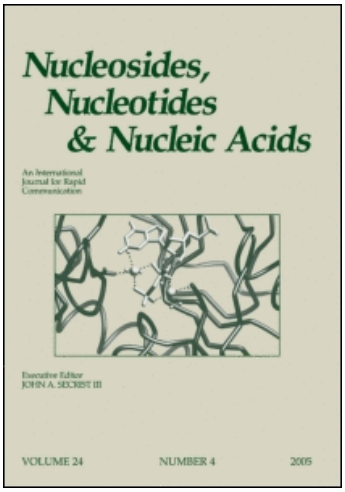

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3'-AZIDO-3'-DEOXY-5'-O-ISONICOTINOYLTHYMIDINE, A NEW PRODRUG OF ZIDOVUDINE. SYNTHESIS, SOLID STATE CHARACTERIZATION AND ANTI HIV-1 ACTIVITY

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ABSTRACT

Synthesis, solid state characterization and anti HIV-1 activity of 3′-azido-3′-deoxy-5′-O-isonicotinoylthymidine (**2**), a new prodrug of zidovudine (AZT, **1**), are described. Two solid forms of **2** prepared by crystallization from ethyl acetate-petroleum ether (*form* α) and from a melt sample of *form* α (*amorphous form*) were characterized by X-ray diffractometry, infrared spectroscopy, differential scanning calorimetry (DSC) and thermogravimetry (TGA) techniques. The novel nucleoside exhibited antiviral activity against standard and resistant strain panels of HIV-1 as well as cytotoxicity similar to that of AZT.

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The discovery 3′-azido-3′-deoxythymidine (Zidovudine, AZT, **1**) a thymidine analog which inhibits replication of human immunodeficiency virus type 1 (HIV-1)[1,2], stimulated considerable interest in the field of this research on pyrimidinic nucleoside derivatives. In view of the serious AZT toxic side effects[3] and resistance[4,5], several investigators have converted this nucleoside analog into a variety of more lipophilic derivatives, in order to circumvent these drawbacks and to enhance its antiviral activity and brain penetration[6–9]. Thus, we have been involved a research program aimed at exploring more convenient novel anti HIV-1 analogs of AZT[10–13]. As part of this program, we here report an efficient short synthesis of 3′-azido-3′-deoxy-5′-O-isonicotinoylthymidine (**2**) a new prodrug of **1**, in order to increase its lipophilicity attaching a heteroaromatic moiety to the 5′-hydroxy function as well as its anti HIV-1 activity and cytotoxicity. This paper also describes the solid state characterization of the title compound because these assays should be undertaken at the earliest phase of a drug molecule development, since many substances have been found to occur in more than one crystalline form, hence leading to significant differences in their physical and thermodynamic properties which affect bioavailability[14–21].

RESULTS AND DISCUSSION

Chemistry

The title compound 3′-azido-3′-deoxy-5′-O-isonicotinoylthymidine (**2**, Fig. 1), was synthesized in a single step at 73% yield by condensation of AZT with isonicotinoyl chloride hydrochloride (**3**) in pyridine and a molar ratio AZT:**3** of 1:1.5 at 70°C during 15 minutes. The progress of the reaction was screened by TLC and no starting materials were detected. When the reaction

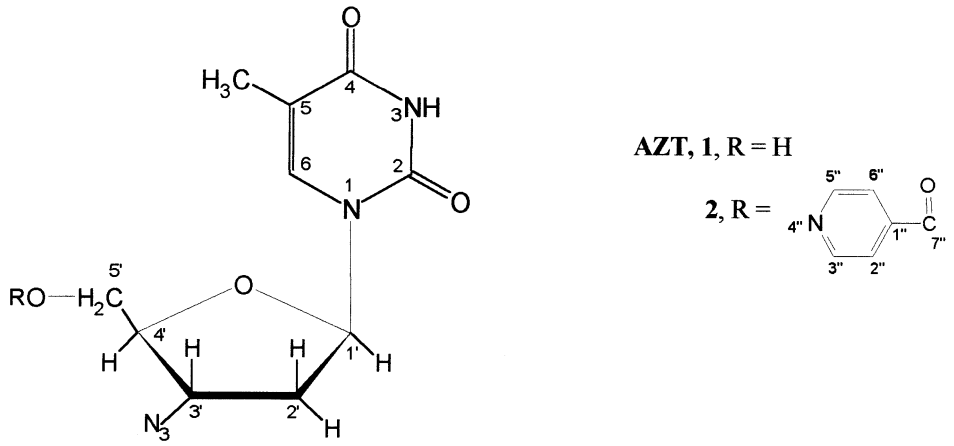

Figure 1. Structure and numeration of 3′-azido-3′-deoxy-5′-O-isonicotinoylthymidine, **2**.

was performed at 0°C for 14 h, **2** was obtained at lower yields detecting **1** in the reaction mixture. Pyridine was used to catalyze the nucleophilic substitution reaction, due to its combination with the hydracide formed during an early step, favoring the desirable product formation[22].

Compound **2** was isolated by flash chromatography, then recrystallized from ethyl acetate-petroleum ether, and characterized by ^{1}H-NMR, ^{13}C-NMR, IR and MS spectroscopic analyses.

In the ^{1}H-NMR spectrum of **2**, the most distinct signals characterizing its structure correspond to pyrimidinic ring. The doublets at δ 8.80 and δ 7.82 which integrate for two protons each, correspond to the downfield chemical shift H-3″ and H-5″, while the upfield one corresponds to H-2″ and H-6″ protons. The signals corresponding to the methyl and H-6 protons appear as singlets at δ 1.58 and δ 7.36, respectively. The chemical shifts of the remaining protons H-1′ (δ 6.11), H-2′$_a$ (δ 2.47), H-2′$_b$ (δ 2.36), H-3′ and H-5′ (δ 4.5–4.7), H-4′ (δ 4.0–4.1), and NH (δ 11.3) were similar to those of AZT. The triplet at δ 5.2 corresponding to OH in **1** is not detectable in the spectrum of **2** because of the ester attachment in position-5′. The addition of D_2O showed that the only exchangeable signal was at δ 11.3, corresponding to one NH imide proton, confirming that no hydroxyl groups were found in the molecule.

The most significant features in the ^{13}C-NMR spectrum were the signals at δ 120.6 (C2″ and C6″), δ 134.5 (C1″) and δ 148.8 (C3″ and C5″) corresponding to the pyridinic carbons as well as the acylic carbon attached to the ring at δ 162.5 (COO^-).

The most characteristic signals in the IR spectrum of **2** were the intense absorption at 3414.3, 2116.6, 1708.3 and 1290.6 cm^{-1} corresponding to NH, azido (N_3), C=O and C-N (N-Ar) group tensions, respectively. It is important to point out that the signal at 3474 cm^{-1} corresponding to OH group in the AZT was not found in **2**.

The ionization mass spectrum of **2** showed the characteristic molecular ion M^+ at m/z 372, as well as M^++1 (m/z 373) M^++2 (m/z 374) ions.

Different Solid Forms

When **2** was crystallized from ethyl acetate-petroleum ether, a solid called *form* α was obtained. The use of different amounts of these solvents did not change the internal structure of the crystalline form. Although this crystalline form displays TLC chromatographic purity, it does not exhibit a definite melting point by the capillary method. If crystalline *form* α is desolvated by heating at 100°C, an *amorphous solid* is obtained. Both thecrystalline and the non-crystalline forms of **2** were characterized by X-raypowder diffraction, thermal analysis, IR spectroscopy and ^{1}H-NMR[14–27].

X-ray Powder Diffractometry

X-ray powder diffractometry analysis allows for an accurate identification between crystalline and amorphous solid states since each diffraction pattern is characteristic for a crystalline lattice and any changes in the inner make up of the crystal lead to a different model[18,21,24,25]. X-ray powder diffraction patterns (XRD) of α and *amorphous forms* of **2** are shown in Fig. 2.

As it can be seen, the XRD profiles were sufficiently distinct to characterize the crystalline and the amorphous forms, whose differences in the corresponding profiles are attributed to alterations in the crystal lattice molecular arrangements. Thus, the XRD of the amorphous solid, is easily identified because as the molecular chaos increases, the fine and sharp peak profile disappears yielding a broad band, since there is no molecular order associated with the amorphous state as in the crystalline *form* α[25].

Thermal Analysis

The TGA, DTGA and DSC analyses of the *form* α and the amorphous state of **2** were carried out against AZT and foscarnet (hexahydrate and anhydrous forms) as reference compounds, which have proved that the employed methodology is adequate to detect nucleosides and hydrate transformations, respectively. Since it was observed that the thermal obtained curves were the same for both nitrogen and air atmospheres, we have only considered the nitrogen ones here.

The TGA, DTGA and DSC curves of *form* α of **2** are shown in Fig. 3 at a heating rate of 10°C/min.

DSC curve of *form* α shows an endothermic peak at 78.6°C attributed to a desolvation process, which is in accordance with the loss of mass in TGA curve (1.85%) at 55–83°C. This feature indicates that ethyl acetate could be occluded in *form* α in a ratio of one molecule of **2** per 0.08 molecules of ethyl acetate, showing a constant non-stoichiometric molar ratio corresponding to inclusion compounds[20,21]. TGA results were an average of four determinations obtained from different batches. DSC curve displays an exotherm attributed to a decomposition process accompanied by a conspicuous mass loss in TGA curve.

Thermal analysis of the *amorphous form* (Fig. 4) was performed by heating *form* α inside the device up to 100°C to achieve desolvation of the sample and its amorphous transformation. The temperature was slowly reduced from 100°C and the amorphous solid was reheated[25].

As it can be seen, the endotherm profiles are not present in TGA-DSC curves, confirming the desolvation process in *form* α. Amorphous state fusion is not evidenced by DSC because it does not require heat to turn from solid

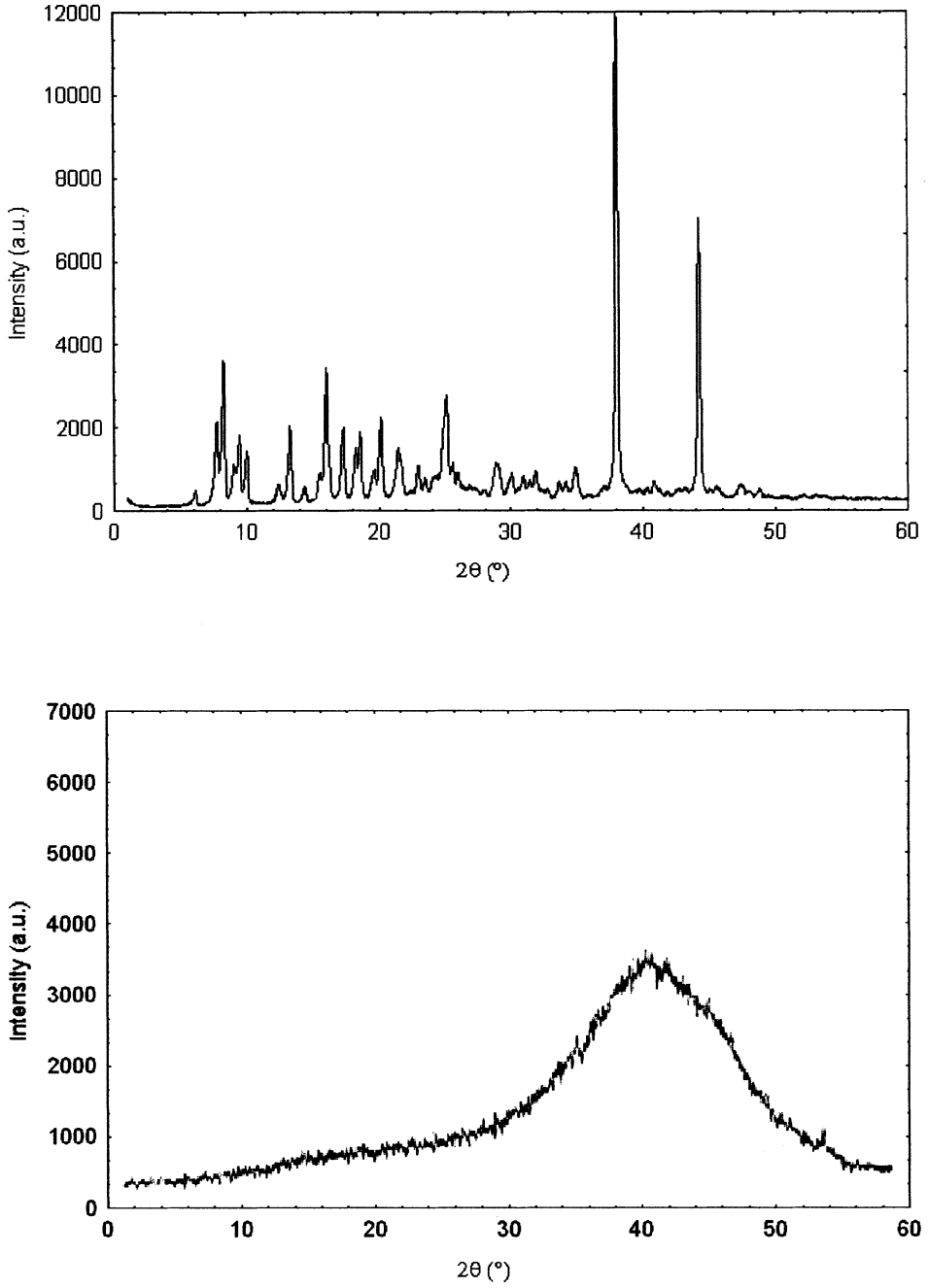

Figure 2. X-ray powder diffraction patterns of **2**. a) *Form* α, b) *Anhydrous form*.

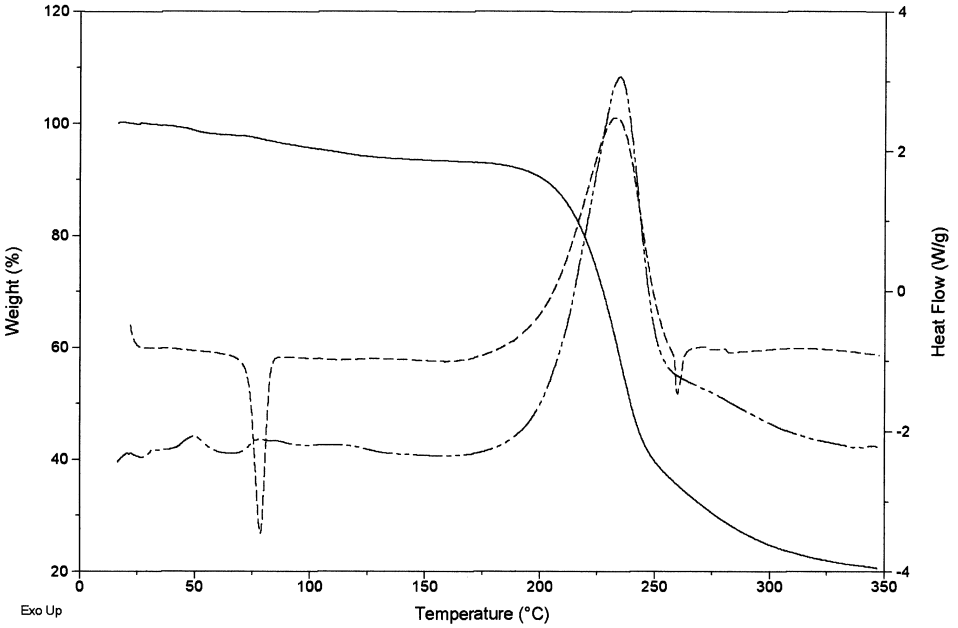

Figure 3. TGA, DTGA and DSC simultaneous curves of *form* α of **2** at 10°C/min. TGA: solid (—); DTGA, broken double dash (— - - —), DSC: short dash (----).

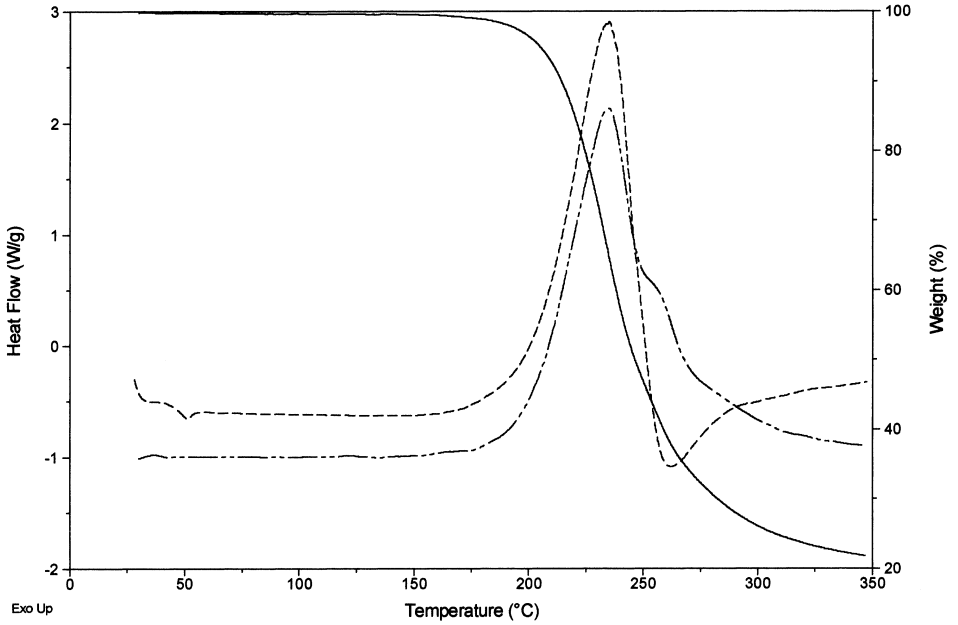

Figure 4. TGA, DTGA and DSC simultaneous curves of *anhydrous form* of **2** at 10°C. TGA: solid (—); DTGA, broken double dash (— - - —), DSC: short dash (----).

into liquid[25]. An amorphous state has more energy than a crystalline one and may consequently undergo a more favorable energetic change. Thus, **2** could occlude ethyl acetate crystallization solvent developing a low energy crystalline compound.

Infrared Spectroscopy

The IR spectra of the two solid forms of **2** are shown in Fig. 5a,b. Noticeable differences can be seen between inclusion compound (Fig. 5a) and *amorphous form* (Fig. 5b), showing recognizable differences in the fine structure and intensities of certain major absorption bands. The method differentiated the solvate from the amorphous solids of **2**, because of the addition of new stretching frequencies resulting from solvation[18–20,26]. The crystalline form exhibits sharp peaks, while the amorphous ones are round. Both azide ($\cong$2100 cm^{-1}) and carbonyl groups display unfolded signals in the crystalline form, whereas a wide band can be seen in the amorphous state.

H^1-NMR

H^1-NMR spectrometry is remarkably effective for occluded solvent detection in molecular adducts[27]. In this way, through H^1-NMR spectrum of

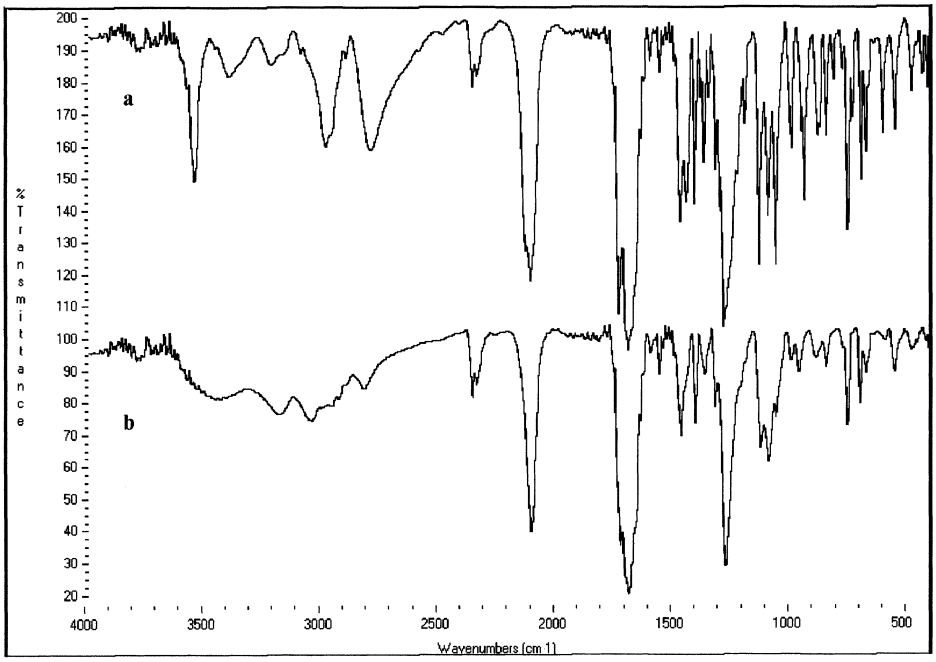

Figure 5. Infrared spectra of **2**. a) *Form* α, b) *Anhydrous form*.

form α, it was possible to detect occluded ethyl acetate, as well as the absence of hexane (arising from petroleum ether). This spectrum shows a triplet (δ 1.12), a singlet (δ 1.93) and a quadruplet (δ 3.98), which confirm the presence of this solvent.

Anti-HIV-1 Activities and Cytotoxicities

The ability of **2** to inhibit p24 antigen synthesis and to reverse transcriptase activity of HIV-1 IIIB in MT4 cell line and Cord Blood Mononuclear Cells (CBMC) was compared with the inhibitory effects of AZT. The novel nucleoside exhibited antiviral activity similar to that of AZT. Nevertheless, this antiviral activity proved to be more significant in MT4 cell line. Moreover, the analysis of cytotoxicity was performed assessing the $CCID_{50}$ for MT-4 and CBMC, and the results obtained showed no significant differences between AZT and **2** (Table 1).

When **2** was tested against the AZT intermediate resistant strains panel obtained from NIH[5,28,29], determinations of IC_{50} were not significantly different from AZT (Table 2).

In our previous work on lipophilic character of pyrimidinic nucleoside derivatives[12], we pointed out that **2** (log P 0.82) was 16.4 times more lipophilic than AZT (log P 0.05). Because of its increased lipophilicity, it might also achieve a higher intracellular concentration in T-lymphocytes than AZT; however, as seen from Tables 1 and 2, in vitro antiviral activity and the $CCID_{50}$ values of **2** were similar to those of AZT.

Stability Assays

The stability assays in water and in isotonic plasma buffer phosphate solution (pH 7.4) during twenty days at 37 °C demonstrated that **2** remained unmodified during all assayed period.

Table 1. Determination of 50% Inhibitory Concentration (IC_{50}) in HIV-1 IIIB and Evaluation of 50% Cell Culture Inhibitory Dose ($CCID_{50}$). Acutely Infected Cells in the Presence of 3′-Azido-3′-deoxythymidine (AZT, **1**) and 3′-Azido-3′-deoxy-5′-O-isonicotinoylthymidine (AZT-Iso, **2**)

	IC_{50} (μM)		$CCID_{50}$ (μM)	
	1	2	1	2
MT-4 cell line	0.007	0.007	80	50
Cord blood mononuclear cells	0.002	0.003	60	105

Table 2. Determination of 50% Inhibitory Concentration (IC_{50}) in Cord Blood Mononuclear Cells with Different 3′-Azido-3′-deoxythymidine **(1)** Resistant Strains Panel of HIV-1

	IC_{50} (μM)			
	AZT Intermediate Resistant Strains Panel from NIH[a]			
Compounds	1073	1074	1075	1082
1	0.02	0.01	0.65	1.3
2	0.03	0.02	0.55	2.0

1; 3′-azido-3′-deoxythymidine (AZT).
2; 3′-Azido-3′-deoxy-5′-O-isonicotinoylthymidine. a; Refs. 5,28,29.

Conclusions

3′-Azido-3′-deoxy-5′-O-isonicotinoylthymidine **(2)**, a new prodrug of AZT was synthesized and characterized. Two different solid forms of **2** (*form* α *and amorphous form*) were prepared. *Form* α (from acetyl acetate-petroleum ether) showed a **2**:ethyl acetate ratio of 1:0.08, whereas the *amorphous form* was obtained from desolvation of *form* α at 100°C. These different solid forms could be characterized by thermal, powder X-ray diffractometric and infrared spectroscopic analyses.

Taking into account probable toxic effects of the occluded ethyl acetate solvent, the amorphous solid state could be the desirable form for pharmaceutical formulations. In addition, the amorphous forms can be generally absorbed in gastrointestinal tract more easily than crystalline ones due to probable solubility differences, since higher energy state in the amorphous form should enhance dissolution properties[25].

EXPERIMENTAL SECTION

Materials

The nucleoside 3′-azido-3′-deoxythymidine (AZT, **1**), and foscarnet (trisodium phosphonoformate; hexahydrate and anhydrous forms) generously supplied by Filaxis (Buenos Aires, Argentina), was used without purification. All chemicals and reagents were of analytical grade. Dimethylformamide (DMF, Sintorgan) was dried with 4 Å molecular sieves and all solvents were purified by distillation prior to use. Standard buffer solutions were purchased from Carlo Erba (Argentina). Silica gel type H, size 10–40 μm, without binder (SIGMA), was used for flash chromatography and precoated 60 F 254 silicagel plates (Merk) for thin layer chromatography (TLC).

Apparatus. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AC 200 spectrometer at 200.13 MHz, using DMSO-d_6 (99%, SIGMA) as solvent and tetramethylsilane (TMS) as internal standard. The assignment of all exchangeable protons (OH, NH) was confirmed by the addition D_2O. IR spectra were obtained from potassium bromide discs on a Nicolet 5 SXC FT-IR. The mass spectra were recorded on a Finnigan Model 3300 F-100 Quadrupole Mass Spectrometer and on a High Resolution (HRMS) ZAB-SEQ4F Mass Spectrometer (UMYMFOR Laboratories, Buenos Aires, Argentina). Ultraviolet spectrophotometric (UV) studies were carried out with a Shimadzu Model UV-160A spectrophotometer, using 1 cm quartz cuvettes. An Orion Model 5A 520 pHmeter was used for measuring the pH of the buffer solutions with a glass-reference electrode.

Powder x-ray diffraction (XRD). The XRD patterns of α and *amorphous forms* were determined with a Rigaku Miniflex desktop X-ray powder diffractometer using Cu Kα radiation. The powder sample was mounted in a flat plate sample holder and the diffraction pattern was recorded with the continuous scan method in the $\theta/2\theta$ mode from 1 up to 60 degrees in 2θ; scan speed 1°/min and sampling width 0.02°. The instrument was set as follows: voltage 30 kV; current, 15 mA; divergence slit: variable slit interlocked with θ axis, maximum 4.2°; receiving slit: 0.3 mm, scattering slit: 4.2°.

Thermal analysis. Melting points were assessed on an Electrothermal 9100 Melting point apparatus and were uncorrected. Thermoanalytical (TGA-DTGA-DSC) measurements were performed with a DSC2920 Modulated DSC and H1-Res Modulated TGA 2950 Thermogravimetric Analyzer (TA instruments) in nitrogen and air atmospheres at a heating rate of 10°C/min.

Synthesis of 3′-azido-3′-deoxy-5′-O-isonicotinoylthymidine (2). To a magnetic stirred solution of 0.3000 g (1.12 mM) of **1** in 3 mL of pyridine at 70°C, 0.3774 g (1.91 mM) of isonicotinoyl chloride hydrochloride was added. The progress of the reaction was monitored by TLC using ethyl acetate-petroleum ether 60–80 (8:2) as solvent system. After 15 min, 25 mL of cool ethyl ether was added and the resulting precipitate, constituted by isonicotinic acid, **2** and pyridine was filtered off under reduced pressure and then redissolved in 20 mL of chloroform. The organic layer was washed with five aliquots of 10 mL each of an aqueous solution of sodium bicarbonate and then dried over Na_2SO_4. Chloroform was then removed *in vacuum* to yield a solid which was exposed to flash chromatography, using a gradient of ethyl acetate-petroleum ether 60–80 as solvent system. After recrystallization from ethyl acetate-petroleum ether (60–80), **2** yielded

0.3041 g (72.96%), M.S.H.R. m/z $M^+ = 372.1179$ (expected: M^+=372.1182), UV (Water): λ_{max} 209.8 nm, 267.5 nm ($\log \varepsilon = 4.00$); (Isopropanol): λ_{max} 265.2 nm ($\log \varepsilon = 4.04$). ^{1}H-NMR (DMSO-d_6); δ: 1.58 (s, 3H, CH_3), 2.36 (m, 1H, H-2′$_b$), 2.47 (m, 1H, H-2′$_a$), 4.0–4.1 (m, 1H, H-4′), 4.5–4.7 (m, 3H, H-3′ and H-5′), 6.11 (t, 1H, H-1′), 7.36 (s, 1H, H-6), 7.82 (d, 2H, H-2″ and H-6″), 8.80 (d, 2H, H-3″ and H-5″), 11.3 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6); δ: 9.9 (CH_3), 33.7 (C2′), 57.8 (C3′), 62.3 (C5′), 78.4 (C4′), 81.7 (C1′), 108.0 (C5), 120.6 (C2″ and C6″), 133.9 (C6), 134.5 (C1′), 148.4 (C2), 148.8 (C3″ and C5″), 161.6 (C4), 162.5 (COO^-). M.S. m/z (%): 374 (M^++2, 2.78), 373 (M^++1, 16.03), 372 (M^+, 5.02), 247 (34.19), 204 (9.29), 127 (8.44), 126 (14.10), 125 (1.12), 106 (82.91), 98 (2.54), 82 (10.10), 81 (100.00), 78 (53.63), 68 (5.13), 55 (10.36), 54 (11.86). IR (KBr) ν_{max} cm^{-1}: 3561.1 and 3414.3 (NH), 2116.6 (N_3), 1708.3 (C=O and NH), 1290.6 (N-Ar).

Preparation of different forms of 2. *Form* α: was prepared by adding 1 mL of cold petroleum ether to a hot solution (70°C) of 30 mg (0.081 mM) of **2** in 1 mL ethyl acetate, showing that the stirring and the temperature were not critical conditions for its preparation. The crystals were isolated, thoroughly washed with cold petroleum ether and dried. The non-crystalline solid (*amorphous form*) was prepared by melting **2** (*form* α) in a stove during 1 h at 100 ± 5°C and then by raising room temperature. Non degradation products were observed by TLC using ethyl acetate-petroleum ether (8:2) as mobile phase.

Cells. Cord blood mononuclear cells (CBMC), obtained from HIV-1 seronegative donors, were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. Cultures were stimulated with phytohemmaglutinin, for 3 days and pools of cells from three CBMC donors were later established and used as feeder cultures[30]. MT-4 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH[4,31]. Cells were cultured at 37°C in RPMI-1640 (Gibco, Burlington, Ontario, Canada) supplemented with 10% fetal calf serum (ICN Chemicals, Montreal, Quebec, Canada), 2 mM glutamine, 100 U/mL penicillin (ICN), and 100 mg/mL streptomycin (ICN).

HIV-1 strains. HIV-1 IIIB was derived from chronically infected H9 cells (kindly supplied by Robert Gallo). An AZT Intermediate Isolate Panel (Catalog Number 1073, 1074, 1075, 1082) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH and was a gift from Dr. Douglas Richman, University of California, San Diego[5,28,29].

Screening and evaluation of single agents for anti HIV-1 activity. Cells were infected with different strains of HIV-1 positive supernatant for 2 h.

The HIV-1 inoculum used to infect MT-4 cells ranged from 20 to 200, 50% tissue culture infective dose ($TCID_{50}$) per 1×10^6 cells; for CBMC, the range was 1000 to 2000 $TCID_{50}$ per 10^6 cells. After infection, cells were washed and dispersed in 96-well plates at various drug concentrations. AZT was used as control, with each experiment performed in duplicate. Culture medium was changed at day 4, so that half of the fluid was replaced with fresh medium containing the original drug concentration. Cell-free supernatant fluids were harvested at day 7, and assayed for production of p24 antigen by ELISA (Abbott Laboratories, North Chicago, Illinois, USA). In the case of CBMC, HIV-1 replication was assayed by measuring levels of reverse transcriptase (RT) activity in culture cell fluid as described[32]. The 50% effective dose (IC_{50}) of each drug was calculated on the basis of p24 antigen levels or RT activity as appropriate.

Effect of 2 on the cellular growth. For toxicity studies, uninfected cells were cultured in duplicate in 96-well plates at varying concentrations of the compounds. Changes of the culture medium were performed on days 4 and 7, and viable cell numbers were determined by trypan blue assay in order to assess the concentration of drug that inhibited cellular growth by 50% ($CCID_{50}$).

Stability assays. Stability assays of **2** were carried out in water and in buffer phosphate solutions of pH 7.4 (Na_2HPO_4-NaH_2PO_4, $\mu = 300$ mOsm), for 20 days at 37°C, using a thermostatic water bath Haake D8 with a precision of ±0.1°C. Assay of the corresponding samples was made in triplicate by HPLC (methanol/water, 80:20 as the mobile phase at a flow-rate of 1 mL/min) and TLC (ethyl acetate-petroleum ether, 80:20) techniques. The HPLC measurements were assayed on a Konik 500 G Chromatograph using an UV detector at λ 265 nm, and a Shimadzu L.C. column packed with a C_{18} (octadecyl silane) chemically bonded non-polar stationary phase. The samples were injected into the column with a Rheodyne (Model 7125) injector of a 20 μL loop.

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