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Original article

Protease-mediated enzymatic hydrolysis and activation of aryl phosphoramidate derivatives of stavudine

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Abstract

Several proteases are capable of hydrolyzing the aryl substituted phosphoramidate derivatives of stavudine resulting in the formation of the active metabolite, alaninyl d4T monophosphate. Subtilisin Protease A, Subtilisin Griseus, Subtilisin Carlsberg, Papaya, Bacillus were amongst the most effective proteases in hydrolyzing stavudine derivatives and specificity of their activity was confirmed using several protease inhibitors to block the hydrolysis of these phosphoramidate derivatives. We found that these proteases exhibit chiral selectivity at the phosphorus center of stavudine derivatives. Our results indicate that cellular proteases may be responsible for the activation of these phosphoramidate derivatives. In addition, we show that the enzymatic hydrolysis takes place at the carboxymethyl ester side chain of these pro-drugs and the direct attack on the phosphorus center by these enzymes does not occur. Finally, we describe a novel activation pathway hitherto unknown for the activation and viral inhibitory characteristic shown by these phosphoramidate derivatives of stavudine.

Keywords: HIV; Stampidine; Proteases; Hydrolysis; Mechanism; Distereoisomers

1. Introduction

The human immunodeficiency virus (HIV)/AIDS pandemic continues its spread at a rate of over 15,000 new infections every day. Three categories of anti-retroviral agents in clinical use are nucleoside analogs, nucleoside reverse transcriptase inhibitors (NRTI), such as zidovudine and stavudine (d4T) [1,2] protease inhibitors and non-nucleoside reverse transcriptase inhibitors (NNRTI) [3–6]. The 5′-triphosphates of 2′,3′-dideoxynucleoside analogs (ddN) which are generated by the action of nucleoside and nucleotide kinases, are potent inhibitors of HIV reverse transcriptase [7,8]. The rate limiting step for the conversion of 3′-azido-3′-deoxy thymidine (AZT) to its bioactive metabolite AZT-triphosphate seems to be the conversion of the monophosphate derivative to the di-phosphate derivative, whereas the rate limiting step for the intracellular generation of the bioactive 2′,3′-dideoxy-2′,3′-didehydro thymidine (stavudine) metabolite stavudine-triphosphate was reported to be the conversion of the nucleoside to its monophosphate derivative [9–11]. Anti-HIV ddN derivatives primarily rely on nucleoside and nucleotide kinases to convert them into the corresponding 5′-triphosphates as discussed before. However, such compounds were found to act as poor substrates for nucleoside kinases. [9,12–15]. Consequently, development of pro-drug strategies were sought to 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 stavudine [10,11,16–25]. Some of these derivatives were found to be potent anti-HIV agents with sub-nanomolar IC_{50} values.

Our lead anti-HIV compound stampidine is a novel phosphoramidate derivative of stavudine [23]. Stampidine was 100-times more active than stavudine and twice as active as zidovudine against nine clinical HIV-1 isolates of non-B envelope subtypes (A, C, F and G) originating from South America, Asia, and sub-Saharan Africa [20]. Stampidine was effective against 20 genotypically and phenotypically nucleoside analog reverse transcriptase inhibitor (NRTI)-resistant and six non-nucleoside inhibitor (NNRTI)-resistant HIV-

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1 isolates at subnanomolar to low nanomolar concentrations [20]. 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 [21]. Orally administered stampidine showed a dose-dependent anti-retroviral effect in chronically FIV-infected cats [22]. 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 [22,24]. 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 non-B envelope subtypes together with its in vivo antiretroviral 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 the alanine side chain of stampidine [10,11,18–20,26–28]. We hypothesized that in various tissue microenvironments the metabolism of stampidine may occur through the action of hydrolytic enzymes other than esterases as well. A recent study implicated lipase-mediated stereoselective hydrolysis of stampidine as an important step in the activation of this prodrug [29]. The purpose of the present study was to evaluate the potential role of proteases in the activation of stampidine. Our experimental results provide unprecedented evidence that stampidine as well as other halogen-substituted phosphoramidate derivatives of stavudine can be metabolized by protease-mediated hydrolysis. To our knowledge this study is the first to provide experimental evidence for a protease-mediated metabolism of a nucleoside phosphoramidate prodrug.

2. Chemistry

2.1. 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 were obtained on a Varian Mercury 300 instrument at ambient temperature in d_6-DMSO. Chemical shifts are reported as δ values in parts per million downfield from tetramethylsilane (δ0.0 ppm) as an internal standard or from the residual dimethylsulfoxide signal (δ 2.49 ppm for ^{1}H NMR or δ 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 m (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 the 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 were previously reported in our earlier publications [18]. Synthesis of chloroethyl substituted stavudine derivatives were accomplished as described below.

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

A RB flask was charged with 20 ml of anhydrous acetonitrile and triethylamine (1.2 eq.) followed by triazole (2.50 eq.). To this mixture was added a solution of appropriately substituted phenol (1.0 eq.) and bis(2-chloroethyl)amine phosphodichloridate (1.00 eq.) 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 eq.) was added to the mixture and the contents of the flask were brought to 50 °C and allowed to stir for 1 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 products in 10% yield as solids.

2.3. Physical constants

2.3.1. 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_2CH_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, 6-H), 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: RT 12.4, 14.1 min.

2.3.2. 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}.

^1H NMR ($CDCl_3$) δ 1.85 (s, 3H, 5-CH_3), 2.32 (s, 3H, CH_3), 3.37–3.60 (m, 8H, NCH_2CH_2Cl), 4.22–4.41 (m, 2H, 5′-H), 5.03 (s, 1H, 4′-H), 5.92 (m, 1H, 1′-H), 6.33 (m, 1H, 2′-H), 6.97–7.14 (m, 5H, Ar-2, 3, 5, 6; 6-H; 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: RT: 21.9, 25.3 min.

2.3.3. 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}.

^1H NMR ($CDCl_3$) δ 1.86 (s, 3H, 5-CH_3), 3.37–3.60 (m, 8H, NCH_2CH_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, 6-H), 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: RT: 14.4, 16.6 min.

2.3.4. 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}.

^1H NMR ($CDCl_3$) δ 1.90 (s, 3H, 5-CH_3), 3.42–3.84 (m, 8H, NCH_2CH_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, 6-H), 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: RT: 31.8, 35.8 min.

2.3.6. 5′-[4-Bromophenyl methoxy alaninylphosphate]-2′,3′-didehydro-3′-deoxylthymidine isomer 1(S)

TLC: Rf: 0.51 [($CHCl_3$/MeOH) (9:1)], m.p. 59 °C; ^1H NMR ($CDCl_3$) δ 8.15(bs), 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(bs), 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 RT: 11.23 min, $[\alpha]_D = -30$.

2.3.7. 5′-[4-Bromophenyl methoxy alaninylphosphate]-2′,3′-didehydro-3′-deoxylthymidine isomer 2 (R)

TLC: Rf: 0.51 [($CHCl_3$/MeOH) (9:1)], m.p. 160 °C; ^1H NMR ($CDCl_3$) δ 8.21(bs), 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(bs), 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 RT: 12.1 min, $[\alpha]_D = -27.5$.

2.4. General procedure for synthesis of substituted phosphoramidate derivatives of stavudine (preparation of 'D' isomers)

Triethylamine (4.2 ml, 30 mmol) was added drop wise to a stirred solution of $POCl_3$ (1.53 g, 10 mmol) and substituted phenol (10 mmol) in anhydrous chloroform (40 ml) at 0 °C. The reaction was allowed to warm to room temperature and stirred for 15 h. Then reaction mixture was cooled to –70 °C and D-alanine methyl ester hydrochloride (1.39 g, 10 mmol) was added to the reaction flask, the reaction was allowed to warm to room temperature and stirred overnight. 1,2,4-Triazole (1.80 g, 25 mmol) was then added to above reaction flask. After stirring at room temperature for 6 h, d4T (0.45 g, 2.0 mmol) was added to above reaction flask and the reaction solution was stirred for 4 days. The solvent was removed under reduced pressure. The crude product was purified by column chromatography (100% $CHCl_3$ followed by 5% MeOH in $CHCl_3$) and it was further purified using preparative TLC to obtain analytically pure compound.

*2.4.1. d4T-5′-*p*-Bromophenyl methyl-D-alaninyl phosphate (**10**)*

^1H NMR (300 MHz, $CDCl_3$) δ 1.33 (t, 3H, J = 7.0 Hz), 1.81 (d, 3H, J = 6.6 Hz), 3.58–4.06 (m, 2H), 3.70 (s, 3H), 4.28 (s, 2H), 5.00 (s, 1H), 5.90 (m, 1H), 6.33 (s, 1H), 7.02 (s, 1H), 7.08 (d, 2H, J = 9.0 Hz), 7.24 (s, 1H), 7.43 (d, 2H, J = 8.1 Hz), 8.61 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.8, 21.3, 50.3, 53.0, 66.7, 67.6, 84.8, 89.9, 111.5, 118.4, 122.1, 127.6, 133.0, 133.5, 136.0, 150.9, 163.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 2.56, 2.97; UV (MeOH) λ_{max}: 266 nm. $[\alpha]_D$ {MeOH} –25.2°: HPLC: 12.72, 12.92; % purity: >98.

*2.4.2. d4T-5′-p-fluorophenyl methoxy-D-alaninyl phosphate (**11**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (q, 3H, J = 4.2 Hz), 1.86 (d, 3H, J = 7.5 Hz), 3.50–4.06 (m, 2H), 3.70 (s, 3H), 4.28 (s, 2H), 5.00 (s, 1H), 5.89 (m, 1H), 6.98–7.04 (m, 3H), 7.13–7.17 (m, 2H), 7.28 (s, 1H), 8.41 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.8, 21.3, 50.3, 53.0, 66.6, 67.5, 84.8, 89.9, 111.5, 116.6, 121.7, 127.6, 133.4, 135.9, 150.8, 163.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 2.96, 3.34; UV (MeOH) λ_{max}: 266 nm. $[\alpha]_D$ {MeOH} –22.2°: HPLC: 10.25, 10.35 min. % purity: 98.0.

*2.4.3. d4T-5′-p-Methylphenyl methoxy-D-alaninyl phosphate (**12**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.32 (t, 3H, J = 7.2 Hz), 1.85 (d, 3H, J = 6.6 Hz), 2.30 (s, 3H), 3.44–4.05 (m, 2H), 3.69 (s, 3H), 4.28 (s, 2H), 5.00 (s, 1H), 5.89 (m, 1H), 6.33 (s, 1H), 7.00–7.12 (m, 5H), 7.30 (s, 1H), 8.35 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.7, 21.3, 30.0, 50.3, 53.0, 66.4, 67.3, 84.8, 89.9, 111.5, 120.0, 127.5, 133.4, 133.5, 136.1, 150.8, 163.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 2.78, 3.27; UV (MeOH) λ_{max}: 266 nm. $[\alpha]_D$ {MeOH} –24.6°: HPLC: 11.33, 11.44 min. % purity: >98.

*2.4.4. d4T-5′-p-Methoxyphenyl methoxy-D-alaninyl phosphate (**13**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (t, 3H, J = 8.7 Hz), 1.81 (d, 3H, J = 6.0 Hz), 3.44–4.06 (m, 2H), 3.70 (s, 3H), 3.78 (s, 3H), 4.28 (s, 2H), 4.99 (s, 1H), 5.88 (m, 1H), 6.34 (s, 1H), 6.83 (d, 2H, J = 9.0 Hz), 7.02 (s, 1H), 7.10 (d, 2H, J = 10.5 Hz), 7.30 (d, 1H, J = 8.4 Hz), 8.41 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.8, 21.4, 50.3, 52.9, 55.9, 66.9, 84.8, 89.8, 111.5, 114.9, 121.3, 127.6, 133.6, 136.2, 150.8, 163.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 3.11, 3.55; UV (MeOH) λ_{max}: 266 nm. HPLC: 10.20, 10.27 min.

*2.4.5. d4T-5′-Phenyl methoxy-D-alaninyl phosphate (**14**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (t, 3H, J = 7.6 Hz), 1.86 (d, 3H, J = 6.6 Hz), 3.44-4.06 (m, 2H), 3.70 (s, 3H), 4.30 (m, 2H), 5.00 (s, 1H), 5.89 (m, 1H), 6.33 (m, 1H), 7.02 (s, 1H), 7.19 (d, 2H, J = 7.5 Hz), 7.28–7.36 (m, 6H), 8.27 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.8, 21.4, 50.4, 53.0, 66.5, 67.4, 84.8, 89.9, 111.5, 120.3, 125.4, 127.5, 133.0, 133.5, 136.0, 150.8, 163.5. ^{31}P NMR (121 MHz, $CDCl_3$) δ 2.17, 2.64; UV (MeOH) λ_{max}: 266 nm. HPLC: 12.71, 12.88 min.

*2.4.6. d4T-5′-p-Chlorophenyl methoxy-D-alaninyl phosphate (**15**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (t, 3H, J = 7.5 Hz), 1.86 (d, 3H, J = 7.8 Hz), 3.55–4.06 (m, 2H), 3.70 (s, 3H), 4.28 (s, 2H), 5.00 (s, 1H), 5.91 (m, 1H), 6.33 (m, 1H), 7.02 (s, 1H), 7.14 (d, 2H, J = 8.1 Hz), 7.24 (s, 1H), 7.29 (d, 2H, J = 8.7 Hz), 8.55 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.8, 21.3, 50.3, 53.0, 66.7, 67.6, 84.8, 89.9, 111.5, 121.7, 127.6, 133.0, 133.4, 136.0, 150.8, 163.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 2.77, 3.16; UV (MeOH) λ_{max}: 266 nm. HPLC: 10.00 min.

3. Results and discussion

Due to the stereochemistry of its phosphorous chiral center, stampidine exists as a mixture of two possible diastereomers (Fig. 1). The TLC profile of the diastereomeric mixture of stampidine shows a single spot with an Rf value of 0.5 using a mixture of chloroform/methanol in the ratio of 9:1. Fig. 2A depicts the ^{1}H NMR spectrum of stampidine with chemical shifts consistent with its structure. Fig. 2B shows the ^{31}P NMR spectrum of stampidine with two sharp peaks confirming the presence of two diastereomers. The HPLC analysis of stampidine shows two distinct peaks corresponding to its putative steroisomers (Fig. 2C). 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, polariometry, HPLC, NMR, and melting point analysis. The IR spectra of the purified diastereomers were identical with a characteristic band at 1690 cm^{-1} due to the C=O unit in their structure. No significant differences in optical rotation were observed (–30° vs. –27.5°) by polarimetric analysis and their individual ^{1}H NMR spectra were identical (Fig. 2D and G). Fig. 2E shows the ^{31}P NMR spectrum of diastereomer #1 with a sharp signal at 3.20 ppm and Fig. 2H shows the ^{31}P NMR spectrum of diastereomer #2 with a sharp signal at 2.56 ppm in agreement with the two peaks observed in the ^{31}P NMR spectrum of the diastereomeric mixture of stampidine. The melting point analysis yielded melting points of 59–60 °C for the mixture, 60 °C for diastereomer #1 and 160 °C for diastereomer #2. The HPLC profiles of individual isomers of stampidine is shown in Fig. 2F and I. 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 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).

We first set out to evaluate the ability of the serine protease Subtilisin Carlsberg to hydrolyze nine different phosphoramidate derivatives of stavudine, including stampidine. The chemical reactions of Subtilisin Carlsberg with phosphoramidate derivatives were monitored using HPLC and the rate of hydrolysis at room temperature was calculated for the two diastereomers of each compound using a first order rate equation. The rate constants are given in Table 1. The selectivity

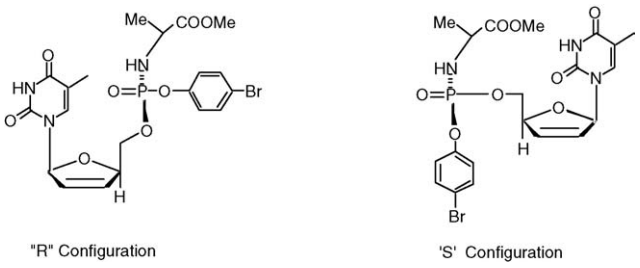


Fig. 1. Structures of two individual isomers of 5′-[4-bromophenyl methoxy alaninylphosphate]-2′,3′-didehydro-3′-deoxylthymidine.

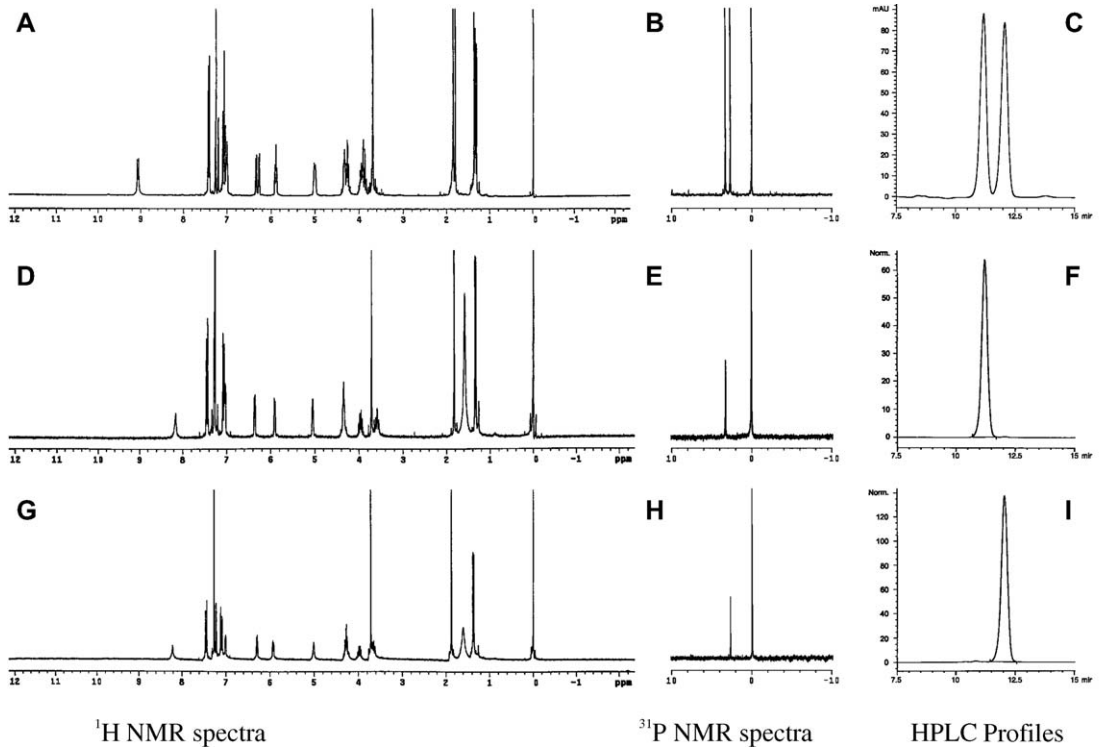
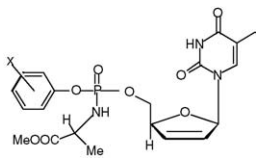


Fig. 2. ^{1}HNMR, ^{31}P NMR and HPLC profiles of distereoisomers as well as separated individual isomers of 4- bromophenyl phosphoramidate (stampdidine).

index indicates the relative rates of hydrolysis for the two diastereomers. Notably, the diastereomers corresponding to the peak#2 in the HPLC chromatograms of these compounds were found to undergo hydrolysis much faster than those corresponding to peak#1 (Fig. 3). Subtilisin Carlsberg was able to differentiate between the two diastereomers of the unsubstituted control phosphoramidate derivative of stavudine showing an almost 10-fold difference in the rate of hydrolysis (0.7 vs. 7.0).

Table 1
Rate Constants for Hydrolysis of Phosphoramidate Derivatives of Stavudine using Enzyme Protease Subtilisin Carlsberg and Carcia Papaya at Room temperature



Compound#	X	Subtilisin Carlsberg			Carcia Papaya		
		#1*	#2*	SI (#1/#2)	#1*	#2*	SI (#1/#2)
1	4-OMe	2.76	5.87	0.5	0.21	0.26	0.8
2	3Br	1.43	3.83	0.4	2.70	0.07	38.6
3	2Cl-4Br	2.07	4.62	0.5	0.52	0.02	26.0
4	4-F	1.34	3.02	0.4	0.68	0.13	5.2
5	2-Br	9.81	16.78	0.6	0.72	0.06	12.0
6	2-Cl	1.36	4.34	0.3	0.29	0.02	14.5
7	H	0.74	6.97	0.1	0.73	0.12	6.1
8	4-Cl	0.78	3.68	0.2	0.86	0.42	2.0
9 Stampidine	4-Br	2.73	6.37	0.4	1.09	0.77	1.4

The rate constants for isomer (#1) and isomer (#2) in h^{-1}. SI indicates the selectivity index calculated as the ratio of the rate constants for #1 and #2.

Hydrolysis with Substilisin Carlsberg was not significantly affected by electron donating or electron withdrawing substituents (Hammett's Sigma) (Spearman's Rank Correlation, Rho = +0.3, $P = 0.4$ for peak 1; Spearman's Rank Correlation, Rho = 0.0, $P = 1$ for peak 2) demonstrating that electronic factors play a minor role during enzymatic hydrolysis with this particular serine protease, unlike chemical hydrolysis. [25] Comparison of the rates of hydrolysis between isomer 1 and isomer 2 among the stavudine derivatives studied showed that most of the compounds had a selectivity index value ranging from 0.1 to 0.5 for Subtilisin Carlsberg, 0.8–26.0 for Carcia Papaya and 0.02–0.22 for alanine racemase. Marked variation in the selectivity index was observed for Carcia Papaya demonstrating that this enzyme discriminated the phosphorus isomers preferentially as compared to other enzymes used in the present study.

We next examined the ability of the cysteine protease, Carcia Papaya to hydrolyze these phosphoramidate derivatives. Table 1 presents the rates of hydrolysis and selectivity index obtained for the substituted phosphoramidate derivatives at room temperature. The rates of hydrolysis of stavudine derivatives were much slower for Carcia Papaya than Subtilisin Carlsberg. The most striking difference is for the preferential hydrolysis of isomer 1 by Carcia Papaya. Fig. 4 shows representative HPLC chromatogram profiles for compound **4** treated with enzyme Carcia Papaya for various time intervals Alanine racemase also hydrolyzed these derivatives and showed a preferential hydrolysis of one isomer over the other (Table 2 and Fig. 5). However, the rate of hydrolysis was much

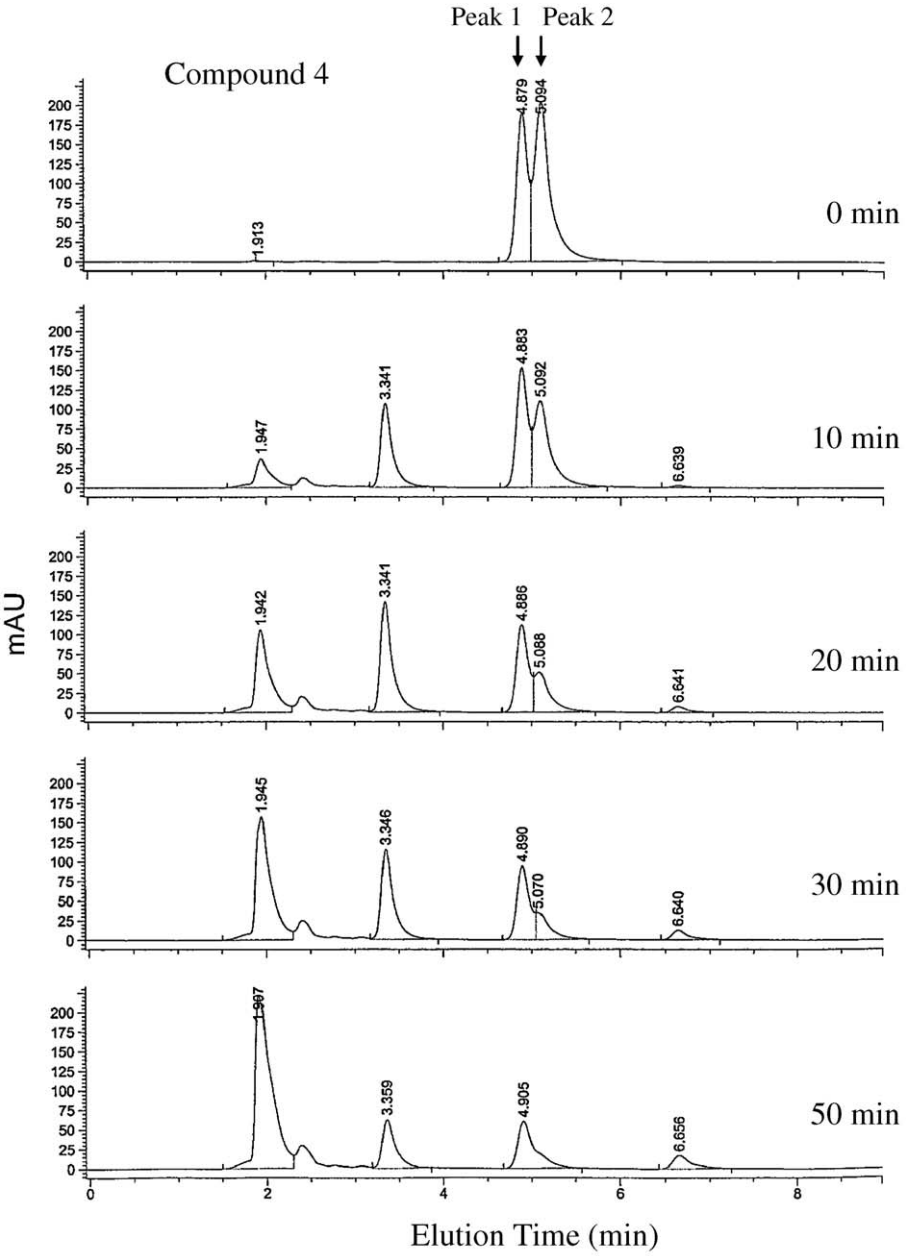


Fig. 3. HPLC profiles of fluoro substituted aryl phosphoramidate derivative compound #4 treated with enzyme Subtilisin Carlsberg for various time intervals.

slower as compared to Subtilisin Carlsberg. Interestingly, there was a significant correlation between electron donating or electron withdrawing substituents (Hammett's Sigma) and the rate of hydrolysis for peak 2 (Spearman's Rank Correlation, Rho = +0.77, $P = 0.01$) in the presence of alanine racemase, suggesting a different mechanism of hydrolysis compared to that catalyzed by the Subtisilin Carlsberg or Carcia Papaya.

Taken together, these findings prompt the hypothesis that proteases may be involved in the enzymatic hydrolysis of stavudine phosphoramidate derivatives necessary for the formation of the active metabolite ala-d4T-MP (Scheme 1). In the above scheme, the hydrolysis of the carbomethoxy ester side chain to form the carboxylic acid represents the first step. We hypothesize that the phosphoramidate derivatives of stavudine undergo enzymatic hydrolysis by the presence of subtilisin or other proteases as follows: The water molecule attacks the phosphorus center of these derivatives resulting a pentacoordinated phosphorus complex having an oxyanion. Similar types of pentacoordinated phosphorus intermediates in enzyme catalyzed reactions has been proposed by Kubiak et al. [30]. In the next step, the negative charge on the oxygen is transferred to eliminate the phenoxy moiety forming d4T-alaninyl methylester monophosphate (Scheme 2). Further hydrolysis by the enzyme yields d4T alanine monophosphate.The scheme also depicts the aminoacid in the protein chain involved in the process. In the case of these proteases, we believe both histidine and serine are involved. This mechanism is in agreement with the proposed hydrolysis of phosphonate esters, by serine proteases as disclosed by Skordalakes et al. [31]. Alternatively, there may be a simultaneous

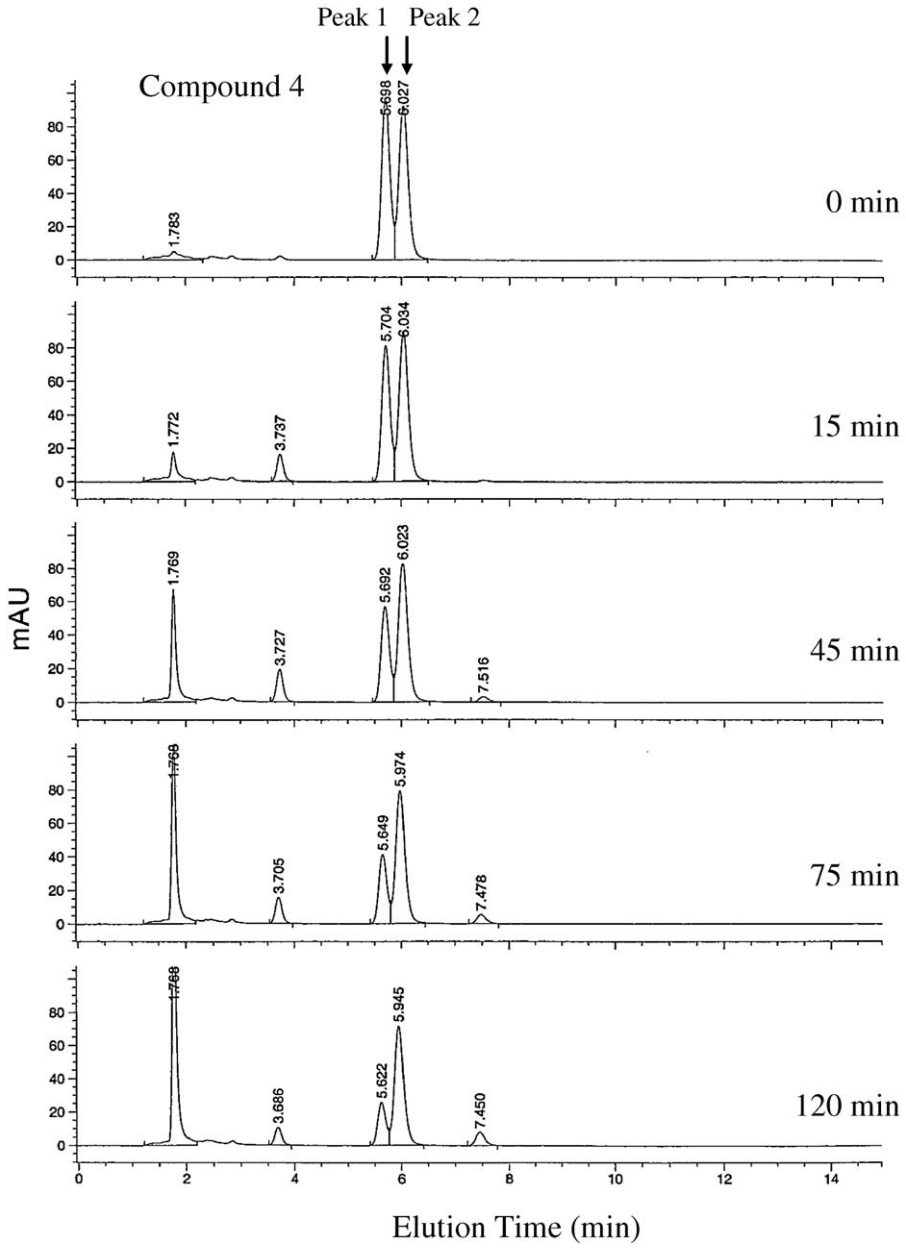


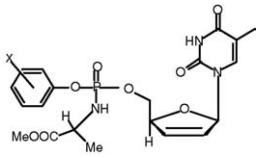
Fig. 4. HPLC profiles of the fluoro- substituted aryl phosphoramidate derivative compound #4 treated with enzyme Carcia Papaya for various time intervals.

attack of two centers in the molecule. Unlike those phosphonate esters reported in the literature [31], these phosphoramidate derivatives of stavudine posses another ester group in their structure. Therefore, we hypothesize that hydrolysis by serine protease enzymes similar to that reported in the previous discussion. However, in the first pathway, we propose the formation of d4T alaninyl methylester attached monophosphate as an intermediate step preceding the formation of the metabolite alanine-d4T-monophosphate.

As discussed above, we have shown that in protease-mediated hydrolysis the methyl ester side chain is converted into the carboxylic acid [B] (see Scheme 1). In the subsequent step the intermediate is converted into the active metabolite [E]. The conversion of [B] to [E] could occur either stepwise, spontaneously or enzymatically.

Evidence from the literature suggests that the conversion of [B] to [E] is likely to be spontaneous [32–36]. The cyclic phosphoramidate immediately hydrolyzes to the active metabolite. In order to confirm this hypothesis, we tried to synthesize the cyclic phosphoramidate following a previously reported method [37–40]. Although we could prepare the simple cyclic phosphoramidate derivatives derived from glycine, several attempts to isolate the stavudine substituted cyclic phosphoramidate failed under our experimental con-

Table 2
Rate constants for hydrolysis of phosphoramidate derivatives of stavudine using enzyme alanine racemase at room temperature



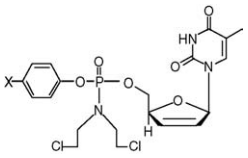
Compound#	X	Isomer 1[a]	Isomer 2[a]	SI (#1/#2)
1	4-OMe	0.070	0.67	0.10
2	3Br	0.02	0.85	0.02
3	2Cl-4Br	0.53	2.39	0.22
4	4-F	0.09	0.73	0.12
5	2-Br	0.17	1.52	0.11
6	2-Cl	0.10	1.25	0.08
7	H	0.01	0.65	0.02
8	4-Cl	0.23	1.02	0.23
9 = Stampidine	4-Br	0.21	1.09	0.20

[a] The rate constants for isomer (#1) and isomer (#2) in h^{-1}. SI indicates the selectivity index calculated as the ratio of the rate constants for #1 and #2.

ditions. 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.

We next set out to experimentally determine if an attack on the carboxymethylester side chain occurs during protease-mediated hydrolysis. To this end, we synthesized chloroethyl substituted stavudine phosphoramidate derivatives that lack the carbomethoxy ester group in their structure (Scheme 3). The rate of protease-mediated hydrolysis was examined for these compounds using Subtilisin Carlsberg. No hydrolysis was observed even after 140 min of incubation with this protease (Table 3). These results indicate that the hydrolysis of these compounds by the protease requires the ester group and does not involve an attack on the phosphorous chiral center alone. We propose that stampidine undergoes rapid enzymatic hydrolysis in the presence of protease according to the following putative pathway (Scheme 1). In the first step, compound B is generated by protease -mediated hydrolysis of the

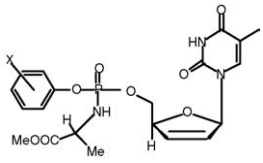
Table 3
Area under curve (HPLC) observed for the chloroethyl substituted aryl phosphoramidate derivatives of stavudine with protease Subtilisin Carlsberg at various time intervals



X	0 min		60 min		140 min		Comments
	#1	#2	#1	#2	#1	#2	
OMe	223	210	226	203	222	217	No hydrolysis
H	180	176	180	178	180	176	″
Br	176	186	168	174	184	186	″
Me	217	209	220	225	218	210	″

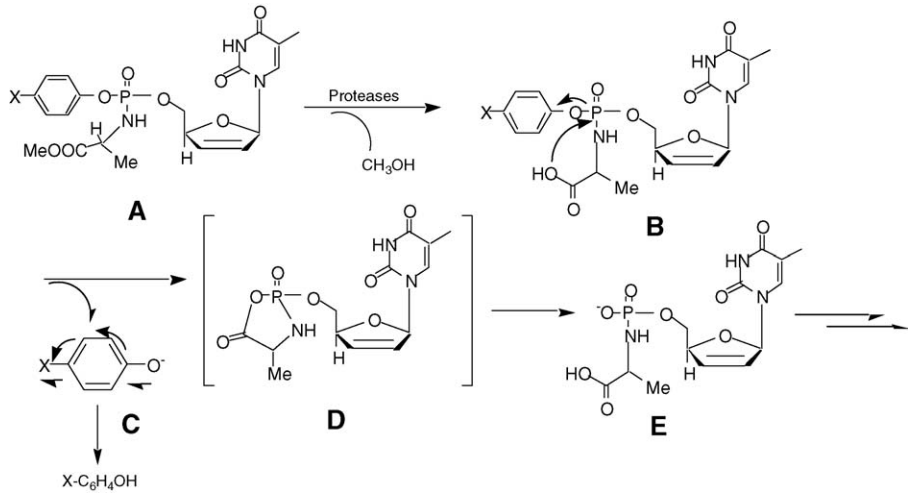
#1 and #2 indicates each of the isomers peak observed in HPLC chromatogram.

Table 4
Data on protease inhibition by protease inhibitors during hydrolysis of phosphoramidate derivatives of stavudine [a]



Compound#	X	Enzyme used	Protease inhibitor	Inhibition
9	4-Br	Subtilisin, Carlsberg	Antipain HCl	Yes
8	4-Cl	Subtilisin, Carlsberg	″	Yes
9	4-Br	Carcia Papaya	″	Yes
8	4-Cl	Carcia Papaya	″	Yes
9	4-Br	Carcia Papaya	Leupeptin	Yes
8	4-Cl	Carcia Papaya	″	Yes
9	4-Br	Carcia Papaya	EDTA free tabs	Yes
8	4-Cl	Carcia Papaya	″	Yes
2	3-Br	Carcua Papaya	″	Yes

[a] No hydrolysis of substrates observed even after several hours at room temperature in presence of protease inhibitors.



Scheme 1. Putative enzymatic hydrolysis pathway for phosphoramidate derivatives of stavudine in the presence of proteases.

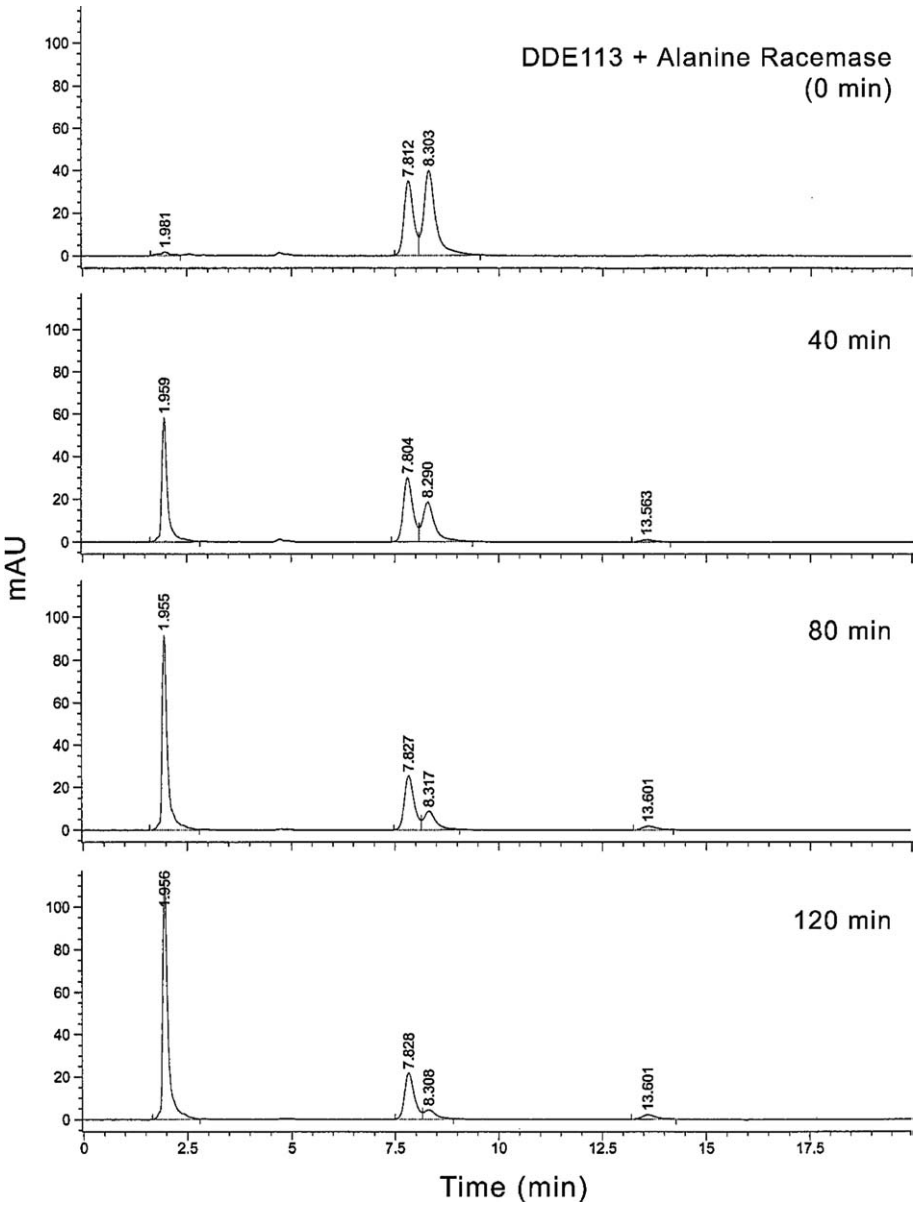
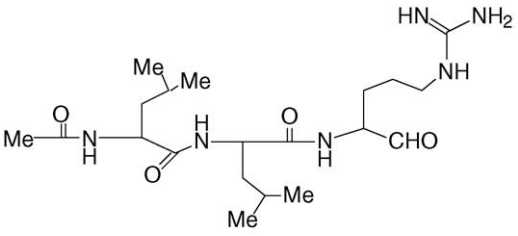
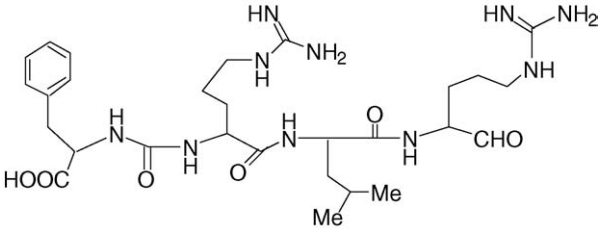


Fig. 5. HPLC profiles of bromo substituted aryl phosphoramidate derivative (9) treated with enzyme alanine racemase at various intervals of time.

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 protease 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 chloroethylsubstituted derivatives of stampidine which possess a chloroethyl linker unit instead of a methyl ester side chain were resistant to protease-mediated hydrolysis which excludes the possibility of a direct hydrolysis of stampidine at the phosphorous center (Table 3). Thus, our model indicates that the protease-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 stereoisomer corresponding to peak#1 in the HPLC chromatogram enters preferentially into the active site of the protease and undergoes rapid hydrolysis to form the cyclic intermediate. 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 order to establish that indeed these protease enzymes are involved and that these phosphoramdiate derivatives act as substrate competitors, we examined the specificity of the interaction using protease inhibitors to block the enzymatic hydrolysis.



Leupeptin

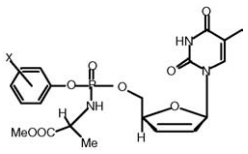


Antipain

Table 4 shows the enzymes used and the protease inhibitors selected for the said study. We chose only two compounds from the series to illustrate the role of protease inhibitors. Both 4-Cl and 4-Br substituted phosphoramidate derivatives of stavudine were found to undergo hydrolysis in the presence of serine proteases; however, introduction of antipain, leupeptin and EDTA-free protease inhibitor cocktail were found to completely block the protease-mediated hydrolysis of these stavudine phosphoramidates.

We were able to show experimentally that protease inhibitors block the hydrolysis of these phosphoramidate derivatives and formation of their active metabolite ala-d4T-MP.

Table 5
First order rate constants for disappearance of starting material using various protease enzymes with arylphosphoramidate derivatives of stavudine at room temperature



Compound#	X	Amino acid configuration	Carlsberg	Carcia Papaya	$HTLVIII_B$ (nM)
9	Br	'L'	9.10	1.86	1 ± 0
10	Br	'D'	0.23	0.01	212 ± 155
4	F	'L'	4.36	0.81	1 ± 0
11	F	'D'	0.20	0.01	851 ± 720
12	Me	'D'	0.04	0.01	427 ± 107
1	OMe	'L'	8.63	0.47	6 ± 3
13	OMe	'D'	0.02	0.01	1290 ± 313
7	H	'L'	7.71	0.85	2 ± 0.6
14	H	'D'	0.03	0.01	1787 ± 215
8	Cl	'L'	4.46	1.28	1 ± 0.3
15	Cl	'D'	0.15	0.02	5837 ± 2304

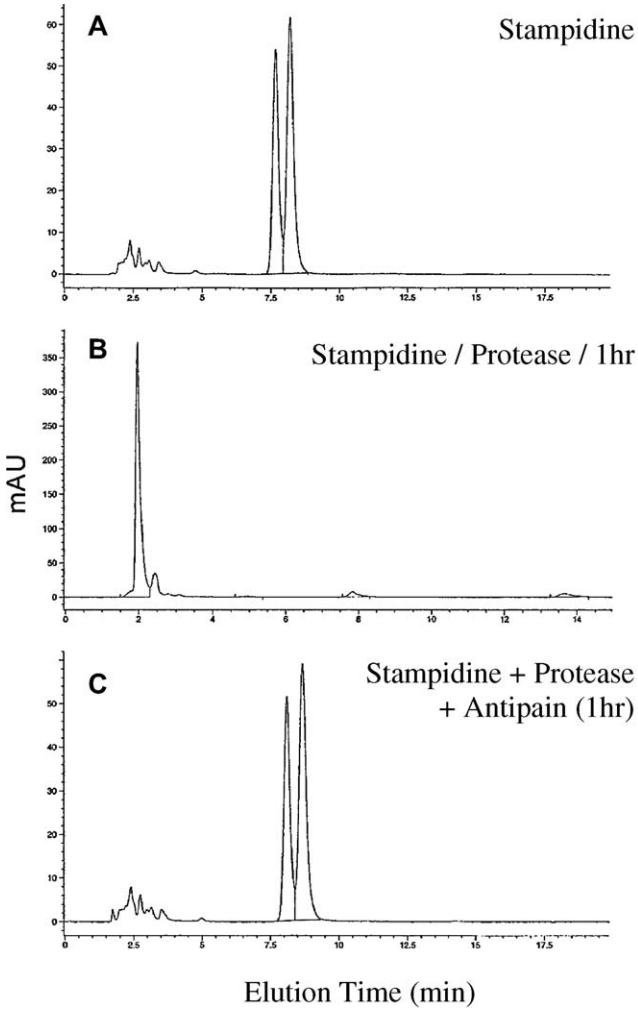
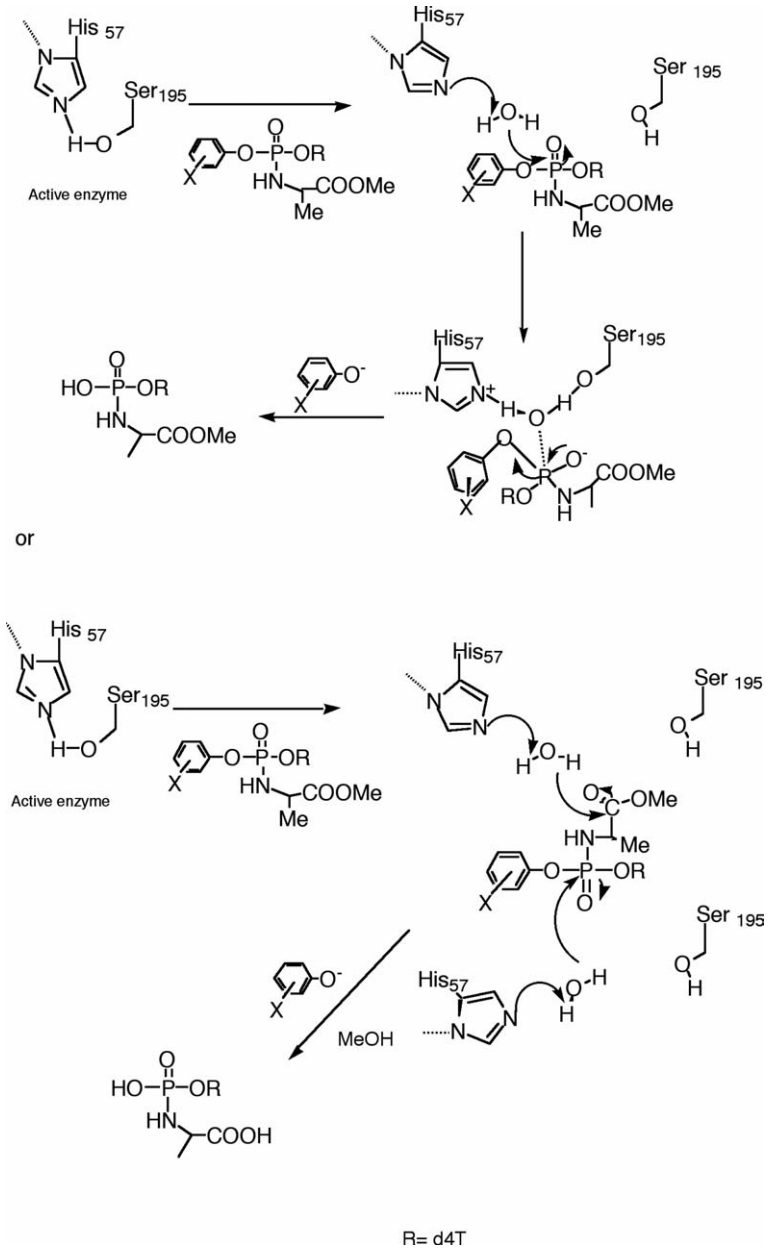


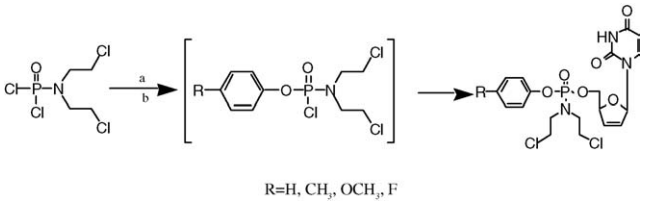
Fig. 6. HPLC profiles of bromo substituted hosphoramidate derivative (9) with Subtilisin Carlsberg in presence of protease inhibitor(antipain). A: Substrate without enzyme, B: substrate with Enzyme C: Substrate with enzyme and protease inhibitor.

Fig. 6 depicts the HPLC profile when a protease inhibitor was added to the reaction medium. Panel A shows the HPLC chromatogram of the 4-bromo-phenyl substituted phosphoramidate derivative of stavudine (=stampidine) before the addition of Subtilisin Carlsberg. Panel B represents the HPLC profile of stampidine 1 h after addition of Subtilisin Carlsberg. Panel C depicts the HPLC profile of the reaction mixture after addition of Subtilisin Carlsberg in the presence of the protease inhibitor antipain.

In an attempt to further confirm the methyl ester group of the L-alanine side chain of the stavudine phosphoramidates as the target site for the proteolytic hydrolysis, we replaced the L-alanine side chain with a D-alanine side chain. As evidenced in Table 5 and Fig. 7, these "D-isomers" (Fig. 8) were resistant to proteolytic hydrolysis by Subtilisin Carlsberg or Carcia Papaya. Furthermore, the D-isomers were substantially less active against HIV than the corresponding L-isomers, which is consistent with the notion that proteolytic hydrolysis at the methyl ester side chain of the sta-



Scheme 2. Proposed mechanism of enzyme catalysis during hydrolysis of phosphoramidate derivatives of stavudine.



Scheme 3. Synthetic scheme for chloroethyl substituted phosphoramidate derivatives of stavudine.

vudine phosphoramidates plays an important role for the activation of these anti-HIV prodrugs.

4. Conclusions

Aryl substituted phosphoramidate derivatives of stavudine were found to undergo hydrolysis in the presence of proteases. We hypothesize that the methoxy ester group on the side chain of these derivatives is first converted into the corresponding carboxylic acid derivative, which then undergoes intramolecular cyclization to form the final active metabo-

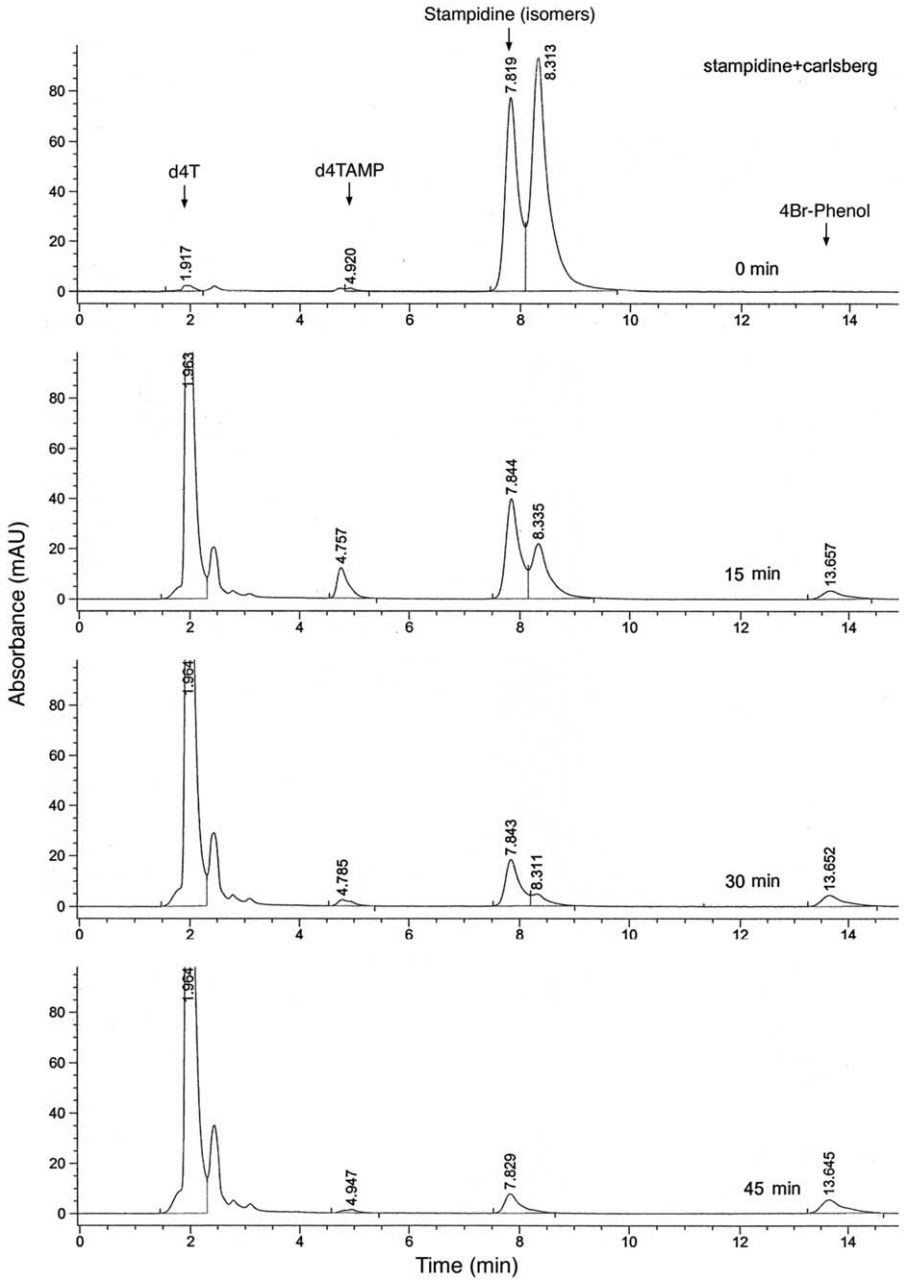


Fig. 7. Protease-mediated hydrolysis profile of compound **9** at various time intervals.

lite. A direct attack of the protease on the phosphorus site of these molecules was ruled out using phosphoramidate derivatives devoid of a carboxymethylester group in their structures. We were able to show experimentally that protease inhibitors block the hydrolysis of these phosphoramidate derivatives and formation of their active metabolite. The mechanism of protease-mediated hydrolysis of phosphoramidate derivatives of stavudine is in agreement with the proposed mechanism of hydrolysis of phosphonate esters. Our model indicates that the protease-mediated formation of a cyclic intermediate is a key step in metabolism of stampidine and relies on the initial configuration of the stereoisomers.

5.1. Experimental conditions for protease enzymatic study

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. Three milliliters of methanol was added to this vial and the contents were vortexed for 2 min until a homogenous solution was obtained. Hundred microliters of this solution was transferred into another scintallation vial and to this was added 950 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 millili-

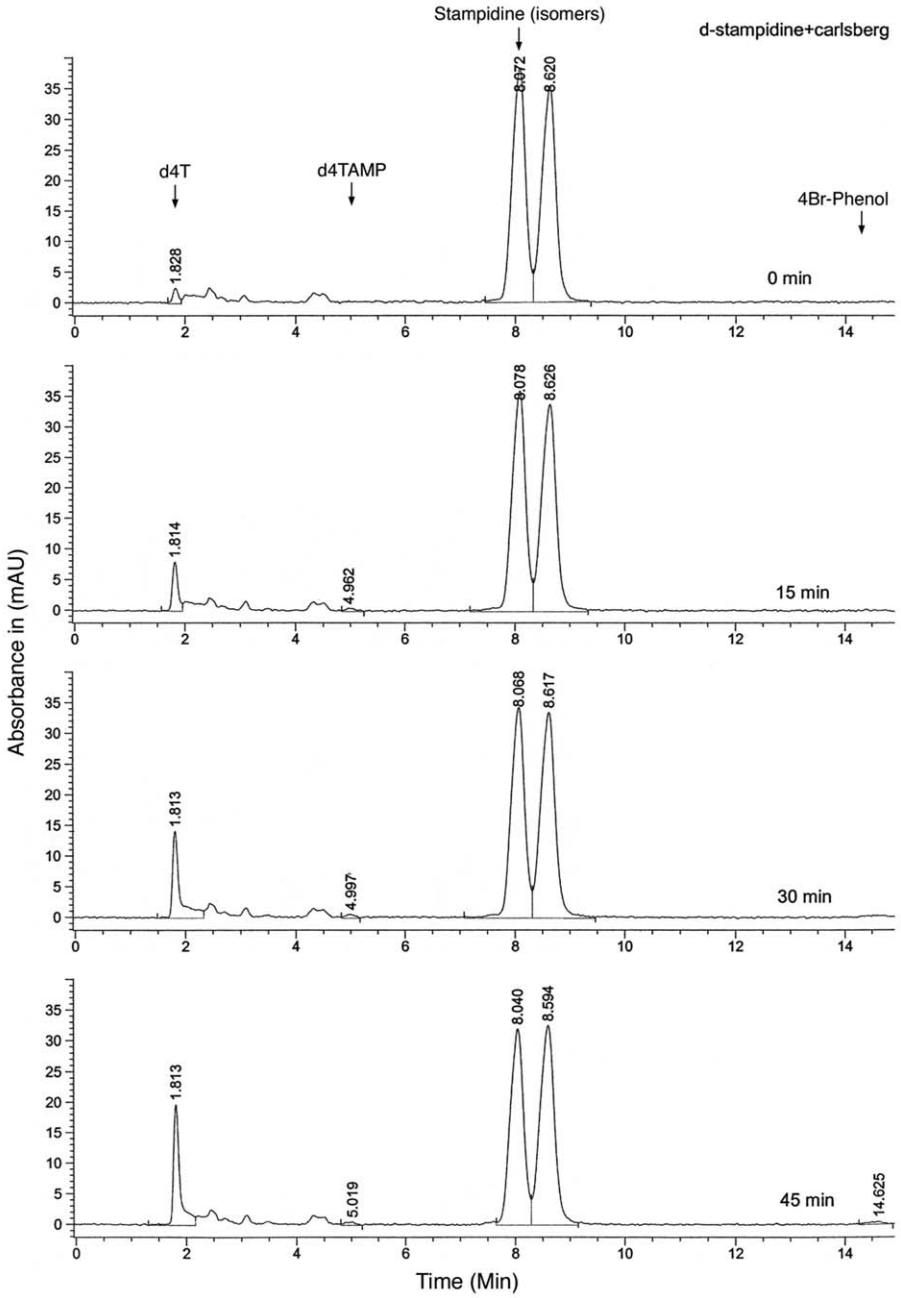


Fig. 8. Protease-mediated hydrolysis profile of compound **10** at various time intervals.

ters 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, 500 μl was pipetted out into another glass vial and to this 500 μl of diluted solution of the enzyme was added and the contents were shaken to form a homogenous solution. From this reaction mixture 50 μl per time point was used for HPLC analysis using a Lichrospher (RP) analytical column of (4 × 250 mm). The eluent used for the HPLC was water/TFA/TEA (0.1%) and CH_3CN with a 65:35 ratio. 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.

5.2. Statistical analysis

Hydrolysis rates were determined by fitting single exponential decay equations to the disappearance of each isomer substrate in the presence of enzyme. 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 per hour since some of the reactions were too slow to obtain meaningful results. Ham-

mett 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 0.05 were considered significant.

5.3. Estimation of products

The amount of products observed during the reaction of these phosphoramidates were 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. Further confirmation of the product structures were obtained using a 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 per hour as some of the reactions were too slow to obtain meaningful results.

5.4. In vitro assays of anti-HIV activity

Normal human peripheral blood mononuclear cells (PBMNC) from HIV-negative donors were cultured 72 h in RPMI 1640 supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS), 3% interleukin-2, 2 mM L-glutamine, 25 mM HEPES, 2 g l^{-1} $NaHCO_3$, 50 mg ml^{-1} gentamicin, and 4 mg ml^{-1} phytohemagglutinin prior to exposure to HIV-1 at a multiplicity of infection (MOI) of 0.1 during a 1 h adsorption period at 37 °C in a humidified 5% CO_2 atmosphere. Subsequently, cells were cultured in 96-well microtiter plates (100 ml per well; 2×10^6 cells per ml) in the presence of various concentrations of d4T phosphoramidates and aliquots of culture supernatants were removed from the wells on the seventh day after infection for p24 antigen assays, as previously described. The applied p24 enzyme immunoassay (EIA) was unmodified kinetic assay commercially available from Coulter Corporation/Immunotech. Inc. (Westbrooke, ME), which utilizes a murine mAb to HIV core protein coated on to microwell strips to which the antigen present in the test culture supernatant samples binds. Percent viral inhibition was calculated by comparing the p24 values from untreated infected cells (i.e. virus controls).

References

[1] M.M. Mansuri, J.E. Starrett Jr., I. Ghazzouli, M.J. M. Hitchcock, R.Z. Sterzycki, V. Brankovan, T.S. Lin, E.M. August, W.H. Prussoff, J.P. Sommadossi, J.C. Martin, J. Med. Chem. 32 (1989) 462.

[2] J.P. Horwitz, J. Chua, M.A. DaRooge, M. Noel, I.L. Klindt, J. Org. Chem. 31 (1996) 205.

[3] F.W. Bell, A.S. Cantrell, M. Hogberg, S.R. Jaskunas, N.G. Johansson, C.L. Jordan, et al., J. Med. Chem. 38 (1995) 4929.

[4] A.S. Cantrell, P. Engelhardt, M. Hogberg, S.R. Jaskunas, N.g. Johansson, C.L. Jordan, J. Kangasmetsa, M.D. Kinnick, P. Lind, J.M. Morin, M.A. Muesing, R. Noreen, B. Oberg, P. Pranc, C. Sahlberg, R.J. Ternansky, R.T. Vasileff, L. Vrang, S.J. West, H. Zhang, J. Med. Chem. 39 (1996) 4261.

[5] R. Vig, C. Mao, T.K. Venkatachalam, L. Tuel-Ahlgren, E.A. Sudbeck, F.M. Uckun, Bioorg. Med. Chem. 8 (1998) 1789.

[6] C. Mao, R. Vig, T.K. Venkatachalam, E.A. Sudbeck, F.M. Uckun, Bioorg. Med. Chem. Lett. 8 (1998) 2213.

[7] P.A. Furman, J.A. Fyfe, M.H. St. Clair, K. Weinhold, J.L. Rideout, G.A. Freeman, et al., Proc. Natl. Acad. Sci. USA 83 (1986) 8333.

[8] Z. Hao, D.A. Cooney, N.R. Hartman, C.F. Perno, A. Fridland, A.L. De Vico, et al., Mol. Pharmacol. 34 (1988) 431.

[9] J. Balzarini, D.A. Cooney, M. Dalal, G.I. Kang, J.E. Cupp, E. De Clercq, et al., Mol. Pharmacol. 32 (1987) 798.

[10] C. McGuigan, D. Cahard, H.M. Sheeka, E. De Clercq, J. Balzarini, J. Med. Chem. 39 (1996) 1748.

[11] C. McGuigan, D. Cahard, H.M. Sheeka, E. De Clercq, J. Balzarini, Bioorg. Med. Chem. Lett. 6 (1996) 1183.

[12] M.G. Starnes, Y.C. Gheng, J. Biol. Chem. 32 (1987) 798.

[13] Z. Hao, D.A. Cooney, D. Farquhar, C.F. Perno, K. Zhang, R. Masood, et al., Mol. Pharmacol. 37 (1990) 157.

[14] M.A. Johnson, G. Ahluwalia, M.C. Connelly, D.A. Cooney, S. Broder, D.G. Johns, et al., J. Biol. Chem. 263 (1988) 15354.

[15] M.A. Johnson, A. Fridlan, Mol. Pharmacol. 36 (1989) 291.

[16] C. McGuigan, R.N. Pathirana, N. Mahmood, K.G. Devine, A.J. Hay, Antiviral Res. 17 (1992) 311.

[17] C. McGuigan, R.N. Pathirana, J. Balzarini, E. De Clercq, J. Med. Chem. 36 (1993) 1048.

[18] R. Vig, T.K. Venkatachalam, F.M. Uckun, Antiviral Chem. Chemother. 9 (1998) 445.

[19] T.K. Venkatachalam, T.L. Tai, R. Vig, C.L. Chen, S.T. Jan, F.M. Uckun, Bioorg. Med. Chem. Lett. 8 (1998) 3121.

[20] F.M. Uckun, S. Pendergrass, T.K. Venkatachalam, S. Qazi, D. Richman, Abtimicrob. Agents Chemother. 46 (2002) 3613.

[21] F.M. Uckun, S. Qazi, S. Pendergrass, E. Lisowaski, B. Waurzyniak, C.L. Chen, et al., Antimicrob. Agents Chemother. 46 (2002) 3428.

[22] F.M. Uckun, C.L. Chen, P. Samuel, S. Pendergrass, T.K. Venkatachalam, B. Waurzyniak, et al., Antimicrob. Agents Chemother. 47 (2003) 1233.

[23] F.M. Uckun, R. Vig, US patent # 6, 030, 957, 2000 Feb.

[24] F.M. Uckun, C.L. Chen, E. Lisowski, G. Mitcheltree, T.K. Venkatachalam, D. Eerbeck, et al., Arzneim-Forsch 53 (2003) 357.

[25] F.M. Uckun, P. Samuel, S. Qazi, C.L. Chen, S. Pendergrass, T.K. Venkatachalam, Antiviral Chem. Chemother. 13 (2002) 73.

[26] A.Q. Siddiqui, C. McGuican, C. Ballatore, O. Wedgwood, E. De Clercq, J. Balzarini, Bioorg. Med. Chem. Lett. 9 (1999) 2555.

[27] A.Q. Siddiqui, C. Ballatore, C. McGuigan, E. De Clercq, J. Balzarini, J. Med. Chem. 42 (1999) 393.

[28] C.R. Wagner, E.J. McIntee, R.F. Schinazi, T.W. Abraham, Bioorg. Med. Chem. Lett. 5 (1995) 1819.

[29] T.K. Venkatachalam, P. Samuel, G. Li, S. Qazi, C. Mao, S. Pendergrass, et al., Bioorg. Med. Chem. 12 (2004) 3371.

[30] R.J. Kubiak, X. Yue, R.J. Hondal, C. Mihai, M.D. Tsai, K.S. Bruzik, Biochemistry 40 (2001) 5422.

[31] E. Skordalakes, G.G. Dodson, D.S. Green, C.A. Goodwin, M.F. Scully, H.R. Hudson, et al., J. Mol. Biol. 311 (2001) 549.

[32] E.J. McIntee, R.P. Remmel, R.F. Schinazi, T.W. Abraham, C.R. Wagner, J. Med. Chem. 40 (1997) 3323.

[33] J. Balzarini, A. Karlsson, S. Aquaro, C.F. Perno, D. Cahard, L. Naesens, et al., Proc. Natl. Acad. Sci. USA 93 (1996) 7295.

[34] J. Balzarini, H. Egberink, K. Hartman, D. Cahard, T. Vahlenkamp, H. Thormar, et al., Mol. Pharmacol. 50 (1996) 1207.

[35] T.W. Abraham, C.R. Wagner, Nucleosides Nucleotides 13 (1994) 1891.

[36] G. Vallette, A. Pompon, J.L. Girardet, L. Cappellacci, P. Franchetti, M. Grifantini, et al., J. Med. Chem. 39 (1996) 1981.

[37] I. Devedjiev, K. Petrova, I. Glavchev, Syn. Commun. 30 (2000) 4411.

[38] I. Devedjiev, G. Borisov, Phosphor. Sulfur Silicon Relat. Elem. 53 (1990) 37.

[39] S.B. Tzokov, I. Devedjiev, D. Petkov, J. Org. Chem. 61 (1996) 12.

[40] S.B. Tzolov, I. Devedjiev, E. Bratovanova, D. Petkov, Angew. Chem. Int. Ed. Engl. 33 (1994) 2302.