inhibitor was omitted in the assay.

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Appendix

Derivation of the equations used for analysis of the kinetic data. The following abbrevations and constants are used:

t:	Time
A:	Absorbance
K_d:	Dissociation constant for the enzyme inhibitor complex
k_2:	Rate constant for the reaction of the enzyme inhibitor complex
k_{obs}:	Apparent overall pseudo-first-order rate constant for the reaction between enzyme and inhibitor

K_m: Michaelis–Menten constant for the reaction between enzyme and substrate

v: Rate of change in absorbance due to hydrolysis of substrate

The calculations are based on the two equations 1 and 2.

$$Enz + Subs \rightleftharpoons Enz{*}Subs \rightarrow Enz + Pro \quad (1)$$

$$Enz + InhX \underset{}{\overset{K_d}{\rightleftharpoons}} Enz{*}InhX \overset{k_2}{\rightarrow} Enz - Inh + X \quad (2)$$

Assuming pseudo-first-order kinetics for the reaction between enzyme and substrate and between enzyme and inhibitor and using K_m for the reaction between enzyme and substrate as an approximate value for the dissociation constant for the enzyme–substrate complex, the following equation can be used:[8]

$$\frac{\Delta t}{\Delta \ln v} = \frac{K_d}{k_2} \times \frac{1}{[Inh](1-\alpha)} + \frac{1}{k_2} \quad (3)$$

$$\alpha = \frac{[Subs]}{K_m + [Subs]} \quad (4)$$

As the reaction between enzyme and inhibitor is pseudo-first-order,

$$v_t = v_0 e^{-k_{obs}t} \quad (5)$$

Setting $dA_t/dt = v_t$ and integrating from time 0 to time t gives

$$A_t = A_0 - \frac{v_0}{k_{obs}}(e^{-k_{obs}t} - 1) \quad (6)$$

When $t = \infty$

$$\frac{v_0}{k_{obs}} = A_\infty - A_0 \quad (7)$$

and when inserted in equation 6 this gives equation 8

$$A_t = A_\infty - (A_\infty - A_0) \times e^{-k_{obs}t} \quad (8)$$

The absorption data is fitted to equation 8 from which k_{obs} is obtained. Assuming $K_m >> [Subs]$, α can be ignored in equation 3, and k_{obs} can be expressed as

$$\frac{\Delta t}{\Delta \ln v} = \frac{t_2 - t_1}{\ln v_2 - \ln v_1} = \frac{t_2 - t_1}{\ln v_0 e^{-k_{obs}t_2} - \ln v_0 e^{-k_{obs}t_1}} = \frac{t_2 - t_1}{-k_{obs}t_2 + k_{obs}t_1} = -\frac{1}{k_{obs}} \quad (9)$$

K_d and k_2 can thus be obtained by plotting k_{obs}^{-1} against $[Inh]^{-1}$:

$$-\frac{1}{k_{obs}} = \frac{K_d}{k_2} \times \frac{1}{[Inh]} + \frac{1}{k_2} \quad (10)$$

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