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Aryl nucleoside H-phosphonates. Part 16: Synthesis and anti-HIV-1 activity of di-aryl nucleoside phosphotriesters

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ABSTRACT

Di-aryl nucleoside phosphotriesters have been explored as a new type of pronucleotides for the purpose of anti-HIV-1 therapy and efficient synthetic protocols, based on H-phosphonate chemistry, have been developed for the preparation of this class of compounds. It was found that anti-HIV-1 activity of the phosphotriesters bearing an antiviral nucleoside moiety (AZT, ddA) and also ddU was due, at least partially, to intracellular conversion into the corresponding nucleoside 5′-monophosphates, and their efficiency correlated well with the pK_a values of the aryloxy groups present.

1. Introduction

To disclose their antiviral activity, nucleoside analogues have to be converted with the help of cellular enzymes, such as nucleoside and nucleotidyl kinases, into the respective nucleoside 5′-triphosphates (NTP).[1] The formation of triphosphates occurs in a stepwise manner and usually the first phosphorylation step, that is, the synthesis of nucleoside 5′-monophosphates, is the crucial step for NTP formation. Since phosphorylation is indispensable for biological activity, nucleoside analogues that are poor substrate for phosphorylating enzymes (e.g., 2′,3′-dideoxyuridine), are usually inactive.[2] Due to this, nucleoside analogues lose their antiviral potency in nucleoside kinase deficient cells.[3]

To by-pass this enzymatic monophosphorylation step, efforts were focused on delivery into the cell 5′-monophosphates of nucleoside analogues[4] (for review[5–8]). Unfortunately, under physiological conditions, nucleoside 5′-monophosphates exist as dianions and cannot cross negatively charged cell membranes.[9] Hence, it was assumed that if a phosphate moiety of mononucleotide is properly masked and became neutral, this should facilitate cell membrane penetration and increase concentration of drug inside the cell. This idea, called pronucleotide approach, triggered studies on various types of nucleotide derivatives, whose intracellular conversion to the desired nucleoside 5′-monophosphates would occur via chemical and/or enzymatic hydrolysis of the phosphate masking groups. Typical examples include cyclic phosphate derivatives (e.g., nucleoside saligenyl phosphates, cyclo-Sal),[10] or nucleoside phosphoramidates.[11,12] Another class of pronucleotides is represented by pivaloyloxymethyl (POM[7]), dithioethyl (DTE[13]) and S-acyl-2-thioethyl (SATE[13–15]) nucleoside phosphotriesters, whose bio-activation is triggered by enzymatic cleavage of a carboxylic acid ester group (POM and SATE), or a disulfide bond of the side chain (DTE), followed by a spontaneous intramolecular conversion into the corresponding nucleoside monophosphates. Synthesis and biological activity of these compounds were widely studied and were subjects of several reviews.[5,6]

Somehow surprisingly, simple alkyl[16] or aryl[17] nucleoside phosphotriesters have not received much attention, most likely due to mediocre anti-HIV-1 activity (exception, bis-4-nitrophenyl AZT phosphate) observed in the early experiments.[18] However, since there is a huge choice of alkyl and particularly aryl groups, with different structural and electronic features, finding a good masking group should be a feasible task.

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In practice, the problem is more complex because an ideal pronucleotide has to fulfil several, sometimes contradictory, criteria. Specifically, (i) a pronucleotide should have proper stability in physiological media and be converted inside the cell into biologically active form with optimal kinetics, (ii) it should be well soluble in water, (iii) it should be neutral and/or sufficiently lipophilic to be able to cross cell membrane and enter the cell, (iv) the pronucleotides and their metabolites should not be toxic.

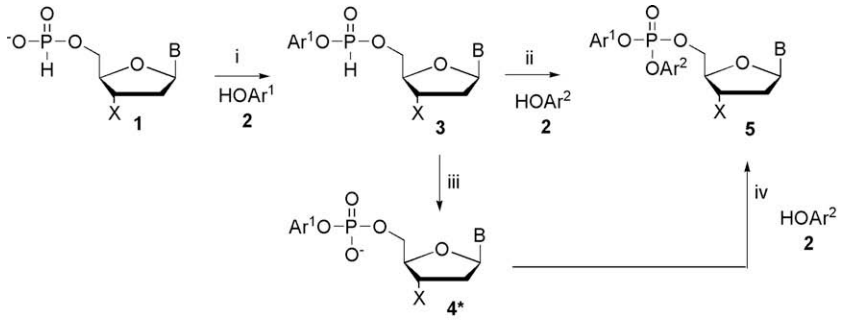
During our studies on anti-HIV-1 activity of uncharged nucleoside α-hydroxyphosphonates,[19] we noticed that compounds, bearing a pyridyl residue in the C-phosphonate fragment of the molecule, were well soluble in water, had significant antiviral activity and were relatively non-toxic. Favourable physicochemical properties prompted us to investigate phosphotriesters bearing a pyridyl protecting group (e.g., AZT, ddA and ddU derivatives), as potential pronucleotides.

2. Results and discussion

2.1. Synthesis of aryl nucleoside phosphodiesters of type 4 and di-aryl nucleoside phosphotriesters of type 5

For the synthesis of nucleoside phosphotriesters of type **5** we investigated two approaches. The first one, a 'step by step' condensation (classical phosphotriester approach[20,21]), consisted of coupling of respective aryl nucleoside phosphodiesters of type **4** and phenols **2**, using TPS-Cl/NMeIm reagent system.[22] This approach was of our interest because of an easy synthetic access in our laboratory to a variety of aryl nucleoside phosphodiesters[23] and a wide commercial offer of phenol or pyridinol derivatives. Preliminary experiments showed (^{31}P NMR analysis) that condensation of 3′-azido-3′-deoxythymidine (AZT) pyridin-3-yl phosphodiester **4af** with phenol **2a** produced rapidly (ca. 15 min) the expected AZT phenyl pyridin-3-yl phosphotriester **5aa** almost quantitatively. Unfortunately, in contrast to this, under the same reaction conditions, condensations of phenyl phosphodiesters **4aa** with pyridin-3-ol **2f** were very slow and produced a complex mixture of products (^{31}P NMR analysis). Although this approach worked well in some cases, it cannot be considered as a general synthetic method for the preparation of nucleoside phosphotriesters.

The second approach studied, namely, an iodine promoted oxidative coupling of aryl nucleoside H-phosphonate diesters of type **3** with 5 M excess of a phenol or pyridinol of type **2**, provided nearly quantitatively (^{31}P NMR analysis) the desired phosphotriesters **5** (Scheme 1). The starting aryl nucleoside H-phosphonates **3**, were easily accessible via a condensation of nucleoside H-phosphonates **1** with various phenols or pyridinols ($OHAr^1$ **2**) (1.2 M - equiv), promoted by diphenyl chlorophosphate (DPCP) or pivaloyl chloride (PvCl), in methylene chloride (or other neutral solvent) in the presence of a limited amount of pyridine.[24] Due to their high



2a, Ar = phenyl
2b, Ar = 4-chlorophenyl
2c, Ar = 4-bromophenyl
2d, Ar = 4-cyanophenyl
2e, Ar = 2,4-dichlorophenyl
2f, Ar = pyridin-3-yl
2g, Ar = pyridin-4-yl
2h, Ar = 2-methylpyridin-3-yl
2i, Ar = 6-methylpyridin-3-yl
2j, Ar = 2-chloropyridin-3-yl
2k, Ar = 5-chloropyridin-3-yl

*- for description of **4** see ***Scheme 2***

3aa, B = 5-methyluracil-1-yl, X = N_3, Ar = phenyl
3ab, B = 5-methyluracil-1-yl, X = N_3, Ar = 4-chlorophenyl
3ac, B = 5-methyluracil-1-yl, X = N_3, Ar = 4-bromophenyl
3ad, B = 5-methyluracil-1-yl, X = N_3, Ar = 4-cyanophenyl
3ae, B = 5-methyluracil-1-yl, X = N_3, Ar = 2,4-dichlorophenyl
3af, B = 5-methyluracil-1-yl, X = N_3, Ar = pyridin-3-yl
3ag, B = 5-methyluracil-1-yl, X = N_3, Ar = pyridin-4-yl
3ah, B = 5-methyluracil-1-yl, X = N_3, Ar = 2-methyl-pyridin-3-yl
3ai, B = 5-methyluracil-1-yl, X = N_3, Ar = 6-methylpyridin-3-yl
3aj, B = 5-methyluracil-1-yl, X = N_3, Ar = 2-chloropyridin-3yl
3ak, B = 5-methyluracil-1-yl, X = N_3, Ar = 5-chloropyridin-3-yl
3ba, B = uracil-1-yl, X = H, Ar = phenyl
3bb, B = uracil-1-yl, X = H, Ar = pyridin-3-yl
3c, B = adenin-9-yl, X = H, Ar = phenyl

5aa, B = 5-methyluracil-1-yl, X = N_3, Ar^1 = phenyl, Ar^2 = pyridin-3-yl
5ab, B = 5-methyluracil-1-yl, X = N_3, Ar^1 = phenyl, Ar^2 = pyridin-4-yl
5ac, B = 5-methyluracil-1-yl, X = N_3, Ar^1 = 4-chlorophenyl, Ar^2 = pyridin-3-yl
5ad, B = 5-methyluracil-1-yl, X = N_3, Ar^1 = 4-bromophenyl, Ar^2 = pyridin-3-yl
5ae, B = 5-methyluracil-1-yl, X = N_3, Ar^1 = 4-cyanophenyl, Ar^2 = pyridin-3-yl
5af, B = 5-methyluracil-1-yl, X = N_3, Ar^1 = 2,4-dichlorophenyl, Ar^2 = pyridin-3-yl
5ag, B = 5-methyluracil-1-yl, X = N_3,Ar^1 = pyridin-3-yl, Ar^2 = pyridin-3-yl
5ah, B = 5-methyluracil-1-yl, X = N_3, Ar^1 = pyridin-3-yl, Ar^2 = pyridin-4-yl
5ai, B = 5-methyluracil-1-yl, X = N_3, Ar^1 =2-methylpyridin-3-yl, Ar^2 = 2-methylpyridin-3-yl
5aj, B = 5-methyluracil-1-yl, X = N_3, Ar^1 =6-methylpyridin-3yl, Ar^2 = 6-methylpyridin-3-yl
5ak, B = 5-methyluracil-1-yl, X = N_3, Ar^1 =phenyl, Ar^2 = 2-methylpyridin-3-yl
5al, B = 5-methyluracil-1-yl, X = N_3, Ar^1 = phenyl, Ar^2 = 6-methylpyridin-3-yl
5am, B = 5-methyluracil-1-yl, X = N_3, Ar^1 =phenyl, Ar^2 = 2-chloropyridin-3-yl
5an, B = 5-methyluracil-1-yl, X = N_3, Ar^1 =phenyl, Ar^2 = 5-chloropyridin-3-yl
5ba, B = uracil-1-yl, X = H, Ar^1 = phenyl, Ar^2 = pyridin-3-yl
5bb, B = uracil-1-yl, X = H, Ar^1 = phenyl, Ar^2 = pyridin-4-yl
5bc, B = uracil-1-yl, X = H, Ar^1 = pyridin-3-yl, Ar^2 = pyridin-4-yl
5c, B = adenin-9-yl, X = H, Ar^1 = phenyl, Ar^2 = pyridin-3-yl

Scheme 1. General scheme for the synthesis of di-aryl nucleoside phosphotriesters of type **5**. Reagents and conditions: (i) diphenyl chlorophosphate (DPCP −1.2 M equiv) in CH_2Cl_2/pyridine 9:1 (v/v); (ii) I_2 (1.5 M equiv) in pyridine (0.3 M solution); (iii) I_2 (1.5 M equiv) in pyridine/water (4:1, v/v); (iv) 2,4,6-tri-isopropylbenzenesulfonyl chloride (TPS-Cl, 3 M equiv) in methylene chloride containing *N*-methylimidazole (NMeIm, 10% by volume).

reactivity,[24] H-phosphonate diesters of type **3** were not isolated, but subjected in situ to the reaction with a second phenol or pyridinol $OHAr^2$ **2** (5 M equiv), in the presence of iodine (1.5–2 equiv). This produced rapidly (<3 min) and cleanly (^{31}P NMR analysis) the desired di-aryl nucleoside phosphotriesters **5**.

In these reactions, 5 M excess of $OHAr^2$ **2** was necessary to ensure rapid formation of unsymmetrical (Ar^1–Ar^2) di-aryl phosphotriester **5**. Under such reaction conditions, symmetrical phosphotriesters (Ar^1–Ar^1) were not formed as judged from the ^{31}P NMR spectra and chromatographic analysis (HRTLC).

These reactions are believed to proceed via iodophosphate intermediates,[25–27] that in the presence of pyridine, reacted rapidly with hydroxylic components to produce di-aryl phosphotriesters of type **5**. Since the whole reaction sequence, from **1** to **5**, proceeded cleanly and nearly quantitatively (^{31}P NMR analysis), one could consider it a simple and efficient one-pot approach to the synthesis of diaryl nucleoside phosphotriesters. After purification by a silica gel short column chromatography[28] and freeze-drying from benzene, compounds **5** were obtained as amorphous solids in satisfactory yields (50–70%). The correctness of their structures and high purity were proven by spectral (^1H, ^{31}P NMR, and HRMS) and chromatographic (HPLC) analyses.

2.2. Decomposition of aryl nucleoside phosphodiesters 4 in RPMI and cell culture media [RPMI/FBS 9:1 (v/v)]

For phosphotriesters of type **5** to act as true pronucleotides, both steps in their intracellular conversion into the corresponding nucleoside 5′-monophosphates, that is, chemical hydrolysis to produce nucleoside phosphodiester, and the subsequent enzymatic hydrolysis to nucleoside 5′-phosphate (Scheme 2), are equally important. In the first step, chemical properties of a phosphotriester masking groups permit usually prediction of its hydrolytic stability. Rates of the removal of protecting groups from phosphodiesters are often difficult to predict, due to enzymatic catalysis usually involved in this step. Considering that the leaving groups are often structurally unrelated to natural substrates of enzymatic reaction (nucleoside or nucleotide), it was necessary to check experimentally susceptibility of nucleoside phosphodiesters of type **4** (Scheme 2) to enzymatic hydrolysis. To this end, we prepared a set of aryl nucleoside phosphodiesters **4aa**–**cb,** which were the expected intermediates during conversion of pronucleotides **5** into the corresponding nucleotide 5′-monophosphates **6**, and examined their stability in RPMI and in RPMI/FBS 9:1 (v/v) media. These experiments should provide information to what extent these compounds were prone to simple chemical hydrolysis (experiments in RPMI), and how good substrates they were to phosphoesterases (experiments in RPMI/FBS media). Although enzymatic activities present in the cell culture media (from FBS) are ca. 10–50 times weaker than those found in cells,[29,30] it is usually assumed that data from the experiments in RPMI and RPMI/FBS can be extrapolated to the analogous metabolic events in the living cell. It was found that all aryl nucleoside phosphodiesters **4aa–cb** (2 μM solutions) were stable within 6 days in RPMI at 37 °C (HPLC analysis). Their stability under analogous conditions but in RPMI/FBS varied and depended on a phosphoester aryl group, type of modification in the sugar ring, and a nucleobase in the nucleoside moiety (Table 1).

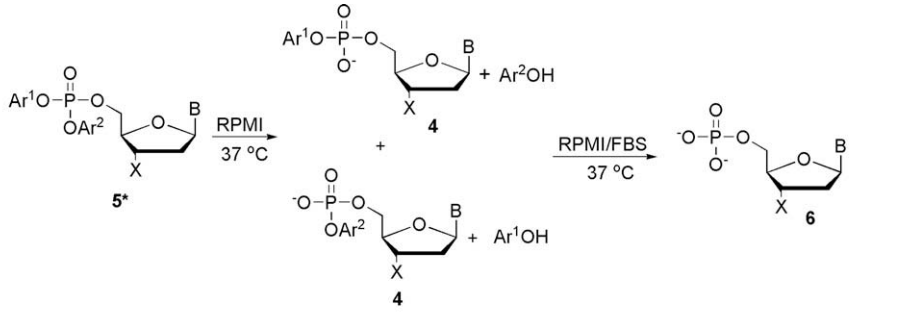
As it is apparent from data in Table 1, the rates of hydrolyses do not vary much along the AZT nucleotide aryl esters **4aa–ak** series, although in the case of phosphodiester **4ae**, it seems that the inductive effect of two chlorine atoms (*o*- and *p*-) made 2,4-dichlorophenol the best leaving group and the presence of chlorine atoms do not affect the interaction with an enzyme active centre during hydrolysis. Considering only the $t_{1/2}$ values within the AZT pyridinyl phosphoesters **4af–ak** series, it seems that best substrates for hydrolytic enzymes are nucleotides, bearing the pyridin-3-yl, pyridin-4-yl and 5-chloropyridin-3-yl groups (**4af**, **4ag** and **4ak**, respectively) and these were nearly equivalent in respect to enzymatic hydrolysis to phenol derivatives **4aa–ad**.

Aryl nucleoside phosphodiesters derived from ddU (**4ba–bc**) and ddA (**4ca**) appeared to be distinctly poor substrates compared to AZT nucleotides, most likely due to weaker interaction of the 2′,3′ dideoxyribose moieties of ddU and ddA with the enzyme active centre. Nevertheless, phosphodiesters **4ba**, **4bc** and **4c** were still good substrates for hydrolytic enzymes of FBS and certainly can be considered as a potentially useful metabolite in the anabolic path to the desired nucleoside 5′-triphosphate in the cell.

It can be tentatively concluded that all of the investigated aryl nucleoside phosphodiesters **4** were substrates for enzymatic activities present in FBS and the observed differences in $t_{1/2}$ values of their hydrolysis can be useful in tuning pronucleotide properties of phosphotriester of type **5** (vide infra).

2.3. Decomposition of di-aryl nucleoside phosphotriesters 5 in RPMI

Next, we examined stability of di-aryl nucleoside phosphotriesters **5** in RPMI. Since no enzymatic activity is present in this medium, the observed decomposition of **5** had to be due to chemical hydrolysis. This was particularly useful in the case of unsymmetrical phosphotriesters of type **5** ($Ar^1 \neq Ar^2$), since it provided infor-



For all **4a, 5a**, **6a** B = 5-methyluracil-1-yl, X = N_3
For all **4b, 5b**,**6b** B = uracil-1-yl, X = H
For all **4c,5c**, **6c** B = adenin-9-yl, X = H

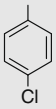
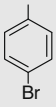
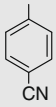
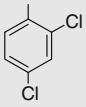
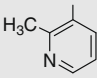
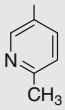
*-for detail descripton of **5** see ***Scheme 1***

4aa Ar^1 = phenyl,
4ab, Ar^1 = 4-chlorophenyl
4ac Ar^1 = 4-bromophenyl
4ad, Ar^1 = 4-cyanophenyl
4ae, Ar^1 = 2,4-dichlorophenyl
4ba, Ar^1 = phenyl,
4ca, Ar^1 = phenyl

4af, Ar^2 = pyridin-3-yl,
4ag, Ar^2 = pyridin-4-yl
4ah, Ar^2 (= Ar^1) = 2-methylpyridin-3-yl,
4ai, Ar^2 (= Ar^1) = 6-methylpyridin-3yl
4aj, Ar^2 = 2-chloropyridin-3-yl
4ak, Ar^2 = 5-chloropyridin-3-yl
4bb, Ar^2 = pyridin-3-yl
4bc, Ar^2 = pyridin-4-yl
4cb, Ar^2 = pyridin-3-yl

Scheme 2. Decomposition of di-aryl nucleoside phosphotriesters **5** in RPMI and aryl nucleoside phosphodiesters **4** in RPMI/FBS 37 °C.

Table 1
Stability of aryl nucleoside phosphodiester **4** in the cell culture media[a]

Compound	**4aa**	**4ab**	**4ac**	**4ad**	**4ae**	**4af**	**4ag**	**4ah**
Ar								
$t_{1/2}$ (min)[b]	568	330	478	425	182	233	484	949
Compound	**4ai**	**4aj**	**4ak**	**4ba**	**4bb**	**4bc**	**4ca**	**4cb**
Ar								
$t_{1/2}$ (min)[b]	1732	976	436	1506	3013	1414	2166	3850

[a] RPMI 1640/FBS 9:1 (v/v), 37 °C.
[b] In each case enzymatic hydrolysis of **4** produced the respective nucleoside (AZT, ddU or ddA)-5′-monophosphate **6**. For all experiments one lot of RPMI and FBS was used.

mation which phosphoester bond, and to what extent, was weaker and in consequence, which phosphodiester **4** would be produced as a major product. In Table 2, nucleotide phosphodiesters, produced during decomposition of nucleoside phosphotriesters **5** in RPMI, are shown. The ratio of different phosphodiesters **4** formed, indicated relative stability of the phosphoester bonds in the studied phosphotriesters **5**.

Data from Table 2 show that the half-lives ($t_{1/2}$) of phosphotriesters **5** in RPMI medium depended on acidity of the phenol moieties in **5**, as it can be seen, for example, along the series of phosphotriesters **5aa**, **5ac**–**5af**. The influence of an aryl group on the stability of the phosphoester bonds was as expected from electronic effects of the substituents in the aromatic ring. For pyridin-3-yl phosphotriesters **5ak**–**5an**, the same rules seemed to operate, although compounds,

Table 2
Stability of di-aryl nucleoside phosphotriester **5** in RPMI[a], their cytotoxicity[b] (CC_{50}) against MT-4 cells and antiviral activity[c] (EC_{50}) against HIV-1_{IIIB}

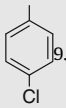
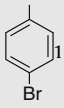
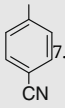
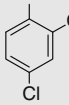
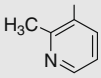
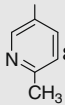
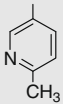
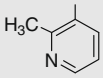
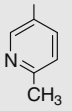
Compounds	Ar^1	Ar^2	RPMI $t_{1/2}$ (min)	Produced **4** and their ratio[d]	CC_{50} (μM)	EC_{50} (μM)	SI
AZT	—	—	—	—	60	0.01	>6000
5aa	 9.98[31]	 8.60[31]	3013	**4aa** > **4af** ~3:1	>100	0.05	>2000
5ab		 7.74[e,f32]	770	**4aa**	>100	0.01	>10000
5ac	 9.38[31]		3300	**4ab** > **4af** ~2:1	⩾100	0.03	>3333
5ad	 10.0[31]		2660	**4ac** > **4af** ~2:1	94	0.03	3133
5ae	 7.95[31]		173	**4af**	58	0.02	2900
5af	 7.85[31]		396	**4af**	>100	0.01	>10,000
5ag			295	**4af**	>100	0.01	>10,000
5ah			513	**4af**	>100	0.02	>5000
5ai	 9.50[33]		1034	**4ah**	>100	0.02	>5000
5aj	 8.90[33]		1283	**4ai**	>100	0.04	>2500

Table 2 (*continued*)

Compounds	Ar[1]	Ar[2]	RPMI $t_{1/2}$ (min)	Produced **4** and their ratio[d]	CC_{50} (μM)	EC_{50} (μM)	SI
5ak			4951	**4aa** > **4ah** ~2:1	>100	0.1	>1000
5al			2665	**4aa** > **4ai** ~2:1	>100	0.09	>1111
5am			963	**4aa**	⩾100	0.03	>3333
5an			1506	**4aa**	100	0.04	2500
ddU	–	–	–	–	>100	>100	1
5ba			3850	**4ba** > **4bb** ~3:1	>100	38	>2
5bb			1610	**4ba**	>100	3.0	>33
5bc			420	**4bb**	>100	14	>7
ddA	–	–	–	–	>100	5.2	>19
5c			5331	**4ca** > **4cb** ~3:1	>100	23	>4

[a] RPMI 1640, 37 °C. Compounds **5** exhibited nearly the same stability also in RPMI/FBS (9:1, v/v: data not shown).
[b] Compound concentration (μM) required to reduce the viability of mock-infected MT-4 cells by 50%, as determined by the MTT method.
[c] Compound concentration (μM) required to achieve 50% protection from virus-induced cytopathogenicity, as determined by the MTT method. Data represent mean values (+SD) for three independent determinations. Variation among triplicate samples was less than 15%.
[d] Calculated on the basis of area of peaks corresponding to particular compounds—not corrected.
[e] pK_a of OH group in respective ArOH.
[f] Corrected value with regard to hydroxypyridine-pyridone tautomerism.[32]

with lipophilic substituent at the *ortho* position (e.g., Cl or CH_3 as in **5ak** and **5am**), were about twice as stable as those bearing the same substituents at the *para* or *meta* position (**5al** and **5an**, respectively). These differences can be explained, at least in part, by less efficient hydration of the leaving aryloxyl group, analogously to what we observed earlier during hydrolysis of α-hydroxyphosphonates.[19] Phosphotriesters **5**, bearing 4-pyridyl group (e.g., **5ab** or **5bb**), were distinctly less stable than the analogous 3-pyridyl protected nucleotides (**5aa** and **5bb**, respectively), which can be attributed to a stronger inductive effect (electron-withdrawing) of an endocyclic nitrogen atom in the position 4 of the pyridine ring.

Another important observation was that ddU and ddA phosphotriesters (**5ba**, **5bb** and **5c**) were apparently more hydrolytically stable than the analogous AZT derivatives (**5aa** and **5ab**, respectively). It is likely that, the inductive effect of the 3-azido function in AZT, although distant, still enhances electrophilicity of the phosphorus center in phosphotriesters **5a**. In the case of phosphotriesters **5ba–bc** and **5c**, the 3′-methylene group may have an electron-donating character and lowers electrophilicity of the phosphorus center. Thus, detail structural features of all the groups attached to the phosphorus center have to be taken into consideration when designing a new type of pronucleotides.

2.4. Cytotoxicity and antiviral activity

All di-aryl nucleoside phosphotriesters investigated disclosed significant anti-HIV-1 potency. Along the AZT phosphotriester **5aa–an** series, the potency, comparable or higher to that of the parent nucleoside (AZT), disclosed the least stable in the RPMI medium compounds, that is, **5ab**, **5af**, **5ag** and also **5ae**, **5ah** and **5ai** ($t_{1/2}$ 300–1000 min). All of them bear at least one aryl group with electron-withdrawing substituents that facilitates chemical hydrolysis to the corresponding nucleoside phosphodiesters of type **4**. Since enzymatic hydrolysis of the resulting phosphodiesters of type **4** proceeded faster than the chemical hydrolysis step, it could be suggested that for the compounds investigated, the antiviral potency depended on stability of phosphotriesters of type **5**. This rule seems to be held also for phosphotriesters **5ba**–**bc** in the 2′,3′-dideoxyuridine (ddU) series and for 2′,3′-dideoxyadenosine (ddA) pronucleotide **5c**.

Antiviral potency of diphenyl AZT phosphotriester is known to be rather poor (EC_{50} 0.3 μM) most likely due to its high stability in RPMI ($t_{1/2}$ > 6 days). Taking this into account, it can be postulated that at least one of the aryloxy groups of a potential pronucleotide of type **5** should have a pK_a value lower than that of unsubstituted phenol (pK_a 9.98), in order to secure efficient generation of phosphodiesters **4**, the first intermediate in anabolic multistep path of enzymatic formation of AZT 5′-triphosphate. It is worth stressing that the above correlation is also valid for ddU-derived phosphotriesters **5ba–bc**, although these compounds were found to be generally more stable (higher the $t_{1/2}$ values, Table 2) than the analogous AZT derivatives.

Since anti-HIV-1 potency of the investigated phosphotriesters **5** correlated mainly with their susceptibility to chemical hydrolysis,

an important question arose, namely, what was the mode of action of these compound? Are they true pronucleotides or are they vehicles for antiviral nucleosides, only?

To address this question, along with AZT nucleotides, the analogous phosphotriesters, bearing the ddU nucleoside moiety, were prepared. It is known that ddU nucleoside is a very poor substrate for thymidine kinase and it cannot be converted into its 5′-monophosphate, and in consequence, into anti-HIV-1 active tri-phosphate.[2] However, when ddU-derived nucleotides with properly masked 5′-phosphate groups were used, they were found to be highly potent anti-HIV-1 agents. This suggested that these compounds could act as pronucleotides and by-pass a thymidine kinase phosphorylation process in the cell.[34]

In these studies we found a significant anti-HIV-1 activity of ddU phosphotriesters **5ba**–**bc**, and this is a strong evidence that these compounds can act, at least partially, as true pronucleotides, delivering into the cell a notable proportion of nucleoside 5′-monophosphates. The same is most likely true for the AZT phosphotriesters (for instance **5ab** and **5ag**) that under similar conditions also exhibited high anti-HIV-1 potency.

Although, most of the compounds investigated were not toxic (CC_{50} >100 μM), some of them (**5ac**, **5ad**, **5ae**, **5am** and **5an)** showed noticeable ($CC_{50} \leqslant 100$ μM) cytotoxicity, which probably came from the known toxicity of halophenols,[35] formed as hydrolytic metabolites (with exception of 4-cyanophenyl phosphotriester **5ae**). In this context rather surprising was the lack of cytotoxicity of phosphotriester **5af**, bearing 2,4-dichlorophenyl phosphoester group. It is also important to note that 2-, 3- and 4-pyridinols, examined separately, were not cytotoxic (CC_{50} >100 μM). These results should be taken into account while designing of new anti-HIV-1 pronucleotides of type **5**.

We have also examined antiviral activity of all di-aryl nucleoside phosphotriesters **5** against HIV-1 variants containing the mutations 181C (N119), 103N+181C (A17), 103R+179D+225H (EFV^R), 67N+70R+215F+219Q (AZT^R) and 41L+74V+106A+215Y (MDR-1). The details of these experiments, which are consistent with those discussed above, are given in Supplementary data.

3. Conclusions

We have prepared a series of aryl nucleoside phosphates **4** and found that all of them are good substrates for a phosphoesterase-type of activity present in FBS, and probably also for similar enzymatic activities in the living cell. These results kindle the hope that, if phosphodiesters of type **4** will be delivered or in situ produced in the cell, they should be converted into nucleoside 5′-monophosphates **6** and further into the corresponding triphosphates, that are true anti-HIV-1 agents. On this basis, we designed di-aryl nucleoside phosphotriesters **5**, bearing AZT, ddU or ddA moiety, as potential anti-HIV-1 pronucleotides. For the preparation of these compounds we developed a highly efficient one-pot synthesis, which consisted of a condensation of nucleoside 5′-H-phosphonates with the appropriate phenol or pyridinol, followed by oxidative coupling of a pyridinol or another phenol, promoted by iodine.

All the compounds investigated showed anti-HIV-1 activity and since it was observed for both AZT- and ddU-derived phosphotriesters **5**, we can assume that these compounds act, at least partially, as true pronucleotides, delivering inside the cells notable amounts of the nucleoside 5′-monophosphates, rather than corresponding nucleosides.

Chemical stability of phosphotriesters **5** determines how easy the corresponding phosphodiesters of type **4** are formed and this may serve for a tentative prediction of anti-HIV-1 activity of these compounds.

4. Experimental

^1H and ^{31}P NMR spectra were recorded on 300 MHz or 400 MHz machines. The ^{31}P NMR (121 MHz) experiments were carried out in 5 mm tubes using 0.1 M solutions of the phosphorus-containing compound. ^{31}P NMR chemical shifts are reported in ppm relative to 85% H_3PO_4 in water, used as an external standard. Mass spectra of phosphodiesters of type **4** were recorded with liquid secondary ion mass technique (LSIMS) using Cs^+ (12 keV) for ionisation. FAB technique was used for measuring mass spectra of phosphotriesters of type **5**. 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 was stored over molecular sieves 4 Å until the amount of water was below 20 ppm. Triethylamine was distilled and stored over CaH_2. For column chromatography Silica gel 60 (Merck) was used. For TLC analysis, the precoated plates (Merck Silica gel 60 F_{254}) were used. Phenols and pyridinols were commercial grade from Aldrich. RPMI-1640 cell culture medium and heat non inactivated foetal bovine serum (FBS) used for studies of stability of compounds were from Sigma (R7256 and F7524, respectively). 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 acetate, pH 7.2; solvent B—A/acetonitrile 4:1 (v/v); events: 5 min A 100%, linear gradient of B 0–100% in 30 min, A 100%—10 min wash. For quantification of peaks Waters Breeze Software was used.

Nucleoside H-phosphonates of type **1** and aryl nucleoside phosphodiesters of type **4** were obtained following procedures described earlier.[36,23] All synthesized compounds were of purity better than 97% as judged from ^1H NMR spectroscopy.

4.1. Biological assays

4.1.1. Compounds

Compounds were dissolved in DMSO at 100 mM and then diluted in culture medium.

4.1.2. Cells and viruses

Cell lines were purchased from American Type Culture Collection (ATCC). The absence of mycoplasma contamination was checked periodically by the Hoechst staining method. $CD4^+$ human T-cells containing an integrated HTLV-1 genome (MT-4) were used to support the multiplication of HIV-1.

4.1.3. Cytotoxicity assays

For cytotoxicity evaluations, exponentially growing cells derived from human haematological tumors [$CD4^+$ human T-cells containing an integrated HTLV-1 genome (MT-4)] were seeded at an initial density of 1×10^5 cells/ml in 96 well plates in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 100 units/mL penicillin G and 100 μg/mL streptomycin. Cell cultures were then incubated at 37 °C in a humidified, 5% CO_2 atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 96 h at 37 °C by the MTT method.[37]

4.1.4. Antiviral assay

Activity of compounds against Human Immunodeficiency virus type-1 (HIV-1) was based on inhibition of virus-induced cytopathogenicity in MT-4 cells acutely infected with a multiplicity of infection (m.o.i.) of 0.01. Briefly, 50 μL of RPMI containing 1×10^4 MT-4 were added to each well of flat-bottom microtitre trays containing 50 μL of RPMI, without or with serial dilutions of test compounds. Then, 20 μL of an HIV-1 suspension containing 100 $CCID_{50}$ were

added. After a 4-day incubation, cell viability was determined by the MTT method.

4.1.5. Aryl nucleoside phosphodiesters of type 4

4.1.5.1. 3′-Azido-3′-deoxythymidyn-5′-yl phenyl phosphate triethylammonium salt (4aa). Yield 0.49 g (94%). ^{1}H NMR ($CDCl_3$) δ 12.24 (br s, 1H, exch. D_2O), 8.00 (br s, exch. D_2O), 7.73 (q, J = 0.9 Hz, 1H), 7.28–7.24 (m, 5H), 6.27 (t, J = 6.7 Hz, 1H), 4.33–4.28 (m, 1H), 4.22–4.18 (m, 2H), 4.03–4.01 (m, 1H), 3.04 (q, J = 7.2 Hz, 6H), 2.33–2.23 (m, 2H), 1.89 (d, J = 0.9 Hz, 3H), 1.32 (t, J = 7.2 Hz, 9H); ^{31}P NMR ($CDCl_3$) δ −5.83 (t, $^3J_{HP}$ = 6.4 Hz); HRMS $[M-Et_3NH^+]^-$: 422.0846, calcd for $C_{16}H_{17}N_5O_7P$: 422.0865.

4.1.5.2. 3′-Azido-3′-deoxythymidyn-5′-yl 4-chlorophenyl phosphate triethylammonium salt (4ab). Yield 0.14 g (81%). ^{1}H NMR ($CDCl_3$) δ 12.02 (br s, 1H, exch. D_2O), 8.76 (br s, exch. D_2O), 7.67 (br s, 1H), 7.19 (s, 4H), 6.26 (t, J = 6.6 Hz, 1H), 4.36–4.31 (m, 1H), 4.24–4.11 (m, 2H), 4.00 (br m, 1H), 3.05 (q, J = 7.2 Hz, 6H), 2.38–2.21 (m, 2H), 1.88 (br s, 3H), 1.33 (t, J = 7.2 Hz, 9H). ^{31}P NMR ($CDCl_3$) δ −5.38 (t, $^3J_{HP}$ = 6.4 Hz); HRMS $[M-Et_3NH^+]^-$: 456.0500, calcd for $C_{16}H_{16}N_5O_7PCl$: 456.0476.

4.1.5.3. 3′-Azido-3′-deoxythymidyn-5'-yl 4-bromophenyl phosphate triethylammonium salt (4ac). Yield 0.16 g (87%). ^{1}H NMR ($CDCl_3$) δ 11.91 (br s, 1H, exch. D_2O), 9.24 (br s, exch. D_2O), 7.64 (br s, 1H), 7.32 (d, J = 8.4 Hz, 2H), 7.12 (d, J = 8.4 Hz, 2H), 6.24 (t, J = 6.6 Hz, 1H), 4.35–4.30 (m, 1H), 4.22–4.10 (m, 2H), 3.98 (br m, 1H), 3.03 (q, J = 7.2 Hz, 6H), 2.35–2.20 (m, 2H), 1.85 (br s, 3H), 1.31 (t, J = 7.2 Hz, 9H); ^{31}P NMR ($CDCl_3$) δ −5.53 (t, $^3J_{HP}$ = 6.4 Hz); HRMS $[M-Et_3NH^+]^-$: 499.9960, calcd for $C_{16}H_{16}N_5O_7PBr$: 499.9971.

4.1.5.4. 3′-Azido-3′-deoxythymidyn-5′-yl 4-cyanophenyl phosphate triethylammonium salt (4ad). Yield 0.15 g (81%). ^{1}H NMR ($CDCl_3$) δ 11.86 (br s, 1H, exch. D_2O), 8.90 (br s, 1H, exch. D_2O), 7.63 (q, J = 0.9 Hz, 1H), 7.54 (d, J = 8.4 Hz, 2H), 7.35 (d, J = 8.4 Hz, 2H), 6.22 (t, J = 6.6 Hz, 1H), 4.33–4.31 (m, 1H), 4.20–4.08 (m, 2H), 3.99 (br m, 1H), 3.06 (q, J = 7.2 Hz, 6H), 2.32–2.21 (m, 2H), 1.88 (d, J = 0.9 Hz, 3H), 1.35 (t, J = 7.2 Hz, 9H); ^{31}P NMR ($CDCl_3$) δ −6.15 (t, $^3J_{HP}$ = 6.4 Hz); HRMS $[M-Et_3NH^+]^-$: 447.0800, calcd for $C_{17}H_{16}N_6O_7P$: 447.0818.

4.1.5.5. 3′-Azido-3′-deoxythymidyn-5′-yl 2,4-dichlorophenyl phosphate triethylammonium salt (4ae). Yield 0.17 g (82%). ^{1}H NMR ($CDCl_3$) δ 11.95 (br s, 1H, exch. D_2O), 8.56 (br s, 1H, exch. D_2O), 7.68 (br s, 1H), 7.57 (d, J = 8.7 Hz, 1H), 7.33 (s, 1H), 7.10 (d, J = 8.7 Hz, 1H), 6.26 (t, J = 6.7 Hz, 1H), 4.40–4.35 (m, 1H), 4.24–4.16 (m, 2H), 4.02–3.98 (m, 1H), 3.07 (q, J = 7.2 Hz, 6H), 2.36–2.24 (m, 2H), 1.88 (br s, 3H), 1.36 (t, J = 7.2 Hz, 9H); ^{31}P NMR ($CDCl_3$) δ −5.88 (t, $^3J_{HP}$ = 6.4 Hz) HRMS $[M-Et_3NH^+]^-$: 490.0065, calcd for $C_{16}H_{15}N_5O_7P$: 490.0086.

4.1.5.6. 3′-Azido-3′-deoxythymidyn-5′-yl pyridin-3-yl phosphate triethylammonium salt (4af). Yield 0.14 g (86%). ^{1}H NMR ($CDCl_3$) δ 11.67 (br s, 1H, exch. D_2O), 9.87 (br s, 1H, exch. D_2O), 8.50 (s, 1H), 8.24 (br s, 1H), 7.65 (m, 2H), 7.18 (m, 1H), 6.20 (t, J = 6.6 Hz, 1H), 4.41–4.32 (m, 1H), 4.26–4.10 (m, 2H), 4.03–3.94 (m, 1H), 3.01 (q, J = 7.2 Hz, 6H), 2.32–2.23 (m, 2H), 1.80 (s, 3H), 1.28 (t, J = 7.2 Hz, 9H); ^{31}P NMR ($CDCl_3$) δ −6.09 (t, $^3J_{HP}$ = 6.4 Hz); HRMS [M-Et_3NH^+]$^-$: 423.0797, calcd for $C_{15}H_{16}N_6O_7P$: 423.0818.

4.1.5.7. 3′-Azido-3′-deoxythymidyn-5′-yl pyridin-4-yl phosphate triethylammonium salt (4ag). Yield 0.22 g (84%). ^{1}H NMR ($CDCl_3$) δ 9.76 (br s, 1H, exch. D_2O), 8.44 (d, J = 5.7 Hz, 2H), 7.63 (q, J = 0.9 Hz, 1H), 7.20 (d, J = 5.7 Hz, 2H), 6.23 (t, J = 6.6 Hz, 1H), 4.38–4.32 (m, 1H), 4.24–4.12 (m, 2H), 4.03–3.97 (m, 1H), 3.02 (q, J = 7.2 Hz, 6H), 2.38–2.22 (m, 2H), 1.89 (d, J = 0.9 Hz, 3H), 1.28 (t, J = 7.2 Hz, 9H); ^{31}P NMR ($CDCl_3$) δ −6.27 (t, $^3J_{HP}$ = 5.5 Hz); HRMS $[M-Et_3NH^+]^-$: 423.0815, calcd for $C_{15}H_{16}N_6O_7P$: 423.0818.

4.1.5.8. 3′-Azido-3′-deoxythymidyn-5′-yl 2-methylpyridin-3-yl phosphate triethylammonium salt (4ah). Yield 0.09 g (54%). ^{1}H NMR ($CDCl_3$) δ 9.35 (br s, 1H, exch. D_2O), 8.17 (d, J = 4.8 Hz, 1H), 7.74 (d, J = 8.1 Hz, 1H), 7.66 (q, J = 0.9 Hz, 1H), 7.00 (dd, J = 8.1 Hz, J = 4.2 Hz, 1H), 6.25 (t, J = 6.6 Hz, 1H), 4.38 (m, 1H), 4.20 (m, 2H), 4.02 (m, 1H), 3.01 (q, J = 7.2 Hz, 6H), 2.53 (s, 3H), 2.33 (m, 2H), 1.86 (d, J = 0.9 Hz, 3H), 1.28 (t, J = 7.2 Hz, 9H); ^{31}P NMR ($CDCl_3$) δ −5.23 (t, $^3J_{HP}$ = 6.4 Hz); HRMS $[M-Et_3NH^+]^-$: 437.0983, calcd for $C_{16}H_{18}N_6O_7P$: 437.0975.

4.1.5.9. 3′-Azido-3′-deoxythymidyn-5′-yl 6-methylpyridin-3-yl phosphate triethylammonium salt (4ai). Yield 0.12 g (73%). ^{1}H NMR ($CDCl_3$) δ 11.89 (br s, 1H, exch. D_2O), 9.17 (br s, 1H, exch. D_2O), 8.40 (s, 1H), 7.66 (q, J = 0.9 Hz, 1H), 7.52 (d, J = 8.4 Hz, 1H), 7.05 (d, J = 8.4 Hz, 1H), 6.26 (t, J = 6.6 Hz, 1H), 4.38–4.33 (m, 1H), 4.27–4.13 (m, 2H), 4.01 (br m, 1H), 3.05 (q, J = 7.2 Hz, 6H), 2.47 (s, 3H), 2.39–2.24 (m, 2H), 1.85 (d, J = 0.9 Hz, 3H), 1.31 (t, J = 7.2 Hz, 9H); ^{31}P NMR ($CDCl_3$) δ −5.46 (t, $^3J_{HP}$ = 6.4 Hz); HRMS $[M-Et_3NH^+]^-$: 437.0993, calcd for $C_{16}H_{18}N_6O_7P$: 437.0975.

4.1.5.10. 3′-Azido-3'-deoxythymidyn-5′-yl 2-chloropyridin-3-yl phosphate triethylammonium salt (4aj). Yield 0.14 g (71%). ^{1}H NMR ($CDCl_3$) δ 11.87 (br s, 1H, exch. D_2O), 9.00 (br s, 1H, exch. D_2O), 8.07 (dd, J = 4.8 Hz, J = 1.5 Hz, 1H), 7.97 (dd, J = 8.4 Hz, J = 1.5 Hz, 1H), 7.66 (q, J = 0.9 Hz, 1H), 7.16 (dd, J = 8.4 Hz, J = 4.8 Hz, 1H), 6.26 (t, J = 6.6 Hz, 1H), 4.46–4.37 (m, 1H), 4.28–4.17 (m, 2H), 4.04–4.01 (m, 1H), 3.06 (q, J = 7.2 Hz, 6H), 2.40–2.25 (m, 2H), 1.89 (d, J = 0.9 Hz, 3H), 1.32 (t, J = 7.2 Hz, 9H); ^{31}P NMR ($CDCl_3$) δ −5.99 (t, $^3J_{HP}$ = 6.4 Hz); HRMS $[M-Et_3NH^+]^-$: 457.0450, calcd for $C_{15}H_{15}N_6O_7PCl$: 457.0429.

4.1.5.11. 3′-Azido-3′-deoxythymidyn-5'-yl 5-chloropyridin-3-yl phosphate triethylammonium salt (4ak). Yield 0.15 g (79%). ^{1}H NMR ($CDCl_3$) δ 11.86 (br s, 1H, exch. D_2O), 8.82 (br s, 1H, exch. D_2O), 8.45 (d, J = 1.5 Hz, 1H), 8.26 (d, J = 1.8 Hz, 1H), 7.71 (dd, J = 1.8 Hz, J = 1.5 Hz, 1H), 7.62 (q, J = 0.9 Hz, 1H), 6.26 (t, J = 6.3 Hz, 1H), 4.40–4.34 (m, 1H), 4.28–4.14 (m, 2H), 4.01 (br m, 1H), 3.06 (q, J = 7.2 Hz, 6H), 2.42–2.25 (m, 2H), 1.88 (d, J = 0.9 Hz, 3H), 1.34 (t, J = 7.2 Hz, 9H). ^{31}P NMR ($CDCl_3$) δ -5.63 (t, $^3J_{HP}$ = 6.4 Hz); HRMS $[M-Et_3NH^+]^-$: 457.0420, calcd for $C_{15}H_{15}N_6O_7PCl$: 457.0429.

4.1.5.12. 2′,3′-Dideoxyuridin-5'-yl phenyl phosphate triethylammonium salt (4ba). Yield 0.07 g (76%). ^{1}H NMR ($CDCl_3$) δ 11.94 (br s, 1H, exch. D_2O), 9.83 (br s, 1H, exch. D_2O), 7.84 (d, J = 8.1 Hz, 1H), 7.22–7.15 (m, 4H), 6.98–6.93 (m, 1H), 6.00–5.96 (m, 1H), 5.51 (d, J = 8.1 Hz, 1H), 4.24–4.18 (m, 2H), 4.07–4.00 (m, 1H), 2.97 (q, J = 7.2 Hz, 6H), 2.29–2.22 (m, 1H), 1.97–1.87 (m, 3H), 1.24 (t,, J = 7.2 Hz, 9H). ^{31}P NMR ($CDCl_3$) δ −5.83 (t, $^3J_{HP}$ = 5.5 Hz); HRMS $[M-Et_3NH^+]^-$: 367.0682, calcd for $C_{15}H_{16}N_2O_7P$: 367.0700.

4.1.5.13. 2′,3′-Dideoxyuridin-5′-yl pyridin-3-yl phosphate triethylammonium salt (4bb). Yield 0.07 g (72%). ^{1}H NMR ($CDCl_3$) δ 11.73 (br s, 1H, exch. D_2O), 10.16 (br s, 1H, exch. D_2O), 8.45 (br s, 1H), 8.2 (d, J = 4.4 Hz, 1H), 7.80 (d, J = 8.4 Hz, 1H), 7.62 (d, J = 8.8 Hz, 1H), 7.17 (dd, J = 8.8 and 4.4 Hz, 1H), 5.97–5.95 (m, 1H), 5.53 (d, J = 8.4 Hz, 1H), 4.21–4.17 (m, 2H), 4.05–4.00 (m, 1H), 2.99 (q, J = 7.2 Hz, 6H), 2.29–2.24 (m, 1H), 1.97–1.87 (m, 3H), 1.24 (t, J = 7.2 Hz, 9H). ^{31}P NMR ($CDCl_3$) δ −5.88 (t, $^3J_{HP}$ = 5.5 Hz); HRMS $[M-Et_3NH^+]^-$: 368.0642, calcd for $C_{14}H_{15}N_3O_7P$: 368.0648.

4.1.5.14. 2′,3′-Dideoxyuridin-5′-yl pyridin-4-yl phosphate triethylammonium salt (4bc). Yield 0.08 g (80%). ^{1}H NMR ($CDCl_3$) δ 11.5 (br s, 1H, exch. D_2O), 9.95 (br s, 1H, exch. D_2O), 7.99 (d, J = 8.4 Hz, 1H), 7.88 (d, J = 7.5 Hz, 2H), 6.59 (d, J = 7.5 Hz, 2H), 6.10–6.04 (m, 1H), 5.86 (d, J = 8.4 Hz, 1H), 4.40–4.22 (m, 2H), 4.17–4.05 (m, 1H), 3.17 (q, J = 7.2 Hz, 6H), 2.51–2.38 (m, 1H), 2.18–1.93 (m, 3H), 1.26 (t, J = 7.2 Hz, 9H); ^{31}P NMR ($CDCl_3$) δ −6.03 (t, $^3J_{HP}$ = 5.5 Hz); HRMS [M−Et_3NH^+]$^-$: 368.0615, calcd for $C_{14}H_{15}N_3O_7P$: 368.0648.

4.1.5.15. 2′,3′-Dideoxyadenosin-5′-yl phenyl phosphate triethylammonium salt (4ca). Yield 0.08 g (84%). ^{1}H NMR ($CDCl_3$) δ 8.55 (s, 1H), 7.29 (d, J = 5.7 Hz, 2H), 7.21 (t, J = 5.7 Hz, 2H), 6.98 (t, J = 5.7 Hz, 1H), 6.34–6.32 (m, 1H), 4.38–4.30 (m, 2H), 4.16–4.10 (m, 1H), 3.04 (q, J = 7.2 Hz, 6H), 2.50–2.41 (m, 1H), 2.37–2.26 (m, 1H), 2.18–2.06 (m, 1H), 2.03–1.96 (m, 1H), 1.32 (t, J = 7.2 Hz, 9H). ^{31}P NMR ($CDCl_3$) δ −6.12 (t, $^3J_{HP}$ = 5.5 Hz); HRMS [M−Et_3NH^+]$^-$: 390.0975, calcd for $C_{16}H_{17}N_5O_5P$: 390.0967.

4.1.5.16. 2′,3′-Dideoxyadenosin-5′-yl pyridin-3-yl phosphate triethylammonium salt (4cb). Yield 0.08 g (81%). ^{1}H NMR ($CDCl_3$) δ 8.58 (s, 1H), 8.53 (d, J = 2.7 Hz, 1H), 8.22 (s, 1H), 8.18 (d, J = 4.8 Hz, 1H), 7.18 (dm, J = 8.7 Hz, 1H), 7.13 (dd, J = 8.7 and 4.8 Hz, 1H), 6.33 (dd, J = 6.6 and 3.6 Hz, 1H), 4.41–4.29 (m, 2H), 4.16–4.10 (m, 1H), 3.06 (q, J = 7.2 Hz, 6H), 2.52–2.40 (m, 1H), 2.38–2.28 (m, 1H), 2.2–2.08 (m, 1H), 1.99–1.92 (m, 1H), 1.24 (t, J = 7.2 Hz, 9H); ^{31}P NMR ($CDCl_3$) δ −6.09 (t, $^3J_{HP}$ = 5.5 Hz); HRMS [M−Et_3NH^+]$^-$: 391.0914, calcd for $C_{15}H_{16}N_6O_5P$: 391.0920.

4.1.6. A typical procedure for the synthesis of bis-aryl nucleoside phosphate triesters 5

Nucleoside-5′-yl H-phosphonate **1** (1 M equiv) and a phenol or pyridinol HOAr[1] **2** (1.2–1.5 M 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 condensation was effected by the addition of diphenyl chlorophosphate (1.2 M equiv) or pivaloyl chloride (1.5 M equiv) to the reaction mixture. When the formation of aryl nucleosid-5′-yl H-phosphonate **3** was completed (ca. 20 min, ^{31}P NMR), the reaction mixture was added to a solution of a pyridinol or another phenol HOAr[2] **2** (5 equiv) to be coupled with, and then iodine (1.5–3 M equiv) in pyridine (1 mL) was added. After the reaction was complete (ca. 5 min, ^{31}P NMR), the excess of iodine was decomposed with ethanethiol, and the solvents were removed by evaporation. The oily residue was dissolved in methylene chloride (3 mL), washed with aqueous 0.1 M KH_2PO_4 buffer (pH 6) and the organic phase was dried over Na_2SO_4. Phosphotriesters **5** were then purified by reversed phase silica gel chromatography using a stepwise gradient of acetone (0–40%) in water, or by a Silica gel 60 column using a stepwise gradient (0–6%) of isopropanol in methylene chloride. Fractions containing pure products **5** were collected and evaporated, yielding non-hygroscopic foams. After freeze-drying from benzene, products were obtained as white amorphous solids.

Mutplicities of signals in ^{1}H and ^{31}P NMR spectra of phosphotriesters of type **5** were often complex due to a mixture of diastereoisomers.

4.1.6.1. 3′-Azido-3′-deoxythymidyn-5′-yl phenyl pyridin-3-yl phosphate (5aa). Yield 0.22 g (89%). ^{1}H NMR (DMSO-d_6) δ 11.35 (br s, 1H, exch. D_2O), 8.51 (br s, 1H), 8.47 (m, 1H), 7.72 and 7.69 (two br s, 1H), 7.49–7.36 (m, 4H), 7.30–7.25 (m, 3H), 6.26 (t, J = 6.6 Hz, 1H), 4.70–4.47 (m, 3H), 4.10–4.02 (m, 1H), 2.45–2.30 (m, 2H), 1.65 (s, 3H). ^{31}P NMR ($CDCl_3$) δ −10.98 and −11.03 [two t (partially overlapping), $^3J_{HP}$ = 6.4 Hz). HRMS [MH]$^+$ 501.1311, calcd for $C_{21}H_{22}N_6O_7P$: 501.1288.

4.1.6.2. 3′-Azido-3′-deoxythymidyn-5′-yl phenyl pyridin-4-yl phosphate (5ab). Yield 0.09 g (40%). ^{1}H NMR (DMSO-d_6) δ 11.37 (br s, 1H, exch. D_2O), 8.59 (two br, s 1H), 8.28 (m, 1H), 7.71 (br s, 1H), 7.44–7.39 (m, 2H), 7.32–7.14 (m, 4H), 7.04–6.92 (m, 1H), 6.17–6.09 (m, 1H), 4.59–4.37 (m, 2H), 4.06–3.95 (m, 2H), 2.51–2.21 (m, 2H), 1.74 and 1.66 (two s, 3H); ^{31}P NMR ($CDCl_3$) δ −12.19 (m); HRMS [MH]$^+$ 501.1319, calcd for $C_{21}H_{22}N_6O_7P$: 501.1288.

4.1.6.3. 3′-Azido-3′-deoxythymidyn-5′-yl 4-chlorophenyl pyridin-3-yl phosphate (5ac). Yield 0.18 g (65%). ^{1}H NMR (DMSO-d_6) δ 11.35 (br s, 1H, exch. D_2O), 8.52 (m, 1H), 8.47 (m, 1H), 7.73 and 7.69 (two m, 1H), 7.50–7.46 (m, 3H), 7.43–7.42 (m, 1H), 7.37–29 (m, 1H), 6.15 (t, J = 6.6 Hz, 1H), 4.62–4.46 (m, 3H), 4.08–4.04 (m, 1H), 2.45–2.30 (m, 2H), 1.65 (s, 3H); ^{31}P NMR ($CDCl_3$) δ −11.12 and −11.14 (m); HRMS [MH]$^+$ 535.0891, calcd for $C_{21}H_{21}N_6O_7P$: 535.0898.

4.1.6.4. 3′-Azido-3′-deoxythymidyn-5′-yl 4-bromophenyl pyridin-3-yl phosphate (5ad). Yield 0.15 g (52%). ^{1}H NMR ($CDCl_3$) δ 8.7 (br s, 1H, exch. D_2O) 8.58–8.48 (m, 2H), 7.60 and 7.55 (two m, 1H), 7.50–7.43 (m, 2H), 7.35–7.23 (m, 1H), 7.21–20 (m, 1H), 7.13–7.08 (m, 2H), 6.15 (t, J = 6.6 Hz, 1H), 4.61–4.44 (m, 2H), 4.35–4.28 (m, 1H), 4.08–4.03 (m, 1H), 2.51–2.32 (m, 2H), 1.64 (s, 3H); ^{31}P NMR ($CDCl_3$) δ −11.86 and −11.91 [two t (partially overlapping), $^3J_{HP}$ = 6.4 Hz); HRMS [MH]$^+$ 581.0376, calcd for $C_{21}H_{21}N_6O_7PBr(81)$: 581.0372.

4.1.6.5. 3′-Azido-3′-deoxythymidyn-5′-yl 4-cyanophenyl pyridin-3-yl phosphate (5ae). Yield 0.12 g (75%). ^{1}H NMR ($CDCl_3$) δ 9.3 (br s, 1H, exch. D_2O), 8.55 (br s, 1H), 7.71–7.65 (m, 2H), 7.61–7.57 (m, 1H), 7.37–7.33 (m, 4H), 7.17 and 7.16 (two q, JJ = 1.2 Hz), 6.08 and 6.07 (two t, JJ = 6.4 Hz), 4.61–4.56 (m, 1H), 4.54–4.49 (m, 1H), 4.37–4.30 (m, 1H), 4.07–4.04 (m, 1H), 2.49–2.45 (m, 2H), 1.83 and 1.82 (two d, JJ = 1.2 Hz, 3H); ^{31}P NMR ($CDCl_3$) δ −12.52 and −12.59 [two t (partially overlapping), $^3J_{HP}$ = 6.4 Hz). HRMS [MH]$^+$ 526.1226, calcd for $C_{22}H_{21}N_7O_7P$: 526.1240.

4.1.6.6. 3′-Azido-3′-deoxythymidyn-5′-yl 2,4-dichlorophenyl pyridin-3-yl phosphate (5af). Yield 0.13 g (77%). ^{1}H NMR ($CDCl_3$) δ 9.0 (br s, 1H, exch. D_2O), 8.60–8.52 (m, 2H), 7.63–7.59 (m, 1H), 7.46–7.44 (m, 1H), 7.36 (s, 1H), 7.34–7.33 (m, 1H), 7.26–7.21 (m, 2H), 6.17–6.13 (m, 1H), 4.66–4.61 (m, 1H), 4.58–4.50 (m, 1H), 4.36–4.31 (m, 1H), 4.08–4.07 (m, 1H), 2.50–2.35 (m, 2H), 1.84 and 1.82 (two s, 3H); ^{31}P NMR ($CDCl_3$) δ −12.20 and −12.29 (m); HRMS [MH]$^+$ 569.0489, calcd for $C_{21}H_{20}N_6O_7P$: 569.0508.

4.1.6.7. 3′-Azido-3′-deoxythymidyn-5′-yl bis-pyridin-3-yl phosphate (5ag). Yield 0.14 g (68%). ^{1}H NMR (DMSO-d_6) δ 11.34 (br s, 1H, exch. D_2O), 8.55 (m, 2H), 8.47 (m, 2H), 7.76 and 7.74 (two m, 2H), 7.51–7.41 (m, 3H), 6.15 (t, J = 6.6 Hz, 1H), 4.67–4.48 (m, 3H), 4.09–4.05 (m, 1H), 2.45–2.30 (m, 2H), 1.65 (s, 3H); ^{31}P NMR ($CDCl_3$) δ −11.63 (t, $^3J_{HP}$ = 6.4 Hz). HRMS [MH]$^+$ 502.1253, calcd for $C_{20}H_{21}N_7O_7P$: 502.1240.

4.1.6.8. 3′-Azido-3′-deoxythymidyn-5′-yl pyridin-3-yl pyridin-4-yl phosphate (5ah). Yield 0.07 g (30%). ^{1}H NMR ($CDCl_3$) δ 9.0 (br s, 1H, exch. D_2O), 8.62 (m, 2H), 8.56 and 8.51 (two m, 1H), 7.62–7.56 (m, 1H), 7.36–7.29 (m, 1H), 7.22–7.17 (m, 3H), 6.15 (m, 1H), 4.63–4.47 (m, 2H), 4.37–4.29 (m, 1H), 4.09–4.02 (m, 1H), 2.47–2.42 (m, 2H), 1.84 and 1.83 (two s, 3H); ^{31}P NMR ($CDCl_3$) δ

−12.72 and 12.75 (m); HRMS [MH]$^+$ 502.1248, calcd for $C_{20}H_{21}N_7O_7P$: 502.1240.

4.1.6.9. 3′-Azido-3′-deoxythymidyn-5′-yl bis-2-methylpyridin-3-yl phosphate (5ai). Yield 0.17 g (62%). ^{1}H NMR ($CDCl_3$) δ 8.4 (br s, 1H, exch. D_2O), 8.38 (m, 2H), 7.62–7.58 (m, 2H), 7.19 (br s, 1H), 7.17–7.10 (m, 2H), 6.09 (t, t, J = 6.6 Hz, 1H), 4.60–4.46 (m, 2H), 4.33–4.27 (m, 1H), 4.08–4.03 (m, 1H), 2.52 (s, 3H), 2.50 (s, 3H), 2.47–2.40 (m, 2H), 1.83 (s, 3H); ^{31}P NMR ($CDCl_3$) δ -10.86 (t, $^3J_{HP}$ = 6.4 Hz); HRMS [MH]$^+$ 530.1571, calcd for $C_{22}H_{25}N_7O_7P$: 530.1553.

4.1.6.10. 3′-Azido-3′-deoxythymidyn-5′-yl bis-6-methylpyridin-3-yl phosphate (5aj). Yield 0.21 g (80%). ^{1}H NMR (DMSO-d_6) δ 11.34 (br s, 1H, exch. D_2O), 8.34 (br s, 2H), 7.63–7.57 (m, 2H), 7.37 (s, 1H), 7.30 and 7.27 (two d, J = 8.9 Hz, 2H), 6.14 (t, J = 6.6 Hz, 1H), 4.62–4.46 (m, 3H), 4.08–4.04 (m, 1H), 2.45 (s, 3H), 2.44 (s, 3H), 2.42–2.31 (m, 2H), 1.63 (s, 3H); ^{31}P NMR ($CDCl_3$) δ −10.28 (t, $^3J_{HP}$ = 7.2 Hz); HRMS [MH]$^+$ 530.1534, calcd for $C_{22}H_{25}N_7O_7P$: 530.1553.

4.1.6.11. 3′-Azido-3′-deoxythymidyn-5′-yl 2-methylpyridin-3-yl phenyl phosphate (5ak). Yield 0.16 g (64%). ^{1}H NMR (DMSO-d_6) δ 11.36 (br s, 1H, exch. D_2O), 8.32 and 8.31 (two d, J = 4.5 Hz, 1H), 7.66 and 7.63 (two s, 1H), 7.45–7.38 (m, 3H), 7.30–7.23 (m, 4H), 6.15 and 6.14 (two t, JJ = 6.6 Hz, 1H), 4.61–4.45 (m, 3H), 4.10–4.04 (m, 1H), 2.44–2.30 (m, 2H), 2.36 (s, 3H), 1.65 (s, 3H); ^{31}P NMR ($CDCl_3$) δ −10.83 and −10.85 (t, J_{HP} = 7.2 Hz); HRMS [MH]$^+$ 515.1455, calcd for $C_{22}H_{24}N_6O_7P$: 515.1444.

4.1.6.12. 3′-Azido-3′-deoxythymidyn-5′-yl 6-methylpyridin-3-yl phosphate phenyl (5al). Yield 0.18 g (71%). ^{1}H NMR (DMSO-d_6) δ 11.33 (br s, 1H, exch. D_2O), 8.35 (br s, 1H), 7.60–7.54 (m, 1H), 7.45–7.39 (m, 3H), 7.30–7.24 (m, 4H), 6.15 (t, J = 6.6 Hz), 4.60–4.46 (m, 3H), 4.06 (m, 1H), 2.44 and 2.43 (two s, 3H), 1.65 (s, 3H); ^{31}P NMR ($CDCl_3$) δ −10.75 and −10.84 (m); HRMS [MH]$^+$ 515.1454, calcd for $C_{22}H_{24}N_6O_7P$: 515.1444.

4.1.6.13. 3′-Azido-3′-deoxythymidyn-5′-yl 2-chloropyridin-3-yl phenyl phosphate (5am). Yield 0.09 g (54%). ^{1}H NMR (DMSO-d_6) δ 11.36 (br s, 1H, exch. D_2O), 8.32–8.30 (m, 1H), 7.90 and 7.87 (two br s, 1H), 7.52–7.39 (m, 4H), 7.30–7.25 (m, 3H), 6.15 and 6.14 (two t, JJ = 6.6 Hz, 1H), 4.67–4.47 (m, 3H), 4.11–4.05 (m, 1H), 2.48–2.30 (m, 2H), 1.66 (s, 3H); ^{31}P NMR ($CDCl_3$) δ −12.18 and −12.33 (two t, J_{HP} = 7.2 Hz); HRMS [MH]$^+$ 535.0906, calcd for $C_{21}H_{22}N_6O_7PCl$ (35): 535.0898.

4.1.6.14. 3′-Azido-3′-deoxythymidyn-5′-yl 5-chloropyridin-3-yl phenyl phosphate (5an). Yield 0.19 g (71%). ^{1}H NMR ($CDCl_3$) δ 9.08 (br s, 1H, exch. D_2O), 8.47–8.40 (m, 2H), 7.64 and 7.61 (two q, JJ = 1.2 Hz, 1H), 7.41–7.31 (m, 2H), 7.28–7.18 (m, 4H), 6.16 and 6.15 (two t, JJ = 6.6 Hz, 1H), 4.62–4.45 (m, 2H), 4.34–4.27 (m, 1H), 4.08–4.03 (m, 1H), 2.50–2.30 (m, 2H), 1.81 and 1.80 (two d, JJ = 1.2 Hz); ^{31}P NMR ($CDCl_3$) δ −11.95 and −12.02 [two t (partially overlapping), J_{HP} = 7.2 Hz]; HRMS [MH]$^+$ 535.0882, calcd for $C_{21}H_{21}N_6O_7PCl$(35): 535.0898.

4.1.6.15. 2′,3′-didehydrouridin-5′-yl phenyl pyridin-3-yl phosphate (5ba). Yield 0.08 g (56%). ^{1}H NMR ($CDCl_3$) δ 9.53 (br s, 1H, exch. D_2O), 8.54 (br s, 1H), 8.49–8.47 (m, 1H), 7.61–7.56 (m, 1H), 7.51 and 7.49 (two d, J = 8.1 Hz, 1H), 7.40–7.18 (m, 6H), 6.07–6.03 (m, 1H), 5.57 and 5.56 (two d, J = 8.1 Hz, 1H), 4.57–4.50 (m, 1H), 4.43–4.28 (m, 2H), 2.46–2.35 (m, 1H), 2.16–1.85 (m, 3H). ^{31}P NMR ($CDCl_3$) δ −11.49 and −11.56 [two t (partially overlapping), $^3J_{HP}$ = 8.2 Hz). HRMS [MH]$^+$ 446.1136, calcd for $C_{20}H_{21}N_3O_7P$: 446.1117.

4.1.6.16. 2′,3′-Didehydrouridin-5′-yl phenyl pyridin-4-yl phosphate (5bb). Yield 0.07 g (49%). ^{1}H NMR ($CDCl_3$) δ 9.51 (br s, 1H, exch. D_2O), 8.56 (br s, 2H), 7.51 and 7.48 (two d, JJ = 8.1 Hz, 1H), 7.40–7.32 (m, 2H), 7.26–7.16 (m, 5H), 6.07–6.03 (m, 1H), 5.58 and 5.57 (two d, JJ = 8.1 Hz, 1H), 4.58–4.50 (m, 1H), 4.43–4.28 (m, 2H), 2.47–2.34 (m, 1H), 2.13–1.87 (m, 3H); ^{31}P NMR ($CDCl_3$) δ −12.59 and −12.61 (m). HRMS [MH]$^+$ 446.1106, calcd for $C_{20}H_{21}N_3O_7P$: 446.1117.

4.1.6.17. 2′,3′-Didehydrouridin-5′-yl pyridin-3-yl pyridin-4-yl phosphate (5bc). Yield 0.07 g (32%). ^{1}H NMR ($CDCl_3$) δ 11.3 (br s, 1H, exch. D_2O), 8.54 (br s, 1H), 8.22–8.11 (m, 3H), 7.72 and 7.71 (two d, JJ = 6.0, 1H), 7.53 and 7.52 (two d, JJ = 6.0 Hz), 7.49 (d, J = 8.4 Hz, 1H), 7.20–7.14 (m, 2H), 6.04 and 6.02 (two d, JJ = 6.8 Hz), 5.61 (d, J = 8.4 Hz, 1H), 4.57–4.52 (m, 1H), 4.45–4.39 (m, 1H), 4.24–4.19 (m, 1H), 2.49–2.40 (m, 1H), 2.36–2.27 (m, 1H), 2.11–1.99 (m, 2H); ^{31}P NMR ($CDCl_3$) δ −12.47 (m). HRMS [MH]$^+$ 447.1059, calcd for $C_{19}H_{20}N_4O_7P$: 447.1070.

4.1.6.18. 2′,3′-Didehydroadenosin-5′-yl phenyl pyridin-3-yl phosphate (5ca). Yield 0.22 g (94%). ^{1}H NMR (DMSO-d_6) δ 8.44 (br s, 1H), 8.43 (m, 1H), 8.27 (two s, 1H), 8.13 (two s, 1H), 7.65–7.60 (m, 1H), 7.41–7.13 (m, 6H), 6.29–6.25 (m, 1H), 4.52–4.45 (m, 2H), 4.44–4.36 (m, 1H), 2.55–2.42 (m, 2H), 2.19–2.12 (m, 2H); ^{31}P NMR ($CDCl_3$) δ −11.09 and −11.14 [two t (partially overlapping), $^3J_{HP}$ = 6.4 Hz). HRMS [MH]$^+$ 469.1372, calcd for $C_{21}H_{22}N_6O_5P$: 469.1390.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.02.033.

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