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Enantioselective Inhibition of microbial lipolytic enzymes by non-racemic monocyclic enolphosphonate analogs of Cyclophostin

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ABSTRACT

Four non-racemic enolphosphonate analogs of Cyclophostin were obtained by asymmetric synthesis, and their absolute configurations at both phosphorus and C-5 carbon chiral centers were unambiguously assigned. The influence of chirality was studied by testing the inhibitory effects of these four stereoisomers towards the lipolytic activity of three microbial lipases: *Fusarium solani* Cutinase, Rv0183 and LipY from *Mycobacterium tuberculosis*. Cutinase was highly diastereoselective for the (S_p) configuration using (S_c) inhibitors whereas no obvious stereopreference at phosphorus was observed with (R_c) compounds. Conversely, Rv0183 exhibited strong enantioselective discrimination for (S_p) configuration regardless of the chirality at the asymmetric carbon atom. Lastly, LipY discriminated only the unusual diastereoisomeric configuration (R_c, R_p) leading to the most potent inhibitor. This work, which provides a fundamental premise for the understanding of the stereoselective relationships between non-racemic enolphosphonates and their inhibitory activity, also opens new prospects on the design and synthesis of highly specific enantioselective antimicrobial agents.

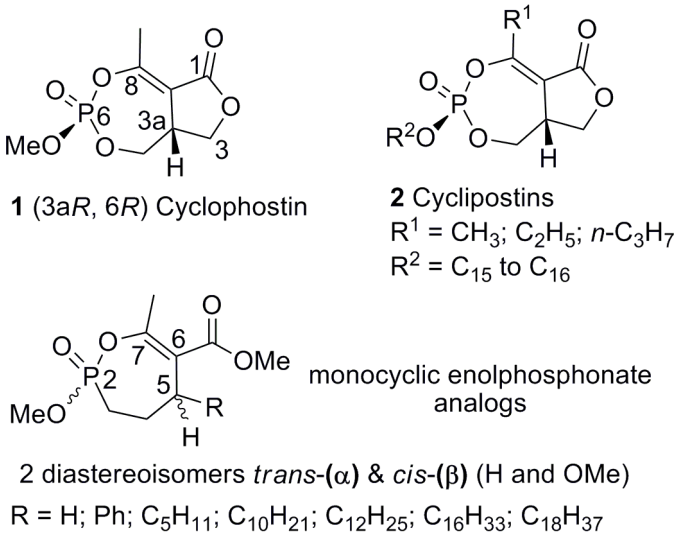
INTRODUCTION

The design and synthesis of specific inhibitors of various animal and microbial lipases is of fundamental value for understanding their mechanism of action as well as for developing novel drugs. Among new phosphorus-containing natural products recently identified as lipase inhibitors, Cyclophostin **1** (Scheme 1) is a bicyclic organophosphate isolated from a fermentation solution of *Streptomyces lavendulae* (strain NK901093).[1] This natural product **1** showed potent inhibition of acetylcholinesterase with a reported IC_{50} in the nanomolar range.[1,2] Based on spectroscopic analysis and X-ray diffraction, the structure of Cyclophostin consisted of a [5.0.3]-bicyclic core with a butyrolactone ring fused to a seven-membered endocyclic enolphosphate ring. The absolute configurations at both C3a and the phosphorus atom (6) were determined to be (**3a*R*, 6*R***). The unusual bicyclic enolphosphate is also found in the family of

structurally related natural products, named the Cyclipostins **2** (Scheme 1).[3] The Cyclipostins **2** possess a core structure similar to that of Cyclophostin, but are phosphate esters of long chain lipophilic alcohols. All members of the Cyclipostins family are very potent inhibitors of hormone-sensitive lipase (HSL),[3] a therapeutic target for type II diabetes. They have also been described as inhibitors of bacterial growth of various mycobacteria (including *Mycobacterium smegmatis*, *Mycobacterium phlei*, *Nocardia abcessus* and *Corynebacterium diphteriae*) with similar minimum inhibitory concentrations than those of Rifampicin and Penicillin G.[4]

Recently, we reported the synthesis of a new series of 18 monocyclic enolphosphonate analogs of Cyclophostin and Cyclipostins (Scheme 1). Their potencies and mechanisms of inhibition toward six representative lipolytic enzymes belonging to distinct lipase families and from various origins were studied.[5] With mammalian gastric and pancreatic lipases no inhibition occurred with any of the compounds tested. Conversely the three microbial enzymes investigated were all fully inactivated. These included *Fusarium solani pisi* Cutinase[6] and two lipolytic enzymes from *Mycobacterium tuberculosis*: Rv0183 an exported monoacylglycerol lipase,[7] and LipY a triacylglycerol lipase belonging to the HSL family involved in triacylglycerols storage or degradation during persistence[8] and in host lipids degradation.[9] The presence of the free or cyclic (lactone) ester functionality in conjugation with the enolphosphonate bond was found essential to the inhibitory activity towards the three microbial lipases. In addition, mass spectrometry analyses confirmed that the enolphosphonate moiety acts as a leaving group by reacting with the active site serine residue of each enzyme, thus leading to the formation of a stoichiometric covalent and irreversible enzyme-inhibitor complex. However, related to their respective substrate specificity, these lipolytic enzymes exhibited different chemoselectivity towards the structures of the tested compounds. In particular, we showed that modulation of the lipophilicity (*i.e.* variation of the R alkyl chain length at C-5 carbon atom) strongly impacted the inhibitory efficiency and could be exploited to significantly either attenuate or increase the affinity of one inhibitor to target a specific lipase over others. In

addition to this chemopreference, the three lipases displayed a high diastereoselectivity for compounds bearing a *cis* configuration (*i.e.* **(β)**-isomers) related to the spatial orientation of H-substituent on C-5 carbon atom and OMe group at phosphorus.[5]



Scheme 1. Structure of Cyclophostin **1**, Cyclipostins **2** and related enolphosphonate analogs[5] recently reported.

Such stereoselectivity at both phosphorus and carbon centers has been observed previously with diastereomeric diacylglycerophosphonates used as inhibitors of either Cutinase[10,11] or human digestive lipases.[12] In each case the inhibition was found highly dependent upon the chirality at the *sn*-2 carbon of the glycerol backbone, the phosphorus atom being in racemic form. Compounds containing the phosphorus moiety at the *sn*-3 position (*i.e.* (R_c)-isomer) were found to be the best inhibitors, therefore reflecting the known stereopreference of these enzymes which preferentially release the fatty acid at the *sn*-3 position of natural triacylglycerols. Assuming that nucleophilic displacement at phosphonate occurred with inversion of stereochemistry,[13] the (S_p) absolute configuration of the most active enantiomeric inhibitor was further established by chemical correlations with the available crystallographic structures of various phosphonate-inhibited enzymes.[11,14-19] The binding of the poorer (R_p)-enantiomer was assumed to be constrained by steric, hydrophobic and

diastereomeric interactions occurring between each enantiomer and the active site of the lipolytic enzyme.[16,18,19]

Since chiral preference of lipases might have important physiological consequences, this phenomenon of enantiomer differentiation therefore deserves further investigation. In addition, understanding their stereoselectivity is a major challenge in view of engineering more potent and specific lipase inhibitors. In particular, the molecular basis of lipase enantioselectivity at phosphorus in combination with the (R_c) or (S_c) configuration at the carbon chiral center still remains not well understood.

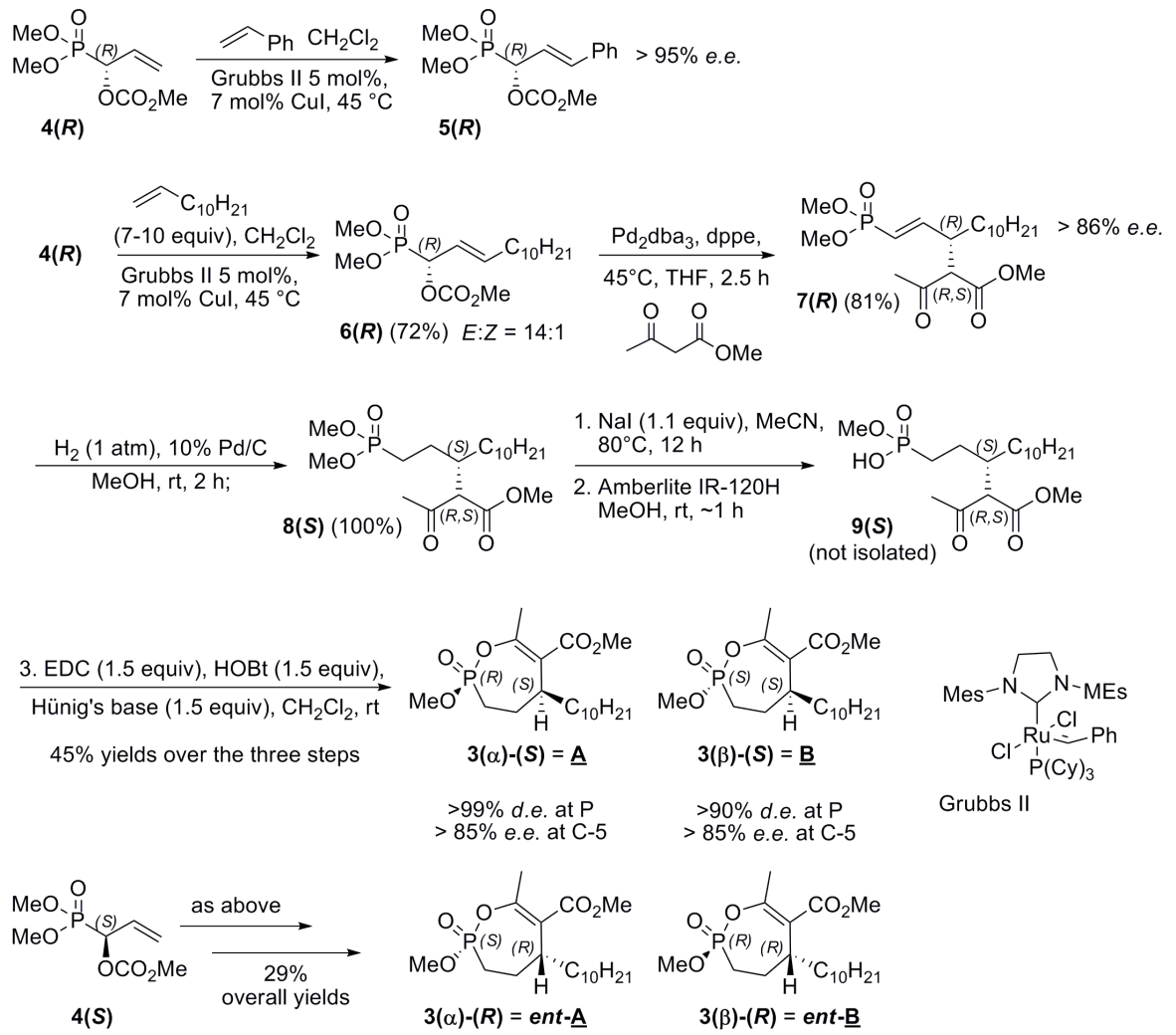
In this context of diastereoselective discrimination and in order to shed more light on the influence of the chirality at both the phosphorus and carbon centers on the inhibition of Cutinase, Rv0183 and LipY, we prepared by asymmetric synthesis the four stereoisomers of monocyclic enolphosphonate **3** (Scheme 2) analog of Cyclophostin. These compounds allowed us to probe and explain the structural elements that determine the previous observed stereoselectivity[5] of the three lipases investigated.

RESULTS AND DISCUSSION

Asymmetric synthesis of monocyclic analogs

The methods employed were adapted to asymmetric synthesis from the recently reported[5] syntheses of racemic monocyclic analogs **3(α)/3(β)** (Scheme 2). First, cross metathesis reaction of the (*R*)[20] or (*S*)[21] 1-(dimethoxyphosphoryl)allyl methyl carbonate **4** with 1-dodecene, using Grubbs second generation catalyst with copper(I) iodide as co-catalyst,[22,23] gave the precursor carbonates **6(*R*)** and **6(*S*)**, respectively, in good yields (70-79%) as mixture of *E* and *Z* isomers in 14:1 ratio (Scheme 2). The absolute configurations of the 1-(dimethoxyphosphoryl)allyl methyl carbonates **4(*R*)** and **4(*S*)** were unambiguously assigned by conversion to the well-characterized enantiomers of (*E*)-1-(dimethoxyphosphoryl)-3-phenylallyl methyl carbonate **5(*R*)** and **5(*S*)** by Grubbs cross metathesis with styrene. Furthermore, the UV absorbing enantiomers

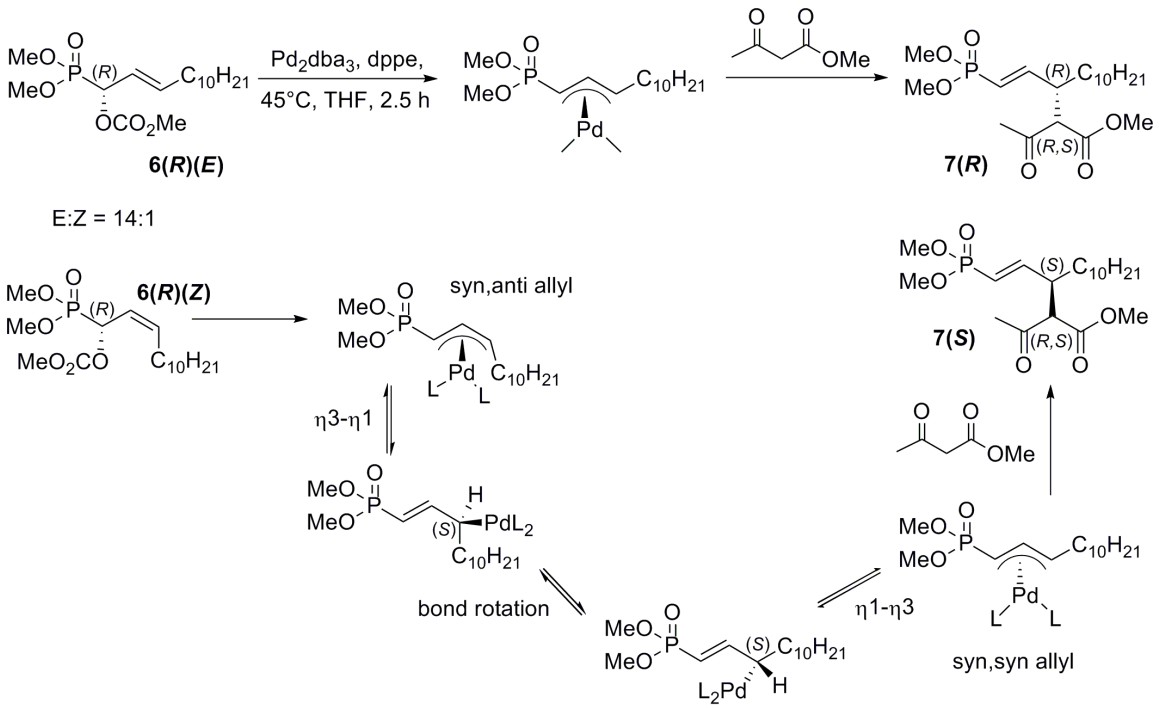
of **5** were easily separable on a chiral stationary phase allowing the enantiomeric excess (> 95% *e.e.*) to be determined.[24,25]



Scheme 2. Asymmetric synthesis of the diastereoisomers of the monocyclic enolphosphonates **3(α)** and **3(β)**.

With the fact that both the absolute configuration on the asymmetric carbon and the enantiomeric excess of the starting phosphonate are preserved upon cross metathesis reaction,[22] the resulting phosphono allylic carbonate **6** therefore displays the same (*R*) configuration with comparable *e.e* to the carbonate **4(*R*)**. Palladium(0)-catalyzed reaction of the carbonates **6** with methyl acetoacetate gave the vinyl phosphonates **7** as a 1:1 diastereoisomeric mixture related to the new acetoacetate carbon stereocenter thus formed. With regards to chirality, we already demonstrated that the palladium-catalyzed addition of nucleophile to such (*E*)-allylic

phosphonates was highly regioselective and stereospecific, taking place exclusively at the 3-position to give the γ-substituted vinyl phosphonates with a predominant (*E*)-alkene geometry and complete chirality transfer.[26-28] In such conditions, the *Z*-isomer (around 6%) also reacts to give the (*E*)-γ-substituted vinyl phosphonate, but with opposite absolute configuration (Scheme 3) resulting in a slight erosion of the enantiomeric excess by approximately 10% to give **7** with > 86% *e.e.* related to the C-3 carbon center.



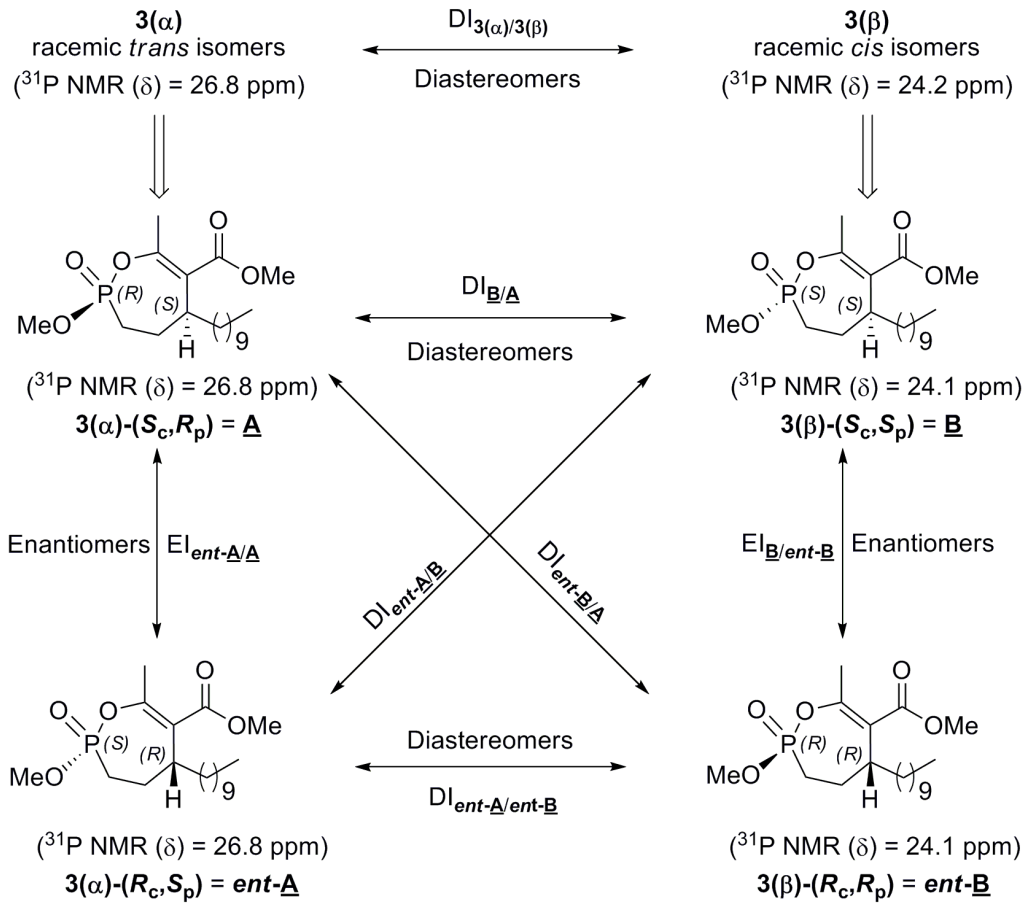
Scheme 3. Mechanism for the palladium(0)-catalyzed reaction of **6(*R*)(*E*)** and **6(*R*)(*Z*)** with methyl acetoacetate.[27]

The treatment of a methanolic solution of these vinyl phosphonates **7** with hydrogen gas over 10% palladium on carbon gave saturated phosphonates **8** in quantitative yields. It is noteworthy that hydrogenation of the double bond in **7** resulted in the inversion of priority rules:[29] a (S_c) designation for the saturated phosphonate **8(*S*)** was indeed obtained starting from **7(*R*)**. This (S_c) stereochemical assignment was conserved after cyclization.

The phosphonates **8** were monodemethylated using sodium iodide (1.1 equiv) in refluxing acetonitrile to give the corresponding sodium salts, which were subsequently converted to

monophosphonic acids **9** by treatment with methanolic solution of Amberlite IR-120H resin. The monophosphonic acids **9** were cyclized without further purification using a combination of EDC, HOBt and Hünig's base (iPr_2NEt) to give the corresponding substituted monocyclic phosphonates *trans*-**3(α)-(*S*)** (= **A**) and *cis*-**3(β)-(*S*)** (= **B**) as diastereoisomeric mixtures in 45-59% yields over the three steps. These two diastereomers **3(α)** and **3(β)**; which varied only by the absolute configuration (R_p or S_p) of the new stereocenter formed at phosphorus; were further separated using silica gel column chromatography. Pure compounds at phosphorus were then obtained with a diastereoisomeric excess determined by ^{31}P NMR of around 99% and 91% for **3(α)** and **3(β)**, respectively. Similarly, the *trans*-**3(α)-(*R*)** (= ***ent*-A**) and *cis*-**3(β)-(*R*)** (= ***ent*-B**) diastereoisomers were synthesized from **4(*S*)**. The HPLC analysis of the *trans* diastereomers **3(α)-(S_c, R_p)** (= **A**) and **3(α)-(R_c, S_p)** (= ***ent*-A**) on chiral stationary phase revealed a full retention of the enantiomeric excess from the (*E*)-vinyl phosphonate **7** with > 85% *e.e* related to the cyclized C-5 carbon center. Similarly, the *cis* diastereomers **3(β)-(S_c, S_p)** (= **B**) and **3(β)-(R_c, R_p)** (= ***ent*-B**) prepared from **4(*S*)** are assumed to have the same enantiomeric excess. These *cis* and *trans* relationships; between the OMe on phosphorus and the H-substituent on C-5 carbon atom; have been unambiguously attributed for each diastereoisomer on the basis of previous structural studies.[5,30]

In that context, taking into accounts the controlled stereochemistry of C-5 carbon atom of **3** as being either (S_c) (from **4(*R*)**) or (R_c) (from **4(*S*)**), on the one hand; and the clearly assigned *cis* or *trans* relationship, on the other hand; it was therefore possible to deduce the absolute configuration at the phosphorus for each isomer (Schemes 2 and 4). The stereochemical relationships between the four separated stereoisomers of **3** are depicted in Scheme 4.



Scheme 4. Stereochemical relationships between the four monocyclic enolphosphonate isomers **3** analogous to Cyclophostin.

In vitro inhibitory evaluation of each stereoisomer of compound 3

In order to investigate the influence of the chirality at both the phosphorus and carbon stereocenters on the inhibitory power, these four stereoisomers have been tested against the activity of Cutinase, Rv0183 and LipY. The stereopreference of these three microbial lipolytic enzymes has been studied on the basis of the Stereoselectivity Index (SI)[5,31] as defined in Eqn. 1; where x_{I50} value is corresponding to the inhibitor molar excess leading to 50% lipase inhibition;[5,32,33] and expressed either as Diastereoselectivity (DI) or Enantioselectivity Index (EI) according to Scheme 4:

$$SI_{(\%)} = \frac{\left|(x_{I50})_{\beta\ isomer} - (x_{I50})_{\alpha\ isomer}\right|}{(x_{I50})_{\beta\ isomer} + (x_{I50})_{\alpha\ isomer}} \times 100 \qquad \text{(Eqn. 1)}$$

Compound **3** bearing a C10-side alkyl chain was chosen for its capacity to efficiently inhibit the three lipases tested (Table 1) with high diastereoselectivity (Table 2) in favor of the racemic *cis*-**(β)** isomer.[5] The inhibition results obtained with the two racemic compounds *trans*-**3(α)** and *cis*-**3(β)** as well as with the four separated stereoisomers **A**, ***ent*-A**, **B** and ***ent*-B** acting on Cutinase, Rv0183 and LipY are reported in Figure 1 and Tables 1 and 2. As expected from the x_{I50} values previously obtained with the racemic isomers,[5] all four stereoisomers strongly inhibit the three lipolytic enzymes. Surprisingly, the inhibition curves depicted in Figure 1 have highlighted clear differences in the stereopreference of each lipase related to the chirality at phosphorus and/or carbon asymmetric centers.

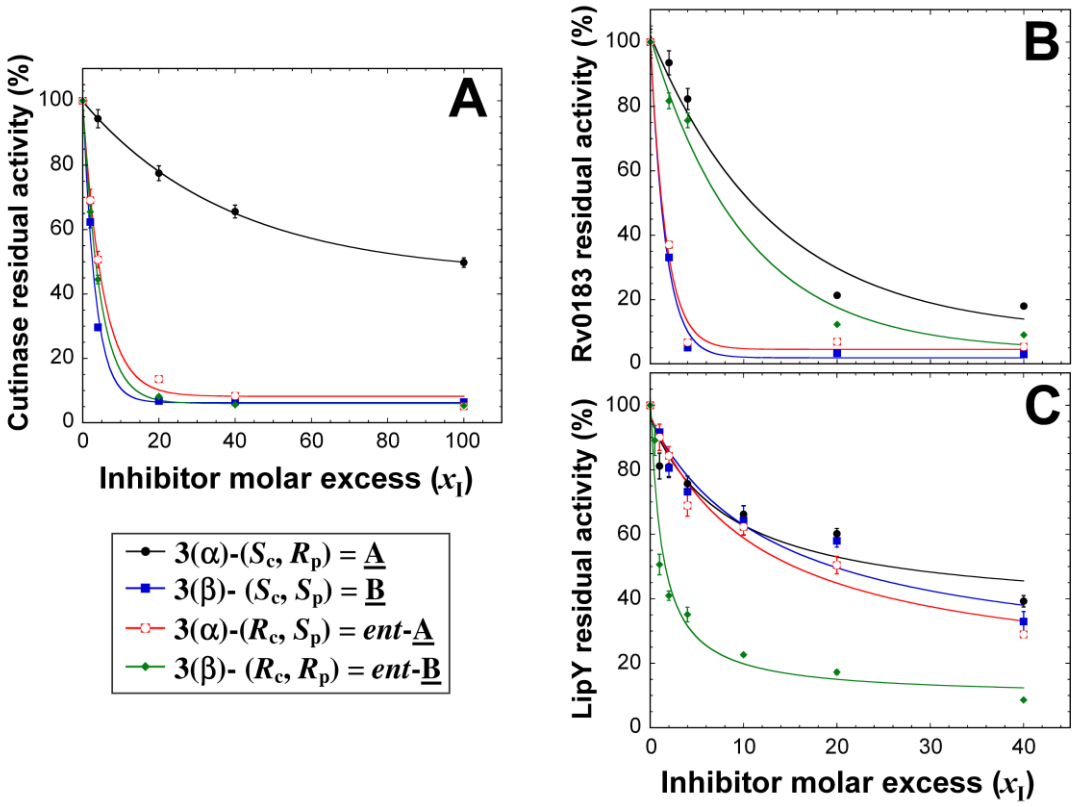


Figure 1: Variation of the residual activities of (**A**) Cutinase, (**B**) Rv0183 and (**C**) LipY as a function of increasing molar excess (x_I) of compounds **3(α)-(S_c, R_p)** (●), **3(β)- (S_c, S_p)** (■), **3(α)-(R_c, S_p)** (□) and **3(β)- (R_c, R_p)** (◆). Each enzyme was pre-incubated at various inhibitor molar excess for 30 min at 25 °C. Kinetic assays were performed as described in **Experimental Section**. Results are expressed as mean values ± standard deviation of at least three independent assays (CV% < 5.0%).

Inhibition of Cutinase

Using (S_c) inhibitors, Cutinase displays high 95.0% (= $DI_{\underline{\mathbf{B}}/\underline{\mathbf{A}}}$) diastereoselectivity in favor of (S_p)-isomer **<u>B</u>** versus the (R_p)-isomer **<u>A</u>**. At the same time, only low stereopreference ($DI_{\underline{\mathbf{B}}/\textit{ent}\text{-}\underline{\mathbf{A}}}$ = 23.5%) between the pair of (S_p)-diastereoisomers ***ent*-<u>A</u>** / **<u>B</u>** was obtained. These results clearly point out a high stereoselective discrimination of Cutinase for the (S_p) configuration at phosphorus whatever the (R_c) (*i.e.* ***ent*-<u>A</u>** isomer) or (S_c) (*i.e.* **<u>B</u>** isomer) configuration at the asymmetric carbon center. However, when using compounds **<u>A</u>** and ***ent*-<u>B</u>** bearing the non-preferential (R_p) configuration, Cutinase is found to strongly differentiate the (R_c)- from the (S_c)-enantiomer with $DI_{\textit{ent}\text{-}\underline{\mathbf{B}}/\underline{\mathbf{A}}}$ of 93.3% in favor of ***ent*-<u>B</u>** isomer. Moreover and as shown by the $DI_{\textit{ent}\text{-}\underline{\mathbf{A}}/\textit{ent}\text{-}\underline{\mathbf{B}}}$ value of 9.3%, no significant stereopreference for the chirality at phosphorus is observed when incubating the lipase with the two (R_c)-isomers, which also exhibit nearly the same potency (mean $x_{I50} = 3.72 \pm 0.347$).

Such chiral differentiation is in good agreement with the work of Mannesse et al.[10,34] Using diastereomeric 1,2-dioctylcarbamoylglycero-*O*-(*p*-nitrophenyl) *n*-alkylphosphonate inhibitors (the alkyl being a methyl or an octyl group - Figure S1 in Supplementary Material) the authors showed that Cutinase reacted 4 to 10-fold faster with the (R_c)- than the (S_c)-enantiomer. They also demonstrated that Cutinase was even more selective for the chirality at phosphorus since one stereoisomer reacted nearly 60-fold slower than the other one.[10] From the crystal structure of Cutinase covalently inhibited by (R_c)-1,2-dibutylcarbamoylglycero-*O*-(*p*-nitrophenyl) *n*-butylphosphonate, Longhi et al.[17,35] showed that only the (R_p)-isomer of the covalently bound inhibitor could be fitted into the observed electron density map starting at the catalytic Ser^{120}-Oγ atom, thus suggesting that only the (S_p)-enantiomer at phosphorus had reacted.

Here, we not only confirm Cutinase (S_p) enantioselectivity at phosphorus postulated so far, but we also provide additional information regarding its stereopreference against four chiral

monocyclic enolphosphonate analogs of Cyclophostin. From the racemic *trans*-**3(α)** and *cis*-**3(β)** isomers, our results suggest a preferential chiral recognition by Cutinase for the (R_c) configuration at the C-5 carbon atom followed by a slight enantiopreference for the (S_p)-isomer. These observations might thus imply, just like in the case of diacylglycerophosphonates, the presence of at least two chiral binding pockets. The first one would fit the C10-acyl chain and also discriminate the two asymmetric carbon atoms; while the second one, close to the catalytic serine, would accommodate the bulky seven-membered ring enolphosphonate cycle with an enantiopreference for the (S_p)-isomer. At present, despite the availability of crystal structures of Cutinase in presence of several inhibitors,[35] such 3D structures however did not help in understanding lipases stereopreference at phosphorus. Explaining such stereoselectivity would require analyzing the enzyme-substrate and/or inhibitor molecular chiral recognition. The mechanism for such enantiomer differentiation could indeed involve the occurrence of preferential interactions (e.g. hydrophobic/hydrophilic interactions, H-bond stabilization, steric hindrance…) between the enzyme and one of the stereoisomers leading, here, to double enantioselective discrimination at both C-5 asymmetric carbon atom and phosphorus.

Inhibition of Rv0183

Regarding Rv0183, a high stereoselectivity is obtained in favor of (S_p)-isomers **B** ($DI_{\mathbf{B}/\mathbf{A}}$ = 81.4% and $EI_{\mathbf{B}/ent\text{-}\mathbf{B}}$ = 73.5%) and ***ent*-A** ($DI_{ent\text{-}\mathbf{A}/ent\text{-}\mathbf{B}}$ = 72.0% and $EI_{ent\text{-}\mathbf{A}/\mathbf{A}}$ = 80.3%), which exhibit the same potent inhibitory power (mean x_{I50} = 1.20 ± 0.0395 and $DI_{ent\text{-}\mathbf{A}/\mathbf{B}}$ = 3.3%). No significant differences are also observed between the two less potent (R_p)-isomers ***ent*-B** and **A** ($DI_{ent\text{-}\mathbf{B}/\mathbf{A}}$ = 19.6%). From these results we can conclude that this monoacylglycerol lipase from *Mycobacterium tuberculosis* displays strong enantioselective discrimination for the (S_p) absolute configuration at phosphorus independently from the (R_c) or (S_c) configuration at the C-5 asymmetric center. Such enantioselectivity for the (S_p)-enantiomer corresponds to the generally-admitted classical enantiopreference of lipases.[13] For instance, both

microbial *Chromobacterium viscosum* and *Rhizopus oryzae* lipases, inhibited by diacylglycero *n*-hexylphosphonate compounds were found to exhibit the same stereopreference for the chirality at the phosphorus regardless of the absolute configuration at the glycerol backbone.[11] Based on the S_N2 nucleophilic attack mechanism of the catalytic serine, the authors postulated that only the (S_p) phosphonate enantiomer reacted with the lipases. Similarly, Kinetic studies on digestive lipases[13,36] supported by the crystal structures of gastric[18,19] and pancreatic[16] lipases in complex with enantiopure alkylphosphonate inhibitors also revealed that these mammalian lipases exhibited a strong enantiopreference for only one stereoisomer which was assumed to be (S_p).

Using the four phosphonate diastereomers **3**, our results (Figure 1B, Tables 1 and 2) clearly demonstrated that Rv0183 reacts preferentially with (S_p)-enantiomers and follows the same "typical" enantiopreference with respect to the phosphorus moiety than classical serine hydrolases. However, contrary to Cutinase where the chirality at the C-5 carbon atom impacts on the inhibition level, Rv0183 stereopreference is independent from the chirality at the asymmetric carbon center and would depend only on enantiomer differentiation at phosphorus.

Inhibition of LipY

LipY, a triacylglycerol lipase belonging to the HSL family, shows a strong stereopreference for the *cis*-**(β)** isomers when dealing with racemic compounds.[5] Testing each of four pure stereoisomers allowed us to refine the influence of both asymmetric centers on this chiral recognition (Figure 1C). Firstly, similar low inhibitory activities have been obtained using phosphonates **A**, **B** and ***ent*-A** (x_{I50} values around 25.9, 20.0 and 15.4, respectively; Table 1), thus resulting in poor stereoselectivity index values between these compounds (Table 2). Surprisingly, LipY shows clear high diastereoselectivity ($DI_{ent\text{-}A/ent\text{-}B}$ = 80.7% and $DI_{ent\text{-}B/A}$ = 88.1%) and enantioselectivity ($EI_{B/ent\text{-}B}$ = 84.9%) only in favor of (R_c, R_p)-***ent*-B** isomer (x_{I50} = 1.64). All these findings highlight the fact that, using these Cyclophostin analogs,

LipY strongly discriminates one single stereoisomer resulting from the combination of both (R_c) and (R_p) absolute configurations leading then to the most potent inhibitor.

To the best of our knowledge, this is the first example of such "unusual" enantioselective recognition for the (R_p) absolute configuration at phosphorus exerted by a lipase. Using the pure enantiomers of ethyl *p*-nitrophenyl *n*-hexylphosphonate as inhibitors, Björkling et al. already observed similar reversed enantioselectivity for the inhibition of the serine protease α-chymotrypsin as compared with *Candida antarctica* and *Rhizomucor miehei* lipases.[37] By comparing the spatial orientation of the active site of each enzyme, the authors indeed showed an opposite orientation (*i.e*. kind of mirror image) of the active site in α-chymotrypsin versus both lipases.[37] In such conditions, they therefore concluded that the (S_p)-alkylphosphonate fitted best in both lipases and the (R_p)-enantiomer in α-chymotrypsin. With the lack of available LipY crystallographic structure or model, we unfortunately are unable to verify the presence of such kind of opposite spatial orientation in the catalytic residues of this lipase. Anyway, this hypothesis has to be kept in mind since it could explain in part this apparent change of stereoselectivity observed for the first time with a lipase.

CONCLUSION

We have reported the enantioselective synthesis of four pure non-racemic monocyclic enolphosphonate analogs of Cyclophostin and assigned without ambiguity the absolute configuration at both asymmetric phosphorous and C-5 carbon centers. Inhibition tests performed with these enantiopure enolphosphonates against the lipolytic activities of Cutinase, Rv0183 and LipY showed that lipases stereopreference was dependent upon the absolute configuration of either phosphorus or C-5 carbon atom; or directly on these two asymmetric centers. This work provides a fundamental premise for the understanding of the stereoselective relationships between enantiopure enolphosphonates and their inhibitory activity towards these three distinct lipases families; thus emphasizing the need for optically active compounds as

specific lipase inhibitors. In particular, modulation of the lipophilicity at the C-5 carbon atom, as previously reported,[5] combined with the unusual high enantiopreference exerted by LipY for the (R_p) and (R_c) associated absolute configurations, should indeed open new prospects in the design of specific inhibitors of mycobacterial lipases. Such specific inhibitors would represent useful probes for better understanding the physiological role of LipY in the growth and pathogenicity of *Mycobacterium tuberculosis*. To support these assumptions, further experiments are currently under way and will be reported in due course.

EXPERIMENTAL SECTION

General Experimental for Synthesis.

All reactions were carried out in oven dried glassware under an atmosphere of argon unless otherwise noted. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 300, 75 and 121 MHz respectively. ^{1}H NMR spectra are referenced to $CDCl_3$ (7.27 ppm), ^{13}C NMR spectra are referenced to the center line of $CDCl_3$ (77.23 ppm) and ^{31}P NMR spectra are referenced to external H_3PO_4. Coupling constants, *J*, are reported in Hz. Analytical thin-layer chromatography (TLC) analyses were performed on silica gel plates $60PF_{254}$. Visualization was accomplished with UV light, $KMnO_4$ solution or iodine. The synthesis of racemic compound **3** and related precursors, have already been reported.[5] The enantiomers of 1-(dimethoxyphosphoryl)allyl methyl carbonates **4** were prepared using previously published methods.[20,21] Target compounds were purified by chromatography and have purity of > 95% as determined by HPLC analysis conducted on an Agilent 1100 system, using a Zorbax Eclipse XBD-C18 column as previously described.[5] The HPLC data was supported by careful analysis of the ^{1}H, ^{13}C and particularly the ^{31}P NMR spectra. Regarding the stereochemical purity at C-5 carbon center, only the *trans*-**(α)** diastereoisomers [^{31}P NMR = 26.8 ppm] were easily analyzed by chiral HPLC. The analyses of the non-racemic enolphosphonates **3(α)-(S_c, R_p)** (= **A**) and **3(α)-(R_c, S_p)** (= ***ent*-A**) were conducted using a chiral (*S*,*S*) Whelk-O column. Elution was performed at a flow rate of 1 mL min^{-1} using isopropanol/heptane 8:92 (v/v) as eluent, and detection at λ = 230 nm.

(*R*, *E*)-1-(Dimethoxyphosphoryl)tridec-2-en-1-yl methyl carbonate 6(*R*).

To a solution of carbonate **4**(***R***) (0.67 g, 3 mmol, > 95% *e.e.*) and 1-dodecene (6.7 mL, 30 mmol) in dry CH_2Cl_2 (6 mL) was added Grubbs 2nd generation catalyst (0.13 g, 0.15 mmol) followed by CuI (0.04 g, 0.21 mmol). After stirring the solution for 2 min at room temperature, the septum was replaced by a condenser and the reaction flask was placed in an oil bath preheated to 45 ^{o}C. After completion of the reaction (TLC and ^{31}P NMR analysis), the solvent was removed under reduced pressure to give the crude product. Purification by column

chromatography (SiO_2, 20-50% EtOAc in hexanes) gave pure **6(*R*)** (0.8 g, 72%, *E*:*Z* = 14:1). All solution spectroscopic data (IR, ^1H and ^{13}C and ^{31}P NMR spectra) were identical to the racemic compound.[5] HRMS (FAB, MH^+) calcd. for $C_{17}H_{34}O_6P$: 365.2092, found 365.2089; $[\alpha]_D^{25}$= + 27.5 (*c* = 1.5, $CHCl_3$).

(*S*, *E*)-1-(Dimethoxyphosphoryl)tridec-2-en-1-yl methyl carbonate 6(*S*).

To a solution of carbonate **4**(***S***) (0.67 g, 3 mmol, > 95% *e.e.*) and 1-dodecene (6.7 mL, 30 mmol) in dry CH_2Cl_2 (6 mL) was added Grubbs 2nd generation catalyst (0.13 g, 0.15 mmol) followed by CuI (0.04 g, 0.21 mmol). After stirring the solution for 2 min at room temperature, the septum was replaced by a condenser connected to an argon bubbler and the reaction flask was placed in an oil bath preheated to 45 ^oC. After completion of the reaction (TLC and ^{31}P NMR analysis), the solvent was removed under reduced pressure to give the crude product. Purification by column chromatography (SiO_2, 20-50% EtOAc in hexanes) gave pure **6(*S*)** (0.8 g, 73%, *E*:*Z* = 14:1). All solution spectroscopic data (IR, ^1H and ^{13}C and ^{31}P NMR spectra) were identical to the racemic compound.[5] HRMS (FAB, MH^+) calcd. for $C_{17}H_{34}O_6P$: 365.2092, found 365.2096; $[\alpha]_D^{25}$ = -26.2 (*c* = 1.5, $CHCl_3$).

(*2R, 3R*)- and (*2S, 3R*)-methyl 2-acetyl-3-((*E*)-2-(dimethoxyphosphoryl)vinyl) tridecanoate 7(*R*).

To a stirring solution of carbonate **6(*R*)** (0.37 g, 1 mmol) and methyl acetoacetate (0.33 mL, 3 mmol) in dry THF (2 mL) was added $Pd_2(dba)_3$ (0.02 g, 0.02 mmol) followed by dppe (0.02 g, 0.05 mmol) and the resulting mixture was stirred at room temperature for 3-4 min. The septum was replaced by a condenser connected to an argon bubbler and the reaction flask was placed in an oil bath preheated to 45 ^oC. After completion of the reaction (TLC and ^{31}P NMR analysis), the reaction mixture was partitioned between brine and EtOAc. The organic layer was collected and the aqueous layer was extracted with additional EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give crude product. Purification by column chromatography (SiO_2, 20-70% EtOAc in hexanes)

gave **7(*R*)** as pale yellow oil (0.33 g, 81%, 1:1 mixture of diastereomers). All solution spectroscopic data (IR, ^{1}H and ^{13}C and ^{31}P NMR spectra) were identical to the racemic compound.[5] HRMS (FAB, MH^+) calcd. for $C_{20}H_{38}O_6P$: 405.2406, found 405.2402.

(*2R, 3S*)- and (*2S, 3S*)-methyl 2-acetyl-3-((*E*)-2-(dimethoxyphosphoryl)vinyl) tridecanoate 7(*S*).

To a stirring solution of carbonate **6(*S*)** (0.61 g, 1.66 mmol) and methyl acetoacetate (0.54 mL, 5 mmol) in dry THF (4 mL) was added $Pd_2(dba)_3$ (0.03 g, 0.03 mmol) followed by dppe (0.03 g, 0.08 mmol) and the resulting mixture was stirred at room temperature for 3-4 min. The septum was replaced by a condenser connected to an argon bubbler and the reaction flask was placed in an oil bath preheated to 45 ^{o}C. After completion of the reaction (TLC and ^{31}P NMR analysis), the reaction mixture was partitioned between brine and EtOAc. The organic layer was collected and the aqueous layer was extracted with additional EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give crude product. Purification by column chromatography (SiO_2, 20-70% EtOAc in hexanes) gave **7(*S*)** as pale yellow oil (0.48 g, 76%, 1:1 mixture of diastereomers). All solution spectroscopic data (IR, ^{1}H and ^{13}C and ^{31}P NMR spectra) were identical to the racemic compound.[5] HRMS (FAB, MH^+) calcd. for $C_{20}H_{38}O_6P$: 405.2406, found 405.2409.

(*2R, 3S*)- and (*2S, 3S*)-methyl 2-acetyl-3-(2-(dimethoxyphosphoryl)ethyl) tridecanoate 8(*S*).

To a solution of vinyl phosphonate **7(*R*)** (0.44 g, 1.08 mmol) in methanol (5 mL) was added palladium on charcoal (0.01 g, 0.11 mmol). The solution was first flushed with argon and then with hydrogen gas. A reservoir of hydrogen was provided by a balloon. After completion of the reaction (^{31}P NMR analysis), the reaction mixture was filtered through Celite which was washed with additional CH_2Cl_2. The solvent was evaporated under reduced pressure to give the saturated phosphonate **8(*S*)** as colorless oil (0.44 g, 100%). All solution spectroscopic data (IR,

^{1}H and ^{13}C and ^{31}P NMR spectra) were identical to the racemic compound.[5] HRMS (FAB, MH^{+}) calcd. for $C_{20}H_{40}O_6P$: 407.2562, found 407.2557.

(*2S, 3R*)- and (*2R, 3R*)-methyl 2-acetyl-3-(2-(dimethoxyphosphoryl)ethyl) tridecanoate 8(*R*).

To a solution of **7(*S*)** (0.46 g, 1.13 mmol) in methanol (4 mL) was added palladium on charcoal (0.01 g, 0.11 mmol). The solution was first flushed with argon and then with hydrogen gas. A reservoir of hydrogen was provided by a balloon. After completion of the reaction (^{31}P NMR analysis), the reaction mixture was filtered through Celite which was washed with additional CH_2Cl_2. The solvent was evaporated under reduced pressure to give the saturated phosphonate **8(*R*)** as colorless oil (0.46 g, 100%). All solution spectroscopic data (IR, ^{1}H and ^{13}C and ^{31}P NMR spectra) were identical to the racemic compound.[5] HRMS (FAB, MH^{+}) calcd. for $C_{20}H_{40}O_6P$: 407.2562, found 407.2561.

(*5S, 2R*)- and (5*S*, 2*S*)-methyl 5-decyl-2-methoxy-7-methyl-2,3,4,5-tetrahydro-1,2-oxaphosphepine-6-carboxylate 2-oxide 3(α)-(S_c, R_p) (= **A**) and **3(β)-(S_c, S_p)** (= **B**).

To a solution of alkyl phosphonate **8(*S*)** (0.21 g, 0.5 mmol) in acetonitrile (1 mL) was added sodium iodide (0.082 g, 0.55 mmol) and the resulting mixture was heated at reflux. After completion of the reaction (^{31}P NMR analysis), the solvent was removed under reduced pressure to give mono-sodium salt as solid. The sodium salt was suspended in methanol, amberlite IR-120H resin (prewashed with methanol) was added and resulting mixture was shaken in an orbit shaker. After completion of the reaction (^{31}P NMR analysis), the resin was removed by filtration and washed with additional methanol. The solvent was removed under reduced pressure to give free monophosphonic acid **9(*S*)** as a red viscous liquid. To a solution of crude monophosphonic acid (0.20 g, 0.5 mmol) in dry CH_2Cl_2 (11 mL) was added EDC (0.14 g, 0.75 mmol) and HOBt (0.1 g, 0.75 mmol) followed by Hunig's base (0.13 mL, 0.75 mmol). After completion of the reaction (^{31}P NMR analysis), the reaction mixture was diluted with additional CH_2Cl_2 and the resulting solution was washed with saturated $NaHCO_3$. The organic

layer was separated and dried over anhydrous Na_2SO_4, then evaporated under reduced pressure to give crude product. Purification by column chromatography (SiO_2, 10-30% EtOAc in hexanes) gave 1:1.1 mixtures of diastereomers (0.08 g, 45%). Further chromatographic separation gave **A** as pale yellow oil; Stereochemical purity ≥ 99% *d.e*. at phosphorus (determined by ^{31}P NMR) and ≥ 86% *e.e* at C-5 carbon center (determined by chiral HPLC, r.t. = 20.052 min); ^{31}P NMR ($CDCl_3$) δ 26.8 ppm; HRMS (FAB, MH^+) calcd. for $C_{19}H_{35}O_5P$: 374.2222, found 374.2227; and **B** also as pale yellow oil; Stereochemical purity ≥ 93% *d.e*. at phosphorus (determined by ^{31}P NMR); ^{31}P NMR ($CDCl_3$) δ 24.1 ppm; HRMS (FAB, MH^+) calcd. for $C_{19}H_{36}O_5P$: 375.2300, found 375.2307; All solution spectroscopic data (IR, ^1H and ^{13}C and ^{31}P NMR spectra) were identical to the racemic compounds.[5]

(*5R, 2S*)- and (5*R*, 2*R*)-methyl 5-decyl-2-methoxy-7-methyl-2,3,4,5-tetrahydro-1,2-oxaphosphepine-6-carboxylate 2-oxide 3(α)-(R_c, S_p) (= ***ent*-A**)and **3(β)-(R_c, R_p)** (= ***ent*-B**).

To a solution of alkyl phosphonate **8(*R*)** (0.46 g, 1.13 mmol) in acetonitrile (3 mL) was added sodium iodide (0.19 g, 1.25 mmol) and the resulting mixture was heated at reflux. After completion of the reaction (^{31}P NMR analysis), the solvent was removed under reduced pressure to give mono-sodium salt as solid. The sodium salt was suspended in methanol, amberlite IR-120H resin (prewashed with methanol) was added and resulting mixture was shaken in an orbit shaker. After completion of the reaction (^{31}P NMR analysis), the resin was removed by filtration and washed with additional methanol. The solvent was removed under reduced pressure to give free monophosphonic acid **9(*R*)** as a red viscous liquid. To the solution of crude monophosphonic acid (0.432 g, 1.1 mmol) in dry methylene chloride (11 mL) was added EDC (0.32 g, 1.65 mmol) and HOBt (0.23 g, 1.65 mmol) followed by Hunig's base (0.29 mL, 1.65 mmol). After completion of the reaction (^{31}P NMR analysis), the reaction mixture was diluted with additional CH_2Cl_2 and the resulting solution was washed with saturated $NaHCO_3$. The organic layer was separated and dried over anhydrous Na_2SO_4, then evaporated under reduced pressure to give crude product. Purification by column chromatography (SiO_2, 10-30%

EtOAc in hexanes) gave 1:1.1 mixture of diastereomers (0.22 g, 53.4%). Further chromatographic separation gave ***ent*-A** as pale yellow oil; Stereochemical purity ≥ 99% *d.e.* at phosphorus (determined by ^{31}P NMR) and ≥ 85% *e.e* at C-5 carbon center (determined by chiral HPLC, r.t. = 18.857 min); ^{31}P NMR ($CDCl_3$) δ 26.8 ppm; HRMS (FAB, MH^+) calcd. for $C_{19}H_{36}O_5P$: 375.2300, found 375.2307; and ***ent*-B** also as pale yellow oil; Stereochemical purity ≥ 90% *d.e.* at phosphorus (determined by ^{31}P NMR); ^{31}P NMR ($CDCl_3$) δ 24.1 ppm; HRMS (FAB, MH^+) calcd. for $C_{19}H_{35}O_5PNa$: 397.2119, found 397.2128.All solution spectroscopic data (IR, ^{1}H and ^{13}C and ^{31}P NMR spectra) were identical to the racemic compounds.[5]

***In vitro* biological evaluation**

***Reagents*.** Glyceryl tributyrate (tributyrin, TC4), 1-Oleoyl-rac-glycerol (1-monoolein, MC18) and sodium taurodeoxycholate (NaTDC) were purchased from Sigma-Aldrich-Fluka Chimie (St-Quentin-Fallavier, France). Sodium chloride (NaCl) and Tris-(hydroxymethyl)-aminomethane (Tris) were purchased from VWR International (Fontenay-sous-Bois, France) and from Euromedex (Mundolsheim, France), respectively. All organic solvents were purchased from Carlo Erba Reactifs-SDS (Val de Reuil, France) and were of HPLC grade.

***Lipases*.** *Fusarium solani pisi* Cutinase was produced and purified according to reported procedure.[38] The lipolytic enzymes from *Mycobacterium tuberculosis*, Rv0183 (monoacylglycerol lipase) and LipY (triacylglycerol lipase) were produced and purified as previously described.[39,40]

***Lipase activity measurements using the pH-stat technique*.** Enzymatic activity was assayed by measuring the amount of free fatty acid (FFA) released from mechanically stirred acylglycerols emulsions at 37 °C using 0.1 N NaOH with a pH-stat (Metrohm 718 STAT Titrino, Switzerland) adjusted to a fixed end point value, as previously described in Point et al.[5] Cutinase and LipY activities were determined using TC4 specific assay emulsions: 0.5 mL TC4 was added to 14.5 mL buffer solution. With Cutinase, the assay solution was 2.5 mM Tris-HCl

(pH 8.0), 150 mM NaCl containing 0.25 mM NaTDC. With LipY, the assay solution was 2.5 mM Tris-HCl (pH 7.5), 300 mM NaCl containing 3 mM NaTDC. With regards to Rv0183, assays were performed with 100 µL MC18 added to 15 mL of 2.5 mM Tris-HCl (pH 8.0), 150 mM NaCl containing 3 mM NaTDC. All experiments were at least performed in triplicate. Activities were expressed as international units: 1 U = 1 µmole FFA released per minute. The specific activities of Cutinase, Rv0183 and LipY, expressed as U per mg of pure enzyme, were found to be: 2517 ±246, 287 ±6.7 and 120 ±2.6 U/mg, respectively.

***Inhibition assays*.** The lipase-inhibitor pre-incubation method was used to test, in aqueous medium and in the absence of substrate, the possible direct reactions between lipases and inhibitors as previously described.[5] Briefly, an aliquot of each enzyme was pre-incubated at 25 °C with each isomer of **3** at various inhibitor molar excess (x_I). A sample of the incubation medium was collected after 30 min. incubation period and injected into the pH-stat vessel to measure the residual lipase activity. The variation in the residual lipase activity allowed determining the inhibitor molar excess which reduced the enzyme activity to 50% of its initial value (x_{I50}).[5,33] In each case control experiments were performed in absence of inhibitor.

Experimental data obtained with soluble lipolytic enzymes acting on insoluble substrates have to be analyzed and processed with care, because the partitioning of the enzyme between the aqueous phase and the substrate interface must be taken into account. Unfortunately, in most experimental set-ups the enzyme activity and the partitioning process cannot be measured simultaneously. In consequence, the Michaelis-Menten–Henri model no longer applies[41] and the K_m, K_i and IC_{50} values often estimated for lipases and expressed in terms of volume concentrations are irrelevant when insoluble substrates are used. In view of this specific mechanism of action, the use of more appropriate kinetic constants such as the x_{I50} values[5,32,33] is therefore recommended. Thereby, a x_{I50} value of 0.5 is synonymous with a 1:1 stoichiometric ratio between the inhibitor and the lipase, and is therefore the highest level of inhibitory activity that can be achieved.

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SUPPORTING INFORMATION AVAILABLE

Figure S1 and ^{1}H, ^{13}C and ^{31}P NMR spectra of compounds **6-8** and racemic **3(α,β)**, as well as HPLC analysis of **3(α)**, **3(α)-(R_c, S_p)** and **3(α)-(S_c, R_p)** are available free of charge at http://pubs.acs.org.

ABBREVIATIONS USED

DI, Diastereoselectivity Index; EI, Enantioselectivity Index; HSL, hormone-sensitive lipase; x_I, inhibitor molar excess related to 1 mol of enzyme; x_{I50}, inhibitor molar excess leading to 50% lipase inhibition.

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Table 1. Inhibition of Cutinase, Rv0183 and LipY by chiral monocyclic enolphosphonate compounds **3** after a 30-min incubation period.[a]

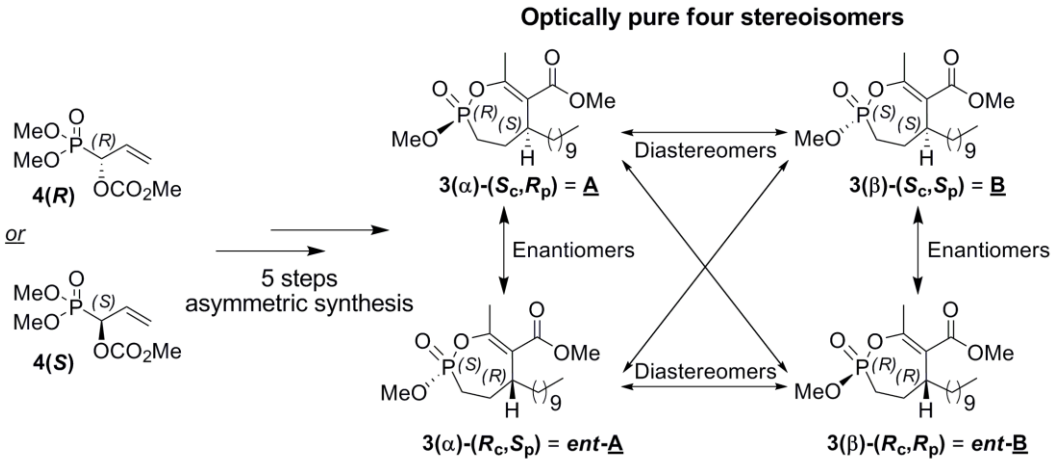
Compound	Cutinase		Rv0183		LipY	
	%inhibition (x_I = 20)	x_{I50}	%inhibition (x_I = 20)	x_{I50}	%inhibition (x_I = 20)	x_{I50}
3(α) [b] Racemic *trans* isomers	57.5% ±1.45%	14.6	92.5% ±1.33%	3.57	43.8% ±1.12%	22.7
3(β) [b] Racemic *cis* isomers	97.3% ±2.47%	1.98	96.1% ±0.870%	1.13	64.1% ±1.21%	2.76
3(α)-(*S*$_c$, *R*$_p$) = **A**	22.5% ±1.04%	97.3	78.7% ±3.09%	11.3	39.8% ±1.02%	25.9
3(β)- (*S*$_c$, *S*$_p$) = **B**	93.2% ±1.23%	2.52	96.6% ±1.45%	1.16	42.1% ±1.41%	20.0
3(α)-(*R*$_c$, *S*$_p$) = ***ent*-A**	86.5% ±1.39%	4.07	93.1% ±1.63%	1.24	49.6% ±2.17%	15.4
3(β)- (*R*$_c$, *R*$_p$) = ***ent*-B**	91.8% ±1.57%	3.38	87.7% ±2.50%	7.61	82.8% ±3.15%	1.64

[a] Inhibition data (%), at an inhibitor molar excess (x_I) of 20 related to 1 mol of enzyme, are derived from Figure 1 and expressed as mean values ± standard deviation of three independent assays (n=3; CV% < 5.0%). The inhibitor molar excess leading to 50% lipase inhibition, x_{I50} values, was determined from inhibition curves as described in **Experimental Section**. [b] Data from reference 5.

Table 2. Stereoselectivity Index values, expressed either as Diastereoselectivity Index (DI) or Enantioselectivity Index (EI), calculated from Eqn. 1 using the x_{I50} values obtained from inhibition tests with Cutinase, Rv0183 and LipY, respectively.

	Stereoselectivity Index		
	Cutinase	Rv0183	LipY
$DI_{\mathbf{3(\alpha)/3(\beta)}}$	76.2%	51.9%	78.3%
$DI_{\underline{\mathbf{B}}/\underline{\mathbf{A}}}$	94.9%	81.4%	12.8%
$DI_{\textit{ent}\text{-}\underline{\mathbf{A}}/\textit{ent}\text{-}\underline{\mathbf{B}}}$	9.3%	72.0%	80.7%
$DI_{\textit{ent}\text{-}\underline{\mathbf{A}}/\underline{\mathbf{B}}}$	23.5%	3.3%	13.0%
$DI_{\textit{ent}\text{-}\underline{\mathbf{B}}/\underline{\mathbf{A}}}$	93.3%	19.6%	88.1%
$EI_{\textit{ent}\text{-}\underline{\mathbf{A}}/\underline{\mathbf{A}}}$	92.0%	80.3%	25.5%
$EI_{\underline{\mathbf{B}}/\textit{ent}\text{-}\underline{\mathbf{B}}}$	14.4%	73.5%	84.9%

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Understanding the stereoselective relationships between non-racemic enolphosphonates and their inhibitory activity towards microbial lipases