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Covalent Inhibition of Digestive Lipases by Chiral Phosphonates

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ABSTRACT

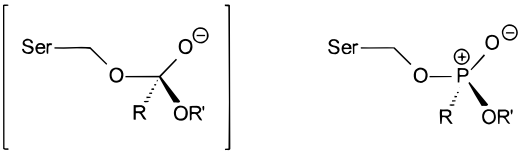
Designing and synthesizing specific inhibitors is of fundamental value for understanding the molecular mechanisms involved in the interfacial adsorption step as well as the catalytic activity of lipases. In this Account, we will review and discuss results obtained mostly at our laboratory concerning the covalent inhibition of human gastric and human pancreatic lipases by chiral phosphonates. Rather than presenting an exhaustive list of compounds tested so far with lipases of animal and microbial origin, we selected recent experimental data illustrating well the specific problems encountered during the covalent inhibition of these digestive lipases.

Introduction

Lipases have potential applications in chemistry,[1−4] biotechnology,[5,6] and medicine.[7] In the latter domain, for instance, conventional weight-reducing strategies have focused largely on controlling the energy intake, but there is a doubt as to the long-term efficacy of these approaches. Reducing the adsorption of dietary fat by prescribing digestive lipases inhibitors holds great promise as an antiobesity strategy.[8−11] Furthermore, designing and synthesizing specific inhibitors is of fundamental interest for understanding the molecular mechanisms involved in the interfacial adsorption step as well as the catalytic activity of lipases.

Among the whole range of esterase inhibitors, phosphonates mimic in both their charge distribution and geometry the first transition state occurring during enzymatic carboxyester hydrolysis. These compounds are thus efficient inhibitors of lipases by formation of a covalent bond between the nucleophilic Oγ of the active serine of the enzyme and the phosphorus atom (Scheme 1).[12−23]

Scheme 1. Comparison between the Structure of the First Tetrahedral Transition State Occurring during Carboxyester Hydrolysis (left) or after Reaction with a Phosphonate Compound (right)



In this Account, we will review and discuss results obtained mostly at our laboratory concerning the covalent inhibition of human gastric (HGL) and human pancreatic

Jean-François Cavalier, born in Nîmes (1969), graduated from the Ecole Nationale Supérieure de Chimie de Marseille (ENSSPICAM) in 1993. He obtained his Ph.D. (1997) from the University of Aix-Marseille III, under the supervision of Professor G. Buono. After one year as a University Teacher Assistant in Professor J.-L. Montero's research group in Montpellier, France, he is currently carrying out postdoctoral studies with Professor J. Marchand-Brynaert at the Université Catholique de Louvain, Belgium.

Gérard Buono was born in Alger, Algeria, in 1944. In 1968 he graduated from the Ecole Supérieure de Chimie de Marseille. He spent a stay (1971) in the group of Professor H. J. Bestmann at the University of Erlangen−Nürnberg (Germany). He received a Ph.D. at the University Aix-Marseille in 1977 on the dynamic stereochemistry of pentacoordinated phosphorus under the direction of Professor G. Peiffer. He became Professor of Organic Chemistry in 1987 at the University of Aix-Marseille (ENSSPICAM). Since 1998, he has been head of the Institut Fédératif de Chimie Moléculaire de Marseille. His research interests are in the fields of asymmetric synthesis, development of novel catalysts (especially chiral catalysts), organophosphorus chemistry and bioorganic chemistry.

Robert Verger was born in 1944 in Maison Carrée, Algeria. He began his study of chemistry at the University of Aix-Marseille, where in 1970 he obtained his Doctorat d'Etat in physics for a thesis under the guidance of Pierre Desnuelle. After a postdoctoral stay under the supervision of Gérard de Haas at the University of Utrecht, The Netherlands, he returned to Marseille and started his scientific career at the Centre National de la Recherche Scientifique (CNRS) in Marseille. Since 1990 he has held the title of "Directeur de Recherche 1ère classe" and has received the bronze and silver medals of the CNRS.

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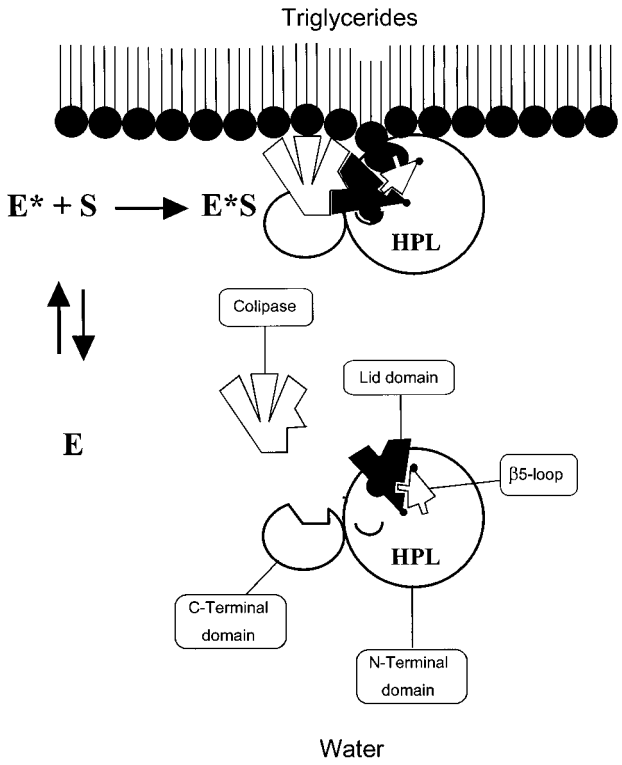


FIGURE 1. Schematic representation of the interfacial activation of the human pancreatic lipase−colipase complex illustrating the conformational changes between the closed (*E*) and the open conformations (*E*S*) induced upon the adsorption of the enzyme to a lipidic interface. Adapted from Carrière et al.[53]

(HPL) lipases by phosphonate compounds. Rather than presenting an exhaustive list of compounds tested so far with lipases of animal and microbial origin, we have selected recent experimental data illustrating well the specific problems encountered during the covalent inhibition of these digestive lipases.

Structure and Mechanism of Action of Human Pancreatic Lipase

The general features of the interfacial catalysis by the pancreatic lipase−colipase complex have been particularly well investigated and refined at a molecular level. The three-dimensional structure of HPL reported by Winkler et al.[24] confirmed the existence of two distinct domains: a larger N-terminal domain and a smaller C-terminal domain (Figure 1). The N-terminal domain, a typical α/β hydrolase fold dominated by a central parallel β sheet, contains the active site with a catalytic triad: Ser152, Asp176, and His263; all of these are conserved in other members of the mammalian lipase family (e.g., in lipoprotein lipase and hepatic lipase). The active site is covered by a surface loop between the disulfide bridge Cys237 and -261. This surface loop includes a short, one-turn α helix with a tryptophan residue in position 252, which is completely buried inside the protein and sitting directly on top of the active site Ser152. Under this closed conformation, the lid prevents the substrate from having access to the active site. The X-ray analyses of cocrystals of HPL−colipase complex in the presence of either mixed bile salt/phospholid micelles, by van Tilbeurgh et al.,[25] or monoalkylphosphonate inhibitor, by Egloff et al.,[15,21] showed that the lid was shifted to one side, exposing a larger hydrophobic surface and giving free access to the active site. This motion is induced when binding of the enzyme to the lipidic interface occurs and is probably the structural basis for interfacial activation of HPL. The open structure of the lipase−colipase complex illustrates how colipase might anchor the lipase at the interface in the presence of bile salts: colipase binds to the noncatalytic β sheet of the C-terminal domain of HPL and exposes the hydrophobic tips of its fingers at the opposite side of its lipase-binding domain. This hydrophobic surface helps to bring the catalytic N-terminal domain of HPL into close contact with the lipid/water interface. Apart from the apolipoprotein CII activating lipoprotein lipase, no protein activator such as colipase has been found in other organs or organisms. Thus, the mechanism discussed above seems to be specific for the HPL−colipase system.

Methods for Lipases Inhibition

To describe the kinetics of a lipolytic enzyme acting at an interface, a simple and versatile model has been proposed by Verger et al.[26] This model consists basically of two successive equilibria. The first describes the reversible penetration of a water-soluble enzyme into an interface ($E \rightleftharpoons E^*$). This is followed by a second equilibrium in which one molecule of penetrated enzyme binds a single substrate molecule, forming the enzyme−substrate complex (E^*S). This is the two-dimensional equivalent of the classical Michaelis−Menten equilibrium. Once the complex (E^*S) has been formed, the catalytic steps take place, regenerating the enzyme in the form (E^*) and liberating the lipolysis products (P). Extensions of the previous kinetic model were proposed by Ransac et al.[27,28] and more recently by Cavalier et al.[16] to account for the competitive inhibition,[28] as well as the covalent inhibition of lipolytic enzymes at a lipid/water interface by two enantiomeric inhibitors (Figure 2).[16,27]

Achieving specific and covalent inhibition of lipolytic enzymes is a difficult task, because of non-mutually exclusive processes such as interfacial denaturation, changes in "interfacial quality",[29,30] and surface dilution phenomena.[31−33] Furthermore, the interfacial enzyme binding and/or the catalytic turnover can be diversely affected by the presence of potential amphipathic inhibitors.[34]

To study the effect of these inhibitors, which can, in principle, modify covalently several essential amino acid residues of the lipases, we have developed a method using mixed substrate/inhibitor monomolecular films (Figure 3).

The Monolayer Technique (Figure 3)

With conventional emulsified systems it is not possible to control the "interfacial quality" and to easily assess the distribution of soluble versus adsorbed amphiphilic molecules. This prompted us to use the monolayer technique based upon surface pressure decrease due to lipid film

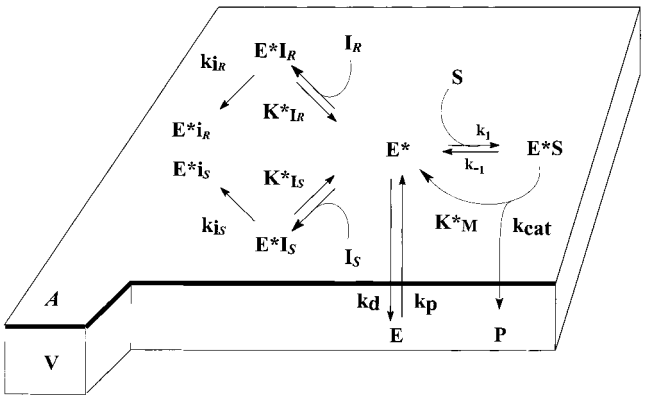


FIGURE 2. Kinetic model illustrating the covalent inhibition of a lipolytic enzyme at a lipid/water interface. Symbols and abbreviations are as follows: A, total interfacial area (surface); V, total volume (volume); E_0, total enzyme concentration (molecule/volume); E, free enzyme concentration (molecule/volume); E^*, interfacial enzyme concentration (molecule/surface); S, interfacial concentration of substrate (molecule/surface); I_R, interfacial concentration of inhibitor-(R) (molecule/surface); I_S, interfacial concentration of inhibitor-(S) (molecule/surface); P, product concentration (molecule/volume); E^*S, interfacial enzyme−substrate complex concentration (molecule/surface); E^*I_R, interfacial enzyme−inhibitor-(R) complex concentration (molecule/surface); E^*I_S, interfacial enzyme−inhibitor-(S) complex concentration (molecule/surface); E^*i_R, interfacial enzyme covalently inhibited by inhibitor-(R) concentration (molecule/surface); E^*i_S, interfacial enzyme covalently inhibited by inhibitor-(S) concentration (molecule/surface); k_d, desorption rate constant (time^{-1}); k_p, penetration rate constant (volume·surface^{-1}·time^{-1}); k_1, rate constant (surface·molecule^{-1}·time^{-1}); k_{-1}, rate constant (time^{-1}); k_{cat}, catalytic rate constant (time^{-1}); k_{i_R} inhibition rate constant for the enzyme−inhibitor−(R) complex (time^{-1}); k_{i_S} inhibition rate constant for the enzyme−inhibitor-(S) complex (time^{-1}); K^*_M, interfacial Michaelis−Menten constant (molecule/surface); $K^*_{I_R}$ interfacial dissociation constant for the enzyme−inhibitor-(R) complex (molecule/surface); $K^*_{I_S}$ interfacial dissociation constant for the enzyme−inhibitor-(S) complex (molecule/surface).[16]

hydrolysis. This technique is applicable to those cases where the lipid forms a stable monomolecular film at the air/water interface and where reaction products are freely soluble and diffuse away rapidly into the aqueous phase.[35]

The principle of this method has been described previously by Verger et al.[36] It involved the use of a "zero-order trough" (Figure 3) with two compartments: a reaction compartment (E) and a reservoir compartment (D), which were connected to each other by a narrow surface canal made of etched glass (B). Enzyme solution was injected into the subphase of the reaction compartment only, whereas the lipid film covered both of them. A mobile barrier (A), automatically driven by the barostat, moved back and forth over the reservoir to keep the surface pressure (π) constant, thus compensating for the substrate molecules removed from the film by the enzyme hydrolysis. The surface pressure was measured on the reservoir compartment with a Wilhelmy plate (C) attached to an electromicrobalance, connected in turn to a microprocessor programmed to regulate the mobile barrier movement. The kinetic recordings obtained with this trough are linear.

There are at least five major reasons for using lipid monolayers as substrates for lipolytic enzymes (the reader

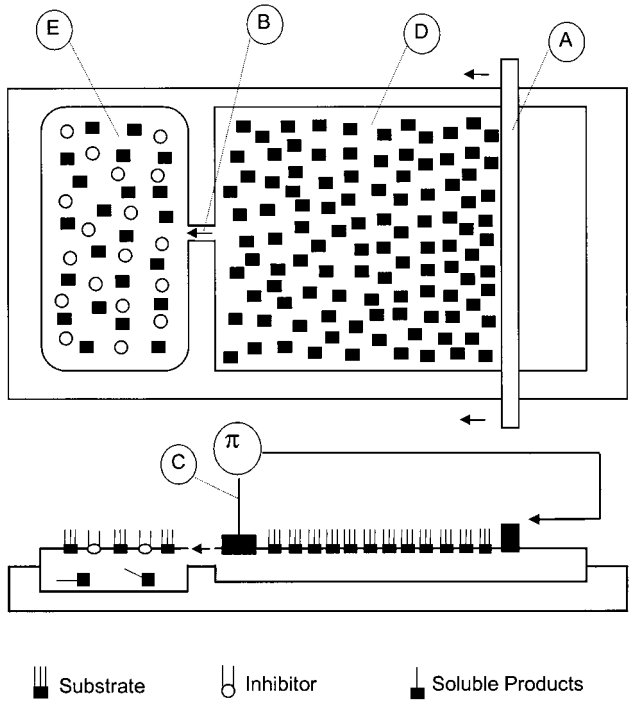


FIGURE 3. Principle of the method for studying lipase inhibition using mixed substrate/inhibitor monomolecular films.[37,38]

is referred to previous reviews[7,14,36−40] for further details): (i) It is easy to follow the course of the reaction monitoring one of several physicochemical parameters characteristic of the monolayer film: surface pressure, potential, density, etc. (ii) Probably the most important basic reason is that it is possible with lipid monolayers to vary and control the "interfacial quality", which depends on the nature of the lipids forming the monolayer, the orientation and conformation of the molecules, the molecular and charge densities, the water structure, the viscosity, etc. (iii) Using the surface barostat balance, the lipid packing of a monomolecular film of substrate can be maintained constant during the course of hydrolysis, and it is therefore possible to obtain accurate pre-steady-state kinetic measurements with minimal perturbation caused by increasing amounts of reaction products. (iv) The monolayer technique is highly sensitive, and very little lipid is needed to obtain kinetic measurements. This advantage can often be decisive in the case of synthetic or rare lipids. (v) Inhibition of lipase activity by water-insoluble substrate analogues can be precisely estimated using a "zero-order" trough and mixed monomolecular films in the absence of any synthetic, nonphysiological detergent. The monolayer technique is therefore suitable for modeling in vivo situations.

Covalent inhibition of lipases was then studied using films of substrate containing amphiphilic inhibitor molecules. The zero-order trough with a reaction compartment containing a mixed substrate/inhibitor film and the reservoir compartment covered only with a film of pure substrate (1,2-dicaprin) is shown in Figure 3.[37−40] The kinetics of hydrolysis were recorded, and the data were analyzed according to previous models.[16,27,28,40]

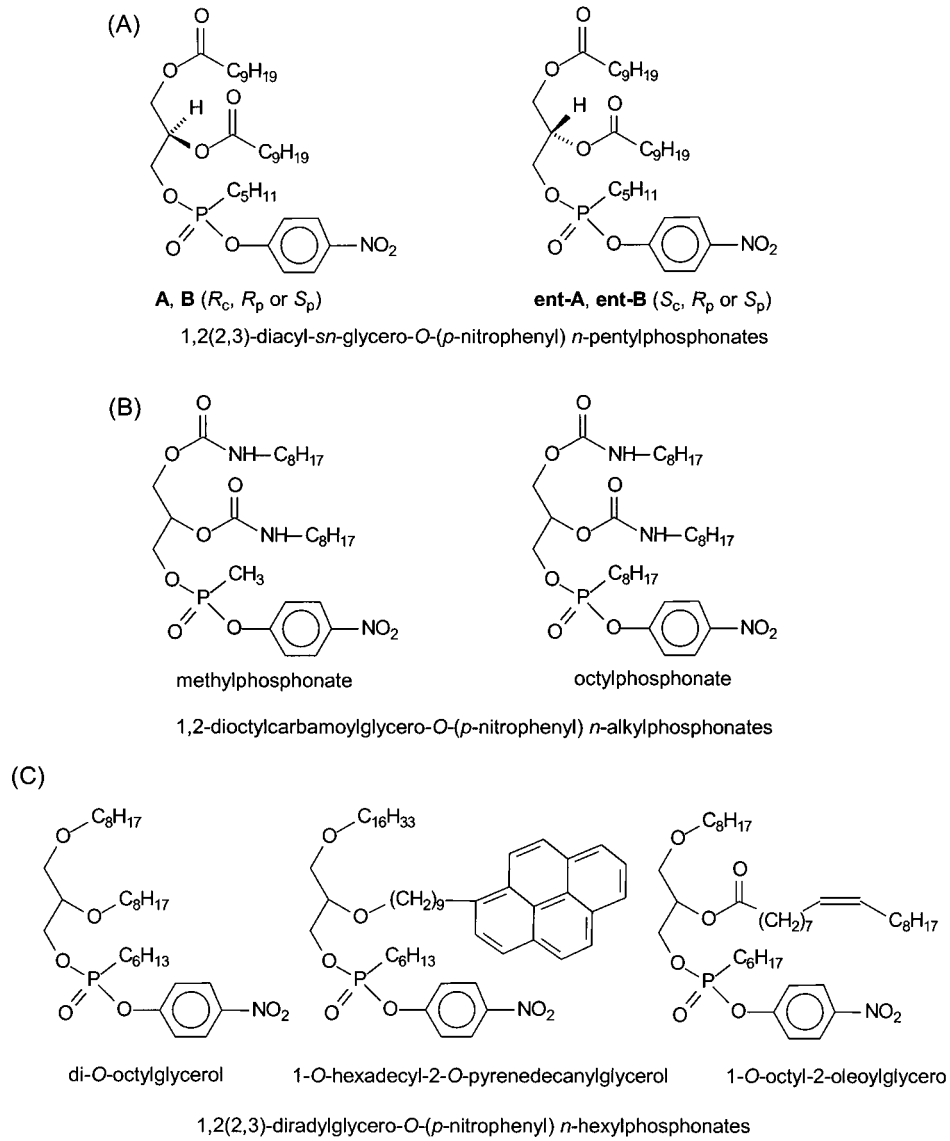


FIGURE 4. (A) Structure of inhibitors analogous to diacylglycerides used in the case of digestive lipases: 1,2(2,3)-diacyl-*sn*-glycero-*O*-(*p*-nitrophenyl) *n*-pentylphosphonates **A**, **B**, **ent-A**, **ent-B**.[18] Structures of inhibitors used in the case of microbial lipases: (B) 1,2-dioctylcarbamoyl glycero-*O*-(*p*-nitrophenyl) *n*-alkylphosphonates[19] and (C) 1,2(2,3)-diradylglycero-*O*-(*p*-nitrophenyl) *n*-hexylphosphonates.[20]

Inhibition of Microbial Lipases by Phosphonate Compounds

Patkar and Björkling[12] reviewed several families of lipase inhibitors including boronic acids, phosphorus-containing inhibitors, and β-lactone-containing inhibitors. For the first time these authors described an enantioselective inhibition using *Candida antartica* and *Rhizomucor miehei* lipases with pure enantiomers of *O*-ethyl-*O*-(*p*-nitrophenyl) *n*-hexylphosphonate as inhibitors.[13] The inhibition could be monitored by the *p*-nitrophenol release, and quantitative analysis of the data indicated that a 1:1 lipase−inhibitor complex was formed during inhibition.

1,2-Dioctylcarbamoylglycero-*O*-(*p*-nitrophenyl) *n*-alkylphosphonates, where the alkyl is a methyl or an octyl group (Figure 4B), were synthesized, and their activity as irreversible inhibitors of cutinase from *Fusarium solani pisi* and *Staphylococcus hyicus* lipase was tested by Mannesse et al.[19] Rapid inhibition of these enzymes occurred with a concomitant release of 1 mol of *p*-nitrophenol per mole of enzyme. Both lipases show great selectivity toward the chirality of these compounds at the glycerol and phosphorus sites: the glycerol moiety with the (*S*) configuration reacted 4−10-fold slower than the (*R*) enantiomer. These results reflect the known positional and stereopreference of these enzymes, which preferentially release the fatty acid at the *sn*-3 position of natural triacylglycerols.

Stadler et al.[20] synthesized 1,2(2,3)-diradylglycero-*O*-(*p*-nitrophenyl) *n*-hexylphosphonates, with the diradylglycerol moiety being di-*O*-octylglycerol, 1-*O*-hexadecyl-2-*O*-pyrene decanylglycerol, or 1-*O*-octyl-2-oleoylglycerol (Figure 4C), and tested for their ability to inhibit lipases from *Chromobacterium viscosum* and *Rhizopus oryzae*. Both lipases exhibited the same preference for the chirality at the phosphorus that was independent from the absolute configuration at the glycerol backbone. Moreover, the

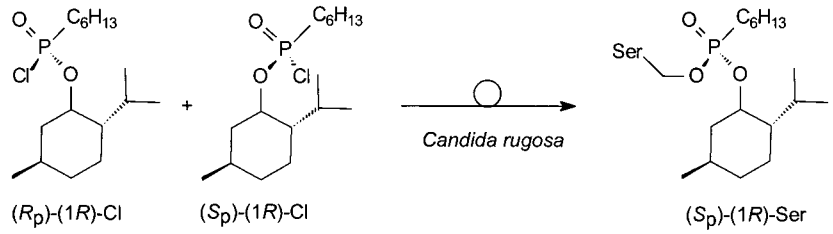
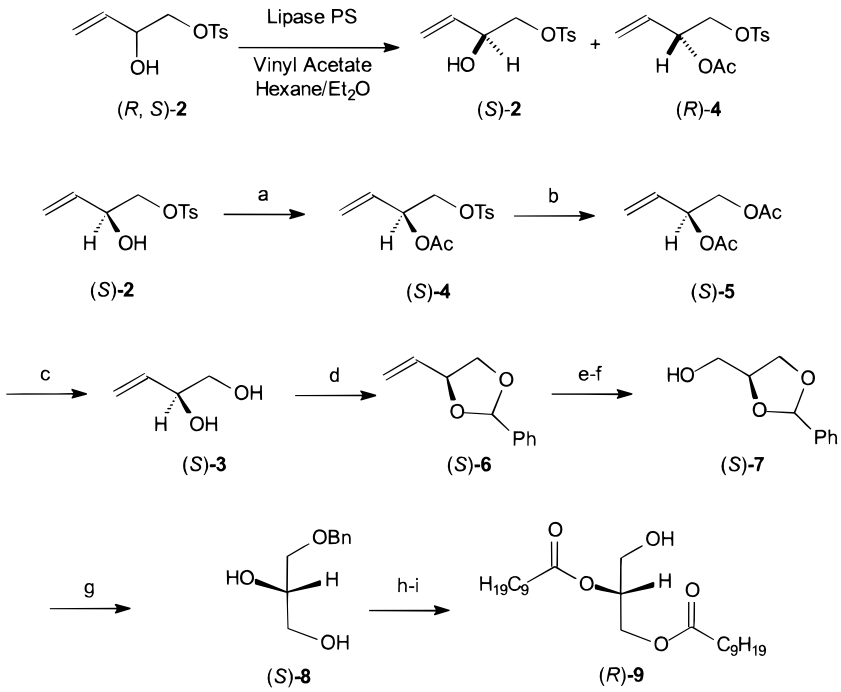


FIGURE 5. Nucleophilic substitution by the catalytic serine of *C. rugosa* lipase using racemic mixture of *O*-(*R*)-menthyl (S_p,R_p)-*n*-hexylchlorophosphonate, with inversion of configuration on the phosphorus atom.[22]

Scheme 2[a]



[a] Reagents and conditions: (a) acetyl chloride, CH_2Cl_2, Pyr, 0 °C; (b) cesium acetate, DMSO, DMF; (c) K_2CO_3, MeOH, H_2O; (d) PhCHO, *p*-TsOH, PhMe, Δ; (e) O_3, Me_2S, CH_2Cl_2, −78 °C; (f) $NaBH_4$, CH_2Cl_2, EtOH, 0 °C; (g) DIBALH, Et_2O, 0 °C to room temperature; (h) decanoic acid, DCC, DMAP, Et_2O; (i) 5% Pd/C, H_2, EtOH.

same authors[41] also studied the influence of substrate hydrophobicity and steric hindrance by variation of the alkyl and acyl chain length at the *sn*-2 position of the glycerol backbone. Hydrolysis of these synthetic substrates demonstrated that minor structural variations at this *sn*-2 position of triacylglycerol strongly affect the stereoselectivity of the lipases tested. They concluded that the carbonyl ester in the non-hydrolyzed *sn*-2 position of a triacylglycerol was responsible for correct positioning of the substrate in the binding site of lipases.

The 3D Structure of Inhibited Microbial Lipases

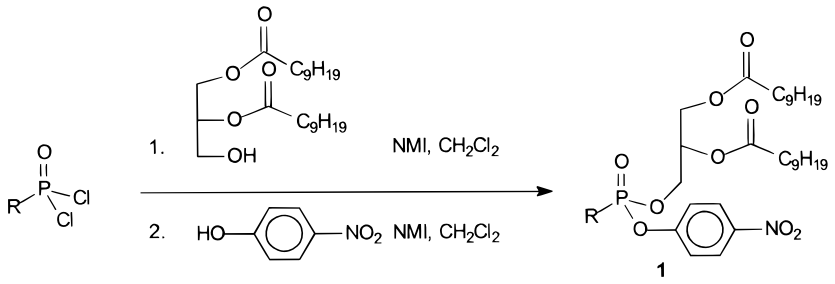
A fungal lipase from *C. rugosa* has been cocrystallized with and without inhibitor, revealing a similar overall structure. However, the helical lid has been found to be shifted in order to unmask a buried active site.[22,42,43] Furthermore, using both diastereomers (S_p,R_p)-(1*R*)-Cl or (S_p, R_p)-(1*S*)-Cl of *O*-menthyl *n*-hexylchlorophosphonate as inhibitors of *C. rugosa* lipase, Cygler et al.[22] have provided a structural basis of the chiral preferences of lipases. It is worth noting that both phosphonate groups were covalently bound to the catalytic Oγ oxygen of Ser209 and presented a (S_p) stereochemistry, assuming that in the original mixture of (S_p,R_p) diastereomers, only the (S_p)-1*R*-Cl and (S_p)-1*S*-Cl diastereomers reacted with inversion of the absolute configuration of the phosphorus atom (Figure 5).

Recently, a cutinase from *F. solani* has been crystallized by Longhi et al.[23] with a triacylglycerol analogue: (*R*)-1,2-dibutylcarbamoylglycero-*O*-(*p*-nitrophenyl) *n*-butylphosphonate. This inhibitor was covalently linked to the active site serine, thus mimicking the first tetrahedral intermediate along the reaction pathway. This structure provides a more realistic model for a complex between a lipolytic enzyme and a triacylglycerol analogue.

Inhibition of HPL and HGL by Phosphonate Compounds

Recently, a lipophilic α-keto amide inhibitor of HPL was synthesized using a lipidic 2-amino alcohol as backbone. The chiral intermediate 2-(*tert*-butyloxycarbonylamino)-D-undecen-5-ol was synthesized starting from D-glutamic acid. Inhibition studies using the monomolecular film

Scheme 3



technique with mixed films of 1,2-dicaprin containing variable proportions of the inhibitor showed a 50% decrease in lipase activity at a 0.14 molar fraction.[44]

Taking then into consideration all the above-mentioned results, and in an attempt to further characterize the catalytic mechanism of digestive lipases by using phosphorus inhibitors, we have focused our research on a new class of specific inhibitors of digestive lipases involving a phosphonate reactive group in a diacylglycerol structure (Figure 4A).[18] The phosphonate moiety was located at an external position, *sn*-1 or *sn*-3 of the glycerol backbone, and bearing a *p*-nitrophenoxy as leaving group. With two chiral centers, these molecules were present as four stereoisomers. Because of the good leaving properties of the *p*-nitrophenoxy group, the strategy of synthesis of these diacylglycerophosphonates (Figure 4A) required the formation of the phosphonate unit during the last chemical step. The elaboration of the diglyceride unit was then realized before the coupling with the phosphorus moiety.

Enantiomerically pure *sn*-1,2- and *sn*-2,3-*O*-didecanoylglycerol compounds **9** (Scheme 2) were then prepared starting from a C-4 chiral synthon: 3-buten-1,2-diol (**3**), obtained by the irreversible enzymatic acylation of (*R*, *S*)-**2** leading to (*S*)-**2** and (*R*)-**4**, respectively. Moreover, it is worth noting that the strategy depicted in Scheme 2 leading to enantiopure diglycerides could also be applied to the synthesis of various mixed diglyceride compounds. The final step in the synthesis was the introduction of the phosphonate group leading to a pair of diastereomeric phosphonates which are acylglycerol analogues (Scheme 3).[18]

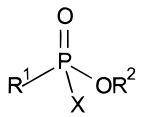
Separation of each phosphonate diastereomer **A**/**B** or **ent-A**/**ent-B** performed by HPLC led to four enantiopure stereoisomers (Figure 4A).

The inhibition of HGL and HPL by the monomolecular film technique using mixed films of each of these chiral diacylglycerophosphonate analogues, **A**, **B**, **ent-A**, **ent-B**, and dicaprin, was then studied (Figure 3). Interfacial lipase binding was evaluated by means of ELISA tests with biotinylated lipases, with which it was possible to measure the surface density of enzymes in the nanogram range.[45,46] The two carboxyl ester linkages were intentionally conserved in order to keep as closely as possible to the structure of acylglycerols (natural substrates of lipases). This choice turned out a posteriori to be rational, since no or only negligible hydrolysis of these compounds occurred during the experiments with HPL and HGL. With both enzymes, kinetic experiments were performed at various molar ratios of dicaprin premixed with each of the four chiral inhibitors. All the four stereoisomers investigated reduced the hydrolysis of dicaprin by HGL and HPL.[45]

With HPL, the four stereoisomers exhibited a rather weak inhibition capacity, and no significant differences were observed among them. With each inhibitor tested, interfacial binding experiments using ELISA tests showed no significant difference in the surface density of HPL, which confirmed the low stereoselectivity of pancreatic lipases using either triacylglycerols[47−49] or triacylglycerol analogues.[50] With respect to gastric lipase, however, the enzyme adsorbed less on each stereoisomeric inhibitor that on the dicaprin substrate. Furthermore, the various organophosphorus enantiomers displayed differential inhibitory effects. The inhibition was highly dependent upon the chirality at the *sn*-2 carbon of the glycerol backbone, while the chirality at the phosphorus atom had no influence. The (R_c,S_p) and (R_c,R_p), which both contain the phosphorus moiety at the *sn*-3 position, were found to be the best inhibitors. This latter finding correlates well with the *sn*-3 preference, during the hydrolysis of triglycerides catalyzed by gastric lipases.[47−51] Moreover, the levels of surface density of gastric lipase differed significantly with each enantiomeric inhibitor used. A clear correlation was observed between the molar ratio (α_{50}) of inhibitor leading to half inhibition and the surface concentration of gastric lipase: the highest enzymatic inhibition was observed with films containing the enantiomeric inhibitor to which HGL was best adsorbed.[21,45]

Other "simple" phosphonate compounds, $R^1P(O)X(OR^2)$, mimicking in both their charge distribution and geometry the first transition state occurring during carboxyester enzymatic hydrolysis, were synthesized and investigated by our group[7,14,16,17] as potential inhibitors of HGL and HPL. Their efficiency as inhibitor was studied on the basis of the alkyl chain length, the nature of the leaving group, and the influence of the ester substituent (Scheme 4).[17]

Scheme 4[a]



[a] $\mathbf{R^1}$ = *n*-C_5H_{11}, *n*-$C_{11}H_{23}$; $\mathbf{OR^2}$ = OCH_3, $O(CH_2)_2OMe$; **X** = Cl, *p*-NO_2PhO, $O(CH_2)_2OMe$; **X**, $\mathbf{OR^2}$ = $O(CH_2)_2O$.

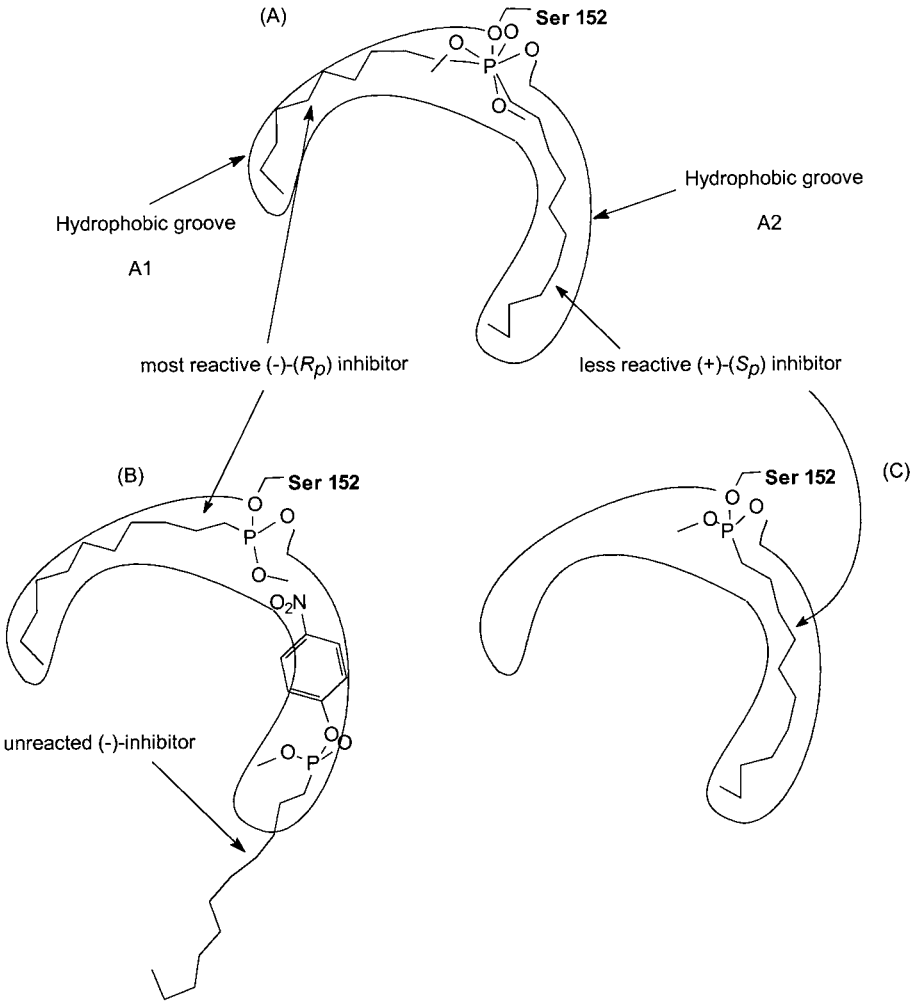


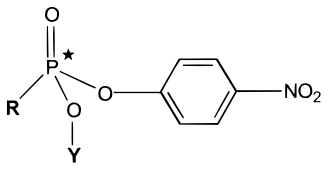
FIGURE 6. Schematic representations of the HPL active site containing chiral C-11 alkylphosphonate compounds: (A) racemic mixture of the two phosphonate enantiomers; (B) the most reactive (−)-(R_p) enantiomer; (C) the less reactive (+)-(S_p) enantiomer. Adapted from Egloff et al.[15]

The released *p*-nitrophenol-to-enzyme ratio indicated the formation of a 1:1 complex. In the absence of substrate, the most powerful inhibitor was *O*-methyl-*O*-(*p*-nitrophenyl) *n*-pentylphosphonate, which possessed a short alkyl chain ($R^1 = n\text{-}C_5H_{11}$), a small methoxy ester substituent ($OR^2 = OCH_3$), and a good leaving group.

In an attempt to further characterize the active site and catalytic mechanism of pancreatic lipase, a C-11 alkylphosphonate compound, the *O*-methyl-*O*-(*p*-nitrophenyl) *n*-undecylphosphonate was selected as an effective inhibitor. The crystal structure of the HPL−colipase complex inhibited by this C-11 phosphonate compound was determined at a resolution of 2.46 Å.[15,21]

The crystal structure of a racemic mixture of this C-11 inhibitor showed that the phosphorus atoms of each enantiomer were covalently bound to the Oγ oxygen of the active Ser152 (Figure 6A). Furthermore, the use of each enantiomer separately in the crystallization medium revealed that the absolute configuration of the phosphorus atom of the most reactive enantiomer, bound to the catalytic serine, was (R_p). The C-11 alkyl chain of this first enantiomer fitted into a hydrophobic groove, mimicking the interactions with the leaving acyl chain of a glycerol substrate and the protein (Figure 6B). The alkyl chain of the second enantiomer (the less reactive bound to the catalytic serine, (S_p) configuration) also possessed an elongated conformation and interacted with hydrophobic patches on the surface of the open conformation of the amphipathic lid (Figure 6C). This was suggestive of the location of a second acyl chain of a glyceride substrate. Finally, the alkyl chains of the two C-11 alkylphosphonate enantiomers superimposed well with the two fatty acyl chains of the phospholipid observed in the ternary phosphatidylcholine−HPL−colipase complex.[25] On the basis of this crystallographic study, the following hypothesis about the binding mode of real substrates and the organization of the active site was proposed: after partly leaving the lipid particle, the scissile acyl chain (*sn*-1 or *sn*-3) of the glyceride substrate may bind to the hydrophobic groove, implying that a triglyceride substrate will adopt a "fork" conformation at the active site.[15,21]

Starting from the above crystallographic observations, and in an attempt to improve the molecular fit into the two hydrophobic clefts of the HPL active site, we designed and synthesized new enantiomerically pure alkylphosphonate inhibitors, RR′P(O)PNP ($R = C_nH_{2n+1}$, R′ = OY,



$\mathbf{R_{11}}$ =	$C_{11}H_{23}$	$\mathbf{Y_{11}}$ =	$C_{11}H_{23}$
$\mathbf{Y_1}$ =	CH_3	$\mathbf{R_1}$ =	CH_3
$\mathbf{Y_4}$ =	C_4H_9	$\mathbf{R_4}$ =	C_4H_9
$\mathbf{Y_6}$ =	C_6H_{13}	$\mathbf{R_6}$ =	C_6H_{13}
$\mathbf{Y_8}$ =	C_8H_{17}	$\mathbf{R_8}$ =	C_8H_{17}
$\mathbf{Y_{10}}$ =	$C_{10}H_{21}$	$\mathbf{R_{10}}$ =	$C_{10}H_{21}$
$\mathbf{Y_{12}}$ =	$C_{12}H_{25}$	$\mathbf{R_{12}}$ =	$C_{12}H_{25}$
$\mathbf{Y_{14}}$ =	$C_{14}H_{29}$	$\mathbf{R_{14}}$ =	$C_{14}H_{29}$
$\mathbf{Y_{16}}$ =	$C_{16}H_{33}$	$\mathbf{R_{16}}$ =	$C_{16}H_{33}$

FIGURE 7. Structure of Alkylphosphonates **(RY)** used to study the inhibition of digestive lipases.[16]

with Y = $C_{n'}H_{2n'+1}$ and $n = n'$ or $n \neq n'$; PNP = *p*-nitrophenoxy), noted **RY**.[16] We varied successively the alkyl or ester phosphonic chain length, R and Y respectively, from 1 to 16 carbon atoms with *p*-nitrophenoxy as leaving group (Figure 7).

The key step was the successive and selective substitution of the chlorine atoms of **2-R**$_{1-16}$ by various alcohols and *p*-nitrophenol in the presence of *N*-methylimidazole (NMI) to lead to the desired alkylphosphonates **(RY)**, as depicted in Scheme 5. We thus obtained various *O*-alkyl-*O*-(*p*-nitrophenyl) *n*-undecylphosphonates **(R**$_{11}$**Y**$_{1-16}$**)** and *O*-undecyl-*O*-(*p*-nitrophenyl) *n*-alkylphosphonates **(R**$_{1-16}$**Y**$_{11}$**)**, with chemical yields ranging from 40 to 70%. All chiral phosphonates were first synthesized in a racemic form and were further purified by silica gel chromatography. Each enantiomer was next separated by performing chiral liquid chromatography.[16]

The inhibitory properties of each phosphonate enantiomer have been tested with the monomolecular film technique using an ELISA, to estimate simultaneously the residual enzymatic activity as well as the interfacial lipase binding. With both lipases, no obvious correlation between the inhibitor molar fraction (α_{50}) leading to half inhibition and the chain length, R or Y, was observed. In each case, the best inhibitors of HPL and HGL were the levorotatory enantiomers: (−)-(**R**$_{11}$**Y**$_{16}$) and (−)-(**R**$_{10}$**Y**$_{11}$), respectively. Furthermore, a highly enantioselective discrimination, with the pure enantiomeric alkylphosphonate inhibitors as well as a scalemic mixture, was observed.

From the kinetic analysis of the inhibition studies performed by using the monomolecular film technique, we can conclude that the overall inhibition can be attributed to both interfacial binding and a chiral interaction of the inhibitor with the lipase active site. The chiral recognition of HPL and HGL may occur not exclusively at the enzyme active site but also during the initial adsorption step. We thus confirmed that the interfacial lipase binding is controlled by a supramolecular chiral recognition process.[16,45]

The kinetic data were then analyzed using both the covalent and the pseudocompetitive inhibition models.[16,27,28] For the first time, the relative interfacial affinities, (K^*_M/K^*_I), of HPL and HGL for each enantiomeric alkylphosphonate inhibitor and 1,2-dicaprin (substrate) were measured.[16]

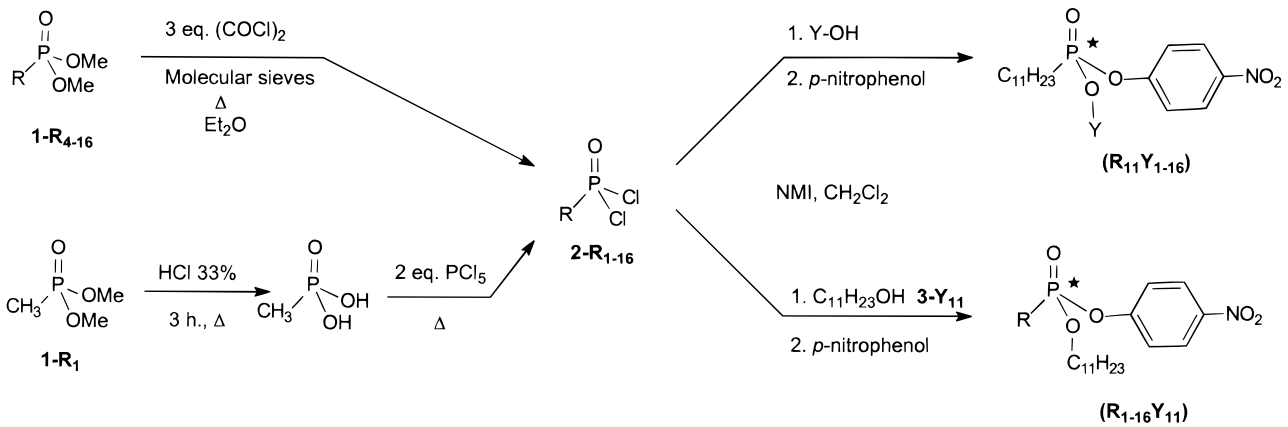
Enantioselective Synthesis of Alkylphosphonates

The need to study various compounds in their pure enantiomeric forms as potential lipase inhibitors prompted us to focus our attention on synthetic methods for optically pure alkylphosphonates. We then developed a new, general, efficient, and highly enantioselective synthetic method for chiral *p*-nitrophenyl alkylphosphonate compounds (Scheme 6).[52]

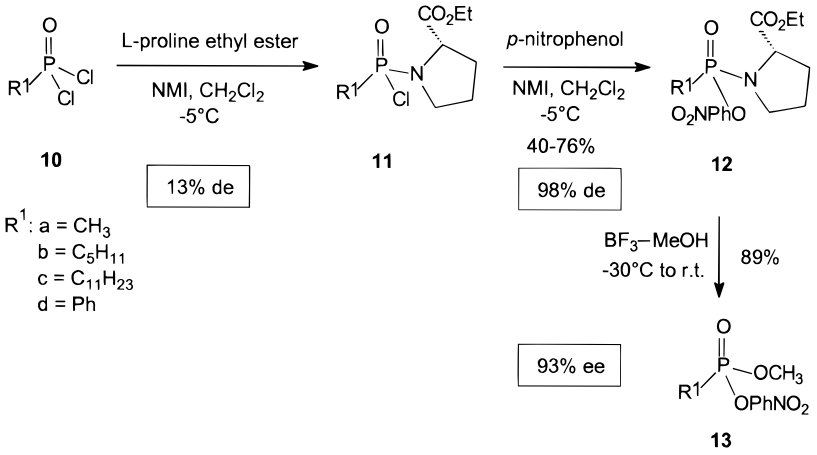
Alkylphosphonic dichloride **10a**−**d** reacted successively with L-proline ethyl ester and *p*-nitrophenol to afford phosphoramidates **12a**−**d** in excellent diastereoselectivity (98% de). Boron trifluoride-catalyzed methanolysis gave compounds **13a**−**c** with a very good enantioselectivity (93% ee). All the phosphonate enantiomers **13a**−**c** obtained by using this procedure were levorotatory, as is the case for the most reactive C-11 phosphonate inhibitor of HPL,[15] compound (−)-**13c**.

The absolute configurations of compounds **13c** and **12c** were derived from the X-ray structure of HPL−colipase

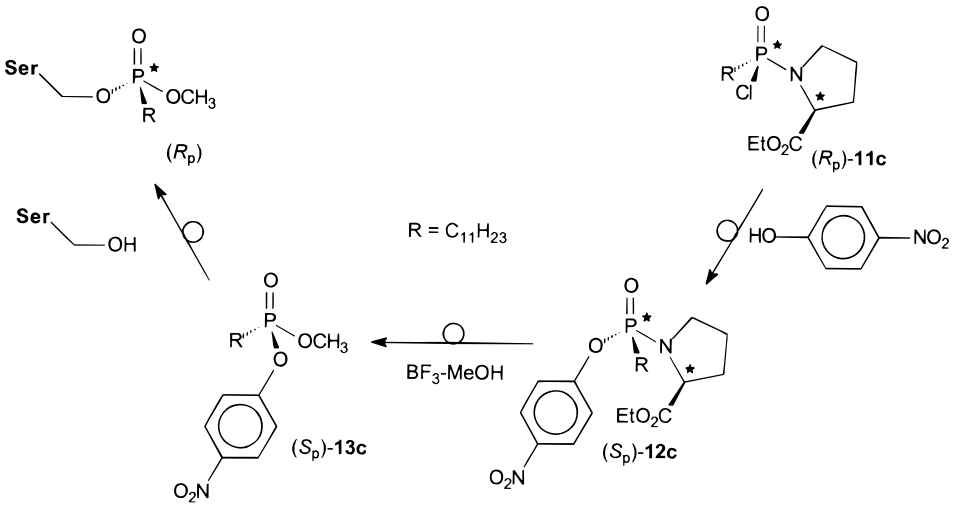
Scheme 5



Scheme 6



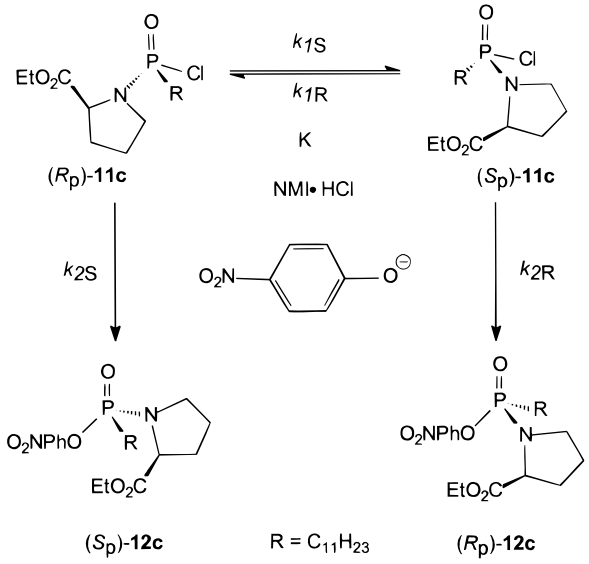
Scheme 7



complexed with (−)-**13c**.[15] We assumed that the nucleophilic substitution of the *p*-nitrophenoxy group by the serine Oγ oxygen, from the lipase active site, proceeds with an inversion of the phosphorus configuration. Since inhibitor (−)-**13c**, once bound to the HPL active site, exhibited an (R_p) absolute configuration, the original configuration of this compound should thus be (S_p). Moreover, compound **13c** was prepared from a methanolysis reaction of **12c** using BF_3−MeOH, which is known to occur with complete inversion of the phosphorus center. Despite this inversion of configuration, the priority rules yield the (S_p) designation for both the starting alkylphosphoramidate **12c** and the inverted alkylphosphonate **13c**. Finally, assuming that the substitution of chlorine by *p*-nitrophenol also proceeds with inversion of configuration in a classical S_N2 mechanism, the configuration of **11c** that led to (S_p)-**12c** should be (R_p) (Scheme 7).[52]

To better understand the origin of the high diastereoselectivity observed for the first time in such types of chemical reactions, dynamic ^{31}P NMR spectroscopy experiments on the crude mixture of the monochlorophosphoramidates **11c** were performed. These NMR studies showed that monochlorophosphoramidates (R_p)- and (S_p)-

Scheme 8



11c undergo fast epimerization. The remarkable diastereoselectivity observed in the formation of (S_p)-**12c** resulted then from a dynamic kinetic resolution process, in which one diastereomer of **11c**, the (R_p) epimer, undergoes

the substitution by the *p*-nitrophenate much faster than the other one ($k_{2S} \gg k_{2R}$, Scheme 8). The two epimers of **11c** being in fast equilibrium (k_{1R}, $k_{1S} \gg k_{2S}$), this reaction obeys the Curtin−Hammett principle, and the high diastereoselectivity in favor of (S_p)-**12c** depends only on the ratio of the substitution rate constants (k_{2S}/k_{2R}).

Conclusion

Glyceride analogues and phosphonate compounds were used as inhibitors of both microbial and human lipases. We have designed and synthesized chiral phosphonate inhibitors which provided us structural and kinetic information about the interactions between the substrate or the inhibitor and the lipase's active site. With the increasingly detailed information available about the kinetic properties of lipases, as well as with the prospect to solve new three-dimensional structures of inhibited lipases with chiral phosphonates, this research area is in constant progress and will remain an exciting frontier.

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