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Lipase-mediated stereoselective hydrolysis of stampidine and other phosphoramidate derivatives of stavudine

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Abstract—Enzymatic hydrolysis of stampidine and other aryl phosphate derivatives of stavudine were investigated using the *Candida Antarctica* Type B lipase. Modeling studies and comparison of the hydrolysis rate constants revealed a chiral preference of the lipase active site for the putative *S*-stereoisomer. The in vitro anti-HIV activity of these compounds correlated with their susceptibility to lipase- (but not esterase-) mediated hydrolysis. We propose that stampidine undergoes rapid enzymatic hydrolysis in the presence of lipase according to the following biochemical pathway: During the first step, hydrolysis of the ester group results in the formation of carboxylic acid. Subsequent step involves an intramolecular cyclization at the phosphorous center with simultaneous elimination of the phenoxy group to form a cyclic intermediate. In the presence of water, this intermediate is converted into the active metabolite Ala-d4T-MP. We postulate that the lipase hydrolyzes the methyl ester group of the L-alanine side chain to form the cyclic intermediate in a stereoselective fashion. This hypothesis was supported by experimental data showing that chloroethyl substituted derivatives of stampidine, which possess a chloroethyl linker unit instead of a methyl ester side chain, were resistant to lipase-mediated hydrolysis, which excludes the possibility of a direct hydrolysis of stampidine at the phosphorous center. Thus, our model implies that the lipase-mediated formation of the cyclic intermediate is a key step in metabolism of stampidine and relies on the initial configuration of the stereoisomers.

1. Introduction

Stampidine is a novel phosphoramidate derivative of stavudine, which is being developed as a potential anti-HIV agent.[1–5] Stampidine was 100-times more active than stavudine and twice as active as zidovudine against nine clinical HIV-1 isolates of nonB envelope subtypes (A, C, F, and G) originating from South America, Asia, and subSaharan Africa.[1] Stampidine was effective against 20 genotypically and phenotypically nucleoside analog reverse transcriptase inhibitor (NRTI)-resistant and six nonnucleoside inhibitor (NNRTI)-resistant HIV-1 isolates at subnanomolar to low nanomolar concentrations.[1] Stampidine was active against HIV-1 isolates with five thymidine analog mutations at subnanomolar concentrations. Orally or intraperitoneally administered stampidine exhibited significant and dose dependent in vivo anti-HIV activity against an NRTI-resistant clinical HIV-1 isolate in severe combined immunodeficient (SCID) mice reconstituted with peripheral blood (PBL) mononuclear cells from seronegative human donors.[4] In the feline immunodeficiency virus (FIV)-infected domestic cat model for AIDS, orally administered stampidine showed a dose-dependent anti-retroviral effect in chronically FIV-infected cats.[5] Stampidine therapy was not associated with any clinical or laboratory evidence of toxicity at dose levels as high as $500\,mg\,kg^{-1}$ or at cumulative dose levels as high as $8.4\,g\,kg^{-1}$. Stampidine exhibited favorable pharmacokinetic behavior in mice, rats, dogs, and cats following oral administration.[6,7] The documented in vitro potency of stampidine against primary clinical HIV-1 isolates with genotypic and/or phenotypic NRTI- or NNRTI-resistance as well as nonB envelope subtypes together with its in vivo anti-retroviral activity in HIV-infected Hu-PBL SCID mice and FIV-infected cats warrants its further development as a new anti-HIV drug.

The generation of the active metabolite of stampidine was originally thought to require the esterase-mediated hydrolysis of the carbomethoxy group associated with

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the alanine side chain of stampidine.[8–13] Recent reviews by Wagner et al.[14,15] and Meier[16–18] illustrates the prodrug methodology for the in vivo delivery of various nucleotides. In addition, Wagner and co-workers[19] have reported the pharmacokinetics of amino acid phosphoramidate monoesters of zidavudine in rats and came to the conclusion that these compounds showed greater tissue distribution and improved pharmacokinetic properties of over zidavudine. We hypothesized that in various tissue microenvironments the metabolism of stampidine may occur through the action of hydrolytic enzymes other than esterases as well. The purpose of the present study was to evaluate the potential role of lipases in the activation of stampidine. Our experimental results provide evidence that stampidine as well as other halogen-substituted phosphoramidate derivatives of stavudine can be metabolized by lipase-mediated hydrolysis. Modeling studies and comparison of the hydrolysis rate constants revealed a chiral preference of the lipase active site for the putative *S*-stereoisomer of these compounds. Our model indicates that the lipase-mediated formation of the cyclic intermediate is a key step in metabolism of stampidine and relies on the initial configuration of the stereoisomers. To our knowledge this study is the first to provide experimental evidence for a lipase-mediated metabolism of a nucleoside phosphoramidate prodrug. A stereoselective hydrolytic activation of such compounds by a lipase has never been reported or even considered. In our earlier publication[7,8] the metabolism of these phosphoramidate derivatives with cellular enzymes such as esterases have been studied. The results show that apart from esterase's other enzymes may be involved in the metabolic pathway of these phosphoramidate derivatives. To further understand the metabolic pathway, we chose *candida* B lipase as a model enzyme in the present study.

2. Results and discussion

2.1. Lipase-mediated hydrolysis of stampidine

It has been postulated that phosphoramidate derivatives of stavudine are activated after an enzymatic hydrolysis mediated by esterase's.[20–22] No other enzymes have yet been proposed as alternative mediators of the hydrolysis of this promising new class of anti-HIV compounds. Since lipases have been shown to be capable of hydrolyzing other chiral compounds with a phosphorous center,[23–28] we sought to determine if the 4-Br substituted phosphoramidate derivative stampidine could be hydrolyzed by a lipase as well. The structurally well-characterized lipase derived from *Candida Antarctica* Type B was selected for the experiments. The porcine liver esterase was included as a control for comparison. Remarkably, lipase-mediated hydrolysis of stampidine was much more efficient than its esterase-mediated hydrolysis (Fig. 1). Furthermore, the unsubstituted compound **1** with 2-fold lower anti-HIV activity showed a 3-fold faster hydrolysis in the presence of the esterase, but 3-fold slower hydrolysis in the presence of the lipase. Thus, the in vitro anti-HIV activity of these two compounds correlated with their susceptibility to lipase-mediated hydrolysis but not esterase-mediated hydrolysis. These findings prompted us to further elucidate the lipase-mediated hydrolysis of stampidine and other 4-halogen substituted phosphoramidate derivatives of stavudine.

2.2. Stereoisomers of stampidine

Due to the stereochemistry of its phosphorous chiral center, stampidine exists as a mixture of two possible diastereomers (Fig. 2). The individual stereoisomers were separated by preparative HPLC due to their distinct retention times of 20.0 and 21.2 min, respectively, lyophilized, and examined by IR, polarimetry, HPLC, NMR, and melting point analysis. No significant differences in optical rotation were observed (−30° vs −27.5°) by polarimetric analysis and their individual ^{1}H NMR spectra were identical. Repetitive HPLC and ^{31}P NMR analyses during a 36-h kinetics experiment confirmed that the separated diastereomers are stable and do not undergo interconversion. The physicochemical constants of the purified diastereomers are described in the experimental section. The in vitro anti-HIV potency of the purified diastereomers (IC_{50}: 0.001 μM for both) was identical to the in vitro anti-HIV potency of the diastereomeric mixture of stampidine (IC_{50}: 0.001 μM). Since there appears to be no difference in the anti-iviral activity of the two distereoisomers, the observed modest differences in substrate specificity (4–5-fold) implies that the lipase discrimination is of little consequence to the biological activity. However, our goal was to establish that Ala-Stav-MP is the key intermediate responsible for its biological activity since this derivative lacks chirality at the phosphorus center and hence both the isomers are expected to show equipotency. We have also established in our earlier studies[7,8] that indeed AlaSTVMP is much more active than STVMP using authentic samples. In addition, we point out that the observed discrimination by lipase occurs at the phosphorus chiral center after initial hydrolysis at the ester group present in the molecule to form the unstable cyclic intermediate.

2.3. Structural model of lipase-mediated hydrolysis of stampidine

The crystal structure of *C. Antarctica* lipase is resolved.[29] Several homologous lipases complexed with various substrate analogs have also been crystallized and their active sites have been extensively explored. The structural basis of the hydrolytic function of the lipase has been deciphered and linked to the spatial arrangement and concerted action of the active site residues[30–44] We obtained the published coordinates from PDB (pdb access code: 1 tcc, 1 tcb, 1 tca)[29] and modeled stampidine and similar stavudine derivatives into the active site using our previously published docking procedures with appropriate modifications.[45] Since these phosphoramidate derivatives of stavudine have multiple rotatable bonds, which can yield several conformations, we first generated up to 255 conformers for each compound and docked them individually into the lipase active site. The

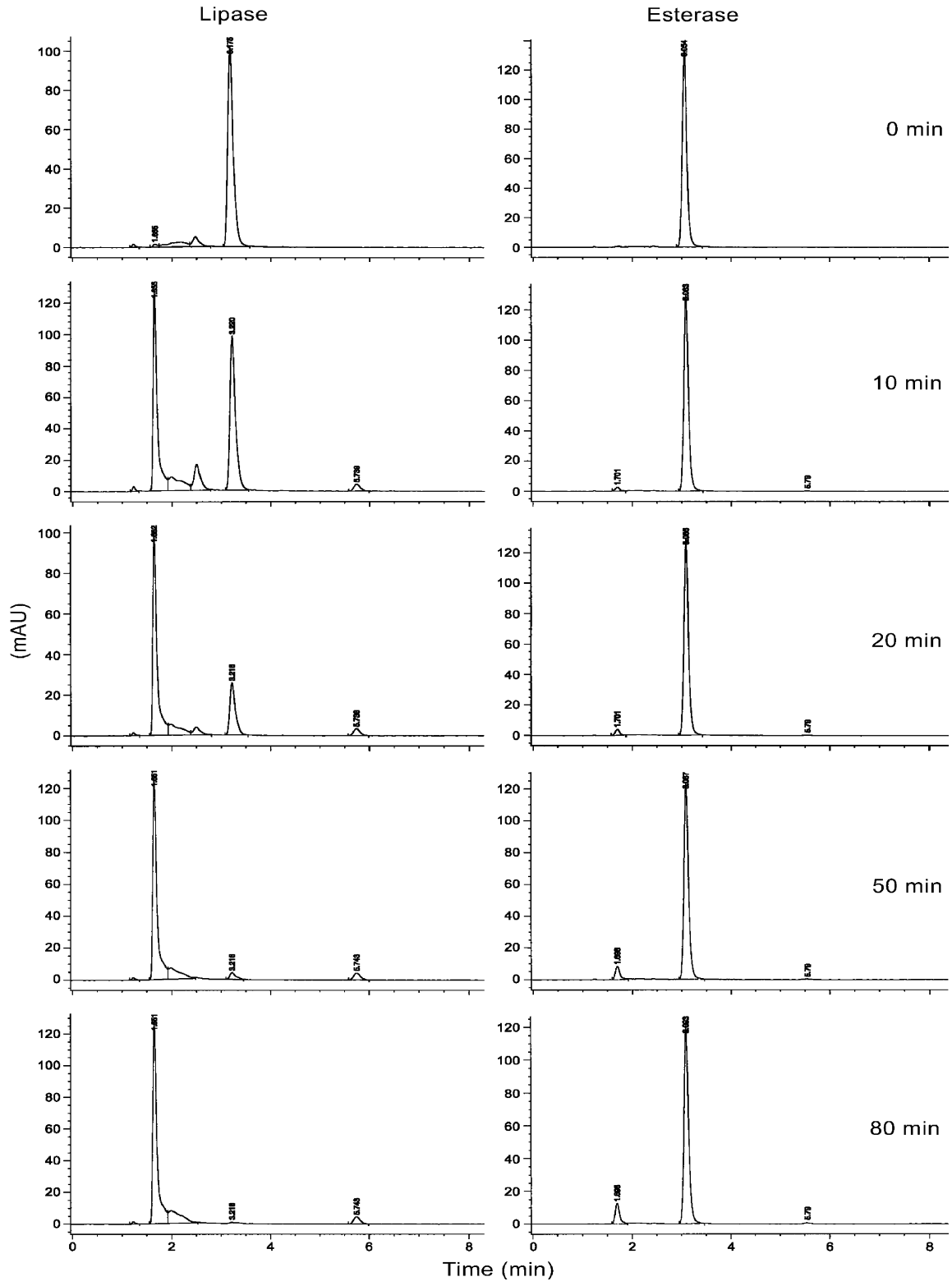


Figure 1. Lipase and esterase mediated hydrolysis of stampidine at various time intervals.

majority of these conformers were excluded during the earliest stages of the docking process because of too many steric collisions with the lipase protein before their carboxylester oxygen atoms would descend toward the protein core at the catalytic center. The final model was determined by sorting and evaluating the energy cost and LUDI interaction score for all pairs.[46–51] The molecules of both *R*- and *S*-stereoisomers of each compound were modeled and compared in order to determine, which enantiomer was better suited for the active site of the lipase. The final model for the interactions of the *S*-stereoisomer of stampidine and lipase is shown in Figure 3A. The phenoxy ring is buried in a tight binding pocket and the stavudine group half buried

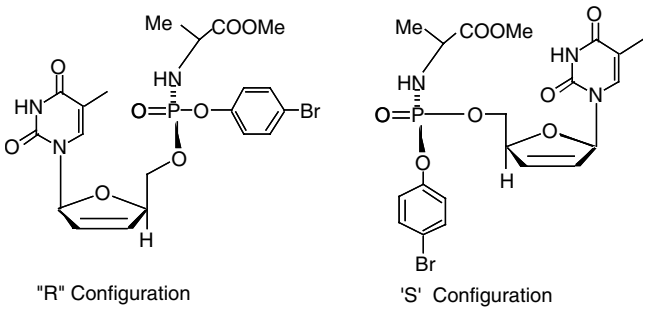


Figure 2. Structures of two individual isomers of stampidine.

in another tight pocket, neither of which is fit to the binding site of the other group. When the *S*-configuration is changed to the corresponding *R*-configuration, the phenoxy ring is switched in position with the stavudine group. Because of significant geometrical differences none of the two groups would remotely fit into the binding site of the other favorably. Because of a major steric 'clash', neither the phenoxy ring nor the stavudine group could be easily accommodated in the binding position for the P═O group. Therefore, the interactions of the *R*-stereoisomer with the lipase active site are energetically less favorable than those of the *S*-stereoisomer. This model predicts a faster and more efficient hydrolysis of the *S*-stereoisomer due to the tight initial binding between this stereoisomer and the lipase active site. The phenoxy ring is fit well in a binding pocket that consists of a protein backbone near Thr40 stacking against with its ring plane and several hydrophobic residues on the sideline including Ile285, Ala281, Ile189, and Leu278 (Fig. 3B). A 2,6-dimethoxy substitution to the phenoxy ring was predicted to significantly impair the binding and to slow down the hydrolysis. There is room for a 4-position substitution that would bring a close contact with the backbone of the 278 residue and thus is favored for halogen substitutions, as suggested by the experiments that 4-position halogens are good for hydrolysis. Interestingly, comparing this lipase active site model with a homologous model of the esterase active site, we observed a larger spatial opening near the entrance of the active site in the esterase than that in the lipase. The larger opening corresponds to roomier binding sites for both stavudine and the phenoxy group. The esterase thus appears to be more forgiving in accommodating a different structure or both enantiomers and thus less discriminative.

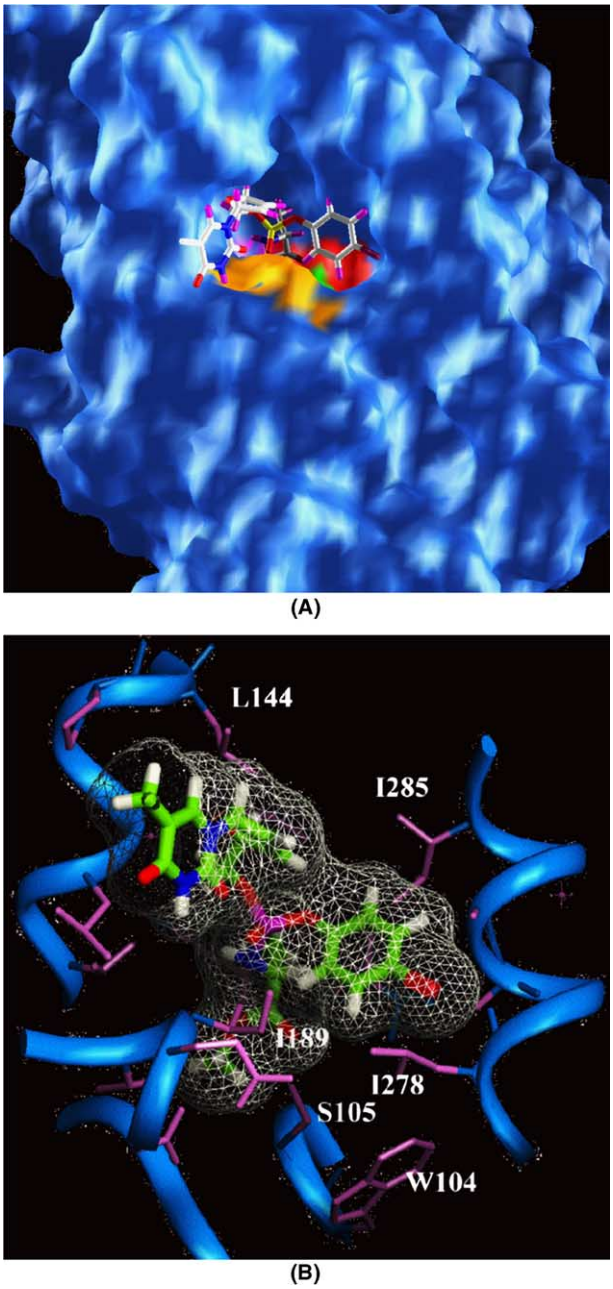
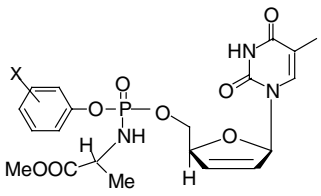


Figure 3. (A) The model of the *S*-stereoisomer of the stampidine molecule in the active site of the *C. Antarctic* lipase, a solid surface representation based on the crystal structure of the protein and the computation of energetically favored conformations for the ligand by a previously published docking procedure.[48–52] (B) The model of the *S*-stereoisomer of the stampidine molecule in the active site of the *C. Antarctic* lipase, based on the crystal structure of the protein and the computation of energetically favored conformations for the ligand by a previously published docking procedure.[48–52] The close-up view of the phenoxy ring interacting with the surrounding protein residues (labeled). Prepared with InsightII.[52]

2.4. Stereoselectivity of lipase-mediated hydrolysis of stampidine

We next set out to determine if stampidine and five other phosphoramidate derivatives of stavudine are hydrolyzed by *C. Antarctica* lipase in a stereoselective manner as predicted from the modeling and docking studies detailed above. The chemical reactions of lipase with these compounds were monitored using HPLC and the rate of lipase-mediated hydrolysis at room temperature was calculated for the two isomers of each compound using a first order rate equation. The rate constants are given in Table 1. The selectivity index indicates the relative rates of hydrolysis for the two isomers. Importantly, the stereoisomers of the four substituted compounds, including stampidine, corresponding to peak #1 in their HPLC chromatograms underwent hydrolysis much faster than their enantiomers corresponding to peak #2. Figure 4 shows representative

Table 1. Enzyme mediated hydrolysis rate constants of various arylphosphoramidate derivatives and their anti-viral activity



Compd	X	Lipase			Esterase			IC_{50} (nM)	
		1	2	SI	1	2	SI	$HTLVIII_B$	RTMDR
1	H	0.9	1.3	0.7	0.8	0.8	1.0	2.0 ± 0.6	534 ± 308
2 (Stampidine)	4Br	3.2	0.8	4.0	0.3	0.2	1.5	1.0 ± 0.0	21 ± 12
3	4Cl	4.9	0.9	5.4	1.8	1.4	1.3	1.0 ± 0.3	62 ± 43
4	4F	1.7	0.9	1.9	1.5	1.2	1.3	1.0 ± 0.0	218 ± 12.6
5	4OMe	1.6	0.6	2.7	2.4	2.6	0.9	4.0 ± 2.0	1014 ± 586
6	2,6-OMe	0.05	0.05	1.0	0.12	0.09	1.3	6.0 ± 3.0	1104 ± 637

1 and 2 represents rate constants/hour for each of the isomers. SI denotes the selectivity index.

HPLC chromatogram profiles for a fluoro substituted phosphoramidate derivative treated with lipase for various time intervals showing the preferential hydrolysis of the isomer corresponding to peak #1, concomitant with the emergence of the peaks corresponding to the metabolites d4T and Ala-d4T-MP. The mechanism through which such metabolite formed has been confirmed by using LC/mass, HPLC profiles, and independent authentic compound synthesis for this purpose.[7,8]

Similar results were obtained from the ^{31}P NMR analysis of the lipase-treated stampidine diastereomer mixture. The signal corresponding to diastereomer #1 (but not the signal corresponding to diastereomer #2) was drastically reduced after lipase treatment consistent with a stereoselective hydrolysis.

Based on the results of the modeling studies, the isomers corresponding to peak #1 undergoing much faster hydrolysis was tentatively designated the '*S*-stereoisomers'. Table 2 indicates the characteristic HPLC and ^{31}P NMR peaks corresponding to the individual stereoisomers. For each compound the two rate constants for the hydrolysis of the stereoisomers were log transformed and correlated with Hammett sigma values. Both rate constants showed a positive association with the Hammett sigma values (*S*-stereoisomer: *R*-square = 0.63, $p = 0.006$; *R*-stereoisomer: *R*-square = 0.71, $p = 0.002$. The rate constants for the *S*-stereoisomers of stampidine ($3.2\,h^{-1}$) and the 4-Cl substituted compound **3** ($4.9\,h^{-1}$) were higher than those of the unsubstituted compound **1** ($0.9\,h^{-1}$) or the 4-OMe substituted compound **4** ($1.6\,h^{-1}$). These two compounds also exhibited more potent anti-HIV activity against the NRTI-sensitive HIV-1 strain HTLVIIIB as well as the NRTI-resistant HIV-1 strain RT MDR than compounds **1** and **4**. As predicted from the modeling studies, compound **5** with 2,6Di-OMe substitution was a poor substrate for the lipase and exhibited 6-fold higher IC_{50} values than stampidine or compound **3**.

The observed association of the higher rate constants with better IC_{50} values prompts the hypothesis that lipase-mediated hydrolytic activation of stampidine and compound **3** may play an important role for their anti-HIV activity. In contrast, we observed no such association for the rate constants of the esterase-mediated hydrolysis. Our lead compound stampidine was a particularly poor substrate for esterase-mediated hydrolysis. These results call in question previous assertions that esterase-mediated hydrolysis of phosphoramidate derivatives of stavudine plays an essential role in their activation. In agreement with the predictions of the modeling studies, esterase-mediated hydrolysis showed less stereoselectivity (Table 1).

2.5. Putative pathway of lipase-mediated hydrolysis of stampidine

We hypothesize that stampidine undergoes rapid enzymatic hydrolysis in the presence of lipase according to the following putative pathway (Fig. 5): In the first step, compound B is generated by lipase-mediated hydrolysis of the methylester side chain of stampidine. The subsequent step involves an intramolecular cyclization step involving the phosphorus center with simultaneous elimination of the phenoxy group to form the cyclic intermediate (D). In the presence of water, this intermediate is converted into the active metabolite (E). We believe that the lipase hydrolyzes the methyl ester group of the L-alanine side chain to form the cyclic intermediate D in a stereoselective fashion. This notion is supported by experimental data showing that chloroethyl substituted derivatives (Scheme 1) of stampidine, which possess a chloroethyl linker unit instead of a methyl ester side chain, were resistant to lipase-mediated hydrolysis, which excludes the possibility of a direct hydrolysis of stampidine at the phosphorous center (Table 3). Although alanine methyl amide analog would have been an alternative, however it would also hydrolyze the compound similar to the stampidine hydrolysis. The object of the present study was to totally eliminate any hydrolyzable group in the structure of the molecule in order to establish whether the enzyme attacks on the phosphorus center only. To further substantiate our

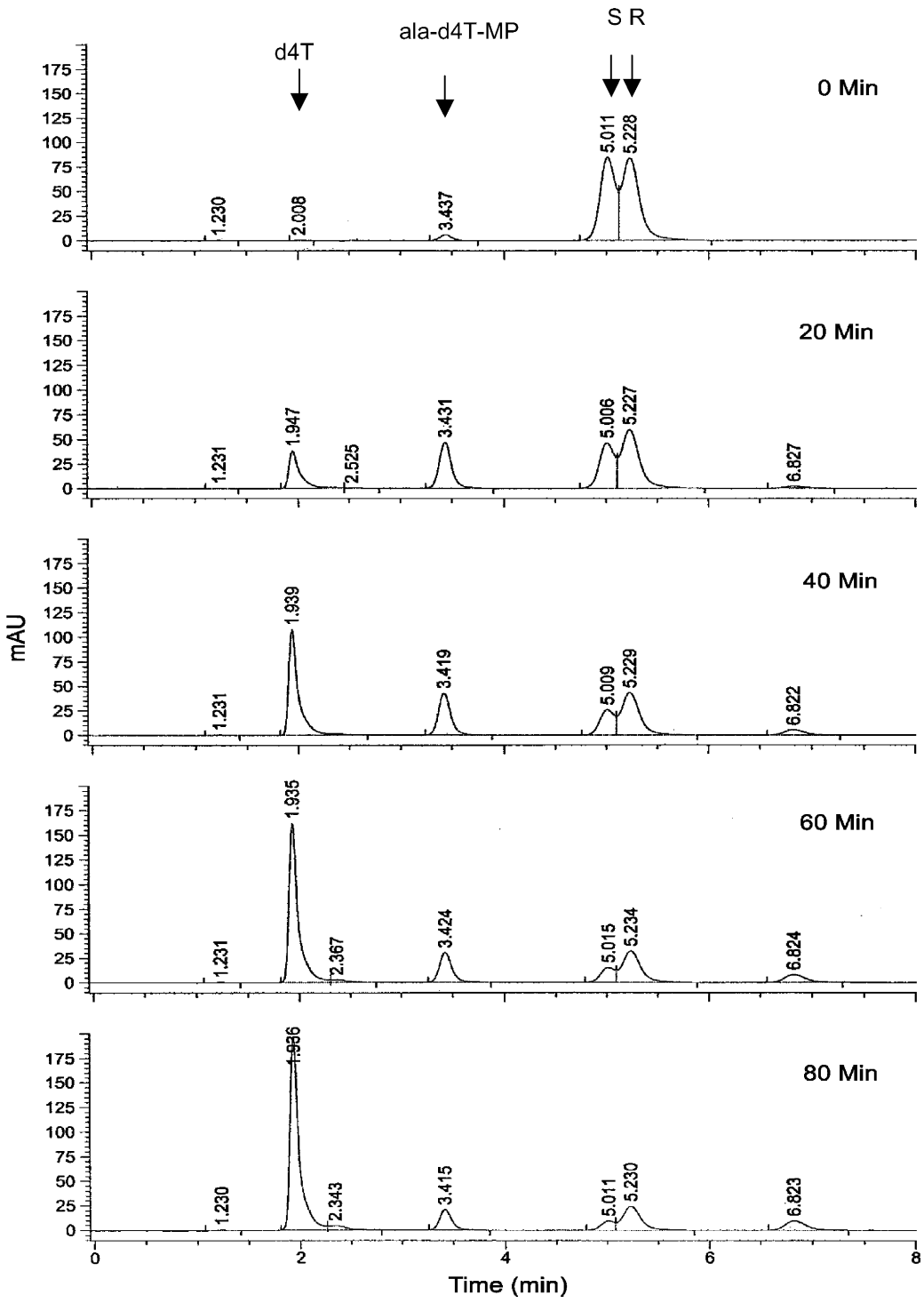
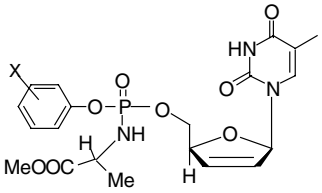


Figure 4. HPLC profiles of 4-fluoro phenylphosphoramidate derivative with lipase at various time intervals at room temperature.

claim, we recently prepared the 'D' isomer of phosphoramidate derivatives and compared their hydrolysis rate with enzymes, and found that the L isomer underwent 10-fold faster hydrolysis as compared to the 'D' isomer Br (L): 3.94; Br (D): 0.40; F (L): 2.60; F(D): 0.27 implying that the hydrolysis occurs at the ester side chain and not at the phosphorus center of the molecule. We indicate that there should be no apparent difference in the electronic charge or steric hindrance between 'L' and 'D' alanine methylester substituted phosphoramdiate derivative and hence the slower rate observed under identical experimental conditions proves beyond doubt that there is no attack on the phosphorus center (Venkatachalam et al., manuscript under preparation). Thus, our model implies that the lipase-mediated formation of the cyclic intermediate is a key step in metabolism of stampidine and relies on the initial configuration of the stereoisomers. The *S*-stereoisomer enters preferentially into the active site of the lipase and undergoes rapid hydrolysis to form the cyclic interme-

Table 2. Proposed configuration, HPLC retention time, and ^{31}P NMR chemical shifts observed for each of the isomers of phosphoramidate derivatives of stavudine using enzymatic hydrolysis profiles and molecular modeling results



Compd	X	Peak #1[a] (R_t) (ppm)	Peak #2[a] (R_t) (ppm)
1	H	*R*(4.47) (2.66)	*S*(4.65) (3.20)
2 (Stampidine)	4-Br	*S*(7.86) (3.11)	*R*(8.37) (2.56)
3	4-Cl	*S*(7.04) (3.57)	*R*(7.45) (2.82)
4	4-F	*S*(5.01) (3.80)	*R*(5.22) (3.22)
5	4-OMe	*S*(4.48) (3.82)	*R*(4.75) (3.11)
6	2,6-OMe	*R*(44.3) (4.28)	*S*(48.3) (4.97)

[a] Peaks are numbered according to the elution time in HPLC. R_t refers their respective retention times and ppm refers to ^{31}P NMR chemical shifts relative to phosphoric acid as standard at 0 ppm.

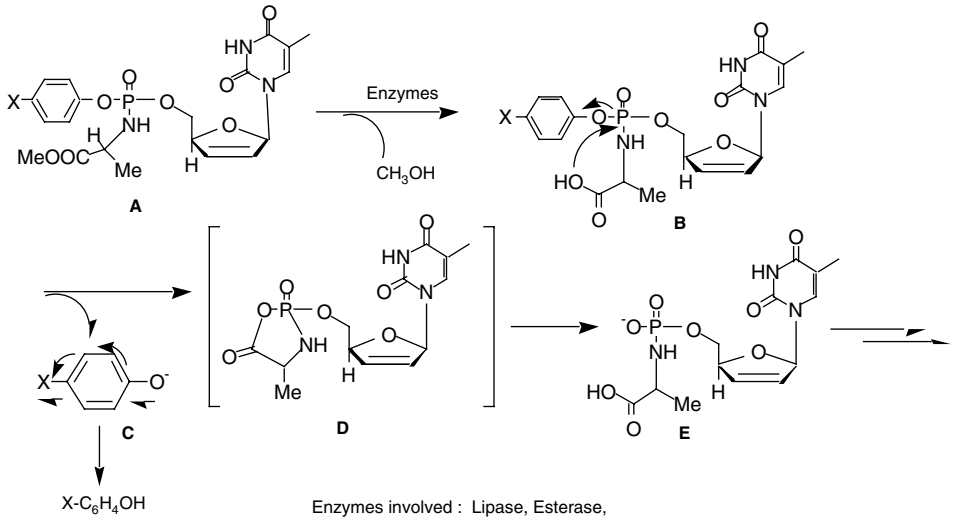
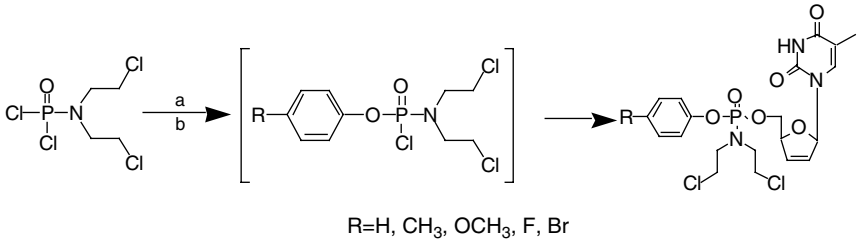


Figure 5. New Pathway for the lipase-mediated hydrolysis of phosphoramidate derivatives of stavudine based on various enzymes involved.

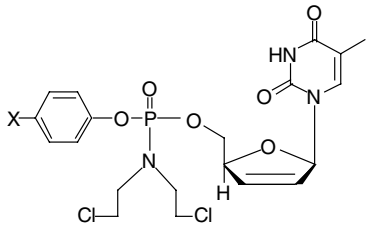


Scheme 1. Synthetic scheme for chloroethyl substituted phosphoramidate derivatives of stavudine. (a) Phenol, triethylamine, 1,2,4-triazole; (b) stavudine.

diate. At subsequent steps of the metabolism, the chirality of the cyclic intermediate at its phosphorous center becomes irrelevant due to the fact that the active metabolite of stampidine, which is the final product of this pathway, is not chiral.

In a previous study, Cygler et al.[25] demonstrated that lipases can distinguish between enantiomers of compounds with phosphorous chiral centers and proposed a general reaction scheme for their selectivity. Using molecular modeling and X-ray diffraction studies, they showed that the '*R*' isomer of methyl hexyl phosphonate fits into the enzyme pocket better than the '*S*' isomer and was more sensitive to the enzymatic action of the lipase. In their studies, the target compound had a halogen atom directly attached to the phosphorous center;

Table 3. Area under curve (HPLC) observed for the chloroethyl substituted aryl phosphoramidate derivatives of stavudine with lipase and esterase at various intervals of time



X	0 min		40 min		120 min		Comments
	#1	#2	#1	#2	#1	#2	
OMe	996	968	985	949	986	956	No hydrolysis
H	387	388	382	378	385	376	No hydrolysis
Br	615	571	627	589	ND	ND	No hydrolysis
Me	640	599	635	599	635	591	No hydrolysis

Lipase was used for the above studies under identical conditions used for alanine substituted phosphoramidate derivatives of stavudine. #1 and #2 indicates each of the isomers peak observed in HPLC chromatogram.

therefore, one could intuitively imagine hydrolysis occurring at this site for chiral recognition by the enzyme. However in our phosphoramidate derivatives, there were two chiral centers, one at the carbon center and the other at the phosphorus center. The chiral recognition of the lipase occurs at the phosphorus center due to the cyclic intermediate formation and there is no direct reaction of the enzyme towards the phosphorus center.

2.6. Possible lipase activation of the prodrug

Based on the results obtained, we hypothesize that along with esterase, lipase may be also involved in the enzymatic conversion of these phosphoramdiate derivatives of stavudine to the metabolite. The enzymatic conversion of Ala-d4TMP is the first step in the process and in the subsequent step it is converted into d4T. However, the interesting aspect is that the above conversion is normally very slows step and is typically rate limiting. In our experiments, it is evident that this conversion is rapid (see Fig. 5) and there was no evidence of d4TMP formation. This is consistent with enzymatic cleavage of the 5′–O–P bond. If this were the case, it would explain the apparent therapeutic advantage of lipase activation. The above discussion is further substantiated by our recent findings that proteases were also found to hydrolyze these phosphoramdiate derivatives (unpublished results).

3. Conclusion

Stampidine and other halogen-substituted phosphoramidate derivatives of stavudine can be metabolized by lipase-mediated hydrolysis. Modeling studies and comparison of the hydrolysis rate constants revealed a chiral preference of the lipase active site for the putative *S*-stereoisomer of these compounds. The in vitro anti-HIV activity of the compounds correlated with their susceptibility to lipase- (but not esterase-) mediated hydrolysis. Our results indicate that the lipase-mediated formation of the cyclic intermediate is a key step in metabolism of stampidine and relies on the initial configuration of the stereoisomers. We postulate that the lipase hydrolyzes the methyl ester group of the L-alanine side chain to form the cyclic intermediate in a stereoselective fashion.

4. Material and methods

All chemicals were purchased from Aldrich (Milwaukee, WI) or Sigma and were used without further purification. Unless otherwise noted, each reaction vessel was secured with a rubber septum, and the reaction was performed under nitrogen atmosphere. ^{1}H and ^{13}C NMR was obtained on a Varian Mercury 300 instrument at ambient temperature in DMSO-d_6 Chemical shifts are reported as $\cdot\delta$ values in parts per million downfield from tetramethylsilane ($\delta = 0.0$ ppm) as an internal standard or from the residual dimethylsulfoxide signal ($\delta = 2.49$ ppm for ^{1}H NMR or $\delta = 39.7$ ppm for ^{13} C NMR). Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad peak. FT-IR spectra were recorded on a Nicolet Protege 460 spectrometer. Mass spectra were performed on a Hewlett Packard MALDI-TOF spectrometer (Model G2025A LD-TOF). Melting points were determined using a Melt John's apparatus and are uncorrected. HPLCs were performed using a Hewlett Packard 1100 series instrument consisting of an automatic sampler, an electronic degasser, a thermostatic control unit, and a diode array detector in conjunction with a Chemstation software assembly. The column used was an analytical RP–18 Lichrospher column 5 mm (4.6×150 μm) and eluent was acetonitrile/water mixture. The flow rate was maintained at 1.0 mL min^{-1} and the detection wavelength was set at 275 nm. The column was maintained at room temperature throughout the analysis. Column chromatography was performed using silica gel obtained from the Baker Company. The solvents used for elution varied depending on the compound and included either one or a combination of the following: ethyl acetate,

methanol, chloroform, hexane, methylene chloride, THF, and ether. Analytical thin-layer chromatography (TLC) was performed on Merck pre-coated glass plates (silica gel 60, F_{254}, 250-μm thick), and visualized under 254-nm UV light. Column chromatography was performed using EM silica gel 60, 230–400 mesh. Synthesis of phosphoramidate analogs of stavudine was achieved starting from substituted phenols and phosphorous oxychloride. The resulting phosphodichloridate was reacted with L-alanine methylester hydrochloride followed by stavudine to furnish the required compounds. The detailed synthetic scheme and methods are already reported in our earlier publications.[1] Synthesis of chloroethyl substituted stavudine derivatives were accomplished as described below:

4.1. General procedure for synthesis of chloroethyl substituted phosphoramidate derivatives of stavudine

The general procedure followed for the synthesis of the above derivatives of stavudine is shown in Scheme 1. A RB flask was charged with 20 mL of anhydrous acetonitrile and triethylamine (1.2 equiv) followed by triazole (2.50 equiv). To this mixture was added a solution of appropriately substituted phenol (1.0 equiv) and bis(2-chloroethyl)amine phosphodichloridate (1.00 equiv) in anhydrous acetonitrile. The contents were stirred at 0 °C by external cooling using ice bath. After addition the contents were brought to room temperature and stirred for 5 h. Upon completion of the reaction, d4T (1.0 equiv) was added to the mixture and the contents of the flask were brought to 50 °C and allowed to stir for one week. After this period, the solvent was evaporated under vacuum and the residue was subjected to column chromatography using chloroform and methanol (10:1) and the appropriate fractions containing the product were pooled together. Evaporation of the solvent and further purification by preparative thin layer chromatography furnished analytically pure product in 10% yield as solids.

4.2. Experimental conditions for lipase-mediated hydrolysis

For the kinetic study, a known amount of the phosphoramidate derivative was carefully weighed (5–7 mg) using a Metller analytical balance and transferred into a scintillation glass vial. Using a pipette man, 3 mL of methanol were added and the contents were vortexed for 2 min until a homogeneous solution resulted. Using another pipette man, 100 μL of the above solution was transferred into another scintillation vial and to this was added 950 μL of water and the contents vortexed. In parallel, 5 mg of solid lipase powder was weighed and transferred to a volumetric flask. To this was added 8 mL of water and the contents were shaken to dissolve the enzyme. The reaction mixture was prepared as follows for the kinetic study: From the stock solution of the compound as mentioned above, 500 μL of the methanolic solution of the phosphoramidate derivative was pipetted out into another glass vial and to this 500 μL of lipase solution was added and the contents were shaken to form a homogeneous solution. From this reaction mixture 50 μL was used for HPLC analysis. The column used was a Lichrospher (RP) analytical column of 4×250 mm. The eluent used for HPLC was water/TFA/TEA (0.1%) and CH_3CN in the ratio of 65:35. The column was maintained at room temperature. The flow rate was maintained at 1 mL min^{-1}, the detection wavelength was adjusted to 265 nm and the reference wavelength was kept at 400 nm. Aliquots of the sample were collected from the reaction vial at various time intervals and analyzed. The amounts of products observed during the reaction of these phosphoramidates were estimated from the area obtained from the HPLC profiles. In addition, authentic samples of the products in most of the cases were run to identify the peaks observed during the reaction. Further confirmation of the product structures was obtained using an LC/mass instrument. The rate of reaction was computed by using first order rate constants and an average of eight to nine time points were used for this estimate. The rate constants reported refers to rate/hour since some of the reactions were too slow to obtain meaningful results.

^{31}P NMR studies with enzyme was conducted using a 5 mm NMR tube. Initially a capillary tube was charged with 1% phosphoric acid in water and fused carefully. The sample was dissolved in either 100 μL of deuterated methanol, followed by D_2O (0.4 mL) and was transferred into the NMR tube using a Pasteur pipette. The capillary tube containing the standard was introduced into the NMR tube. The NMR spectrum of the substrate was taken under these conditions and the tube was taken out of the machine, using a pipette man, 100 μL of the esterase solution was introduced into the NMR tube. The contents were carefully shaken for a minute the spectrum was run and periodically monitored to ascertain the disappearance of the starting material.

4.3. Statistical analysis

Hydrolysis rates were determined by fitting single exponential decay equations to the disappearance of each isomer substrate in the presence of enzyme. Hammett Sigma values were correlated to the log transformed hydrolysis rate constants using a linear regression model (JMP Software, SAS Institute Inc.). All *p*-values less than 5% were deemed significant. The effect of sigma was tested for the fast rate constant, the slow rate constant and the selectivity index (fast/slow).

4.4. Physical constants for the chloroethyl substituted phosphoramdiate derivatives

4.4.1. 5′-(2′,3′-Didehydro-3′-deoxythymidine)phenyl *N*-bis(2-chloroethyl)phosphoramidate. IR (KBr) 3184, 3061, 2365, 2329, 1690, 1490, 1455, 1249, 1087, 934, 774, 699 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.86 (d, 3H, 5-CH_3), 3.39–3.61 (m, 8H, NCH_2 CH_2Cl), 4.23–4.40 (m, 2H, 5′H), 5.03 (s, 1H, 4′H), 5.87–5.96 (m, 1H, 1′H), 6.28–6.37 (m, 1H, 2′H), 7.00 (m, 1H, 3′H), 7.17–7.20 (m, 2H,

Ar-2, 6), 7.21 (s, 1H, 6H), 7.31–7.34 (m, 2H, Ar-3, 5), 8.55 (s, 1H, 3-NH); ^{13}C NMR (CD_3OD): δ 12.90, 42.19, 49.67, 67.95, 84.79, 90.13, 111.61, 120.24, 125.66, 127.70, 130.10, 133.25, 135.71, 150.79, 163.60; ^{31}P NMR δ 4.66, 5.27; HPLC: R_t: 12.4, 14.1 min.

4.4.2. 5′-(2′,3′-Didehydro-3′-deoxythymidine)4-methylphenyl *N*-bis(2-chloroethyl)phosphoramidate. IR (KBr) 3037, 2959, 2360, 1692, 1507, 1465, 1248, 1089, 941, 818, 756 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.85 (s, 3H, 5-CH_3), 2.32 (s, 3H, CH_3), 3.37–3.60 (m, 8H, NCH_2 CH_2Cl), 4.22–4.41 (m, 2H, 5′H), 5.03 (s, 1H, 4′H), 5.92 (d, 1H, J = 18.0 Hz, 1H), 6.33 (m, 1H, J = 18.0 Hz, 2′H), 6.97–7.14 (m, 5H, Ar-2, 3, 5, 6; 6H; 3′H), 8.23 (br, 1H, 3-NH); ^{13}C NMR (CD_3OD): δ 12.89, 21.11, 42.21, 49.71, 67.32, 84.79, 90.11, 111.59, 119.93, 127.67, 130.53, 133.32, 135.41, 135.77, 150.70, 163.45; ^{31}P NMR ($CDCl_3$): δ 4.79, 5.42; HPLC: R_t: 21.9, 25.3 min.

4.4.3. 5′-(2′,3′-Didehydro-3′-deoxythymidine)4-methoxyphenyl *N*-bis(2-chloroethyl)phosphoramidate. IR (KBr) 3066, 2958, 2360, 2339, 1695, 1506, 1462, 1249, 1091, 1034, 936, 838 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.86 (s, 3H, 5-CH_3), 3.37–3.60 (m, 8H, NCH_2 CH_2Cl), 3.79 (s, 3H, OCH_3), 4.22–4.39 (m, 2H, 5′H), 5.03 (s, 1H, 4′H), 5.92 (m, 1H, 1′H), 6.33 (m, 1H, 2′H), 6.83–6.87 (m, 2H, Ar-2, 6), 7.09–7.14 (m, 2H, Ar-3, 5), 7.34 (s, 1H, 6H), 7.27 (m, 1H, 3′H), 8.14 (s, 1H, 3-NH); ^{13}C NMR (CD_3OD): δ 12.90, 42.22, 49.69, 55.95, 67.83, 84.79, 90.11, 114.99, 121.14, 127.68, 133.31, 135.36, 135.76, 150.70, 163.80; ^{31}P NMR ($CDCl_3$): δ 5.09, 5.69; HPLC: R_t: 14.4, 16.6 min.

4.4.4. 5′-(2′,3′-Didehydro-3′-deoxythymidine)4-bromophenyl *N*-bis(2-chloroethyl)phosphoramidate. IR (KBr) 3171, 3046, 2361, 2339, 1693, 1485, 1467, 1251, 1090, 930, 837, 778 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.90 (s, 3H, 5-CH_3), 3.42–3.84 (m, 8H, NCH_2 CH_2Cl), 4.18–4.40 (m, 2H, 5′H), 5.04 (s, 1H, 4′H), 5.93–5.96 (m, 1H, 1′H), 6.29–6.37 (m, 1H, 2′H), 7.00 (m, 1H, 3′H), 7.07–7.12 (m, 2H, Ar-2, 6), 7.34 (s, 1H, 6H), 7.44–7.47 (m, 2H, Ar-3, 5), 8.36 (s, 1H, 3-NH); ^{13}C NMR (CD_3OD): δ 12.90, 42.19, 49.62, 67.83, 84.87, 90.18, 122.02, 127.71, 133.12, 134.47, 135.27, 135.58, 136.13, 150.67, 163.80; ^{31}P NMR ($CDCl_3$): δ 4.71, 5.22; HPLC: R_t: 31.8, 35.8 min.

4.5. Physical constants of individual separated individual stereoisomers of DDE113

4.5.1. 5′-[4-Bromophenyl methoxy alaninylphosphate]-2′,3′-didehydro-3′-deoxylthymidine, isomer 1 (*S*). TLC: R_f: 0.51 ($CHCl_3$/MeOH, 9:1), mp 59 °C. ^{1}H NMR ($CDCl_3$): δ 8.15 (b s), 7.45–7.42 (m), 7.42–7.27 (m), 7.26–7.19 (m), 7.08–7.03 (m), 6.36–6.35 (d), 5.91–5.89 (d), 5.04 (s), 4.36–4.32 (m), 4.0–3.92 (m), 3.71–3.65 (m), 3.61–3.54 (m), 1.82 (s), 1.58 (b s), 1.34–1.32 (m); ^{31}P NMR ($CDCl_3$) 3.11; IR ν 3228, 3064, 2918, 2848, 1743, 1689, 1485, 1385. 1246, 1223, 1153, 1113, 1088, 1036, 928, 837 cm^{-1}; HPLC R_t: 11.23 min, $[\alpha]_D$ −30.

4.5.2. 5′-[4-Bromophenyl methoxy alaninylphosphate]-2″,3′-didehydro-3′-deoxylthymidine, isomer 2 (*R*). TLC: R_f: 0.51 ($CHCl_3$/MeOH, 9:1), mp 160 °C; ^{1}H NMR ($CDCl_3$): δ 8.21(b s), 7.45–7.43 (m), 7.42–7.27 (m), 7.26–7.11 (m), 7.11–7.09 (m), 7.01–6.99 (m), 6.31–6.28 (d), 5.94–5.91(d), 5.01(s), 4.30–4.26 (m), 3.96–3.62 (m), 1.88 (s), 1.59 (b s), 1.40–1.35 (m); ^{31}P NMR ($CDCl_3$) 2.56; IR ν 3423, 3242, 2924, 2852, 1741, 1693, 1589, 1485, 1385, 1248, 1223, 1153, 1112, 1090, 1038, 1018, 930 cm^{-1}; HPLC R_t: 12.1 min, $[\alpha]_D$ −27.5.

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