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Synthesis and anti-HIV activity of some novel arylphosphate and *H*-phosphonate derivatives of 3′-azido-2′,3′-dideoxythymidine and 2′,3′-didehydro-2′,3′-dideoxythymidine

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Abstract

Phosphate and *H*-phosphonate derivatives of anti-HIV nucleoside analogues (AZT and d4T) were prepared as potential prodrugs of the bio-active free nucleotide and they were evaluated for their inhibitory effects on the replication of HIV-1 in several cell culture systems. One compound exhibited an important anti-HIV-1 activity and proved to be significantly more efficient than the parent nucleoside. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: AZT/d4T/Phosphate derivatives; Prodrugs; Nucleoside H-phosphonates; Nucleotide; HIV chemotherapy

1. Introduction

Nucleoside analogues, e.g. 3′-azido-2′,3′-dideoxythymidine (AZT) and 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T) are HIV reverse transcriptase (RT) inhibitors that are used as antiviral agents in the treatment of AIDS (De Clercq, 1992). As with all other nucleoside analogues, AZT and d4T do not exert directly antiviral activity, but they are rather prodrugs of active phosphorylated metabolites which are synthesized by action of various cellular kinases (Balzarini et al., 1989). The monophosphorylation step that involves cellular nucleoside kinases, is generally considered to be the most restrictive and this may explain the low anti-HIV activity of some of ddN compounds (Hitchcock, 1991). Moreover, the dependence on phosphorylation for activation of the particular nucleoside analogue may be a problem in cells where the nucleoside kinase activity is known to be low or even lacking (i.e. monocyte/macrophage), (Sommadossi, 1993). On the other hand, because of the structural differences of d4T compared to natural nucleosides, the first

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thymidine kinase catalyzed phosphorylation into d4T-monophosphate is the limiting step of the metabolization in CEM and MT-4 cells (Balzarini et al., 1989; Ho and Hitchcock, 1989). Several approaches have been developed to overcome this dependence on nucleoside kinase activation, particularly the pronucleotide strategy (Krayevsky et al., 1992; McGuigan et al., 1994a,b; Girardet et al., 1995; Lefebvre et al., 1995; Meier et al., 1995; Balzarini et al., 1996; Meier et al., 1997). The viability of such an approach is based on the ability to suitably modify the phosphate structure of a membrane-soluble masked nucleotide to enable intracellular delivery to release the free phosphate form. Therefore, the direct introduction of neutral lipophilic derivatives of ddN-MP should bypass the limiting step and it should have advantages for the biological activity.

As part of a program to mimic nucleoside monophosphates, we have prepared chemically stable phosphate and *H*-phosphonate analogues as isosteres of AZT and d4T monophosphates (Cardona et al., 1998a; Cardona et al., 1998b). We have noted significant and selective anti-HIV activity for the *H*-phosphonate derivatives.

In this work, we report the synthesis and the properties of this class of phosphonate, which has exhibited potent HIV activity ($EC_{50} = 0.014$ μM) for *H*-phosphonate derivatives of AZT and ($EC_{50} = 0.034$ μM) for *H*-phosphonate derivatives of d4T, without any sign of toxicity in MT-4 cells. Moreover, the latter compound retains marked antiviral activity in TK^- (thymidine kinase deficient) mutant CEM cells in which d4T was poorly active. Particularly interesting is the observation that this *H*-phosphonate monoester may act by an additional mechanism of action.

2. Results and discussion

2.1. Chemistry

As shown in Schemes 1–3, the synthesis of the compounds started with the preparation of d4T from thymidine essentially by the method of Horwitz (Horwitz et al., 1966; Horwitz and Chua, 1968) noting the latter comments of Mansuri et al. (1989). AZT was obtained by a method described by Czernecki and Valéry (1991).

Compounds (1), (2) and (3) were synthesized using the following procedure: N-succinimide chloride (NCS) reacted with commercially available dibenzyl or diethyl phosphite to give the corresponding monochloride derivatives (A) as intermediates; reaction of AZT or d4T with (A) gave the desired products in moderate to good yields. Compounds (4) and (5) were obtained by reaction of precursor (B) which was prepared by reaction of dibenzylphosphite in 10 ml of anhydrous toluene with 1 equivalent of 1,4-diazabicyclo-[2.2.2] octane (DABCO) followed by reaction with oxalyl chloride to give (C) and finally by reaction with AZT or d4T.

D4T-MP (6) was prepared by reaction of 4 equivalent of phosphorous oxychloride with d4T according to the previously described method. In our hands, this method led to compound (6) as triethylammonium salt in 62% yield.

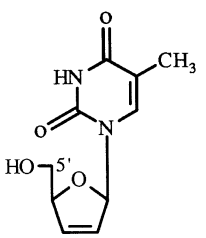
2.2. Antiretroviral evaluations

All derivatives were studied concerning their inhibitory and cytotoxicity effect on HIV-1 induced cytopathicity CEM-SS cells as well as against HIV-1 induced cytopathicity in thymidine kinase-deficient CEM/TK^- cells. The tested compounds were free of traces of the parent nucleoside (AZT and d4T) as verified by means of analytical high performance liquid chromatography (HPLC).

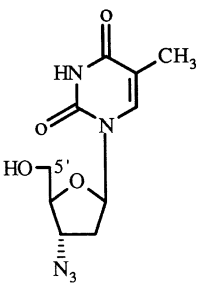
The results are displayed in Table 1. All compounds showed activity againt HIV-1 replication. In some cases (compounds 4 and 5) the inhibitory effect was even more pronounced than that of the parent nucleoside d4T or AZT. In addition, the 50% cytotoxic concentration (CC_{50}) of compounds (4) and (5) was above 100 μM and thus appeared to be of comparable toxicity to AZT or d4T. Consequently, the selectivity index of (4) is at least as high as that of d4T. Moreover, compound (4) proved to be 10-fold more effective than d4T in inhibiting HIV-1 replication in thymidine kinase positive MT-4 and the same compound (4) showed a more pronounced inhibitory effect (50-fold) against HIV-1 replication

in the thymidine kinase-deficient CEM-TK$^-$ cell line. It was showed that d4T is weakly active against HIV-1 replication in thymidine kinase-deficient CEM cells with a 50% effective concentration (EC_{50}) value at 14 μM. The neutral *H*-phosphonate derivative of d4T emerged as a potent inhibitor with an EC_{50} value at 0.3 μM. However, in the same cells, lacking the ability to express thymidine kinase, compound (5) displayed a poor activity similarly to the parent nucleoside (AZT). Thus it is apparent that, whereas the *H*-phosphonate of d4T is able to act as a nucleotide delivery form, the *H*-phosphonate of AZT is probably only able to act as prodrug of the free nucleoside.

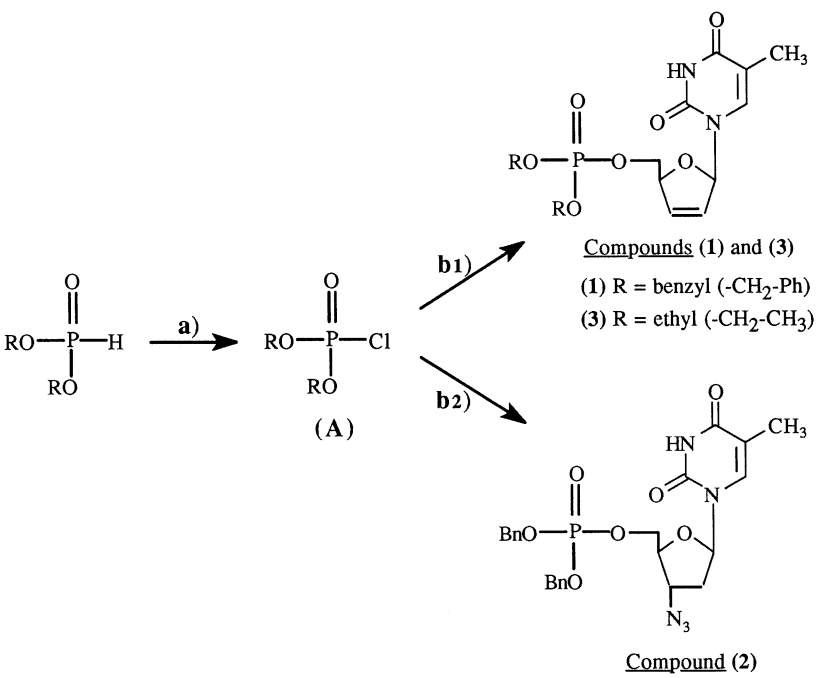
These results confirmed for the first time, the intracellular delivery of a d4T 5′-monophosphate bioactive metabolite from an *H*-phosphonate



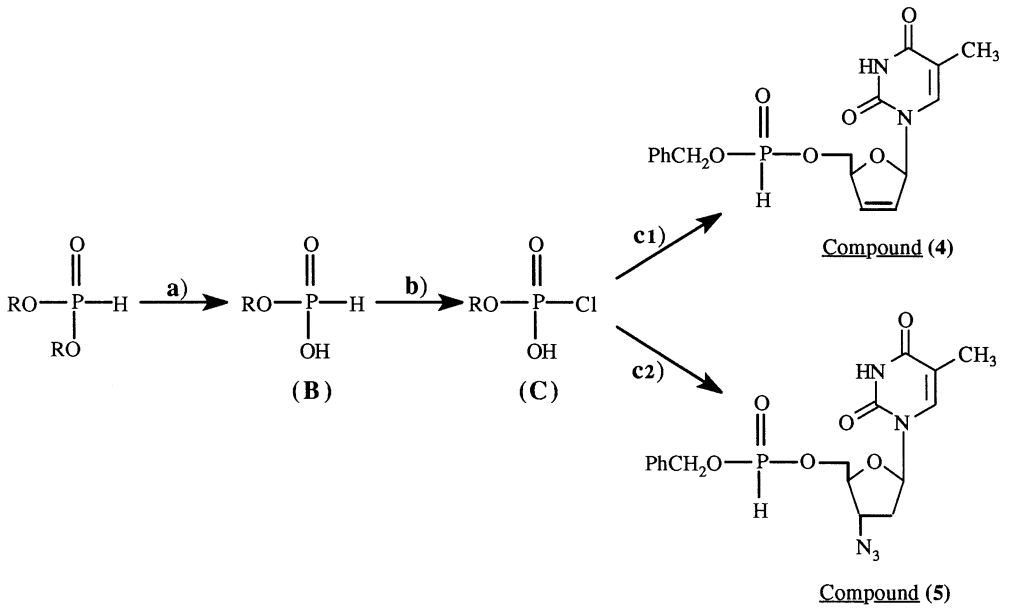
d4T (Stavudine)



AZT (Zidovudine)



Scheme 1. Synthetic route to compounds (1), (2) and (3). Reaction conditions: (a) NCS, toluene, dibenzyl phosphite (1 eqivalent) *or* diethyl phosphite (2 eqivalent), 20°C, N_2, 1.5–2.5 h. (b1) d4T, pyridine, 20°C, 18 h (for compound (3): 4.5 h). (b2) AZT, pyridine, 20°C, 19 h.



Scheme 2. Synthetic route to compounds (4) and (5). Reaction conditions: (a) DABCO, toluene, dibenzyl phosphite, reflux, N_2, 3.5 h. (b) Oxalyl chloride, DMF, 0°C, 45 min. (c1) d4T, pyridine, 0°C, 15 min and 20°C, 4 h. (c2) AZT, pyridine, 0°C, 15 min and 20°C, 4 h.

derivative of the nucleoside. Further work is currently in progress in our laboratory to explore this pro-nucleotide concept.

3. Experimental section

All experiments involving water-sensitive compounds were conducted under dry conditions. Solvents were dried and purified by distillation before used. D4T was dried at elevated temperature in vacuo. Proton, carbon and phosphorus nuclear magnetic resonance spectra were recorded on a Brucker W-200 spectrometer operating at 200 MHz. All NMR spectra were recorded in $CDCl_3$ or D_2O at room temperature. Chemical shifts were quoted in ppm relative to the solvent signal as internal standard. *J* (Hz) values refer to coupling constants, and signal splitting patterns are described as singlet (s), broad singlet (bs), doublet (d), triplet (t), quartet (q), multiplet (m), or combinations thereof. Mass spectra were recorded on a Finnigan Mat TSQ 7000 quadrupole instrument (ESI: Electrospray ionization). Elemental analyses were performed by the Service de Microanalyse du CNRS (Vernaison, France).

Reactions were monitored by TLC: silica gel 60F-254 aluminium plates (0.25 mm; Merck containing fluorescent indicator). Spots were visualized under 254 nm UV light or with molybdenum blue reagent spray by heating. Liquid chromatography was carried out using Merck silica gel 60H (40–63 μm) as stationary phase. Ion exchange chromatography was performed using Dowex 50 H^+X2 (50–100) or Sephadex DEAE A-25 (40–120 μm). HPLC studies were conducted on a Merck Interchrom 203110 C18 column (particule size 2.5 μm; 2503. × 9 mm) or DEAE 5PW column (75 × 7.5 mm) and motored by UV at λ_{max} 254 nm. The products were eluted with methanol/water, acetonitrile/water, or phosphate, bicarbonate or formate buffers.

3.1. General procedure for the preparation of the dibenzyl phosphate derivatives

To a solution of 423 mg (3.17 mmol) of dry N-chlorosuccinimide (NCS) in 6.5 ml of anhy-

drous toluene, 690 μl (1 equivalent) of dibenzyl or diethyl phosphite was added with stirring. The mixture was stirred at room temperature for 1.5–2.5 h under nitrogen. The solid was filtered off and the solvent was evaporated under vacuum to lead to a viscous liquid (2 ml) which was dissolved in 0.7 ml of freshly distilled pyridine. This solution was slowly added to a solution of 200 mg (0.89 mmol) of 2′,3′-didehydro-2′,3′-dideoxythymidine in 2 ml of pyridine cooled at −5°C. Stirring under dry conditions was continued for 18–19 h. Water was added. The organic layers were treated with a solution of hydrochloric acid (1 M) and neutralized with sodium bicarbonate solution and dried over sodium sulfate. After evaporation of the solvents, the residue was purified by chromatography on a silica gel column using a gradient of dichloromethane/methanol (0–10%). Fractions containing the desired product were collected and lyophilized. Yields ranged from 62 to 92%.

All compounds obtained were characterized as described below, and found to be pure by rigourous HPLC analysis, high-field multinuclear nuclear magnetic resonance (NMR) spectroscopy, high-resolution mass spectroscopy and elemental analysis.

3.1.1. 2′,3′-Didehydro-2′,3′-dideoxythymidine-5′-dibenzyl phosphate (1)

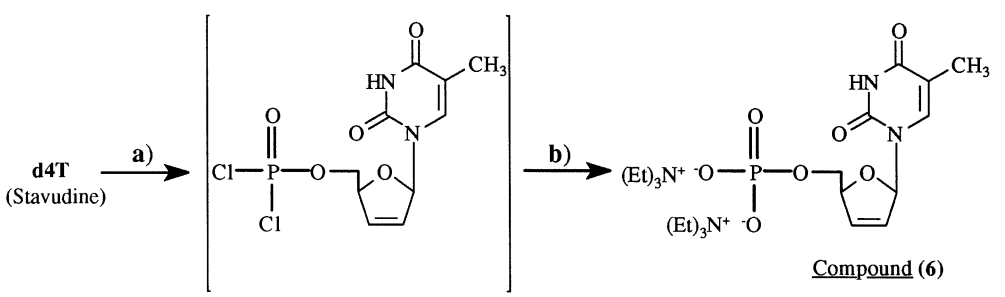
This compound was obtained as an oil in 84% yield after purification by HPLC on a C18 column (tr = 8.5 min) using a water/acetonitrile mixture (50/50 v/v) as eluent; TLC: $R_f = 0.58$ (CH_2Cl_2/CH_3OH: 92/8 v/v). ^{1}H NMR ($CDCl_3$) $\delta = 8.21$ (s, 1H, NH), 7.27 (m, 10H, C_6H_5), 7.20 (s, 1H, H_6), 6.91 (m, 1H, H'_1), 6.08 (d, 1H, $J^3 = 6$, $H_{3'}$), 5.75 (d, 1H, $J^3 = 6$, $H_{2'}$), 4.81 (d, 4H, $J^2 = 8.6$, $C_6H_5CH_2$), 4.83 (m, 1H, $H_{4'}$), 4.08 (m, 2H, $H_{5'}$), 1.70 (s, 3H, CH_3). ^{31}P NMR (^{1}H decoupled) $\delta = -0.08$. MS: 485$(M+H)^+$, 507$(M+Na)^+$, 523$(M+K)^+$. Anal. Calc. for $C_{24}H_{25}N_2O_7P$: C 59.50; H 5.20; P 6.39. Found: C 59.64; H 5.18; P 6.40

3.1.2. 3′-Azido-2′,3′-dideoxythymidine-5′-dibenzyl phosphate (2)

This compound was obtained as an oil in 62% yield after purification by HPLC on a C18 column (tr = 11 min) as above; TLC: $R_f = 0.59$ (CH_2Cl_2/CH_3OH: 95/5 v/v). ^{1}H NMR ($CDCl_3$) $\delta = 7.95$ (s, 1H, NH), 7.38 (m, 10H, C_6H_5), 7.24 (s, 1H, H_6), 6.10 (m, 1H, $H_{1'}$), 5.00 (d, 4H, $J^2 = 10.4$, $C_6H_5CH_2$), 4.02–3.86 (m, 4H, $H_{3'}$, $H_{4'}$, $H_{5'}$), 2.55 (m, 2H, $H_{2'}$), 1.80 (s, 3H, CH_3). ^{31}P NMR (^{1}H decoupled) $\delta = -0.11$. MS: 528$(M+H)^+$, 550$(M+Na)^+$. Anal. Calc. for $C_{24}H_{26}N_5O_7P$: C 54.65; H 4.97; P 5.87. Found: C 54.76; H 5.03; P 5.82.

3.1.3. 2′,3′-Didehydro-2′,3′-dideoxythymidine-5′-diethyl phosphate (3)

This compound was prepared as described by McGuigan et al. (1990). It was isolated as an oil in 92% yield after purification by HPLC on a C18 column (tr = 8.7 min) using a water/acetonitrile mixture (60/40 v/v) as eluent; TLC: $R_f = 0.50$ (CH_2Cl_2/CH_3OH: 92/8 v/v). ^{1}H NMR ($CDCl_3$)



Scheme 3. Preparation of d4TMP (6). Reaction conditions: (a) $POCl_3$, $PO(OCH_3)_3$, 0°C, N_2, 15 min and room temperature 2.5 h. (b) H_2O/NH_3 2N.

Table 1
Inhibitory and cytotoxicity effect of compounds (1), (2), (3), (4), (5) and (6) against HIV-1 replication in CEM-SS, MT4 and CEM-TK$^-$ cells[a]

Compounds	CEM-SS		MT4		CEM-TK$^-$	
	EC_{50}[a]	CC_{50}[a]	EC_{50}[a]	CC_{50}[a]	EC_{50}[a]	CC_{50}[a]
4d4T	0,049	>100 (30%)[b]	0,33	47	14	>100 (6%)[b]
AZT	0,004	>100	0,7	>100	>100	>100
(1)	4,4	>100	>100	74	5,5	>100
(2)	0,010	>100	0,98	10	>100	>100
(3)	3,5	>100	47	32	ND	ND
(4)	0,016	>100 (44%)[b]	0,034	24	0,3	>100 (34%)[b]
(5)	<0,005	>100 (38%)[b]	0,014	>100	>100	>100
(6)	0,73	>100	0,71	42	24	>100

[a] EC_{50}, 50% effective concentration (in μM) or concentration required to inhibit the replication of HIV-1 by 50%, and CC_{50}, 50% cytotoxic concentration (in μM) or concentration required to reduce the viability of uninfected cells by 50%.
[b] Percent reduction of viable cells at the indicated highest concentration tested (100 μM).

$\delta = 7.86$ (s, 1H, NH), 7.31 (s, 1H, H_6), 6.97 (m, 1H, $H_{1'}$), 6.26 (d, 1H, $J^3 = 6$, $H_{3'}$), 5.82 (d, 1H, $J^3 = 6$, $H_{2'}$), 4.95 (m, 2H, $H_{4'}$), 4.20 (m, 2H, $H_{5'}$), 4.10 and 4.08 (2q, 4H, CH_2CH_3), 1.90 (s, 3H, CH_3), 1.28 and 1.26 (2t, 6H, CH_2CH_3). ^{31}P NMR (^{1}H decoupled) $\delta = -0{,}30$. MS: $361(M+H)^+$, $383(M+Na)^+$. Anal. Calc. for $C_{14}H_{21}N_2O_7P$: C 46.67; H 5.87; P 8.60. Found: C 47.03; H 5.90; P 8.57

3.2. General procedure for the preparation of H-phosphonate derivatives

To a solution of 628 μl (2.84 mmol) of dibenzylphosphite in 10 ml of anhydrous toluene, 318 mg (1 equivalent) of 1,4-diazabicyclo-[2.2.2] octane was added with stirring under nitrogen at room temperature. The mixture was then heated under reflux for 3.5 h. The solvent was evaporated under reduced pressure and the residue was taken up in a 5% aqueous hydrochloric acid solution. The mixture was extracted with dichloromethane (2 × 20 ml). The organic layers were dried under anhydrous sodium sulfate and filtered. After evaporation of the solvents, O-benzyl *H*-phosphonate acid was obtained in 74% yield as liquid and used in the next step without further purification. To a solution of 258 mg (1.5 mmol) of the above intermediate in dried toluene cooled to 0°C, 262 ml (3 mmol) of oxalyl chloride in 5 ml of dimethylformamide under nitrogen. The resulting mixture was stirred at this temperature for 45 min and the reaction was allowed to warm up to room temperature. The solid which was formed, was filtered off and the solvents were evaporated under vacuum. The residue was taken up in 2.5 ml of freshly distilled pyridine. To this solution cooled at 0°C, was added with stirring and under nitrogen, 112 mg (0.5 mmol) of 2′,3′-didehydro-2′,3′-dideoxythymidine in 4 ml of the same solvent. The mixture was stirred at room temperature for 4 h. After evaporation of the solvents under reduced pressure the residue was applied to a silica gel column chromatography. The product was eluted with CH_2Cl_2/CH_3OH (9/1 v/v) and purified again by semi-preparative HPLC (C18 column, eluent H_2O/CH_3CN 60/40 isocratic mixture).

3.2.1. 2′,3′-Didehydro-2′,3′-dideoxythymidine-5′-benzyl H-phosphonate (4)

This compound was obtained as an oil in 56% yield after purification by HPLC on a C18 column (tr = 5.8 min) using a water/acetonitrile (60/40 v/v). TLC: $R_f = 0.40$ (CH_2Cl_2/CH_3OH: 92/8 v/v). ^{1}H NMR ($CDCl_3$) $\delta = 8.45$ (s, 1H, NH), 7.32 (m, 5H, C_6H_5), 7.18 (s, 1H, H_6), 7.00 (m, 1H, $H_{1'}$), 6.20 (d, 1H, $J^3 = 6.2$, $H_{3'}$), 5.82 (d, 1H, $J^3 = 6.2$, $H_{2'}$), 5.10 (d, 2H, $J^2 = 11.6$, $C_6H_5CH_2$), 4.88 (m, 1H, $H_{4'}$), 4.16 (d, 2H, $J^2 = 7.1$, $H_{5'}$), 1.81 (s, 3H, CH_3). ^{31}P NMR (^{1}H decoupled) $\delta = 8.50$

(d, $J^1 = 708$). MS: 379$(M + H)^+$, 401$(M + Na)^+$, 417$(M + K)^+$. Anal. Calc. for $C_{17}H_{19}N_2O_6P$: C 53.97; H 5.06; P 8.19. Found: C 53.84; H 5.11; P 8.1

3.2.2. 3′-Azido-2′,3′-dideoxythymidine-5′-benzyl H-phosphonate (5)

This compound was obtained as an oil in 52% yield after purification by HPLC on a C18 column (tr = 5.6 min) using water/acetonitrile (60/40 v/v). TLC: $R_f = 0.48$ (CH_2Cl_2/CH_3OH: 95/5 v/v). ^{1}H NMR ($CDCl_3$) $\delta = 8.10$ (s, 1H, NH), 7.35 (m, 5H, C_6H_5), 7.20 (s, 1H, H_6), 6.08 (m, 1H, $H_{1'}$), 5.10 (d, 2H, $J^2 = 12.4$, $C_6H_5CH_2$), 4.12 (m, 2H, $H_{5'}$), 3.87 (m, 2H, $H_{3'}$ and $H_{4'}$), 2.40 (m, 2H, $H_{2'}$), 1.82 (s, 3H, CH_3). ^{31}P NMR (^{1}H decoupled) $\delta = 8.67$ (d, $J^1 = 890$). MS: 422$(M + H)^+$, 444$(M + Na)+$. Anal. Calc. for $C_{17}H_{20}N_5O_6P$: C 48.46; H 4.78; P 7.35. Found: C 48.32; H 4.79; P 7.28

3.2.3. Preparation of d4T monophosphate triethylammonium salt (6)

This product was obtained by a method described by Mansuri et al. (1989). The analytical data of the isolated product corresponded to those of earlier studies. HPLC on a C18 column (tr = 29.6 min) using a 0.15 M ammonium formate buffer (pH = 7.3). TLC: $R_f = 0.54$ (eluent H_2O/i-PrOH/NH_4OH 2N: 1/6/3 v/v/v). ^{1}H NMR (D_2O) $\delta = 7.57$ (s, 1H, H_6), 6.91 (s, 1H, $H_{1'}$), 6.42 (d, 1H, $J^3 = 6$, $H_{3'}$), 5.88 (d, 1H, $J^3 = 6$, $H_{2'}$), 5.03 (m, 1H, $H_{4'}$), 3.98 (dd, 2H, $J^2 = 10$, $J^3 = 5$, $H_{5'}$), 1.84 (s, 3H, CH_3). ^{31}P NMR (D_2O, ^{1}H decoupled) $\delta = 0{,}87$. MS: 303 $(M + H)^+$. Anal. Calc. for $C_{10}H_{11}N_2Na_2O_7P.0{,}5\ H_2O$: C 33.61; H 3.36; P 8.68. Found: C 34.15; H 3.42; P 8.55

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