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Synthesis and metabolism of naphthyl substituted phosphoramidate derivatives of stavudine

T. K. Venkatachalam, S. Qazi and F. M. Uckun*

Departments of Chemistry and Virology, Parker Hughes Institute, Roseville, MN 55113, USA
Paradigm Pharmaceuticals, White Bear Lake, MN 55110, USA

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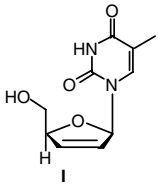
Abstract—The synthesis of naphthylphosphoramidate derivatives of stavudine was achieved using a four-step procedure. The derivatives were subjected to several different enzymes including lipase, esterase, Subtilisin Carlsberg, and *Carica papaya*, and their hydrolysis rates were determined. Based on the rates of hydrolysis, we were able to differentiate between the chiralities at the phosphorus center of the phosphoramidate compounds. In addition, lipase was found to distinguish between both α and β forms of the compounds. The superior chiral selectivity shown by lipase toward the naphthyl substituted phosphoramidate derivatives is attributed to the restrictive binding pocket of the lipase.

1. Introduction

Acquired immunodeficiency syndrome (AIDS) affects a significant part of the world population.[1–7] Most antiretroviral treatment regimens in clinical use employ nucleoside reverse transcriptase inhibitors (NRTI), such as zidovudine and stavudine (d4T, **1**).[4–7]



The 5′-triphosphates of 2′,3′-dideoxynucleoside analogs (ddN) that are generated by nucleoside and nucleotide kinases are potent inhibitors of HIV reverse transcriptase.[8,9] The rate limiting step for the conversion of 3′-azido-3′-deoxythymidine (AZT) to its bioactive metabolite, AZT-triphosphate, seems to be the conversion of the monophosphate derivative to the diphosphate derivative, whereas the rate limiting step for the intracellular generation of the bioactive metabolite of **1**, stavudine-triphosphate, was reported to be the conversion of the nucleoside to its monophosphate derivative.[10–12] The activation of anti-HIV ddN analogs primarily relies on nucleoside and nucleotide kinases to convert these NRTI into the corresponding 5′-triphosphates as discussed before. However, NRTI were found to act as poor substrates for nucleoside kinases.[10,13–16] Consequently, development of pro-drug strategies was sought that could bypass the initial nucleoside kinase activation. In an attempt to overcome the dependence of ddN analogs on intracellular nucleoside kinase activation, we and others have prepared aryl phosphate derivatives of zidovudine and **1**.[11,12,17–21] Although the putative hydrolysis product would still need to undergo kinase activation, the initial rate limiting step of phosphorylation of the nucleoside to its monophosphate is overcome by use of the pro-drug strategy. Recently Siccardi et al.[22] have investigated the stereospecific chemical and enzymatic stability of phosphoramidate triester prodrugs of d4T in vitro and came to the conclusion that the stereoselective metabolic rate of the pronucleotide series can affect the activity or toxicity due to preferred body distribution of one diastereoisomeric form. Recently, Congiatu et al.[23] have prepared several naphthyl substituted phosphoramidates and evaluated their anti-cancer activity. In a subsequent study they have successfully separated the individual phosphorus isomers and assigned the absolute stereochemistry using modeling and NMR techniques.[24] Some of these derivatives were found to be potent anti-HIV agents with subnanomolar IC_{50} values.

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* Corresponding author. Tel.: +1 651 329 2486; fax: +1 651 653 3262; e-mail: fatih_uckun@ih.org

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Previously, we examined the hydrolysis profile of phenyl substituted phosphoramidate derivatives of stavudine (**1**) in the presence of various enzymes (lipase, esterase, and proteases). We found that all the substituted phosphoramidate derivatives underwent facile hydrolysis and the phenyl substituted derivative (**4**) had a hydrolysis rate of 0.9 and 1.3/h, respectively, for the two isomers in presence of lipase.[25] In the case of esterase-mediated hydrolysis, the values were 1.52 and 1.54/h, respectively, for the two isomers. Treatment with protease enzyme, such as Subtilisin Carlsberg, yielded hydrolysis rates of 1.3 and 13.4/h for the individual isomers. Additionally, we found that both lipase and protease showed chiral selectivity. On the other hand, esterase did not show chiral selectivity for (**4**).

If hydrolysis were the key step in the formation of the active metabolites of phenyl substituted phosphoramidate derivatives of stavudine, introduction of electron-withdrawing groups on the phenyl ring and any other structural factors that can influence on the stability of the phenoxy anion should favor the formation of active metabolites. In this regard, we have already demonstrated in our earlier studies that electron-withdrawing substituents improve the hydrolysis rate and formation of the active metabolites.[20,21] The purpose of the present study was to examine if introducing a bicyclic ring to the structure of a phosphoramidate had any beneficial effects on its hydrolysis. To this end, we prepared naphthyl substituted phosphoramidate derivatives of stavudine and determined their enzyme-mediated hydrolysis profiles. We hypothesized that the introduction of a naphthyl ring rather than a phenyl group in the structure should stabilize the anion better due to the delocalization of the negative charge to both the rings. We also envisioned that the lipophilicity of a bicyclic ring may also improve the intracellular entry of these phosphoramidate derivatives and thereby improve their potency.

In our study, we also sought to determine whether the lipase enzyme could accommodate the naphthyl ring in comparison with phenyl ring due to its tight binding pocket geometry. Furthermore, the regiochemistry associated with α- and β-naphthyl groups could also affect the interactions with the lipase enzyme due to the restrictive binding pocket associated with lipase's structure. We observed that the lipase was able to differentiate between these two isomers although the groups attached are similar unlike the naphthyl compound.

2. Results

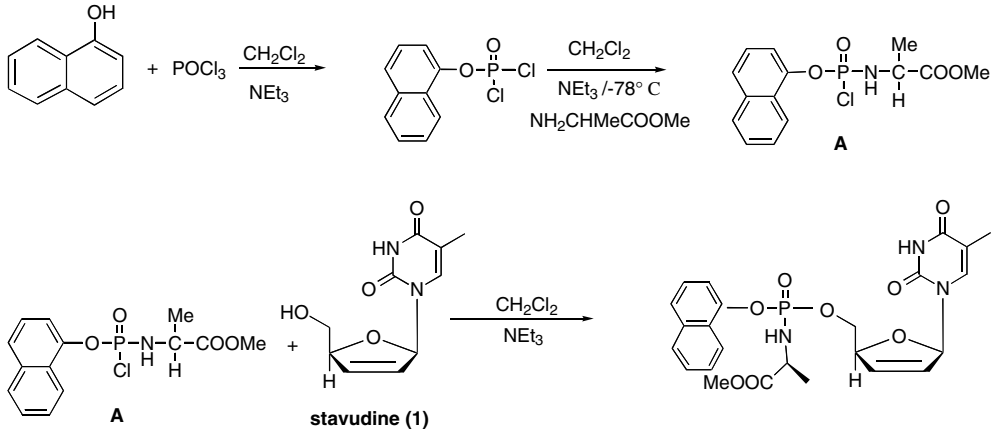
The synthesis of naphthylphosphoramidate derivatives was achieved following Scheme 1, which is based on a previously described method for the general synthesis of arylphosphoramidate derivatives of stavudine.[6,11,12,17,23,24] Both α- and β-naphthol were used to prepare the phosphoramidate derivatives in order to determine whether hydrolyzing enzymes could differentiate between these two isomers. Scheme 1 shows a representative synthetic procedure adopted for α-naphthyl substituted phosphoramidate of stavudine. A similar procedure was adopted for the preparation of β-naphthyl substituted phosphoramidate derivative of stavudine.

In brief, naphthol was condensed with phosphorus oxychloride in methylene chloride to yield a naphthylphosphorodichloridate. This was further condensed with L-alanine methylester hydrochloride to obtain the monochloridate (**A**). Treatment of monochloridate (**A**) in methylene chloride and triethylamine with stavudine (d4T, **1**) furnished the desired naphtholphosphoramidate derivatives **2** (α) and **3** (β), which were then characterized.

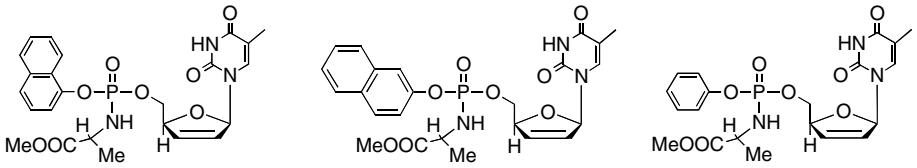
Due to the stereochemistry[11,12,17,18] of its phosphorus chiral center, naphthyl substituted phosphoramidate derivatives of stavudine exist as a mixture of two possible diastereoisomers as evidenced from their ^{1}H, ^{13}C, and ^{31}P NMR spectra and the HPLC analysis.

2.1. Esterase-mediated hydrolysis profile of naphthyl substituted stavudine phosphoramidate derivatives

α-Naphthyl methoxy alaninyl stavudine phosphoramidate derivative **2**, similar to the other phosphoramidate derivatives discussed in this paper, has two peaks in its HPLC profile with a retention time of 11.1 and



Scheme 1. Synthetic procedure for α-naphthyl substituted phosphoramidate of stavudine.



2 3 4

12.1 min, respectively, under the conditions that were used. This is owing to the stereochemistry at the phosphorus center of the molecule as described earlier. The isomer numbers were arbitrarily assigned based on their relative HPLC retention times with the earlier first peak labeled as fast eluting isomer (fe) and the second peak labeled as slow eluting isomer (se). Figure 1 shows the hydrolysis profile of **2** after treatment with esterase for various time intervals. The compound was readily hydrolyzed to form stavudine **1** and α-naphthol, as evidenced by their respective retention times 2.67 and 23.2 min, respectively. The peak at 6.88 min was found to increase with time. This peak has a different retention time than stavudine alaninyl monophosphate. At present the structure of this intermediate is unknown and further work is needed to identify the structure of this intermediate. The observed rate constants of hydrolysis were 2.76/h for fast eluting isomer (fe) and 4.36/h for slow eluting isomer (se) (Table 1). These results demonstrate that esterase is able to differentiate between the two phosphorus isomers of compound **2**.

We next examined the hydrolysis of the corresponding β-naphthyl methoxy alaninyl stavudine phosphoramidate derivative **3** in presence of esterase under identical experimental conditions (Table 1). We found that fast eluting isomer (fe) of compound **3** was hydrolyzed slightly slower than the slow eluting isomer (se) (4.24 and 5.15/h, respectively) (Fig. 2) reminiscent of our results with compound **2**. The rate of hydrolysis was found to be higher for both isomers in comparison with the hydrolysis rates for compound **2** (Table 1).

2.2. Lipase-mediated hydrolysis profile of naphthyl substituted stavudine phosphoramidate derivatives

We continued our studies by using lipase as the hydrolyzing enzyme (Fig. 3). As with the esterase-mediated hydrolysis, we found that slow eluting isomer (se) of compound **2** underwent hydrolysis faster than the fast eluting isomer (fe) (6.77/h vs 1.02/h). Figure 4 depicts the HPLC profiles of hydrolysis products of compound **3** during lipase-mediated hydrolysis. The fast eluting isomer (fe) underwent slightly faster hydrolysis compared to the slow eluting isomer (se) (3.07 and 2.43/h, respectively).

2.3. Protease-mediated hydrolysis profile of naphthyl substituted stavudine phosphoramidate derivatives

We next evaluated the ability of the serine protease, Subtilisin Carlsberg, on the hydrolysis of compounds **2** and **3**. Figure 5 shows the HPLC profiles of the hydrolysis products obtained for α-naphthyl derivative **2**. The rate of protease-mediated hydrolysis was much faster than the rates of hydrolysis observed with esterase or lipase enzymes. The fast eluting isomer (fe) underwent hydrolysis at a rate of 32.3/h and the slow eluting isomer (se) underwent hydrolysis at a rate of 10.3/h. Similarly, the β-naphthyl derivative **3** underwent hydrolysis in presence of protease enzyme (Fig. 6). The rates of hydrolysis for the fast eluting isomer (fe) and the slow eluting isomer (se) were 9.02 and 5.4/h, respectively. Comparison of the hydrolysis rates indicates that compound **2** undergoes hydrolysis 2–3 times faster than compound **3**. The phenyl derivative **4** showed an opposite trend in which the fast eluting isomer (fe) was hydrolyzed much slower than the slow eluting isomer (se) (1.3 and13.4/h, respectively). However, caution should be exercised since a reversal in order of elution in HPLC can also result in such opposite trend of these isomers. In general, the rate of protease-mediated hydrolysis was much faster than the rates of esterase or lipase-mediated hydrolysis. This result is in agreement with our earlier results showing that Subtilisin Carlsberg hydrolyzed a series of phenyl phosphoramidate derivatives at a 2- to 3-fold faster rate than esterase for lipase.[26]

We next examined the hydrolysis of the naphthyl derivatives in the presence of the cysteine protease, *Carica papaya*. Fast eluting isomer (fe) of compound **2** underwent slow hydrolysis (0.11/h), while the slow eluting isomer (se) was not hydrolysable under our experimental conditions (Fig. 7). Additionally, we found that the rate of hydrolysis of compound **3** was also very slow for both the fast eluting isomer (fe) (0.09/h) and the slow eluting isomer (se) (0.07/h) (Fig. 8). In contrast to the naphthyl derivatives, the phenyl derivative **4** yielded a slightly faster rate of hydrolysis (Fig. 9). The fast eluting isomer (fe) of compound **4** had a hydrolysis rate of 0.85/h and the slow eluting isomer (se) had a hydrolysis rate of 0.17/h (Table 1). The smaller phenyl derivative is likely to enter the enzyme pocket more efficiently resulting in a higher rate of hydrolysis.

Based on the results, we propose that the naphthyl oxyanion can also stabilize more efficiently by resonance stabilization. However, we point out that leaving group's ability is directly related to the pK_a of the resulting anion and furthermore the relative leaving group potentials of these groups would only be expected to be predictive in a non-enzymatic process. We previously reported that the phenyl substituted methoxy alaninyl stavudine phosphoramidate derivative **4** underwent hydrolysis with rates of 1.52 and 1.54/h for fast eluting isomer (fe) and the slow eluting isomer (se),

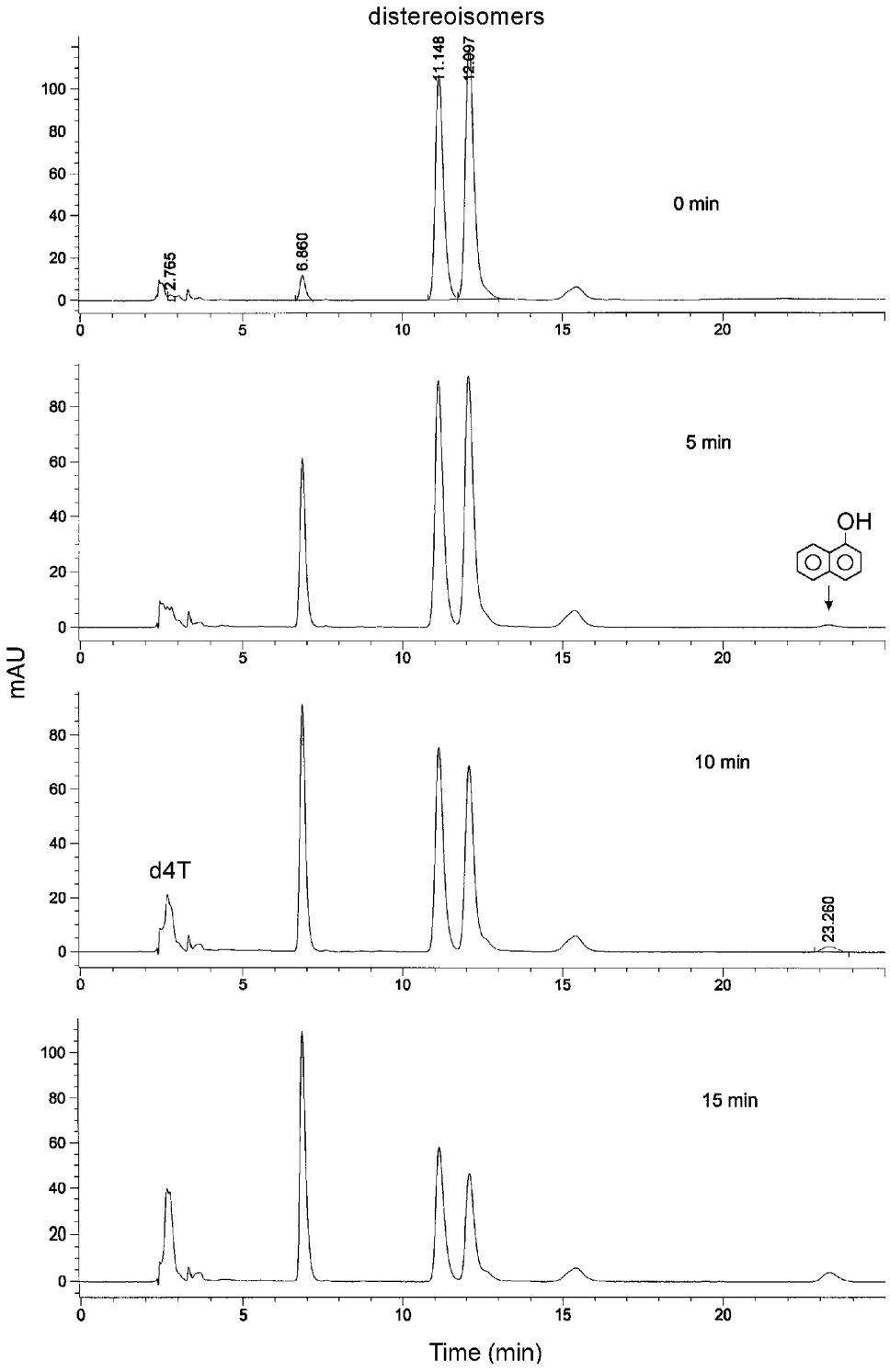


Figure 1. Hydrolysis profile of α-naphthylmethoxy alaninyl phosphoramidate **2** treated with esterase at various time intervals.

Table 1. Hydrolysis rate of phosphoramidate derivatives in presence of various enzymes*

Compound	Esterase		Lipase		S. Carlsberg		*Carica papaya*	
	(fe)	(se)	(fe)	(se)	(fe)	(se)	(fe)	(se)
2	2.76 ± 0.08	4.36 ± 1.4	1.02 ± 0.15	6.77 ± 1.0	32.2 ± 6.5	10.3 ± 1.5	0.11 ± 0.01	0
3	4.24 ± 0.3	5.1 ± 0.3	3.07 ± 0.9	2.43 ± 0.8	9.02 ± 1.1	5.4 ± 0.6	0.09 ± 0	0.07 ± 0
4	1.52 ± 0.04	1.54 ± 0.003	0.83 ± 0.08	1.15 ± 0.1	1.3 ± 0.8	13.4 ± 9	0.85 ± 0.1	0.17 ± 0.05

*Rates are expressed per hour. (fe) and (se) represent fast and slow eluting isomers.

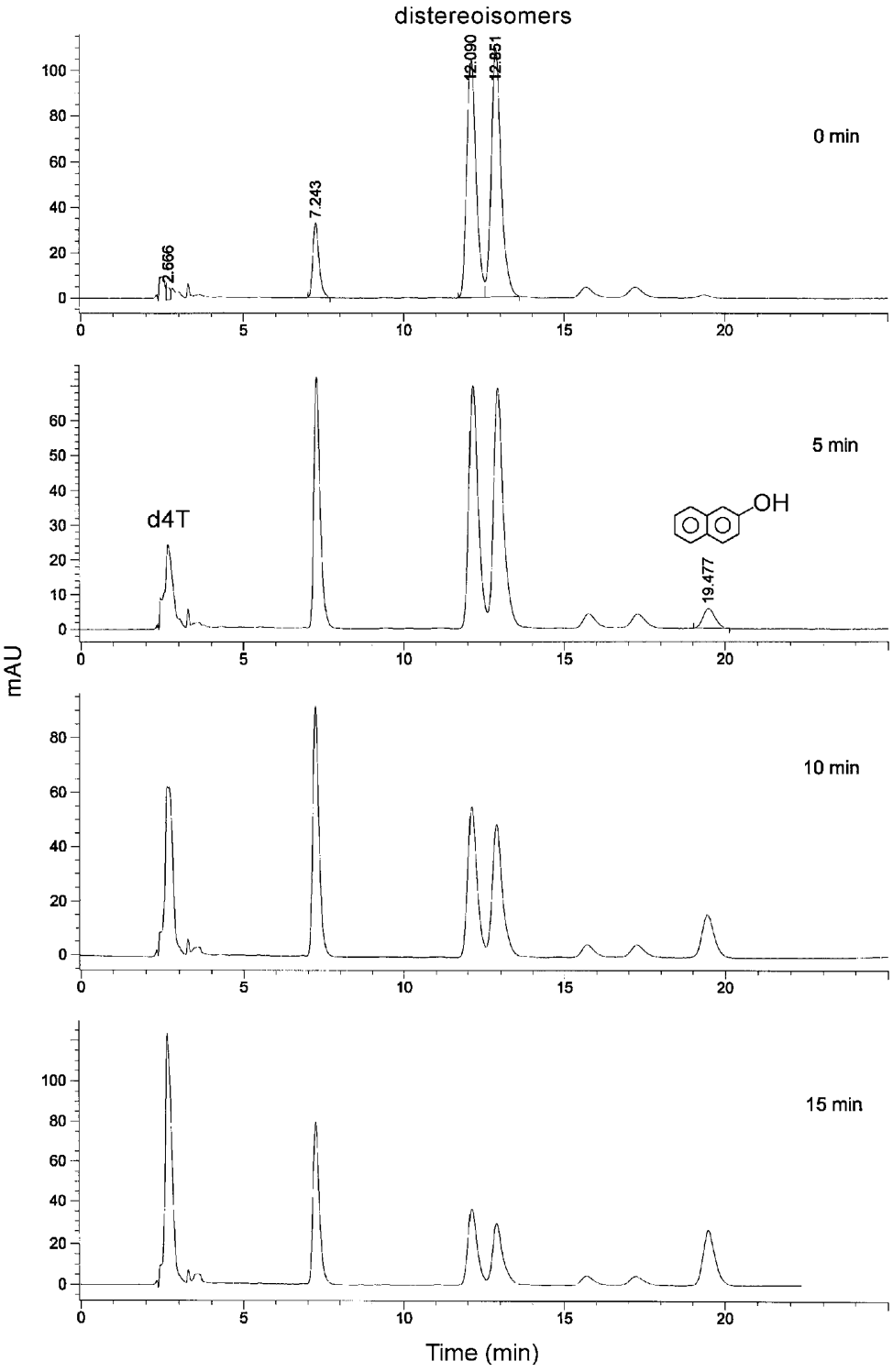


Figure 2. Hydrolysis profile of β-naphthylmethoxy alaninyl phosphoramidate **3** treated with esterase at various time intervals.

respectively.[22] These features of the naphthyl oxyanion may explain why the naphthyl derivatives underwent hydrolysis 3–4 times faster when compared to the previously reported phenyl substituted derivative in lipase-, esterase-, and Subtilisin Carlsberg enzyme-mediated hydrolysis. However in *C. papaya* enzyme-mediated hydrolysis, the phenyl derivative showed a faster rate of hydrolysis as compared to naphthyl derivatives (Table 1). Taken together, these results provide experimental evidence that esterase and lipase enzymes can differentiate between the individual isomers of compound **2** as well as compound **3**.

Again for comparison, the phenyl derivative **4** indicated a hydrolysis rate of 0.83 and 1.15/h for the fast eluting isomer (fe) and the slow eluting isomer

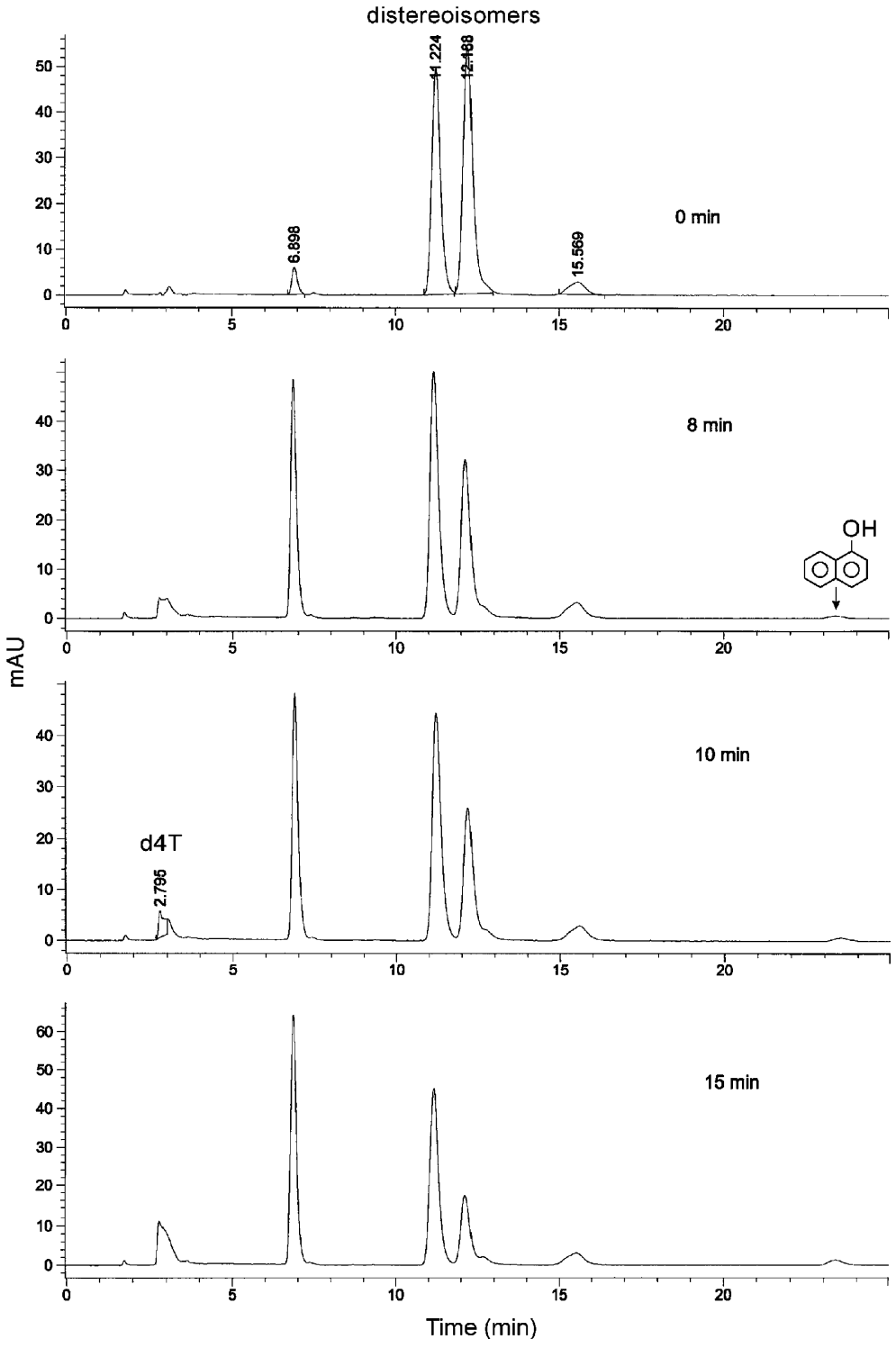


Figure 3. Hydrolysis profile of α-naphthylmethoxy alaninyl phosphoramidate **2** treated with lipase at various time intervals.

(se), respectively, for lipase-mediated hydrolysis. This is 4-fold slower than that observed for compound **3** and 1.2-fold slower than that obtained for compound **2** with the same enzyme. As with esterase we found that the rates of esterase-mediated hydrolysis for naphthyl derivatives were higher than those of the phenyl derivative **4** (Table 1).

3. Discussion

In previous studies, we have examined the hydrolysis profile of phenyl substituted phosphoramidate derivatives of stavudine **1** in presence of various enzymes (lipase, esterase, and proteases). We found that all the substituted phosphoramidate derivatives undergo hydrolysis in the

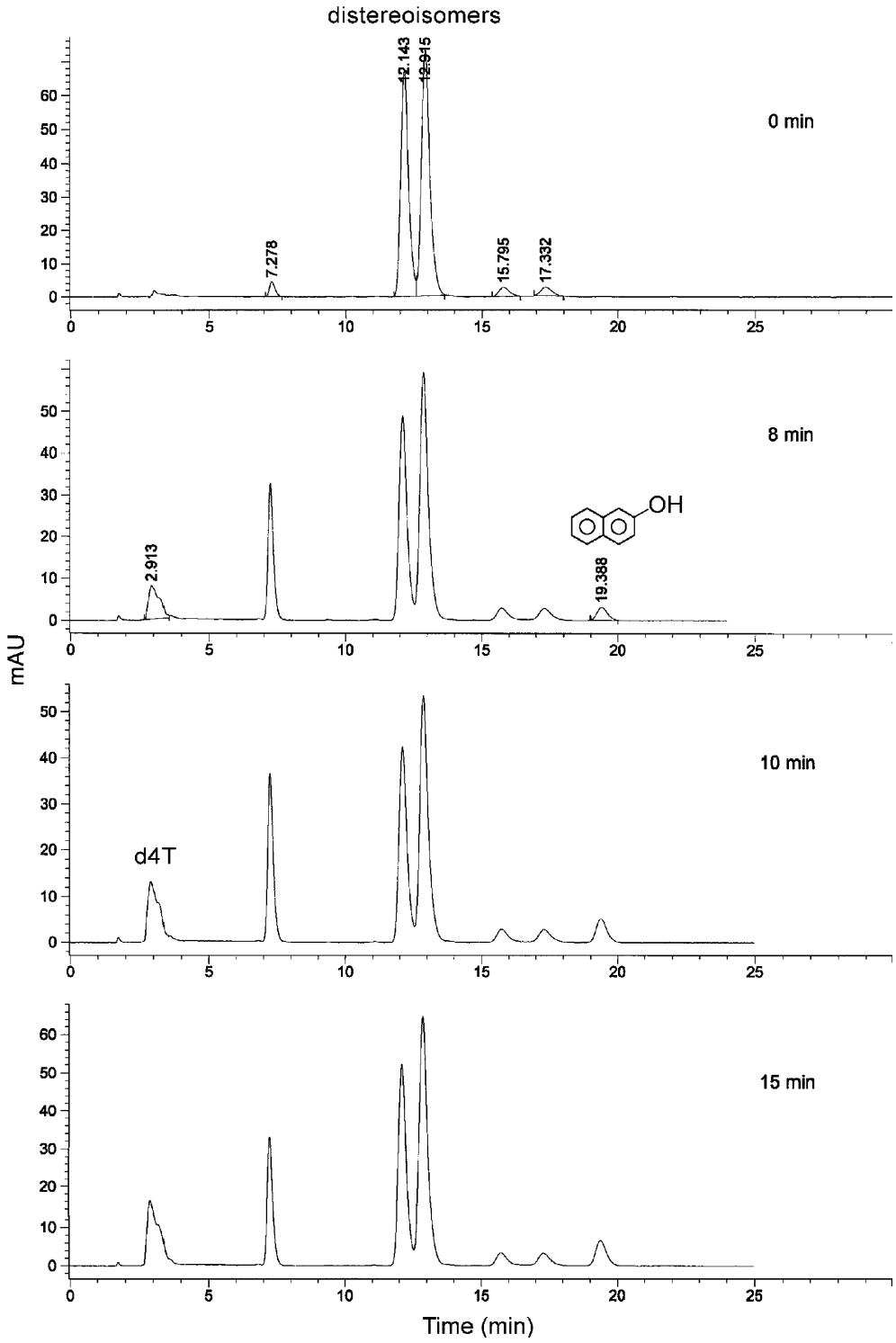


Figure 4. Hydrolysis profile of β-naphthylmethoxy alaninyl phosphoramidate **3** treated with esterase at various time intervals.

presence of lipase and the phenyl substituted derivative **4** had a hydrolysis rate of 0.9 and 1.3/h for the individual isomers.[22] In the case of esterase-mediated hydrolysis, the values were 1.52 and 1.54/h for each of the isomers. Treatment with protease enzyme, such as Subtilisin Carlsberg, gave a value of 1.3 and 13.4/h for the individual isomers. Additionally, we found that both lipase and protease showed chiral selectivity. On the other hand esterase did not show chiral selectivity for **4**.

3.1. Selectivity

In the previous discussion, we have examined the ability of each of the enzymes in hydrolyzing phosphoramidate derivatives of stavudine. We have identified that these enzymes hydrolyze each one of the phosphorus isomers (Rp or Sp) at different rate. A summary of their selectivity indexes, a ratio of the hydrolysis rate constants for slow eluting isomer (se) vs the fast eluting isomer (fe), can be

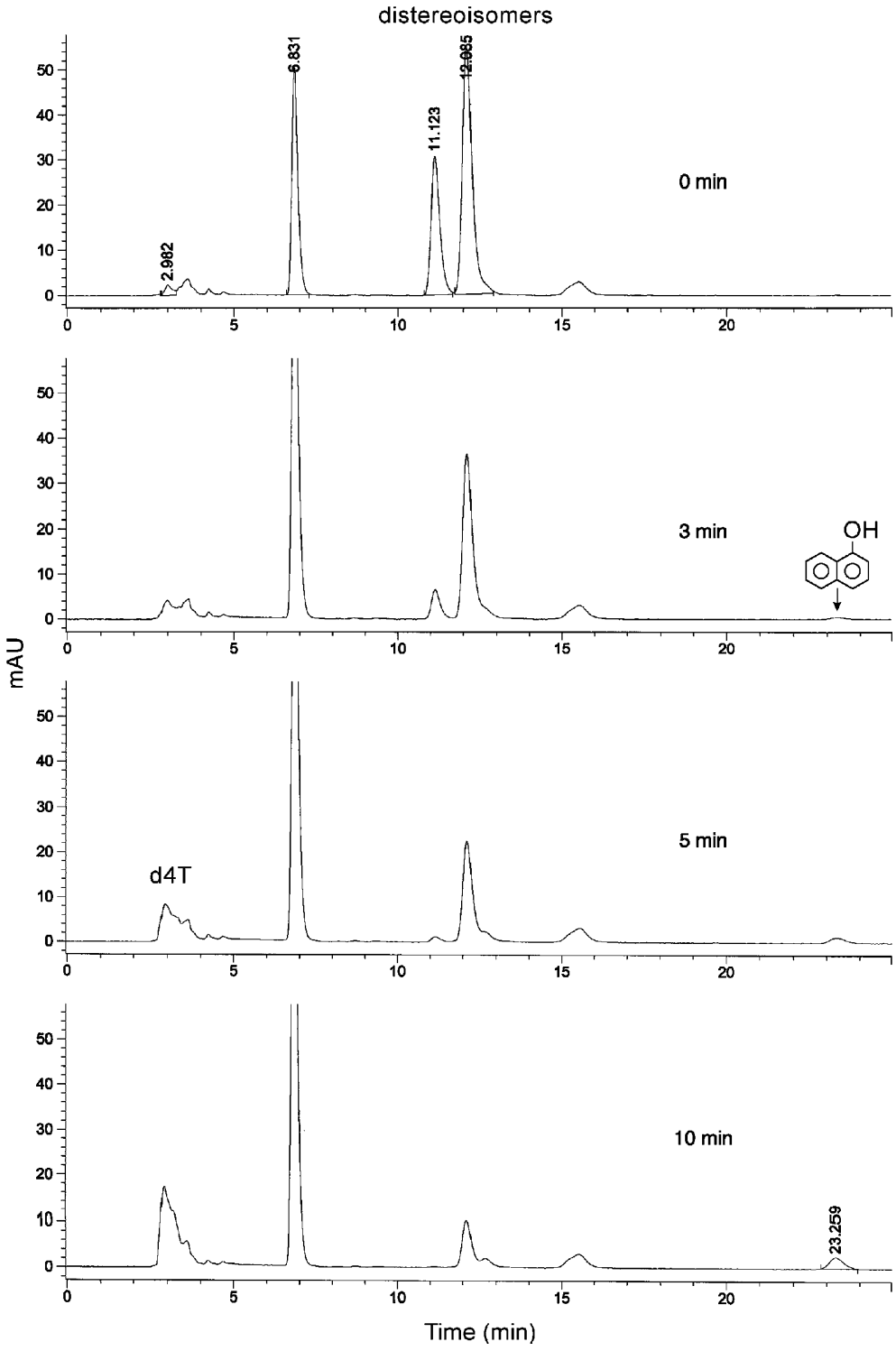


Figure 5. Hydrolysis profile of α-naphthylmethoxy alaninyl phosphoramidate **2** treated with Subtilisin Carlsberg at various time intervals.

found in Table 2. Overall, esterase did not show any chiral selectivity in the case of phenyl derivative, while it showed marginal chiral selectivity in the case of naphthyl derivatives. Lipase was able to differentiate the Rp and Sp isomers of **2** very efficiently compared to **3**. This is attributed due to the change in the conformation associated with α- and β-naphthyl group. This result is in agreement with our earlier studies, where lipase was found to show the highest chiral selectivity. In the case of protease enzyme, Subtilisin Carlsberg indicated similar behavior concerning **2** and **3**. However, **4** had a selective index value of 10.3 demonstrating the strong selectivity toward this compound. This may be due to the size of the phenyl ring as compared to the naphthyl ring associated with the other two compounds. Finally, we examined the chiral selectivity of *C. papaya*. Unfortunately, with the isomers

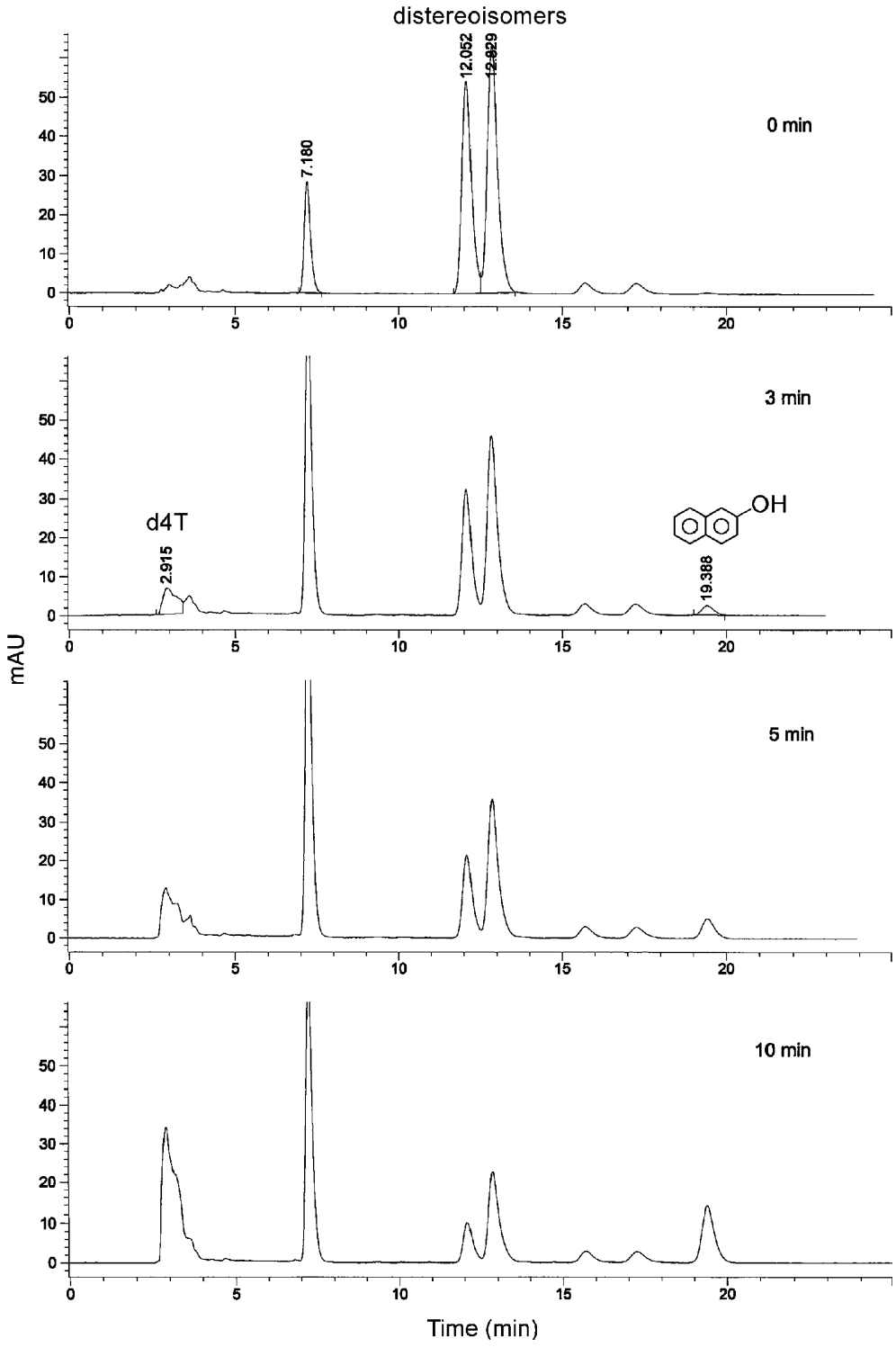


Figure 6. Hydrolysis profile of β-naphthylmethoxy alaninyl phosphoramidate **3** treated with Subtilisin Carlsberg at various time intervals.

of **2**, the slow eluting isomer (se) was not hydrolyzed, while the fast eluting isomer (se) underwent hydrolysis with a very low rate. So estimation of its chiral selectivity index was not possible. The isomers of **3** indicated only a slight difference between them (0.8). On the other hand, again the phenyl substituted derivative gave a value of 0.2. From the results of serine protease and *C. papaya* toward phenyl derivative, it showed good chiral selectivity index.

Furthermore, we suggest that depending on the enzyme recognition of the individual isomers of these phosphoramidate derivatives, the rate of conversion to active metabolites may vary. This is an important aspect for the development of prodrugs. Large-scale separation of both diastereoisomeric isopropylalainates of phosphonates with adenine as the nucleoside allowed the investigation of the anti-HIV potency of pure diastereoisomers.[27,28] A variation in the anti-HIV activity was

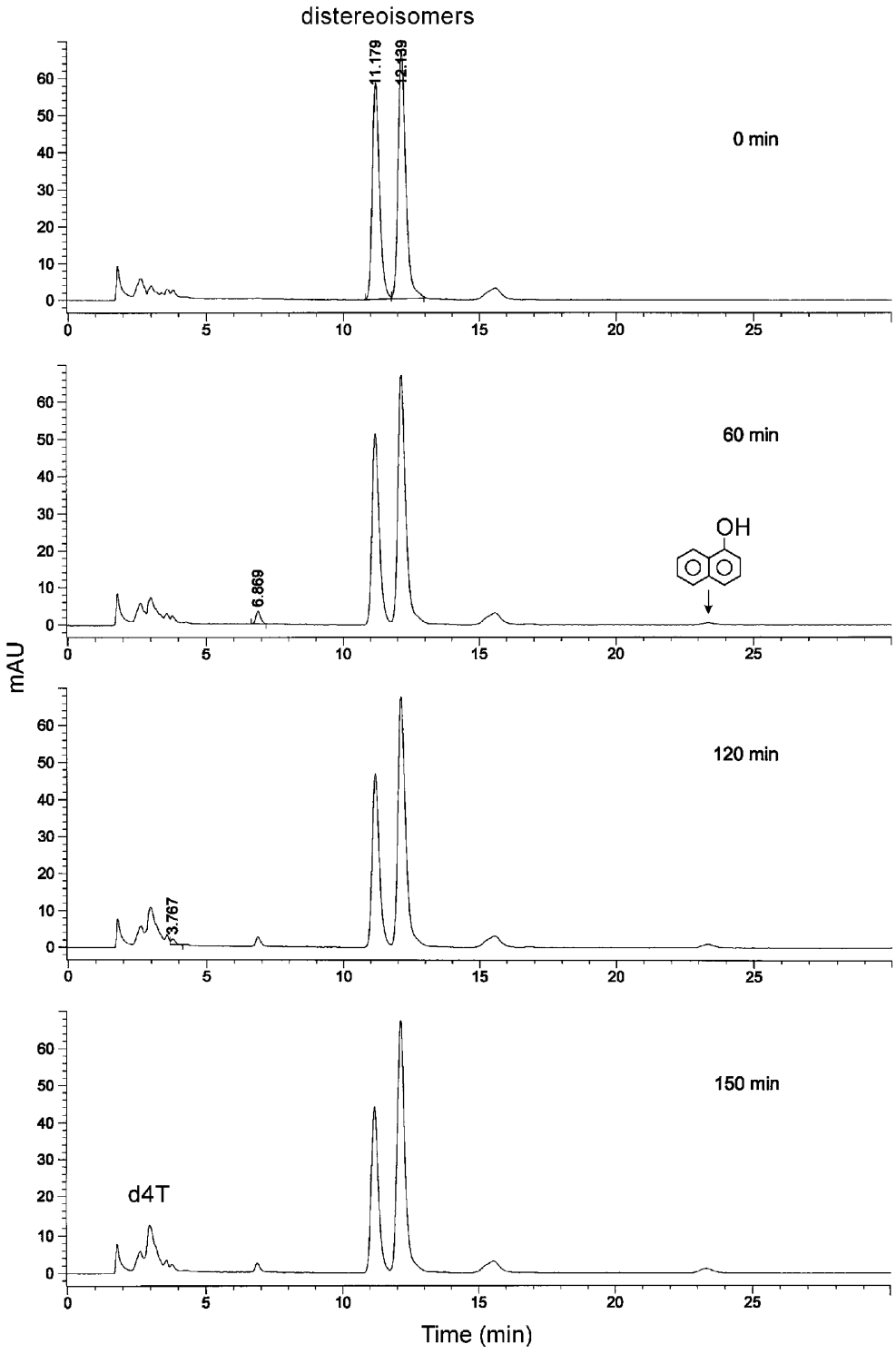


Figure 7. Hydrolysis profile of α-naphthylmethoxy alaninyl phosphoramidate **2** treated with *Carica papaya* at various time intervals.

observed in the above case between Sp and Rp compounds. Similar differences were reported for cyclosal pronucleotides.[29,30] In contrast partially separated Rp or Sp diastereoisomers of moderately effective hexyl phosphovaline isopropyl ester of AZT did not exhibit significant differences in their anti-viral activity.[31] We have recently isolated individual stereoisomers of phosphoramidate derivatives of stavudine and found that there was no significant difference existing between the anti-HIV activities.[32] One of the factors that were not previously considered for explanation of these results is a different behavior of the stereoisomers in passage through chiral membrane channels. However, our recent experiments on direct and lysed cell treatment point out that each of the isomers is preferentially hydrolyzed.[33] This result is a one-step forward explanation of why each of the isomers gets metabolized preferentially in the biological systems.

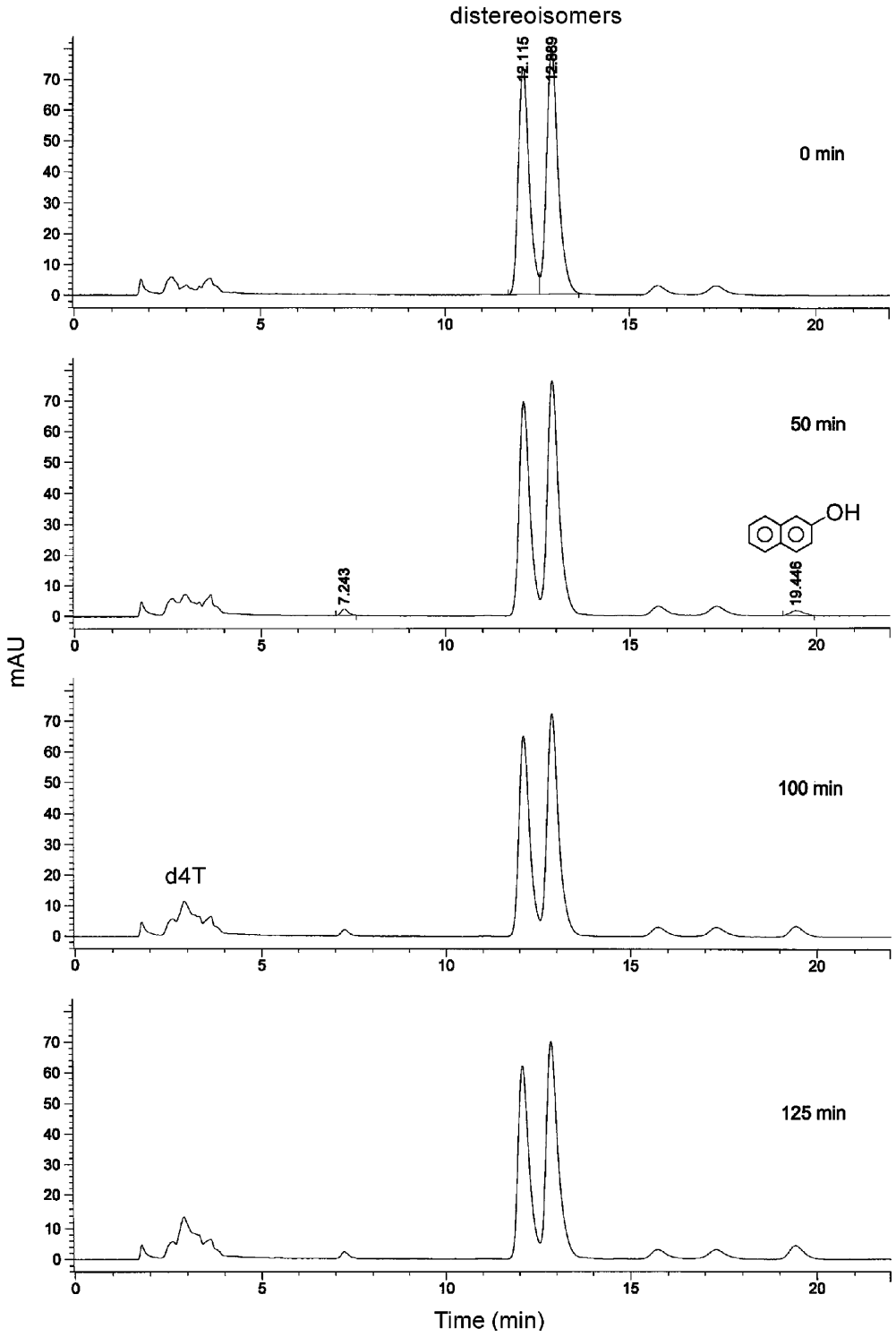


Figure 8. Hydrolysis profile of β-naphthylmethoxy alaninyl phosphoramidate **3** treated with *Carica papaya* at various time intervals.

3.2. Mechanism of hydrolysis in presence of enzymes

The mechanism of hydrolysis of these phosphoramidate derivatives in presence of enzymes follows a well-known literature pathway.[9–17] The products formed in Scheme 2 are alanine d4T monophosphate, d4T, naphthol, phosphoric acid, and alanine. Detailed work on the structural identification of these products has been previously reported.[9–17] However, at present we are not certain whether conversion of (B) to (E) occurs either stepwise, spontaneously or enzymatically. Evidence from the literature suggests that the conversion of (B) to (E) is likely to be spontaneous.[34–37] The cyclic phosphoramidate immediately hydrolyzes to the active metabolite. In order to confirm this hypothesis, we have attempted to synthesize the cyclic phosphoramidate intermediate following previously reported method for amino acid cyclic phosphoramidates.[38–41] Although

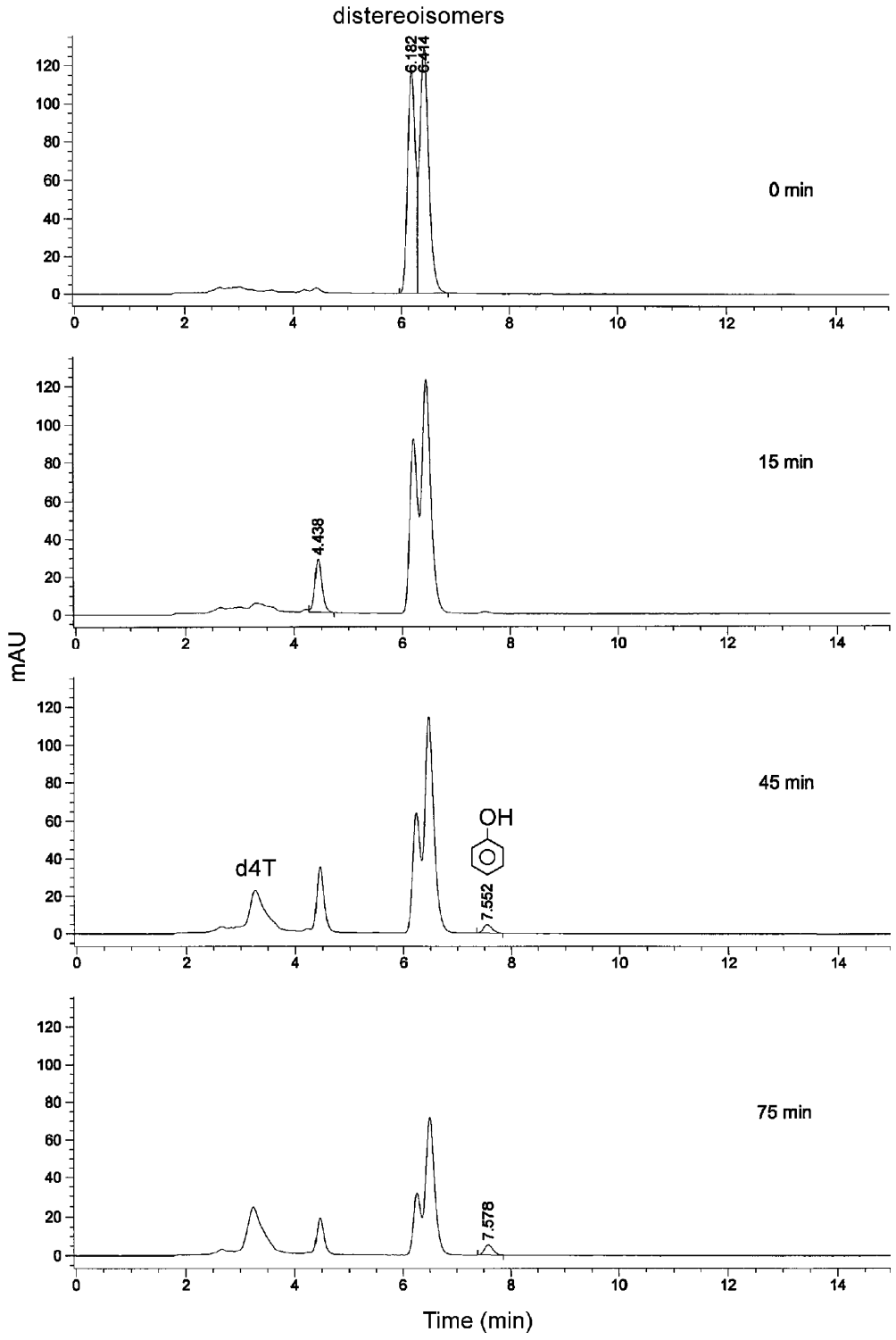


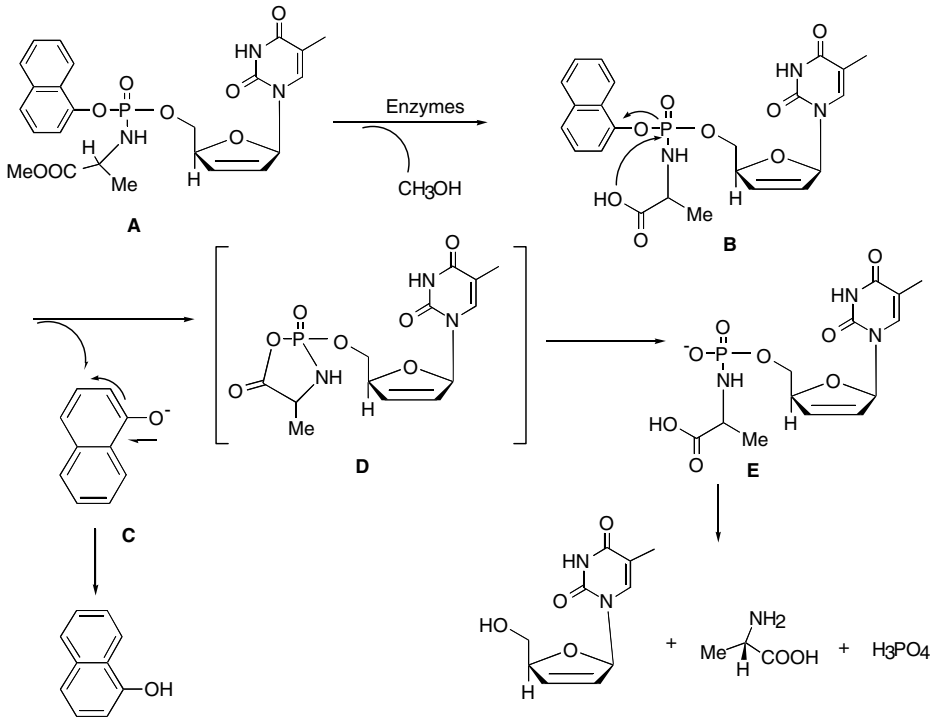
Figure 9. Hydrolysis profile of phenylmethoxy alaninyl phosphoramidate **4** treated with *Carica papaya* at various time intervals.

Table 2. Selectivity indices for enzyme-mediated hydrolysis of phosphoramidate derivatives of stavudine

Compound	Esterase	Lipase	S. Carlsberg	*Carica papaya*
2	1.6	6.6	0.3	—
3	1.2	0.8	0.6	0.8
4	1.0	1.4	10.3	0.2

Selectivity index: hydrolysis rate constant for se/fe.

we could prepare the simple cyclic phosphoramidate derivatives derived from glycine, several attempts to isolate the stavudine substituted cyclic phosphoramidate failed under our experimental conditions. This result suggests that the conversion of (B) to (E) must be fast. At this time it is not known whether or not enzymatic influence is required for this step. Based on the results obtained using bacterial enzymes, we propose that



Scheme 2. Putative pathway for hydrolysis of naphthyl substituted phosphoramidate derivative of stavudine.

hydrolyzing enzymes may take part in a particular step of the activation process.

Additionally, 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 step is normally very slow and is typically rate limiting. In our experiments, it is evident that this conversion is rapid in some of the HPLC profiles. This is consistent with the enzymatic cleavage of the 5′-O–P bond. An example of the proposed mechanism of enzyme catalysis during hydrolysis of naphthyl substituted phosphoramidate derivative is shown in Scheme 3. Based on our earlier report[26] we suggest that the naphthyl phosphoramidate derivative undergoes activation from His 57 to Ser 195. In the first part of the scheme we have indicated direct attack on the phosphorus center to form a pentacoordinated phosphorus intermediate which eliminates the naphthyl unit forming a methylester substituted stavudine phosphate. In the second case, the enzyme attacks simultaneously the ester side chain as well as the phosphorus center eventually forming the carboxylic acid of the phosphate derivative of stavudine. Furthermore, it is possible that a stepwise mechanism is taking place during the enzyme catalysis. It is evident from these schemes that various intermediate products can be formed.

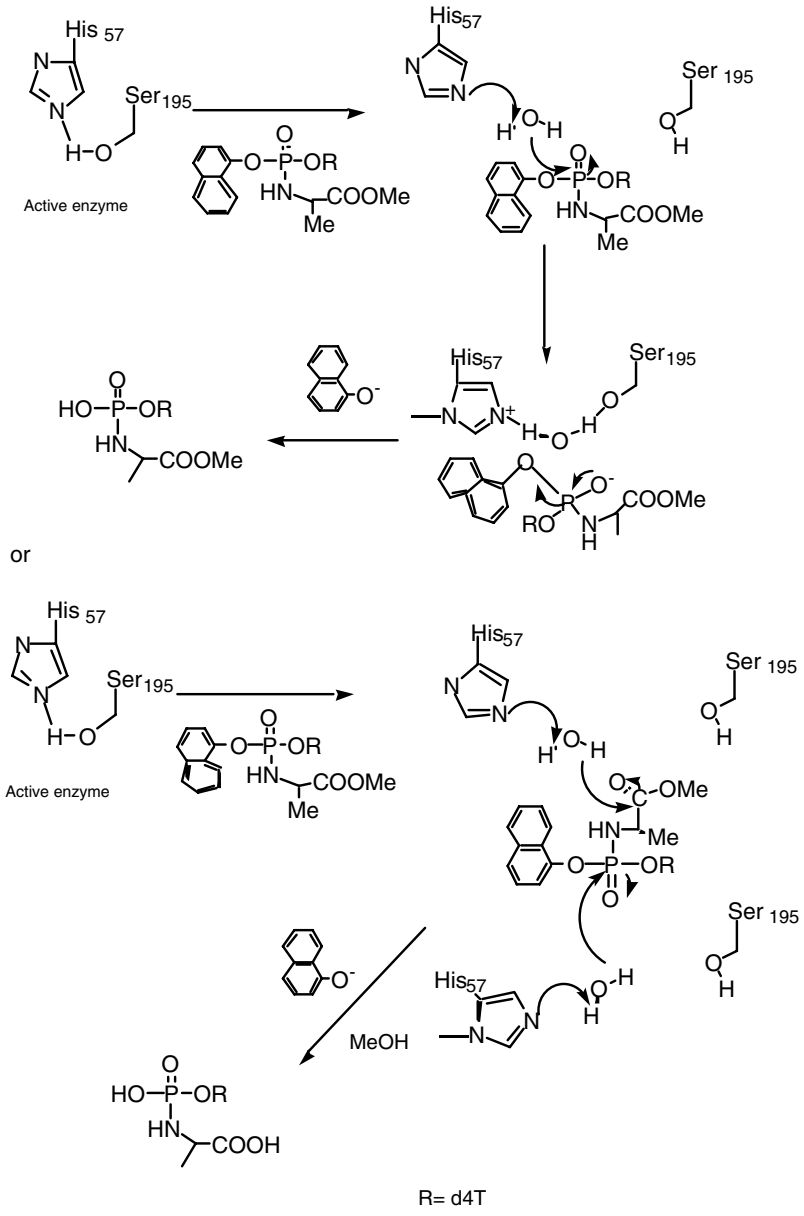
From the foregone discussion, we propose that the enzyme-mediated hydrolysis of these phosphoramidate derivatives is a relatively complex multi-step process. We also believe that enzymes hydrolyze the methylester group of the L-alanine side chain to form the cyclic intermediate in a stereoselective fashion. This notion is supported by our recent findings that the same enzymes does not hydrolyze effectively 'D' alanine substituted phosphoramidate derivatives.[25] Furthermore, we have recently demonstrated that the site of attack is the methylester side chain by varying the alkyl groups on the ester portion of the molecule whereby *t*-butyl group substituted compound underwent extremely slow rate of hydrolysis in presence of enzymes.[32]

4. Conclusion

In summary, synthesis of α- and β-naphthyl substituted phosphoramidate derivatives of stavudine was achieved using a four-step procedure. Examination of enzyme-mediated hydrolysis of these two derivatives demonstrated that the enzymes could differentiate between the Rp and Sp isomers. Lipase was found to differentiate these isomers as well as the α and β forms of the compounds. Lipase was also found to show highest chiral selectivity among the enzymes studied. This is attributed due to the tight binding pocket geometry of the lipase.

5. Materials and methods

All chemicals were purchased from Aldrich (Milwaukee, WI) or Sigma and were used without further purification. Porcine liver esterase was purchased from Sigma Chemical Company and was used without further purification. Lipase (Candida Antarctica, type B), Subtilisin Carlsberg, and *C. papaya* were all purchased from Biocatalytics Inc., USA, as solid powder form and used as such 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



Scheme 3. Proposed mechanism of enzyme (proteases) catalysis during hydrolysis of naphthyl substituted phosphoramidate derivatives of stavudine.[26]

Varian Mercury 300 instrument at ambient temperature in $CDCl_3$. Phosphorus NMR studies were done using 0.1% phosphoric acid as an internal standard in a capillary tube and referenced to 0 ppm. Chemical shifts are reported as δ values in parts per million downfield from tetramethylsilane (δ ppm) as an internal standard. 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 Protégé 460 spectrometer. HPLC was 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 μm, 4.6×250 mm) and eluent was an acetonitrile/water mixture. The isocratic flow rate was maintained at 1.0 mL/min 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 naphthols and phosphorus 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.[6,11,12,17]

5.1. General procedure for synthesis of naphthyl phosphoramidate derivative of stavudine

Triethyl amine (4.2 mL, 30 mmol) was added dropwise to a stirred solution of phosphorus oxychloride (1.53 g, 10 mmol) and α-naphthol (1.44 g, 10 mmol) in anhydrous methylene chloride (75 mL). The reaction flask was externally cooled by ice bath throughout the addition of the base to ensure that the temperature does not raise beyond 0 °C. After completion of addition of the base, the reaction mixture was allowed to warm to room temperature and stirred for additional 20 h. In the second step, the reaction mixture was cooled to −70 °C by dry ice/acetone bath and using a dry syringe, 50 mL of anhydrous methylene chloride was introduced followed by L-alanine methylester hydrochloride (1.39 g, 10 mmol). The reaction was allowed to warm up gradually to room temperature and stirred overnight. Additional triethylamine (4.2 mL, 30 mmol) was then added to the reaction mixture and the contents were allowed to stir. After stirring at room temperature for 3 h, stavudine (0.45 g, 2.0 mmol) was added to the above reaction flask and the reaction mixture was stirred at room temperature for 7 days. During this period the flask was covered with an aluminum foil to avoid exposure to light. The contents of the flask were filtered to remove any precipitate and were washed with 2×25 mL of anhydrous methylene chloride. The combined methylene chloride extract was subjected to rotary evaporation under reduced pressure. The crude product was purified by column chromatography (100% $CHCl_3$ followed by 5% MeOH in $CHCl_3$) over silica gel. Fractions were collected and those fractions containing the product (as evidenced from TLC) were pooled together and the solvent was removed once again under vacuum. The product was further purified using preparative TLC to obtain analytically pure compound.

5.2. Experimental conditions for the lipase-mediated hydrolysis experiments

For the kinetic study, a known amount of the phosphoramidate derivative was carefully weighed (5–7 mg) using a Mettler analytical balance and transferred into a scintillation glass vial. Using a pipetman, 3 mL of methanol was added and the contents were vortexed for 2 min until a homogeneous solution resulted. Using another pipetman, 100 μL of the above solution was transferred into another scintillation vial and to this was added 900 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: for each kinetic run, from the stock solution of the compound as mentioned above, 50 μL of the methanolic solution of the phosphoramidate derivative was pipetted out into another glass vial and to this 50 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 (5 μm, 4.6×250 mm). The eluent used for HPLC was water (0.1% TFA + 0.1% TEA) and acetonitrile in the ratio of 65:35. The column was maintained at room temperature. The flow rate was maintained at 1 mL/min, 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. 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 refer to rate/h since some of the reactions were too slow to obtain meaningful results.

5.3. Experimental conditions for the esterase-mediated hydrolysis experiments

For the kinetic study, a known amount of the phosphoramidate derivative was carefully weighed (5–7 mg) using a Mettler analytical balance and transferred into a scintillation glass vial. Three milliliters of methanol was added and the contents vortexed for 2 min until a homogeneous solution was obtained. Hundred microliters of this solution was transferred into another scintillation vial and to this was added 900 μL of water and the contents vortexed. For each of the runs, 50 μL of the above solution was transferred into a vial and 5 μL of carboxy esterase solution (20,000 U/5.6 mL) was added and the contents were shaken and HPLC runs were done using the method described as above in the lipase study.

5.4. Experimental conditions for the protease-mediated hydrolysis experiments

For the kinetic study, a known amount of the phosphoramidate derivative was carefully weighed (5–7 mg) using a Mettler analytical balance and transferred into a scintillation glass vial. Three milliliters of methanol was added to this vial and the contents were vortexed for 2 min until a homogeneous solution was obtained. Hundred microliters of this solution was transferred into another scintillation vial and to this was added 900 μL of water and the contents vortexed. In parallel, 5–6 mg amounts of the respective proteases were weighed and transferred to a volumetric flask. Eight milliliters of water was added and the contents were shaken to dissolve the protease. The reaction mixtures for the kinetic study were prepared as follows: from the stock solution of the compound, 50 μL was pipetted out into another glass vial and to this 50 μL of diluted solution of the enzyme was added and the contents were shaken to form a homogeneous solution. From this reaction mixture 50 μL/time point was used for HPLC analysis using the method previously described for the lipase-mediated hydrolysis.

5.5. Estimation of products

The amount of products observed during the reaction of these phosphoramidates was estimated from the area obtained from the HPLC profiles. Authentic samples of the products when possible were run to identify the peaks observed during the reaction. Additionally confirmation of the product structures in the past was obtained using an LC/MS 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 refer to rate/h as some of the reactions were too slow to obtain meaningful results.

5.6. Statistical analysis

Hydrolysis rates were determined by fitting single exponential decay equations to the disappearance of each isomer substrate in the presence of enzyme. For simplicity the reaction was considered to be first order. An average of two to three runs were done for each of the enzymes and the standard deviation calculated.

5.7. Physical constants of compounds

5.7.1. Stavudine-5′-α-naphthyl methoxy L-alaninyl phosphate (2). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.34 (m, 3H, ala Me), 1.78 (m, 3H, 5-Me), 3.64 (m, 3H, OMe), 4.0–4.08 (m, 2H, Ala NH, AlaCH), 4.31–4.43 (m, 2H, H-5′), 5.00–5.05 (m, 1H, H-4′), 5.89–5.92 (m, 1H, H-3′), 6.26–6.36 (m, 1H, H-2′), 7.00–7.02 (m, 1H, H-1′), 7.24–7.27 (m, 3H, H-6, Ar), 7.47–7.54 (m, 2H, Ar), 7.65–7.67 (m, 1H, Ar), 7.83–7.84 (m, 1H, Ar), 8.01–8.07 (m, 1H, Ar), 8.41 (br s, 1H, NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.1, 12.3, 20.8, 21.0, 21.1, 50.2, 50.8, 52.5, 52.6, 66.8, 67.4, 84.6, 84.7, 89.7, 89.9, 111.4, 115.0, 115.1, 121.1, 121.2, 125.1, 125.2, 125.4, 125.5, 126.5, 126.8, 127.3, 127.4, 127.9, 133.1, 133.4, 134.8, 135.5, 135.7, 150.7, 163.4, 174.0; ^{31}P NMR (121 MHz, $CDCl_3$) δ 2.94, 3.40; UV (MeOH) λ_{max}: 266 nm. HPLC: 7.10, 7.80 min. % purity: 98.0.

5.7.2. Stavudine-5′-β-naphthyl methoxy L-alaninyl phosphate (3). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.38 (m, 3H, ala Me), 1.88 (m, 3H, 5-Me), 3.68 (m, 3H, OMe), 4.0–4.05 (m, 2H, Ala NH,AlaCH), 4,30–4.39 (m, 2H, H-5′), 5.02–5.05 (m, 1H, H-4′), 5.84–5.92 (m, 1H, H-3′), 6.30–6.37 (m, 1H, H-2′), 7.0–7.02 (m, 1H, H-1′), 7.26–7.31 (m, 3H, H-6, Ar), 7.47–7.50 (m, 2H, Ar), 7.76–7.83 (m, 3H, Ar), 8.21 (br s, 1H, NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.34, 12.36, 21.04, 21.10, 50.1, 50.2, 52.6, 66.6, 67.15, 84.5–84.7, 89.8, 89.6, 111.4, 116.5, 116.6, 116.8, 119.9, 150.6, 163.3, 174.1; ^{31}P NMR (121 MHz, $CDCl_3$) δ 2.66, 3.21; UV (MeOH) λ_{max}: 266 nm; HPLC: 8.75, 9.4 min. % purity: 98.0.

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