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Aryl nucleoside *H*-phosphonates. Part 15: Synthesis, properties and, anti-HIV activity of aryl nucleoside 5′-α-hydroxyphosphonates

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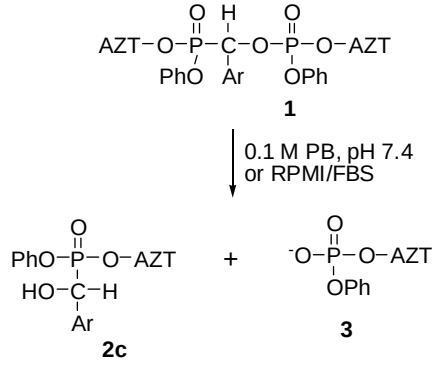
Abstract—Aryl nucleoside 5′-*H*-phosphonates **4** bearing AZT or 2′,3′-dideoxyuridine moieties were subjected to reaction with various aromatic aldehydes to produce nucleoside 5′-α-hydroxyphosphonate derivatives **2** as potential anti-HIV agents. Stability of the title compounds in cell culture media was investigated and three distinct decomposition pathways were identified. The anti-HIV activity of hydroxyphosphonates **2** correlates well with the type and extent of their chemical or enzymatic degradation in culture medium (RPMI 1640 containing 10% FBS), suggesting that aryl nucleoside 5′-hydroxyphosphonates **2** act as depot forms of the parent antiviral nucleosides.

1. Introduction

Nucleoside analogues currently used in AIDS therapy caused by HIV infections are pro-drugs that, following uptake by the cells, are stepwise phosphorylated into the corresponding 5′-triphosphates. As such, they are substrates for the HIV reverse transcriptase (RT) and, upon incorporation into the growing DNA chain, terminate the synthesis of the viral DNA.[1,2] Since phosphorylation of nucleoside analogues is indispensable for their antiviral activity, efforts have been focused to prepare derivatives that might efficiently enter the cell and be converted into bioactive forms.[3–11]

The approach of using precursors, which could deliver into the cell two identical or different phosphorus-linked antiviral nucleosides, has received considerable attention.[12–25] Ex vivo studies have shown that, in many instances, such precursors are endowed with considerable anti-HIV activity and that synergistic potentiation may be obtained following simultaneous delivery of two nucleosides, for example, 2′,3′-dideoxyinosine (ddI) and 3′-azido-3′-deoxythymidine (AZT).[12]

Based on these promising results, we recently developed dinucleoside phosphonate-phosphates[26] of type **1** (Scheme 1) as potential anti-HIV agents. In



AZT = 3'-deoxy-3'-azidothymidin-5'-yl
Ph = phenyl
Ar = 4-methoxyphenyl
PB = 0.1 M phosphate buffer pH 7.4
FBS = fetal bovine serum
RPMI = RPMI 1640

Scheme 1. Decomposition of **1** in PB, pH 7.4, and RPMI/FBS 10% (v/v) 37 °C.

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HIV-1-infected MT-4 cells, 3′-azido-3′-deoxythymidin-5′-yl phenyl (3′-azido-3′-deoxythymidin-5′-yl phenyl phosphinyl)-4-methoxybenzyl phosphate **1** showed anti-HIV potency (EC_{50} = 0.01 μM) equal to that of AZT (EC_{50} = 0.01 μM). The decomposition pathway of compound **1** in cell culture medium (RPMI 1640 containing 10% heat-inactivated foetal bovine serum FBS) indicated the rapid formation (HPLC, $t_{1/2} < 5$ min) of 3′-azido-3′-deoxythymidin-5′-yl phenyl hydroxy(4-methoxyphenyl)methanephosphonate **2** and 3′-azido-3′-deoxythymidin-5′-yl phenyl phosphodiester **3** in equal amounts (Scheme 1).[27]

Phosphodiester **3** can be metabolized to provide the corresponding nucleoside monophosphate. However, since the anionic structure of the latter one may prevent efficient crossing of the cell membrane, it is likely that the observed anti-HIV activity of **1** was due to the generation of uncharged AZT phenyl hydroxyphosphonate **2c** that might undergo further enzymatic transformation into biologically active species. To verify this assumption, we synthesised a series of nucleoside aryl α-hydroxyphosphonates of type **2** (Scheme 2) and investigated their chemical and anti-HIV properties.

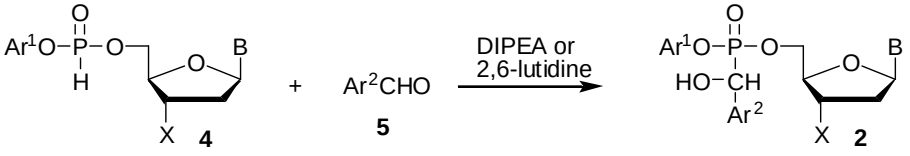
2. Results and discussion

2.1. Synthesis of α-hydroxyphosphonates of type 2 via aryl *H*-phosphonate four intermediates

Recent studies by Meier et al. have shown that dinucleoside analogues of hydroxybenzylphosphonates bearing two AZT moieties exhibit potent antiviral activity in HIV-infected cells.[16,18,19,28] In this context, it was interesting to investigate the antiviral properties of hydroxyphosphonates of type **2** (Scheme 2) as their structural features should permit a precise modulation of their chemical and pharmacokinetic properties by a proper choice of two aromatic units Ar^1 and Ar^2.

Dinucleoside hydroxyphosphonates can be prepared by the addition of an aldehyde to the corresponding *H*-phosphonate diesters in the presence of base, but this usually requires high temperature, large excess of base and prolonged reaction times.[28] Analogous synthesis of aryl nucleoside hydroxyphosphonates **2** would call for the corresponding aryl nucleoside *H*-phosphonates **4** as intermediates, but these are much more labile than dinucleoside *H*-phosphonates and show high propensity towards disproportionation under basic conditions.[29] Indeed, even under relatively mild reaction conditions, for example, addition of an aldehyde to *H*-phosphonate diesters in THF in the presence of triethylamine,[28] formation of significant amount of side products was observed in the case of aryl *H*-phosphonate diesters (^{31}P NMR spectroscopy). For this reason, it was necessary to develop a new synthetic protocol that would permit efficient synthesis of aryl nucleoside hydroxyphosphonates **2**.

To this end, AZT phenyl *H*-phosphonate **4a** [generated in situ by coupling of AZT *H*-phosphonate and phenol in methylene chloride in the presence of pyridine (10 molar equiv) and diphenyl chlorophosphate (DPCP)][30] was allowed to react with benzaldehyde **5a** (5 molar equiv) in the presence of a slight excess of diisopropyl ethylamine (DIPEA, 2.5 molar equiv in respect to **4a**), instead of



4a; B = thymin-1-yl, X = N_3, Ar^1 = phenyl
4b; B = thymin-1-yl, X = N_3, Ar^1 = 4-methoxyphenyl
4c; B = thymin-1-yl, X = N_3, Ar^1 = 2,6-dimethylphenyl
4d; B = uracil-1-yl, X = H, Ar^1 = phenyl

5a; Ar^2 = phenyl
5b; Ar^2 = 4-methylphenyl
5c; Ar^2 =4-methoxyphenyl
5d; Ar^2 = 4-chlorophenyl
5e; Ar^2 = 4-nitrophenyl
5f; Ar^2 = pyridin-3-yl

DIPEA = diisopropylethylamine

2a; B = thymin-1-yl, X = N_3, Ar^1 = Ar^2 = phenyl
2b; B = thymin-1-yl, X = N_3 Ar^1 = phenyl, Ar^2 = 4- methylphenyl
2c; B = thymin-1-yl, X = N_3 Ar^1 = phenyl, Ar^2 = 4-methoxyphenyl
2d; B = thymin-1-yl, X = N_3 Ar^1 = phenyl, Ar^2 = 4-chlorophenyl
2e; B = thymin-1-yl, X = N_3 Ar^1 = phenyl, Ar^2 = 4-nitrophenyl
2f; B = thymin-1-yl, X = N_3 Ar^1 = phenyl, Ar^2 = pyridin-3-yl
2g; B = thymin-1-yl, X = N_3 Ar^1 = 4-methoxyphenyl, Ar^2 = pyridin-3-yl
2h; B = thymin-1-yl, X = N_3 Ar^1 = 2,6-dimethylphenyl, Ar^2 = pyridin-3-yl
2i; B = uracil-1-yl, X = H Ar^1 = phenyl, Ar^2 = 4-methoxyphenyl

Scheme 2. Scheme of synthesis of aryl nucleoside α-hydroxy(aryl)methanephosphonates of type **2**.

Table 1. Octanol/water partition coefficient (log *P*), ^{31}P NMR and HPLC data of hydroxyphosphonates **2** and their decomposition products

Compound	δ_P[a,c](ppm)	t_R[b,c] [min]	Log *P*
2a	18.72, 18.76, 18.97, 19,05	27.2, 27.7	1.49
2b	18.88, 18.96, 19.11, 19.24	28.2, 28.3, 28.8	1.88
2c	18.82, 18.85, 18.98, 19,09	27.8, 28.1, 28,31, 28.46	1.48
2d	17,96, 18.05, 18,08, 18,26	29.5, 29.9	1.53
2e	17.10, 17.14, 17.31, 17.54	28.9, 29.1, 29.2	0.99
2f	18.05, 18.22, 18.43, 18.59	23.4, 23.7	−0.14
2g	17.80, 17.98, 18.08, 18.36	23.4, 23,6	−0.23
2h	17.54, 17.68, 17.81, 18.12	25.4, 26.0	0.56
2I	18.96, 19.12	26.4, 26.7, 26.9	1.27
6	5.41d of t, $^1J_{HP}$ = 631 Hz, $^3J_{HP}$ = 5.5 Hz	13.9	
8a	16.08, 16.27	17.4	—
8b	15.09, 15.31	14.6	—
8c	15.81, 16.05	14.5	—
9	−6.26, −6.29	32.0, 32.2	—
3	−6.00	18.1	—
10	0.44	12.1	—
11	52.95, 53.45 (2t, $^3J_{HP}$ = 6.4 Hz)	—	—
AZT	—	—	0.06

[a] Chemical shifts in spectra recorded with heteronuclear decoupling.
[b] t_R, retention time; Hypersil ODS, 4.6 × 250 mm (for details, see Section 4).
[c] Multiplicity of signals and peaks is due to the presence of diastereoisomers.

triethylamine (Scheme 2). ^{31}P NMR spectroscopy revealed that the reaction was completed within 10 min and the corresponding α-hydroxyphosphonate **2a** was the only nucleotidic product observed. In the ^{31}P NMR spectra, α-hydroxyphosphonates **2** gave rise to a group of four signals centred at ca. 18 ppm, due to the presence of four diastereomers (two chiral centres: at the phosphorus atom and at the phosphonate carbon centre) (Table 1). These conditions were also suitable for the reactions of other aldehydes with electron-donating (**5a**–**c**) or weakly electron-withdrawing substituents (**5d**), and afforded the corresponding hydroxyphosphonates **2a**–**d** in 70–80% yields after silica gel column chromatography.

Aldehydes with strong electron-withdrawing substituents (**5e** and **5f**) in the presence of DIPEA reacted faster with *H*-phosphonates **4** than with aldehydes **5a**–**c**, but produced several side products resonating at δ_P 27–32 ppm (ca. 20%). To suppress these side reactions, we replaced DIPEA (pK_a > 11) with a weaker base 2,6-lutidine (pK_a 6.6).[31] This caused the condensation reactions to proceed slowly (overnight), however, the corresponding hydroxyphosphonates **2e**–**h** were formed without noticeable contamination of side products (^{31}P NMR spectroscopy).

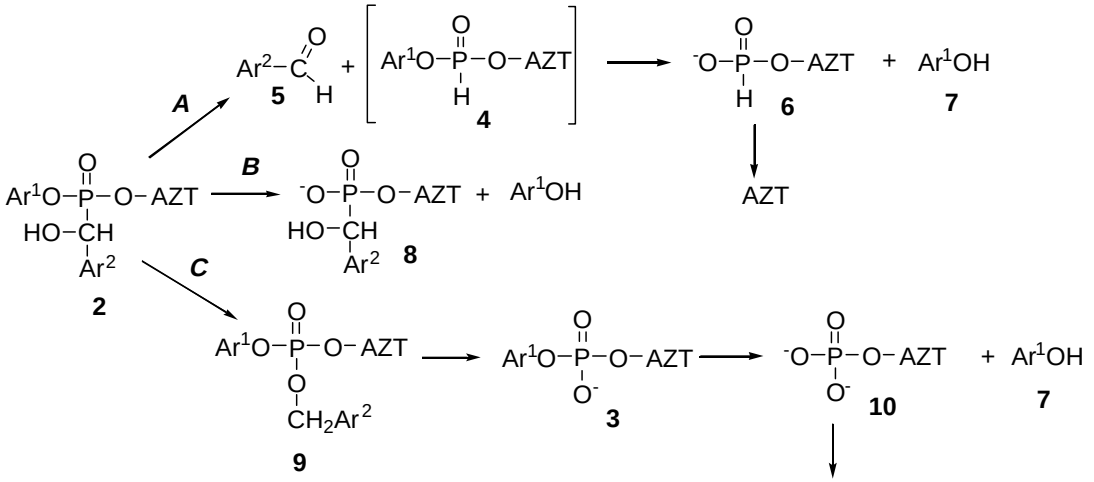
Structure and purity of all synthesised AZT (or ddU) aryl hydroxyphosphonates **2** were established by ^{1}H, ^{31}P and HRMS spectroscopy, and also by chromatographic methods (TLC, HPLC).

2.2. Stability of hydroxyphosphonates 2 in cell culture medium

Stability of all hydroxyphosphonates **2** was investigated in the same cell culture medium (RPMI 1640 containing 10% FBS, pH 7.6), in which cultivation of T-lymphocytes was carried out during in vitro cytotoxicity and anti-HIV assays. To this end, 2 mM solutions of each compound of type **2** in the above culture medium containing 5% DMSO were incubated at 37 °C and the formation of metabolites was monitored by HPLC. Since hydroxyphosphonates **2** were stable in acidic media (vide infra), 0.01 M triethylammonium trifluoroacetate, pH 3.2, was used as a polar solvent for HPLC analyses. The products formed upon decomposition of **2** were unambiguously identified by co-elution with authentic samples obtained by other methods (vide infra and Section 4).

All aryl nucleoside α-hydroxyphosphonates **2** appeared to be rather unstable ($t_{1/2}$ 10–30 min) in the cell culture medium and decomposed in different ways depending on the aryl moiety Ar^2 in the hydroxy(aryl)methylene part of the molecule, and on the aryl group Ar^1 bound to the phosphorus centre (Scheme 3). Three decompositions pathways (***A***, ***B*** and ***C***), primarily determined by the electron density in Ar^2, were identified: (i) path ***A***, which is likely to be initiated by a spontaneous dissociation of hydroxyphosphonates **2** into the parent *H*-phosphonate diesters **4** and the corresponding aldehydes **5**; (ii) path ***B***, which results from hydrolysis of the phosphoester aryl moiety and represents, under these reaction conditions, a dead-end product; (iii) path ***C***, which involves as an initial step a phosphonate-phosphate rearrangement to produce the corresponding phosphotriesters **9**. The relative contribution of pathways ***A***, ***B*** and ***C*** for a particular hydroxyphosphonate **2** is determined by electronic nature combination of Ar^1 and Ar^2 groups present in the compound.

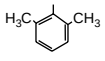
Decomposition paths of phenyl esters **2a**–**f** bearing different Ar^2 substituents were investigated. α-Hydroxyphosphonates **2a**–**c** bearing electron-donating substituents on Ar^2 decomposed exclusively according to pathway ***A*** (Scheme 3, Table 2) and rapidly produced AZT *H*-phosphonate **6**, which then slowly underwent dephosphonylation to AZT most likely by



7a; Ar^1 = phenyl
7b; Ar^1 = 4-methoxyphenyl
7c; Ar^1 = 2,6-dimethylphenyl
8a; Ar^2 = 4-chlorophenyl
8b; Ar^2 = 4-nitrophenyl
8c; Ar^2 = pirydin-3-yl
9; Ar^1 = phenyl, Ar^2 = 4-nitrophenyl
3; Ar^1 = phenyl

Scheme 3. Decomposition paths of AZT aryl hydroxyphosphonates of type **2** in cell culture media.

Table 2. Stability and decomposition paths of hydroxyphosphonates of type **2** in cell culture media[a] and their anti-HIV potency[b]

Compound	Ar^1	Ar^2	RPMI/FBS $t_{1/2}$ (min)	Path			CC_{50} (μM)	EC_{50} (μM)	SI
				A (%)	***B*** (%)	***C*** (%)			
2a			27.2	100	—	—	49	0.06	817
2b			19.4	100	—	—	42	0.05	840
2c			13.1	100	—	—	49	0.04	1225
2d			13.1	92	8	—	50	0.05	1000
2e			9.0	24	47	29	37	0.05	740
2f			15.9	55	45	—	>100	0.04	>2500
2g			26.3	58	42	—	27	0.01	2700
2h			57.6	100	—	—	49	0.01	4900
2I			25.8	100	—	—	>100	>20	5
6			1175	—	—	—	55	0.01	5500
8b			Stable 6 days	—	—	—	nd[c]	nd[c]	—
8c			Stable 6 days	—	—	—	>100	2.4	42
9			9901	—	—	—	27	0.01	2700
AZT			—	—	—	—	50	0.01	5000

[a] RPMI 1640/FBS 9:1 (v/v), 37 °C.
[b] MT-4 cells, HIV IIIB strain.
[c] Not determined.

the 5′-nucleotidase-like but not phosphatase-like[32] activity of FBS. Since ^{31}P NMR spectroscopy proved formation of phosphonic rather than phosphoric acid at this step, it seems that AZT *H*-phosphonate **6** does not need to be oxidized to the phosphate form to be substrate of FBS 5′-nucleotidase-like enzymatic activity as it has been suggested previously.[33] This lent support to the opinion that AZT *H*-phosphonate **6** is rather a nucleoside vehicle,[15] although some data indicated that **6** to some extent can also act as a pronucleotide.[34]

In the case of hydroxyphosphonates **2d**–**f**, in which the Ar^2 group is electron deficient due to the presence of electron-withdrawing substituents, two additional decomposition routes, ***B*** and ***C***, became available. α-Hydroxyphosphonate **2d** predominantly (92%) produced AZT *H*-phosphonate **6** via path ***A***, although the formation of α-hydroxyphosphonate monoester **8a** (8%) through path ***B*** became evident. The contribution of path ***B*** to the decomposition of **2f** bearing pyridin-3-yl as an Ar^2 group was higher (45%), approaching that of path ***A*** (55%). In the case of α-hydroxyphosphonate diester **2e** with the most electron-withdrawing 4-nitrophenyl group as Ar^2, the third decomposition path ***C***, triggered by the phosphonate-phosphate rearrangement,[35–37] could be observed (29% of **9**). The produced AZT 4-nitrobenzyl phenyl phosphotriester (**9**) was slowly (Table 2) but chemoselectively hydrolysed to AZT phenyl phosphodiester **3**, followed by transformation to AZT monophosphate **10** and, ultimately, to AZT (Table 2). The last two transformations, that is, formation of AZT monophosphate **10** and its dephosphorylation to AZT, are apparently enzymatic reactions catalysed by FBS phosphodiesterases and phosphatases, respectively, because compounds **3** and **10** were stable for at least 6 days in RPMI lacking FBS.

It was also interesting to know to what extent electronic properties of an aryl ester group Ar^1 can modify a relative contribution of the decomposition pathways of hydroxyphosphonates **2** in cell culture media. To this end, the stability in cell culture medium of α-hydroxyphosphonates **2f**–**h** with Ar^2 = pyridin-3-yl and Ar^1 = phenyl (pK_a = 9.98) (**2f**), 4-methoxyphenyl (pK_a = 10.20) (**2g**) or 2,6-dimethylphenyl (pK_a = 10.59)[31] (**2h**) was investigated.

The replacement of a phenyl (compound **2f**) by a *p*-methoxyphenyl group (compound **2g**) had only a marginal effect on the decomposition mode (Table 2); however, the introduction of a 2,6-dimethylphenyl group (hydroxyphosphonate **2h**) completely prevented degradation through the hydrolytic pathway ***B***. The fact that **2h** decomposes exclusively according to path ***A*** might explain its high antiviral potency. From the point of view of tuning chemical and biological properties of α-hydroxyphosphonates of type **2**, a potentially important feature is that the introduction of an electron rich aryl ester group (Ar^1) increases the stability of these compounds, as it is apparent from the corresponding $t_{1/2}$ values: **2f**, Ar^1 = phenyl, 15.9 min; **2g**, Ar^1 = 4-methoxyphenyl, 26.3 min; **2h**, Ar^1 = 2,6-dimethylphenyl 57.6 min (Table 2).

2.3. Chemistry underlying the *A*, *B* and *C* decomposition pathways

In 0.1 M phosphate buffer (pH 7.4), the decomposition of compounds **2a**–**i** followed the same initial steps observed in culture medium, although at 5- and 6-fold lower rates (data not shown). These findings are consistent with data by Meier et al. concerning the decomposition of dinucleoside hydroxybenzylphosphonates.[28]

Factors influencing decomposition of dinucleoside hydroxyphosphonates according to path ***C*** have been discussed in detail by Meier et al.[16] and seem to apply also to the decomposition of the aryl nucleoside α-hydroxyphosphonates investigated in this study. Formation of phosphotriesters **9** occurs most likely *via* a phosphonate-phosphate rearrangement[35,37] and involves an intramolecular nucleophilic attack of the hydroxyl function on the phosphorus centre of hydroxyphosphonates **2**, followed by the P–C bond scission. Since the latter process occurs in a rate-determining step and consists of movement of electrons towards the benzylic carbon of the hydroxyphosphonate, the carbanion-stabilizing substituents in Ar^2 should facilitate this process. Consistent with this mechanism, the pathway ***C*** was observed only for hydroxyphosphonate **2e** bearing the 4-nitrophenyl group (Ar^2).

The path ***B***, a $S_N2(P)$ reaction, is characteristic for hydroxyphosphonates bearing a good leaving group at the phosphorus and for this reason it was observed for aryl nucleoside α-hydroxyphosphonates **2**, but not for dinucleoside derivatives.[16] For the hydroxyphosphonates investigated, the decomposition pathway ***B*** became apparent only for compounds **2d**–**g**. All of them have electron-deficient Ar^2 groups (4-chlorophenyl, 4-nitrophenyl and 3-pyridyl) which apparently facilitate the nucleophilic attack at the phosphorus centre with expulsion of the phenoxy (for **2d**–**f**) or the *p*-methoxyphenoxy group (for **2g**). As expected, phenyl esters of α-hydroxyphosphonates **2** with electron-rich Ar^2 groups (e.g., **2a**–**c**, Ar^2 = phenyl, 4-methylphenyl and 4-methoxyphenyl, respectively) did not undergo this kind of reaction. A decreasing contribution of the hydrolytic path ***B*** within the series **2f** > **2g** > **2h** is consistent with the pK_a values of the leaving groups involved (phenyl, 4-methoxyphenyl, 2,6-dimethylphenyl, respectively; vide supra) and, in the case of the 2,4-dimethylphenolyl ester **2h**, with steric factors which may interfere with solvation of the 2,6-dimethylphenoxy moiety and thus lower its leaving group ability.

From the point of view of the potential antiviral activity of α-hydroxyphosphonates, the most important decomposition pathway is, apart from ***C***, path ***A*** which, ultimately, generates nucleoside *H*-phosphonate monoesters and the corresponding nucleosides. This seems to be a general pathway for decomposition of hydroxyphosphonates under hydrolytic conditions and is observed for both dinucleoside and aryl nucleoside hydroxyphosphonates.

Spontaneous generation of an aldehyde and the corresponding *H*-phosphonate diester, postulated by Meier

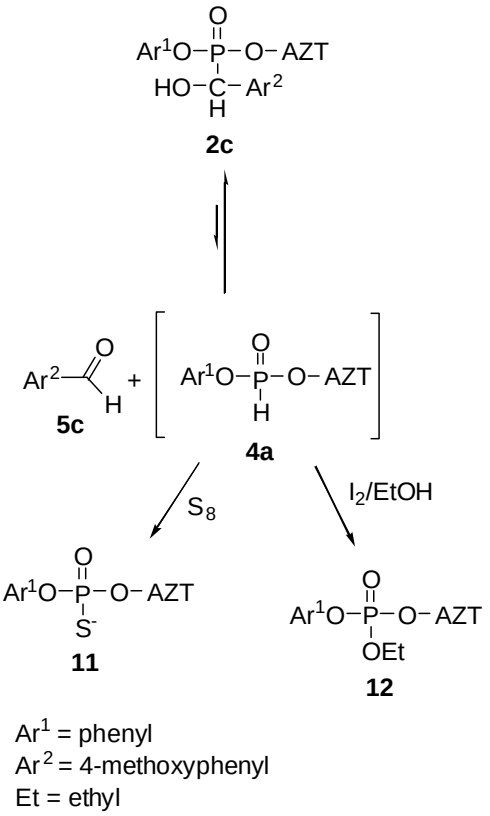
et al. for dinucleoside hydroxyphosphonates, was also observed with the aryl nucleosides **2** (Scheme 3, path ***A***). The most likely reason for this is that α-hydroxyphosphonates are adducts of *H*-phosphonate diesters and aldehydes, and this reaction is apparently reversible with the equilibrium shifted to the left. Under mildly basic hydrolytic conditions, an equilibrium concentration of an *H*-phosphonate diester is rapidly depleted due to fast hydrolysis of *H*-phosphonate diesters, and the equilibrium shifts to the right. This results in decomposition of α-hydroxyphosphonate derivatives according to pathway ***A***. For hydroxyphosphonates **2** (e.g., for compounds **2a**–**c**, **2h** and **2i**) for which this route was the only reaction pathway available, ^{31}P NMR spectroscopy revealed the presence of only starting material **2** and *H*-phosphonate monoester **6**.

To provide evidence for the existence of a true equilibrium between α-hydroxyphosphonate and the corresponding substrates, a trapping experiment was designed to show intermediacy of the *H*-phosphonate diester **4**, generated from a hydroxyphosphonate **2** under anhydrous conditions. For this purpose, α-hydroxyphosphonate **2c** was dissolved in methylene chloride containing elemental sulfur and left overnight. The ^{31}P NMR spectra of the reaction mixture showed that the starting material **2c** (four signals at ca. 19 ppm) disappeared and two new resonances in a region typical for aryl nucleoside phosphorothioate diesters[38] (δ_P 52.8 and 53.5 ppm; two diastereoisomers) appeared. After work-up and column chromatography, two compounds were isolated from the reaction mixture: the aldehyde **5c** and the AZT phenyl phosphorothioate **11** (^{1}H, ^{31}P NMR, TLC, comparison with authentic samples). In another experiment, α-hydroxyphosphonate **2c** was treated in acetonitrile with ethanol (10 molar equiv) and iodine (2 molar equiv) in the presence of triethylamine. After an overnight standing, the ^{31}P NMR spectroscopy revealed that **2c** was completely converted into AZT ethyl phenyl phosphate **12**. These results are consistent with the generation of *H*-phosphonate diester **4a** which, in the presence of elemental sulfur, underwent: sulfurization to the corresponding phosphorothioate **11**, or in the presence of iodine underwent oxidative coupling with ethanol to produce the phosphotriester **12**. These results support the postulated equilibrium between α-hydroxyphosphonate **2** and its substrate **5** + **4** (Scheme 4) involved in the decomposition of α-hydroxyphosphonates of type **2** according to path ***A***.

Although the above equilibria for all hydroxyphosphonates **2** investigated are completely to the left (equilibrium concentrations of the corresponding *H*-phosphonate diesters are usually below the detection level of ^{31}P NMR spectroscopy), it is apparent that the electronic nature of the aryl groups Ar^1 and Ar^2 may influence both the position of the equilibria and the rates with which these are established, and ultimately may determine the decomposition pathways of these compounds.

2.4. Antiviral potency of type 2 hydroxyphosphonates

α-Hydroxyphosphonates **2** were evaluated for their in vitro cytotoxicity and anti-HIV-1 activity in MT-4-based assays. The antiviral activity was determined against wild type HIV-1, strain IIIB using AZT as reference compound.



Scheme 4. Reactions of AZT phenyl hydroxy(4-methoxyphenyl)methanephosphonate with elemental sulfur under anhydrous conditions and with ethanol in the presence of iodine.

Taking into account the observed decomposition pathways of α-hydroxyphosphonates **2** in RPMI/FBS and assuming that both AZT *H*-phosphonate **6** and AZT monophosphate **10** probably act as depots for AZT,[15,39] it was expected that all α-hydroxyphosphonates **2** that decompose according to paths ***A*** and ***C*** should show similar EC_{50} indices. Data in Table 2 are in agreement with this general consideration. Small variation in antiviral activity (EC_{50} 0.01–0.06 μM) might be attributed to different rates and decomposition pathways of α-hydroxyphosphonates **2** to AZT *H*-phosphonate **6** (or phosphate **10**) in cell culture medium. The latter compound, being dephosphonylated by phosphatase-like activity of FBS, released ($t_{1/2}$ = 1175 min) the active pro-drug AZT. AZT phenyl α-hydroxyphosphonate **2e** (Ar^2 = 4-nitrophenyl), which decomposed via ***A***, ***B***, and ***C*** paths, did not show an improved selectivity index. Both activity and cytotoxicity for this compound may come from the produced AZT *H*-phosphonate **6** as well as from AZT 4-nitrobenzyl phosphotriester **9**, which itself was highly active but also very cytotoxic.

The family of α-hydroxyphosphonates **2f**–**h** bearing an electron-deficient Ar^2 group (pyridin-3-yl) and different aryl ester groups Ar^1 (phenyl, 4-methoxyphenyl and 2,6-dimethylphenyl, respectively) deserves some comments. α-Hydroxyphosphonate **2f** (Ar^1 = phenyl), which decomposed according to paths ***A*** and ***B***, showed high

anti-HIV potency (EC_{50} = 0.04 μM) and the lowest cytotoxicity (CC_{50} > 100 μM) among the compounds tested. These results were somewhat surprising in the light of the very low antiviral activity of **8** and also of the low antiviral potency but high cytotoxicity of nicotine aldehyde that was generated during the decomposition of **2f** (Table 2). The α-hydroxyphosphonate **2g** (Ar^1 = 4-methoxyphenyl) (EC_{50} = 0.01 μM) was more potent than **2f** (EC_{50} = 0.04 μM) but, at the same time, highly cytotoxic (CC_{50} = 27 μM), probably due to the release of 4-methoxyphenol (CC_{50} = 39 μM). When 4-methoxyphenyl was replaced by 2,6-dimethylphenyl as an Ar^1 group, compound **2h** underwent decomposition exclusively according to path ***A*** (vide supra) and, due to the low cytotoxicity of the generated 2,6-dimethylphenol (CC_{50} > 100 M), CC_{50} and EC_{50} indices for **2h** were identical to those of AZT alone. For this reason, compound **2h** may be interesting as a potential fragment of a more complex molecular framework because of its low rate of decomposition in cell culture medium.

Finally, we examined the anti-HIV potency of α-hydroxyphosphonate **2i** bearing 2′,3′-dideoxyuridine (ddU). Since ddU is not phosphorylated by cellular kinases,[40] it shows antiviral activity only when ddUMP is externally delivered.[41–43] The low anti-HIV potency observed for compound **2i** (Table 2) indicates that the α-hydroxyphosphonates **2** investigated herein act probably as nucleoside vehicles and not as pro-nucleotides.

3. Conclusions

We have developed efficient synthetic protocols for the preparation of aryl nucleoside α-hydroxyphosphonates **2** which allow the introduction of various combinations of aryl ester (Ar^1) and α-arylmethylphosphonate (Ar^2) groups. The method makes use of easily available nucleoside *H*-phosphonate monoesters which, upon in situ conversion into the corresponding aryl nucleoside *H*-phosphonate diesters **4**, are subjected to the reaction with aromatic aldehydes **5** to produce α-hydroxyphosphonates **2** with high yields.

Aryl nucleoside α-hydroxyphosphonates **2** can be considered as potential antiviral drugs, whose reactivity can be modulated by the proper choice of Ar^1 and Ar^2 groups. Stability studies in cell culture medium showed that, depending on the electronic structure of the aryl groups, α-hydroxyphosphonates **2** may undergo decomposition via three reaction pathways, ***A***, ***B*** and ***C***, to produce the corresponding nucleosides, nucleotides, phenols, and aldehydes.

Biological studies showed that the antiviral activity of α-hydroxyphosphonates **2** mostly correlated with the released AZT *H*-phosphonate monoester, and AZT as a final metabolite. This is in line with the observed chemical decomposition pathways for the investigated compounds. All α-hydroxyphosphonates **2** showed high antiviral activity and it seems that their cytotoxicity can be controlled (and probably improved) by a proper combination of Ar^1 and Ar^2 groups. At the present stage of investigations, α-hydroxyphosphonates **2f–h** bearing a 3-pyridyl moiety as Ar^2 and different aryl ester groups Ar^1 are promising candidates for incorporation into a more complex molecular framework to be evaluated for their antiviral activity.

Finally, it is worth noting that all α-hydroxyphosphonates **2** studied herein are very stable under acidic conditions (e.g., phosphate buffer, pH 2.0, >6 days; HPLC analysis). This property, together with high lipophilicity of α-hydroxyphosphonates **2** (Table 1), makes these types of compounds attractive candidates for oral administration in AIDS therapy.

4. Experimental

^1H and ^{31}P NMR spectra were recorded on a 300 MHz spectrometer. The ^{31}P NMR experiments were carried out in 5 mm tubes using 0.1 mol/mL concentration of phosphorus-containing compounds and using H_3PO_4 as external reference (inner tube). For column chromatography, Kieselgel 60 Merck was used. The amount of water in solvents was measured with Karl Fisher coulometric titration. Methylene dichloride was dried over P_2O_5, distilled and kept over molecular sieves 4 Å until the amount of water was less than 10 ppm. Pyridine and 2,6-lutidine were stored over molecular sieves 4 Å until the amount of water was below 20 ppm. Triethylamine and diisopropylethylamine were distilled and stored over CaH_2. Aldehydes **5a–f** were of commercial grade from Aldrich. RPMI-1640 cell culture medium and foetal bovine serum (FBS) used for studies of stability of compounds were from Sigma. HPLC analysis was carried out on a Hypersil ODS column (4.6 × 250 mm, 5 μm); flow rate 1.5 mL/min; solvent A—0.01 M triethylammonium trifluoroacetate, pH 3.02; solvent B—A/acetonitrile 4:1 (v/v); events: 5 min A 100%, linear gradient of B 0–100% in 40 min, A 100%—15 min wash. For quantification of peaks LKB 2221 integrator was used. Octanol/water partition coefficients were determined using the published procedure.[44]

The reference compounds used for the identification of some reaction products or intermediates were obtained as follows: AZT *H*-phosphonate **6** and ddU *H*-phosphonate in the reaction of 3′-azido-3′-deoxythymidine or 2′,3′-dideoxyuridine with pyrophosphonic acid in pyridine;[45] AZT phenyl phosphorothioate **11** was obtained following the earlier described procedure;[38] phosphotriesters **9** and **12** were obtained by condensation of phosphate diester **3** with 4-nitrobenzyl alcohol or ethanol aided with 2,4,6-triisopropylbenzenesulfonyl chloride in methylene chloride in the presence of *N*-methylimidazole.[46]

4.1. Antiviral assay procedures

4.1.1. Compounds. Compounds subjected to biological assays were solubilized in DMSO at 200 mM and then diluted in culture medium.

4.1.2. Cells and viruses. MT-4, C8166, and H9/IIIB cells were grown at 37 °C in a 5% CO_2 atmosphere in RPMI 1640 medium, supplemented with 10% foetal calf serum

(FCS), 100 IU/mL penicillin G and 100 μg/mL streptomycin. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco). Human immunodeficiency viruses type-1 (HIV-1, IIIB strain) were obtained from supernatants of persistently infected H9/IIIB cells. The HIV-1 stock solutions had titres of 4.5×10^6 50% cell culture infectious dose ($CCID_{50}$)/mL.

4.1.3. HIV titration. Titration of HIV was performed in C8166 cells by the standard limiting dilution method (dilution 1:2, four replica wells per dilution) in 96-well plates. The infectious virus titre was determined by light microscope scoring of syncytia after 4 days of incubation. Virus titres were expressed as $CCID_{50}$/mL.

4.1.4. Anti-HIV assays. The activity of test compounds against multiplication of wt HIV-1, in acutely infected cells, was based on the inhibition of virus-induced cytopathicity in MT-4 cells. Briefly, 50 μL of culture medium containing 1×10^4 cells was added to each well of flat-bottom microtiter trays containing 50 μL culture medium with or without various concentrations of test compounds. Then 20 μL of HIV suspensions (containing the appropriate amount of $CCID_{50}$ to cause complete cytopathicity at day 4) was added. After incubation at 37 °C, cell viability was determined by the 3-(4,5-dimethylthiazol-1-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Alternatively, p24 levels were determined by an immunoenzymatic kit (Abbott). The cytotoxicity of test compounds was evaluated in parallel with their antiviral activity and was based on the viability of mock-infected cells, as monitored by the MTT method.

4.2. General procedure for the synthesis of aryl nucleoside α-hydroxyphosphonates of type 2

Nucleosid-5′-yl *H*-phosphonate (1 molar equiv) and the respective phenol (1.5 molar equiv) were rendered anhydrous by co-evaporation of added pyridine and then were dissolved in methylene chloride containing pyridine [10% (v/v)] (0.1 mmol/1 mL). The coupling was effected by the addition of diphenyl chlorophosphate (DPCP, 3 molar equiv) to the reaction mixture. When the formation of aryl nucleosid-5′-yl *H*-phosphonate **4** was completed (ca. 20 min., ^{31}P NMR), aldehyde **5** (5 molar equiv) and diisopropylethylamine (DIPEA, 2.5 molar equiv; for aldehydes **5a**–**d**) were added. After the reactions were completed (10–80 min, ^{31}P NMR) the reaction mixture was diluted with methylene chloride (three times the initial volume) and acetic acid (1 molar equiv) was added to neutralize excess amine. The resulting mixture was washed with water, organic layer separated, and after drying (Na_2SO_4 anhyd) it was evaporated under vacuum.

When instead of DIPA, 2,6-lutidine [10% (v/v)] was used as a base in condensation of nucleosid-5′-yl *H*-phosphonates and phenols the reactions were carried out in methylene chloride containing 2,6-lutidine [10% (v/v)] (0.1 mmol/1 mL) using the same ratios of reagents (condensations were usually completed in 20 min, ^{31}P NMR). To produce aryl nucleosid-5′-yl *H*-phosphonates **4**, a respective aldehyde **5** was added (5 molar equiv) and the reaction mixture was left standing overnight at rt. After completion of the reaction (^{31}P NMR), the mixture was diluted with methylene chloride, washed with aqueous 1 M KH_2PO_4, pH 4.0, and worked-up as described above.

α-Hydroxyphosphonates **2** were isolated by a silica gel 60 column chromatography using a stepwise gradient (0–10%) of isopropanol in methylene chloride. Fractions containing pure products were collected and evaporated yielding non-hygroscopic foams. After freeze–drying from benzene, compounds **2** were obtained usually as white amorphous solids (yellow for *p*-nitrobenzyl derivatives).

4.3. 3′-Azido-3′-deoxythymidin-5′-yl phenyl hydroxy(phenyl)methanephosphonate (2a)

Obtained from *H*-phosphonate diester **4a** and benzaldehyde **5a**. The addition reaction in the presence of DIPEA was completed in 10 min. Yield 0.18 g (70%). ^1H NMR ($CDCl_3$) δ 7.54–7.05 (m, 11H), 6.13–6.03 (m, 1H), 5.54, 5.26, 5.22 (3d, 1H, $J = 3.3$, 2.7, and 2.7 Hz), 4.34–3.89 (m, 4H), 2.36–1.90 (m, 2H), 1.83, 1.81, 1.78, 1.77 (4d, 3H, $J = 1.2$ Hz); HRMS $[MH]^+$: 514.1486, calcd for $C_{23}H_{25}O_7N_5P$: 514.1491.

4.4. 3′-Azido-3′-deoxythymidin-5′-yl phenyl α-hydroxy (4-methylphenyl)methanephosphonate (2b)

Obtained from *H*-phosphonate diester **4a** and 4-methylbenzaldehyde **5b**. The addition reaction in the presence of DIPEA was completed in 40 min. Yield 0.38 g (77%). ^1H NMR ($CDCl_3$) δ 7.41–7.06 (m, 10H), 6.15–6.04 (m, 1H), 5.24–5.20 (m, 1H), 4.32–3.89 (m, 4H), 2.33 (s, 3H), 2.29–1.85 (m, 2H), 1.80, 1.78, 1.75 (3s, 3H); HRMS $[MH]^+$: 528.1650, calcd for $C_{24}H_{27}O_7N_5P$: 528.1648.

4.5. 3′-Azido-3′-deoxythymidin-5′-yl phenyl α-hydroxy (4-methoxyphenyl)methanephosphonate (2c)

Obtained from *H*-phosphonate diester **4a** and 4-methoxybenzaldehyde **5c**. The addition reaction in the presence of DIPEA was completed in 80 min. Yield 0.46 g (73%). ^1H NMR ($CDCl_3$) δ 7.44–6.88 (m, 10 H), 6.16–6.05 (m, 1H), 5.19–5.14 (m, 1H), 4.36–3.90 (m, 4H), 3.82, 3.80, 3.79 (3s, 3H), 2.39–1.90 (m, 2H), 1.85, 1.83, 1.80, 1.79 (3d, 3H, $J = 1.2$, 1.2, and 1.2 Hz); HRMS $[MH]^+$: 544.1584, calcd for $C_{24}H_{27}O_8N_5P$: 544.1597.

4.6. 3′-Azido-3′-deoxythymidin-5′-yl phenyl hydroxy (4-chlorophenyl)methanephosphonate (2d)

Obtained from *H*-phosphonate diester **4a** and 4-chlorobenzaldehyde **5d**. The addition reaction in the presence of DIPEA was completed in 10 min. Yield 0.42 g (77%). ^1H NMR ($CDCl_3$) δ 7.46–7.03 (m, 10H), 6.08–5.98 (m, 1H), 5.27, 5.24, 5.23, 5.19 (4s, 1H), 4.40–4.10 (m, 3H), 3.99–3.90 (m, 1H), 2.43–2.05 (m, 2H), 1.83, 1.80, 1.79 (s, 2d, 3H, $J = 1.2$ and 0.9 Hz). HRMS $[MH]^+$ 548.1106, calcd for $C_{23}H_{24}O_7N_5\,{}^{35}ClP$: 548.1102.

4.7. 3′-Azido-3′-deoxythymidin-5′-yl phenyl hydroxy(4-nitrophenyl)methanephosphonate (2e)

Obtained from *H*-phosphonate diester **4a** and 4-nitrobenzaldehyde **5e**. The addition reaction in the presence of 2,6-lutidine was completed in 45 min. Yield 0.92 g (80%). ^{1}H NMR ($CDCl_3$) δ 8.22–8.19, 7.75–7.68 (2m, 4H), 7.26–7.04 (m, 6H), 5.98–5.88 (m, 1H), 5.45–5.35 (m, 1H), 4.47–4.23 (m, 3H), 4.01–3.89 (m, 1H), 2.54–2.27 (m, 2H), 1.85, 1.84, 1.82 (3d, 3H, J = 1.2, 0.9, and 1.2 Hz); HRMS $[MH]^+$: 559.1337, calcd for $C_{23}H_{24}O_9N_6P$: 559.1342.

4.8. 3′-Azido-3′-deoxythymidin-5′-yl phenyl hydroxy(pyridin-3-yl)methanephosphonate (2f)

Obtained from *H*-phosphonate diester **4a** and pyridin-3-aldehyde **5f**. The addition reaction in the presence of 2,6-lutidine was completed in 24 h. Yield 1.16 g (55%). ^{1}H NMR ($CDCl_3$) δ 8.70–8.67 (m, 1H), 8.52–8.50 (m, 1H), 7.90–7.84 (m, 1H), 7.30–7.02 (m, 7H), 5.92 (m, 1H), 5.30–5.25 (m, 1H), 4.44–4.16 (m, 3H), 3.91–3.90 (m, 1H), 2.30–2.24 (m, 2H), 1.82–1.77 (m, 3H); HRMS $[MH]^+$: 515.1427, calcd for $C_{22}H_{24}O_7N_6P$: 515.1444.

4.9. 3′-Azido-3′-deoxythymidin-5′-yl 4-methoxyphenyl hydroxy(pyridin-3-yl)methanephosphonate (2g)

Obtained from *H*-phosphonate diester **4b** and pyridin-3-aldehyde **5f**. The addition reaction in the presence of 2,6-lutidine was completed in 24 h. Yield 0.19 g (52%). ^{1}H NMR ($CDCl_3$) δ 8.68 (br m, 1H), 8.50 (br m, 1H), 7.90–7.82 (m, 1H), 7.23–6.67 (m, 6H), 5.90 (br m, 1H), 5.26–5.23 (m,1H), 4.37–4.15 (m, 3H), 3.93–3.88 (m, 1H), 3.70, 3.69 (2s, 3H), 2.26 (br m, 2H), 1.77 (s, 3H). HRMS $[MH]^+$: 545.1552, calcd for $C_{23}H_{26}O_8N_6P$: 545.1550.

4.10. 3′-Azido-3′-deoxythymidin-5′-yl 2,6-dimethylphenyl hydroxy(pyridin-3-yl)methanephosphonate (2h)

Obtained from *H*-phosphonate diester **4c** and pyridin-3-aldehyde **5f**. The addition reaction in the presence of 2,6-lutidine was completed in 48 h. Yield 0.15 g (60%). ^{1}H NMR ($CDCl_3$) δ 8.78–8.75 (m, 1H), 8.52 (s, 1H), 8.06–7.7 (m, 1H), 7.38–6.93 (m, 7H), 6.02–5.79 (m, 1H), 5.44–5.30 (m, 1H), 4.45–4.31 (m, 1H), 4.21–4.09 (m, 2H), 3.97–3.83 (m, 1H), 2.43–2.28 (m, 2H), 2.14, 2.13 (2s, 6H), 1.90, 1.86, 1.85 (3d, 3H, J = 0.6, 0.9, and 1.2 Hz). HRMS $[MH]^+$: 543.1748, calcd for $C_{24}H_{28}O_7N_6P$: 543.1757.

4.11. 2′,3′-Dideoxy-2′,3′-didehydrouridin-5′-yl phenyl α-hydroxy(4-methoxyphenyl)methanephosphonate (2i)

Obtained from *H*-phosphonate diester **4d** and 4-methoxybenzaldehyde **5c**. The addition reaction in the presence of DIPEA was completed in 2 h. Yield 0.22 g (63%). ^{1}H NMR ($CDCl_3$) δ 7.46–6.87 (m, 10H), 6.06–5.91 (m, 1H), 5.66, 5.62, 5.54, 5.51 (4d, 1H, J = 8.1, 7.5, and 8.7 Hz), 4.32–4.15 (m, 3H), 3.81, 3.80, 3.80, 3.79 (4s, 3H), 2.40–1.64 (m, 4H). HRMS $[MH]^+$: 488.1342, calcd for $C_{23}H_{25}N_2O_8P$: 488.1349.

4.12. Synthesis of reference nucleoside α-hydroxyphosphonates 8

Aryl nucleoside α-hydroxyphosphonate **2** (0.5 mmol) was dissolved in a mixture of acetonitrile/water/triethylamine 2:1:1 (v/v) (5 mL). After the reaction was completed (^{31}P NMR), the solvents were evaporated and the residue dissolved in methylene chloride was applied into the silica gel column prepared in isopropanol. Reaction mixtures consist of variable amounts of **8** (**8a** 47%, **8b** 27%, and **8c** 82%) and other decomposition products. Compounds **8a**–**c** were isolated using a stepwise gradient of water [0–6% (v/v)] in isopropanol containing triethylamine [3% (v/v)]. Fractions containing pure products **8** were collected and evaporated yielding a white foam. After freeze–drying from benzene/methanol, compounds **8a**–**c** were obtained as non-hygroscopic white amorphous solids.

4.13. 3′-Azido-3′-deoxythymidin-5′-yl hydroxy(4-chlorophenyl)methanephosphonate triethylammonium salt (8a)

^{1}H NMR ($CDCl_3$) δ 7.69,7.63 (2d, 1H, J = 0.9 and 1.2 Hz), 7.52–7.47 (m, 2H), 7.28–7.26 (m, 2H) 6.19–6.12 (m, 1H) 4.92, 4.90, 4.88, 4.85 (4s, 1H), 4.41–4.37 (m, 1H), 4.30–3.94 (m, 3H), 2.92 (q, J = 7.4 Hz, 6H) 2.38–2.26 (m, 2H),1.93–1.92 (m, 3H), 1.18 (t, J = 7.4 Hz, 9H). HRMS $[M]^-$: 470.0646, calcd for $C_{17}H_{18}O_7N_5P$: 470.0632.

4.14. 3′-Azido-3′-deoxythymidin-5′-yl hydroxy(4-nitrophenyl)methanephosphonate (8b)

^{1}H NMR ($CDCl_3$) δ 8.18–8.15 (m, 2H), 7.78–7.73 (m, 2H), 7.66, 7.62 (2d, J = 1.2 and 0.9 Hz, 1H), 6.15–6.08 (m, 1H), 5.08, 5.06, 5.03, 5.00 (4s, 1H), 4.40–4.37 (m, 1H), 4.30–3.91 (m, 3H), 2.94 (q, J = 7.4 Hz, 6H), 2.41–2.28 (m, 2H), 1.93 (s, 3H), 1.17 (t, J = 7.4 Hz, 9H); HRMS $[M]^-$: 481.0888, calcd for $C_{17}H_{18}O_9N_6P$: 481.0873.

4.15. 3′-Azido-3′-deoxythymidin-5′-yl hydroxy(pyridin-3-yl)methanephosphonate (8c)

^{1}H NMR ($CDCl_3$) δ 8.76–8.75 (m, 1H), 8.46–8.44 (m, 1H), 7.95–7.89 (m, 1H), 7.69, 7.65, (2d, J = 1.2 and 1.2 Hz), 7.27–7.23 (m, 1H), 6.18–6.11 (m, 1H), 4.98, 4.96, 4.94, 4.91 (4s, 1H), 4.44–4.40 (m, 1H), 4.30–4.02 (m, 3H), 2.87 (q, J = 7.4 Hz, 6H), 2.44–2.24 (m, 2H), 1.92–1.91 (m, 3H), 1.13 (t, J = 7.4 Hz, 9H); HRMS $[M]^-$: 437.0983, calcd for $C_{16}H_{18}O_7N_6P$: 437.0975.

4.16. 3′-Azido-3′-deoxythymidin-5′-yl 4-nitrobenzyl phenyl phosphate (9)

Yield 0.185 g (73%). ^{1}H NMR ($CDCl_3$) δ 8.78–8.76 (m, 1H), 8.23–8.17 (m, 2H), 7.51–7.15 (m, 8H), 6.19–6.15 (m, 1H), 5.30,5.28, 5.27, 5.25 (4s, 2H), 4.48–4.24 (m, 3H), 4.03–4.01 (m, 1H), 2.48–2.24 (m, 2H), 1.86, 1.82 (2d, J = 1.2 and 1.2 Hz); HRMS $[MH]^+$: 559.1322, calcd for $C_{23}H_{24}O_9N_6P$: 559.1342.

4.17. 3′-Azido-3′-deoxythymidin-5′-yl phenyl phosphorothioate triethylammonium salt (11)

Yield 0.069 g (64%). ^{1}H NMR ($CDCl_3$) δ 8.16 (s, 1H), 7.83, 7.75 (2d, J = 1.2 and 1.2 Hz), 7.15–7.09 (m, 4H), 6.95–6.93 (m, 1H), 6.33–6.27 (m, 1H), 4.43–4.28 (m, 3H), 4.09–4.08 (m, 1H)3.15 (q, J = 7.4 Hz, 6H), 2.37–2.25 (m, 2H), 1.93, 1.90 (2d, J = 1.2 and 1.2 Hz, 3H), 1.28 (t, J = 7.4 Hz, 9H); HRMS $[M]^-$: 438.0625, calcd for $C_{16}H_{17}O_6N_5P$: 438.0637.

4.18. 3′-Azido-3′-deoxythymidin-5′-yl ethyl phenyl phosphate (12)

Yield 0.195 g (86%). ^{1}H NMR ($CDCl_3$) δ 8.59 (s, 1H), 7.40–7.19 (m, 6H), 6.26–6.20 (m, 1H), 4.67–4.21 (m, 5H), 4.06–4.02 (m, 1H), 2.45–2.19 (m, 2H), 1.88, 1.86 (2d, J = 0.9 and 1.2 Hz, 3H), 1.40–1.33 (m, 3H); HRMS $[MH]^+$: 451.1263, calcd for $C_{18}H_{22}N_5O_7P$: 451.1257.

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