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Aryl *H*-Phosphonates 17: (*N*-Aryl)phosphoramidates of Pyrimidine Nucleoside Analogues and Their Synthesis, Selected Properties, and Anti-HIV Activity

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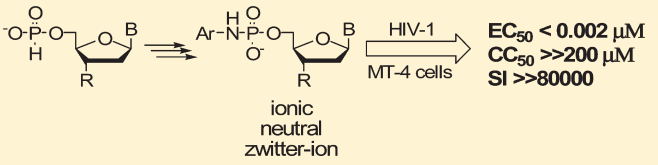
Supporting Information

ABSTRACT: New synthetic protocol for the preparation of nucleoside 5′-(*N*-aryl)phosphoramidate monoesters **4** was developed. It consisted of a condensation of the corresponding nucleoside 5′-*H*-phosphonates with aromatic- or heteroaromatic amines promoted by diphenyl phosphorochloridate, followed by oxidation of the produced *H*-phosphonamidates with iodine/water. 5′-(*N*-Aryl)phosphoramidate monoesters derived from 3′-azido-3′-deoxythymidine (AZT) or 2′,3′-dideoxyuridine (ddU) nucleosides and various aromatic and heteroaromatic amines were evaluated as potential anti-HIV drugs. It was found that these compounds act most likely as pronucleotides and that some of them have therapeutic indices superior to those of the reference AZT.



INTRODUCTION

Modifications at the phosphorus center of natural products was proved to be an exceptionally valuable tool in studies of reaction mechanisms particularly those catalyzed by enzymes.[1−3] In most cases, P-modified analogues of natural compounds exhibit new, sometimes unique chemical and biological properties. This was particularly well demonstrated for compounds bearing phosphoramidic bonds, which found many applications in molecular biology,[4−8] medicinal chemistry,[9−11] and in cancer therapy (e.g., cyclophosphamide).[12] In nucleotide and oligonucleotide chemistry, phosphoramidites and phosphoramidates occupy a well established position. While nucleoside phosphoramidites[13] are commonly used as basic building blocks in modern chemical synthesis of oligonucleotides, modified oligonucleotides bearing either 3′-5′ P−N bonds[14,15] or internucleotide aminoalkyl phosphoramidate moieties (cationic oligonucleotides)[16] are well established as potential antisense or antigene agents. Phosphor- and phosphonamidates derived from nucleoside analogues were subjects of intensive studies due to their promising pro-drug activity against various viruses (particularly HIV)[9,17−19] and in cancer therapy.[20−23] These compounds fulfill the criteria of so-called pro-nucleotides, i.e., they are (i) uncharged, (ii) highly lipophilic, (iii) reasonably soluble in water, (iv) stable in physiological environment, (v) enter the target cells without affecting the nucleotide skeleton, and after this (vi) can be converted chemically or by means of cellular enzymes into the respective nucleotide and subsequently into their 5′-triphosphates that inhibit proliferation of viruses. Although some of the above requirements for pro-nucleotides are contradictory (e.g., lipophilicity vs solubility in water or stability vs susceptibility to metabolism), several types of pro-nucleotides fulfill to some extent these criteria and play an important role in drugs used for treating of viral infections or cancer. Among these compounds, nucleoside pivaloyloxymethyl phosphotriesters (POM),[24] nucleoside *S*-acetyl-thioethyl phosphotriesters (SATE),[25] nucleoside cyclosaligenyl phosphotriesters (cycloSal nucleotides),[26] and phosphoramidate diesters have received the most attention,[27,28] which hopefully will expand the range of antiviral drugs. It is worth noting that until now there was only one pro-nucleotide (tenofovir disoproxil fumarate, VIREAD)[29] that was used in AIDS and chronic hepatitis B therapy.

The findings by C. R. Wagner et al., that nucleoside amino acid derived phosphoramidate monoesters are exceptionally highly active against HIV,[6] caused some confusion in the pro-nucleotide concept and criteria since being pronucleotides, they were inactive in HIV infected TK$^-$ cells. Later, it was found that phosphoramidic pro-nucleotides derived from simple amines are also very active against HIV.[30] These findings suggested that

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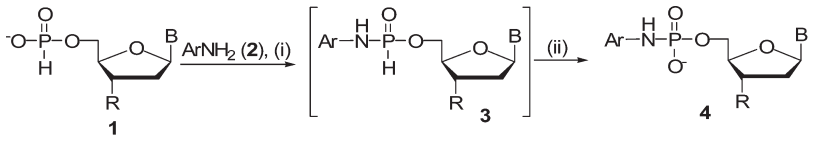
Scheme 1. Synthesis of Nucleoside (*N*-Aryl)phosphoramidates 4[a]



1a, R = N_3; B = thymin-1-yl
1b, R = H; B = uracil-1-yl
2a, Ar = phenyl
2b, Ar = 4-methoxyphenyl
2c, Ar = 4-chlorophenyl
2d, Ar = pyridin-2-yl
2e, Ar = pyridin-3-yl

3, 4aa, B = thymidin-1-yl, R = N_3, Ar = phenyl
3, 4ab, B = thymidin-1-yl, R = N_3, Ar = 4-methoxyphenyl
3, 4ac, B = thymidin-1-yl, R = N_3, Ar = 4-chlorophenyl
3, 4ad, B = thymidin-1yl, R = N_3, Ar = pyridin-2-yl
3, 4ae, B = thymidin-1`yl, R = N_3, Ar = pyridin-3-yl
3, 4ba, B = uracil-1-yl, R = H, Ar = phenyl
3, 4bd, B = uracil-1-yl, R = H, Ar = pyridn-2-yl
3, 4be, B = uracil-1-yl, R = H, Ar = pyridin-3-yl

[a] Reagents and conditions: (i) DPCP (1.5 molar equiv with respect to **1**) in DCM/pyridine 2: 1 (v/v);. (ii) I_2 (2.0 molar equiv) in pyridine containing water (50 molar equiv).

Table 1. ^{31}P NMR Data and Times of Oxidation of Nucleoside *H*-Phosphonamidates 3 to the Respective Nucleoside Phosphoramidates 4

cpd	δ_P[ppm]	$^1J_{HP}$[Hz]	$^2J_{HP}$[Hz]	$^3J_{HP}$[Hz]	t[a][min]
3aa	7.68, 7.99[b,c]	654.9, 656.7	7.3	7.3	<5
3ab	7.91, 8.23[b,c]	655.7, 654.0	7.4	7.4	30
3ac	7.43, 7.73[b,c]	655.1, 653.4	7.9	7.9	<5
3ad	7.32, 8.42[b,d]	664.0, 670,4	n. o.[e]	8.3	<5
3ae	7.43, 7.89[b,d]	656.0, 653.0	n. o.[e]	8.3	<5
3bd	7.52, 7.88[b,d]	671.6, 673.4	n. o.[e]	8.2	<5
3be	6.62, 7.05[b,d]	656.1, 656.9	n. o.[e]	8.2	<5
4aa	−1.73 dt[f]		7.3	7.3	
4ab	−1.38 dt[f]		6.4	6.4	
4ac	−2.13 dt[f]		7.3	7.3	
4ad	−4.47 dt[f]		6.4	6.4	
4ae	−2.17 dt[f]		7.0	7.0	
4af	−4.43 dt[f]		6.4	6.4	
4bd	−3.82 dt[f]		5.4	5.4	
4be	−2.50 dt[f]		6.4	6.4	
4bf	−4.29 dt[f]		6.4	6.4	

[a] Time required for the completion of the oxidation of **3** into **4**. [b] Two diastereomers. [c] In DCM/pyridine 1: 9 (v/v), doublet of doublets of triplets. [d] In DCM/pyridine 1: 9 (v/v), doublet of triplets. [e] n.o.: not observed. [f] In DMSO-d_6. Upon the addition of D_2O, due to the exchange of the hydrogen atom of the bridging phosphoramidate group, a triplet was observed in the ^{31}P NMR spectra.

nucleoside phosphoramidates may constitute a unique group of nucleotide derivatives with high antiviral or anticancer potency. Some efforts were devoted to recognize this problem, but they were focused on rather narrow groups of compounds, especially amino acids or some simple *N*-alkylamine derived phosphoramidates.[10,17]

In the course of our studies on nucleoside *H*-phosphonates, we have investigated the synthesis and properties of nucleoside (*N*-alkyl)phosphonamidates.[31] In this article, we present our studies on nucleoside (*N*-aryl)phosphon- and (*N*-aryl)phosphoramidates, which have properties distinguishing them from other phosphoramidates and which can make them attractive anti-HIV agents. Among aryl amines investigated, the main emphasis was placed on 2-, 3-, and 4-pyridynylamines due to their electronic and physicochemical properties, as well as their favorable lipophilic/hydrophilic character.

RESULTS AND DISCUSSION

Chemistry. In our previous studies on the synthesis and properties of nucleoside (*N*-alkyl)-*H*-phosphonamidates, we observed that formation of the P—N bond strongly depended on the condensing agent used and the basicity of the reaction media.[31] It was also found that a competing reaction of *N*-alkylamines with a condensing agent significantly affected yields of the desired nucleoside (*N*-alkyl)-*H*-phosphonamidates.

In contrast to these, we found that *N*-arylamines **2** used in this study [except, 4-aminopyridine (**2f**), *vide infra*] reacted smoothly with nucleoside *H*-phosphonate in the presence of DPCP as condensing agent, producing rapidly (<5 min) and nearly quantitatively (^{31}P NMR) putative (*N*-aryl)-*H*-phosphonamidates **3** (Scheme 1), whose structures were tentatively assigned on the basis of their chemical shifts, splitting pattern, and coupling constancies recorded in ^{31}P NMR spectra (Table 1). Unfortunately, isolation of the above species was impossible due to their partial instability during workup and/or silica gel chromatography. Coupling reactions were performed under mild conditions, i.e., at room temperature, in DCM containing 10% (v/v) of pyridine using a slight excess of an *N*-arylamine **2** and DPCP (both 1.5 molar equiv). As was mentioned above, the reaction worked well for all aromatic amines investigated except for 4-aminopyridine, for which a complex mixture of products was formed (^{31}P NMR spectroscopy).

These differences between *N*-alkylamines and *N*-arylamines investigated herein, can be attributed, at least partly, to the disparity in their pK_a values. For *N*-alkylamines (pK_a >10), high basicity was responsible for the side reactions observed and high nucleophilicity for a partial loss of chemoselectivity during the aminolysis of the pivalic-*H*-phosphonic mixed anhydride. Consequently, for *N*-alkylamines specific synthetic protocols had to be developed depending on the reactivity of the amine used.

In contrast to these, *N*-arylamines **2a**—**e** are much weaker bases (pK_a for **2a** — 4.6; **2b** — 4.58; **2c** — 3.99; **2d** — 6.71; **2e** — 6.03),[32] and thus, undesired side-reactions with activated *H*-phosphonate[31] (*vide supra*) or subsequent disproportionation[33] were significantly suppressed. However, their relatively low nucleophilicity was apparently sufficient to allow rapid and quantitative formation of the desired nucleoside (*N*-aryl)-*H*-phosphonamidates **3**.

The basicity of 4-aminopyridine (**2f**) (pK_a 9.13) markedly differs from those of 2- and 3-aminopyridines (**2d**, **2e** respectively)

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as well as of aniline (**2a**) and its derivatives (**2b**−**c**) (*vide supra*). This was probably a critical factor responsible for the low efficiency of synthesis of *H*-phosphonamidate of type **3** via a direct coupling of 4-aminopyridine and nucleoside *H*-phosphonate **1**, similar to that observed for highly basic *N*-alkylamines ($pK_a > 9.0$).[32] Thus, to obtain (*N*-pirydin-4-yl)phosphoramidate **4af** required for this study, another approach, which secured higher yield, was developed.

Since the attempted isolation of the intermediate species of type **3** failed (*vide supra*), the putative nucleoside (*N*-aryl)-*H*-phosphonamidates **3** were subjected *in situ* to oxidation. In contrast to nucleoside (*N*-alkyl)-*H*-phosphonamidates,[31] which were resistant to oxidation with iodine under the standard conditions commonly used for oxidation of *H*-phosphonate diesters,[34,35] we assumed that this reaction might work well in the case of analogous *N*-aryl phosphonamidate analogues **3**. Considering that oxidation of *H*-phosphonates with iodine proceeds via their tricoordinated tautomers ($\lambda^3\sigma^3$), we expected that the presence of an electron-withdrawing aryl group should make the (*N*-aryl)-*H*-phosphonamidates more prone to oxidation. Indeed, when these compounds were treated with an iodine−water system, all *H*-phosphonamidates **3** investigated yielded the respective (*N*-aryl)phosphoramidates **4** quantitatively. The influence of an aryl group on the oxidation of **3** could be observed along the series of **3a**−**c**, in which *H*-phosphonamidate **3b** bearing the least electron-withdrawing aryl group (4-methylphenyl one) required the longest time (30 min), while the other *H*-phosphonamidates **3** were oxidized much faster (<5 min, Table 1). All products **4** were isolated by silica gel flash column chromatography and after freeze-drying were obtained as white solids with high yields (>60%). Their purity and structure were confirmed with chromatography (TLC and HPLC) and spectral (^{1}H, ^{13}C and ^{31}P NMR spectroscopy) analyses.

Since both steps in the synthesis of nucleoside aryl phosphoramidates **4** (Scheme 1) proceeded smoothly and nearly quantitatively (^{31}P NMR), the above approach could be recommended as a convenient method for the synthesis of *N*-arylphosphoramidates derived from amines of $pK_a < 8$ (for synthesis of 4-aminopyridine derivative **4af** and **4bf**, *vide infra*).

During the purification and isolation of phosphoramidates **4**, we noticed an interesting phenomenon, namely, that triethylammonium or pyridinium salts of these compounds upon evaporation from polar solvents (particularly water) were rapidly loosing the cationic parts forming the corresponding acids (^{1}H NMR spectroscopy). Such a phenomenon was not observed for the triethylammonium salt of 2-, 3- or 4-pyridyl phosphodiesters (AZT derivatives)[36] that did not show a detectable (^{1}H NMR) loss of triethylammonium cation even upon repeated (several times) evaporation from aqueous solutions.

This behavior of phosphoramidates can be related to the presence of the P−N bond, which, due to back-donation of electrons from the nitrogen to phosphorus center,[37] should make these compounds less acidic than the corresponding phosphate derivatives. Unfortunately, pK_a values of phosphoramidate monoesters are usually unavailable,[38] but the acidity of *N*-(*n*-butyl) phosphoramidic acid (pK_a 2.92 and 9.88)[39] vs *n*-butyl phosphoric acid (pK_a 1.80 and 6.84)[32] seems to support this assumption.

The low acidity of phosphoramidate monoesters **4** can make these compounds under physiological conditions exist as an equilibrium mixture of different charged and uncharged forms (e.g., **I**−**IV** in Figure 1), although the exact preference for O- vs

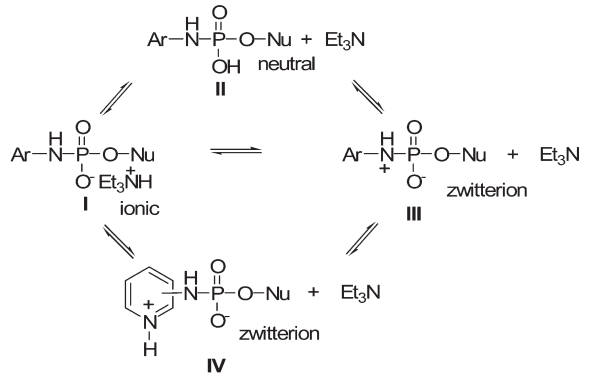
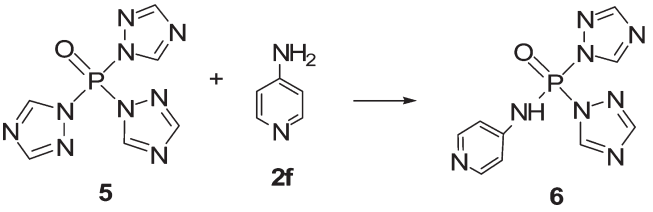


Figure 1. Some possible ionic structures of phosphoramidates **4** in solution.

Scheme 2. Synthesis of Di(1*H*-1,2,4-Triazol-1-yl)-[*N*-(pyridin-4-yl)]phosphoramidate (6)



various N-site protonation is unknown. Structural and electronic features of these species may have important biological bearing for chemical decomposition of (*N*-aryl)phosphoramidates *in vivo* as well as for their cellular up-take. Under physiological conditions, a fraction of neutral (**II**) and zwitter-ionic (**III** and **IV**) forms is probably not very high, but this may be sufficient to enable their uptake via passive diffusion (neutral molecule **II**) or the flip−flop mechanism[40] (zwitter-ion **III** and **IV**). One should note that although various phosphoramidates have been proposed as drugs or prodrugs, the importance of their pK_a values and sites of protonation for the potential biological activity has never been discussed.

In contrast to amines **2a**−**e**, coupling the 4-aminopyridine **2f** with nucleoside *H*-phosphonates **1** appeared to be ineffective in any of the several variants of this method examined. Thus, it was necessary to find another method for the synthesis of nucleoside [*N*-(pyridin-4-yl)]phosphoramidates **4f**. For this purpose, we turned our attention to phosphoryl tris-triazole[41] **5**, which in reaction with an equimolar amount of 4-aminopyridine (**2f**) in dioxane at room temperature produced di(1*H*-1,2,4-triazol-1-yl)-[*N*-(pyridin-4-yl)]phosphoramidate (**6**), which can be considered as a new phosphorylating agent for the introduction of basic aromatic amines into 5′-monophosphate nucleosides (Scheme 2).

The reaction proceeded smoothly, and it was complete within ca. 15 min (^{31}P NMR spectroscopy). Product **6** precipitated from the reaction mixture as a white solid, and after filtering off, washing, and drying, pure triamide **6** was obtained in ca. 90% yield. Its structure and purity were determined by spectral methods (^{1}H, ^{13}C and ^{31}P NMR, HRMS).

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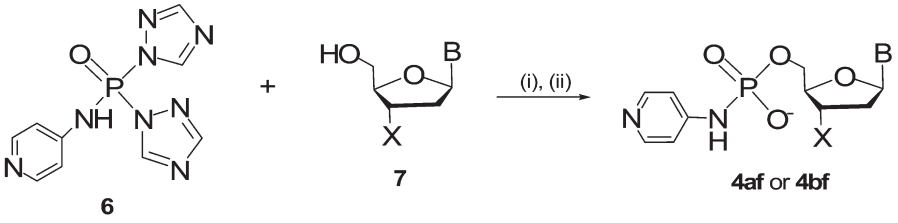
Scheme 3. Synthesis of Nucleosid-5′-yl-[*N*-(pyridin-4-yl)]phosphoramidates 4af and 4bf[a]



4af, X = N_3, B = thymin-1-yl
4bf, X = H, B = uracil-1-yl
7a, X = N_3, B = thymin-1-yl
7b, X = H, B = uracil-1-yl

[a] Reagents and conditions: (i) **6** (2 M equiv) and **7** (1 M equiv) in pyridine, 75 °C, 10 min. (ii) H_2O excess, 1 h.

This new phosphorylating reagent was applied for the introduction of the (*N*-pyridin-4-yl)phosphoramidate moiety into the 5′-position of the nucleoside analogues used in this study (Scheme 3).

To this end, to a suspension of di(1*H*-1,2,4-triazole)-[*N*-(pyridin-4-yl)]phosphoramidate (**6**) (2 molar equiv) in pyridine at 75 °C, nucleoside **7** (1 molar equiv) dissolved in pyridine was added stepwise with vigorous stirring. After 10 min, the reaction was quenched with the added excess of water to hydrolyze all reactive species present in the reaction mixture (excess of phosphorylating reagent and the intermediate nucleoside (*N*-pyridyn-4-yl)phosphotriazolide generated). This furnished nucleoside [*N*-(pyridin-4-yl)]phosphoramidate **4af** and **4bf** that after workup and isolation by silica gel column chromatography were obtained in 60%−70% yields. This simple protocol can be considered as a convenient approach for a chemoselective introduction of the [*N*-(pyridin-4-yl)]phosphoramidate moiety into nucleosides, even those that are thermally rather fragile (for instance ddU).

It is worth noticing that [*N*-(pyridin-4-yl)]phosphoramidates **4af** and **4bf** after chromatography were obtained as free acids, similar to the *N*-pyridin-2-yl and *N*-pyridin-3-yl regio isomers **4ad**−**ae** and **4bd**−**be** discussed above.

Biological Evaluation. Anti-HIV activity of nucleoside (*N*-aryl) phosphoramidates **4** and their decomposition in cell culture media were evaluated (Table 2).

These data indicate the high anti-HIV potency of AZT (*N*-aryl) phosphoramidates **4aa**−**af** but among them aminopyridinyl derivatives **4ad**−**af** stood out clearly. A striking feature of all compounds **4ad**−**f** is their very low cytotoxicity, which was not observed even when the cells were grown in cell culture media enriched with the tested compounds up to a concentration of 200 μM. High selectivity indices, SI_{50}, significantly exceeding a value of 80000 for all AZT (*N*-pyridinyl)phosphoramidates **4ad**−**f**, make these compounds exceptionally good candidates for prodrugs in AIDS therapy. It is worth noticing that compounds **4ad**−**f** retained their high anti-HIV potency during several days of the experiments and during this time their cytotoxicity was below a measurable level (data not shown). The concentration-dependent anti-HIV activity of ddU [*N*-(pyridin-4-yl)]phosphoramidate **4bf** can be considered as proof (at least partially) that this type of nucleotide analogues are pronucleotides, i.e., they enter the infected cell and release the corresponding nucleotides, which are subsequently converted by cellular kinases into nucleoside triphosphates. Since ddU is not a substrate for cellular kinases, the observed anti-HIV activity of compound **4bf** has to be attributed to its internalization and its further conversion into the respective 2′,3′-dideoxyuridin-5′-yl phosphate.[43] Because the acute cytotoxicity of AZT is due to the accumulation of AZT 5′-monophosphate (AZTMP) in cells, one can speculate that nucleoside phosphoramidates **4** have favorable pharmacokinetic characteristics (slower release of the corresponding nucleoside monophosphate than its conversion into di- and triphosphates) that maximize the therapeutic effect. Finally, a very low cytotoxicity of all aminopyridines **2d**−**f** (Table 2) (the expected metabolites of (*N*-pyridinyl)phosphoramidates **4**) can be certainly the added value when considering phosphoramidates **4** as potential candidates for anti-HIV drugs.

Unfortunately, a pronucleotide mode of action of nucleoside (*N*-pyridinyl)phosphoramidates **4** was not verified in the experiments with CEM TK^- HIV-1 infected cells, in which none of the examined compounds (of AZT or ddU series, **4a** and **4b**, respectively) showed detectable HIV inhibitory activity. It is symptomatic that similar observations were reported by C. R. Wagner et al., and J.-L. Imbach et al. during studies on another type of nucleoside phosphoramidates, namely, nucleoside amino-acid derived phosphoramidates[44] and nucleoside SATE [*N*-alkyl(or *N*-aryl)]phosphoramidates,[45] respectively. They found that these compounds in normal cells acted as pronucleotides whereas in TK^- cells were inactive against HIV. It was suggested, that the lack of anti-HIV activity in TK^- cells was due to the reduced substrate affinity of certain nucleoside phosphoramidates bearing an aromatic *N*-substituent (for instance phenyl group) for phosphoramidases; thus, the conversion of this type of nucleoside phosphoramidates to the respective nucleoside 5′-phosphate derivatives was prevented. It is possible that for the same reasons phosphoramidates **4ad**−**af** bearing heteroaromatic pyridinyl group studied herein also did not show anti-HIV activity in thymidine kinase deficient cells, while they were highly active in TK^+ HIV infected cells.

Stability of Nucleoside Phosphoramidates 4 in Cell Culture Media. In order to have a deeper insight into the mode of action of compounds **4** as potential pro-nucleotides, first we checked their stability and decomposition pathways in cell culture media. To find out the chemical factors involved in a possible metabolism, nucleoside (*N*-aryl)phosphoramidates **4aa**−**f** and **4bd**−**f** were incubated in RPMI at 37 °C, and progress of the decomposition was monitored by HPLC analysis. Under these conditions, all investigated compounds remained unchanged for at least 6 days, proving their stability in RPMI. In contrast, in cell

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Table 2. Stability and Anti-HIV Potency of Nucleoside (*N*-Aryl)phosphoramidates 4

Cpds	Nucl.	Ar	*r. t.*[a] [min]	$t_{1/2}$[b] [h]	C_{max}[c] [(μM]	CC_{50}[d] [μM]	CC_{90}[d] [μM]	EC_{50}[e] [μM]	EC_{90}[e] [μM]	SI_{50}[f] [μM]
4aa	**AZT**		15.5	> 5 days	200	>200	>>200	0.6	>10	>300
4ab	**AZT**		17.4	> 5 days	200	>200	>>200	<0.02	<0.02	>10000
4ac	**AZT**		17.3	> 5 days	360	290	360	0.02	0.1	14500
4ad	**AZT**		13.9	116.7	200	n. o.[g]	n. o.[g]	0.0024	0.029	>>83500
4ae	**AZT**		13.1	73.3	200	n. o.[g]	n. o.[g]	0.0023	0.009	>>87000
4af	**AZT**		11.8	>5 days	200	n. o.[g]	n. o.[g]	0.0011	0.006	>>182000
4bd	**ddU**		11.6	22.1	30	n. o.[g]	n. o.[g]	>10	>10	<3
4be	**ddU**		11.0	27.9	30	n. o.[g]	n. o.[g]	>10	>10	<3
4bf	**ddU**		10.7	32.1	50	n. o.[g]	n. o.[g]	0.2	1.35	>>250
AZT	-	-	13.5	-	200	>200	>>200	<0.002	0.005	>100000
ddU	-	-	9.8	-	-	-	-	-	-	-
AZTMP[h]	-	-	10.5	3.6	-	~100	-	0.01	-	~10000
ddUMP	-	-	8.8		-	-	-	-	-	-
2d	-	-		-	200	n. o.[g]	n. o.[g]	>20	>20	<10
2e	-	-		-	200	n. o.[g]	n. o.[g]	>20	>20	<10
2f	-	-		-	200	n. o.[g]	n. o.[g]	>20	>20	<10

[a] Retention time in HPLC analysis. For details, see the Experimental Section. [b] In RPMI/FBS 9:1 (v/v), 37 °C; concn 2 mM. [c] C_{max}: maximal concentration used in the assay. [d] Concentration (μM) required to reduce the viability of mock-infected CEM-T4 cells by 50% or 90%, respectively, as determined by the MTT method. [e] Concentration (μM) required to achieve 50% and 90% (respectively) protection from virus-induced cytopathogenicity. [f] SI_{50}: selectivity index CC_{50}/EC_{50}. SI values were rounded off respectively. [g] n. o.: in the tested range of concentrations cytotoxicity was not observed. [h] See ref 42.

culture media [RPMI/FBS 9:1 (v/v), 37 °C], which contained enzymatic activities (including those of phosphoesterase type),[36] the examined compounds **4** underwent slow decomposition with half-life times ($t_{1/2}$) varying from 22 h (**4bf**) to several days (**4af**) (Table 2). The observed decomposition can be attributed to enzymatic activities introduced into cell culture media with FBS, and the differences in $t_{1/2}$ are probably due to different substrate affinities of the AZT (**4a** series) and ddU (**4b** series) derivatives.

The main products observed (HPLC) during the hydrolysis of all the investigated nucleoside (*N*-pyridinyl)phosphoramidates **4** were the respective nucleosides (AZT or ddU) and their 5′-monophosphate derivatives (NMPs) as minor products. This might suggest that compounds **4** decomposed by hydrolysis of the phosphoramidic bond to produce NMP and/or by hydrolysis of phosphoester bond to produce the respective nucleoside and (*N*-pyridinyl)phosphoramidate. To clarify this issue, in a separate experiment AZTMP was incubated in RPMI/FBS, and it was found (HPLC) that it was readily ($t_{1/2}$ 3.6 h, Table 2) dephosphorylated with phosphatase-like activity present in FBS. This may suggest that decomposition of the examined compounds **4** proceeded via the respective NMPs, which were subsequently dephosphorylated and produced the observed AZT or ddU. Because in cell culture media compounds **4** decomposed very slowly (Table 2), the suggestion that they enter the cell unchanged and are metabolized into biologically active nucleotide (most likely nucleosid-5′-yl phosphate) seems plausible.

In order to recognize a potential involvement of cell surface components or other factors of cellular origin, which potentially may influence the metabolism of the examined compounds, additional experiments were carried out, in which the stability of nucleoside (*N*-pyridinyl)phosphoramidates **4ad**−**af** in the cell culture media in the presence of MT-4 cells was investigated. Under such conditions, no essential differences in the degradation rates and decomposition paths of nucleoside (*N*-pyridinyl)-phosphoramidates **4ad**−**af** as compared to the neat cell culture media were observed (results are not shown). The above results indicated that in *in vitro* experiments these compounds preserved their structure and that HIV infected cells are exposed for a long period of time to unchanged compounds **4ad-af.**

Trying to find possible degradation paths of the studied compounds, we looked for enzymes that potentially may be involved in the conversion of (*N*-pyridinyl)phosphoramidates

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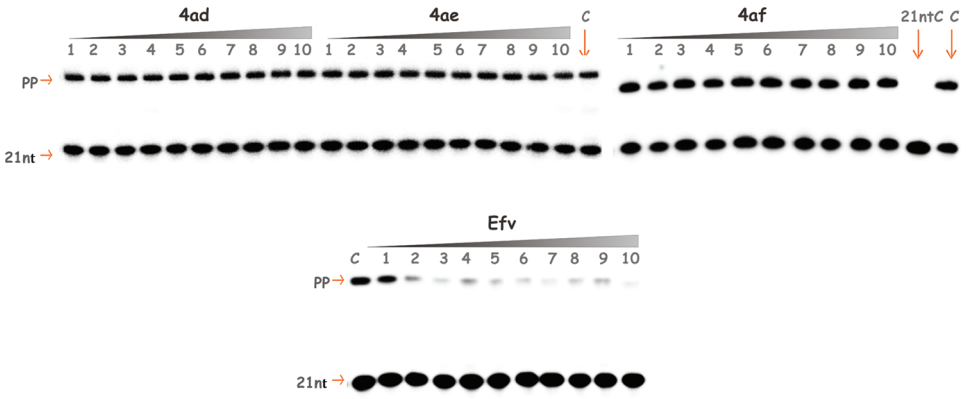

Figure 2. Influence of nucleoside (*N*-pyridinyl)phosphoramidates **4ad**−**af** on the HIV-1 RT polymerase activity.

4ad−**af** into AZTMP. To this end, the studied compounds were treated with different HIT-proteins, some of which exhibited the nucleoside phosphoramidase activity (Fhit proteins[46] and some Hint proteins[47]). Unfortunately, we found that none of the above nucleoside (*N*-pyridinyl)phosphoramidates was hydrolyzed by these enzymes (results not shown). To find out a possible origin of exceptionally high anti-HIV activity of the investigated compounds, their HIV-1 RT inhibitory potency was also investigated (*vide infra*).

Nucleoside (*N*-Pyridinyl)phosphoramidates 4 As Potential HIV-1 RT Inhibitors. Theoretically, nucleoside (*N*-pyridinyl)phosphoramidates **4** can inhibit a polymerase activity of HIV RT by mechanical blocking its active site or via allosteric interaction(s). Considering the high stability of compounds **4** in cell culture media (and possibly also in the cell), one might suspect that they could inhibit viral polymerase without conversion into the respective nucleoside triphosphates. To verify the above hypothesis, the inhibitory capacity of AZT (*N*-pyridinyl)-phosphoramidates **4ad**−**f** was tested in the standard *in vitro* reverse transcription reaction involving recombinant HIV-1 RT. The reaction mixtures contained DNA template, 5′(^{32}P)-radiolabeled primer, all four natural nucleosid-5′-yl triphosphates and variable amounts of the examined compounds **4ad**−**f**. After incubation for 1 h at 45 °C, the reaction mixtures were analyzed by electrophoresis in a 12% denaturating polyacrylamide gel. The efficacy of DNA synthesis was determined by autoradiography. As a positive control, an analogous series of the reactions with efavirenz (well established non-nucleoside HIV RT inhibitor) was carried out. A negative control was a standard reaction mixture without any inhibitor. The obtained data clearly showed (Figure 2) that none of the investigated compounds demonstrated inhibitory activity in concentrations ≤3 μM. The above results might strongly suggest that it is not nucleoside (*N*-pyridinyl)phosphoramidates **4** but their metabolites [e.g., AZT 5′-triphosphate (AZTTP)][48] that inhibit viral RT in the cell culture experiments.

The tested compounds are indicated above each set of analytical runs. Lanes 1−10 correspond to the increasing concentration of each compound: 0.03; 0.06; 0.15; 0.3; 0.45; 0.6; 1.2; 1.5; 2.1 and 3 μM, respectively. Lane C, control, reaction without inhibitor; 21nt, primer, 21 nucleotide long; 21ntC, primer control lane; PP, polymerization product; Efv, efavirenz.

Conclusions. In conclusion, we found that nucleoside (*N*-aryl)-*H*-phosphonamidates **3** can be effectively generated (the only product observed in the ^{31}P NMR spectra) in neutral solvents (e.g., DCM) containing a controlled amount of pyridine and then can be oxidized with an iodine/water system into the respective nucleoside (*N*-aryl)phosphoramidates **4**. The rate of the latter reaction depended on electronic features of the aryl group present, and for more electron-withdrawing aryls, oxidation was faster. In this respect, nucleoside (*N*-aryl)phosphoramidates **4** differ from their *N*-alkyl counterparts that are very resistant to oxidation.[31] Hence, the procedures described herein provide a versatile and efficient approach for the synthesis of (*N*-aryl)phosphoramidates. The exceptions were 4-aminopyridine derivatives, which were synthesized using the new phosphorylating agent, di(1*H*-1,2,4-ditriazol-1-yl)-[*N*-(pyridin-4-yl)]phosphoramidate **6**. This reagent can be recommended for the introduction of [*N*-(pyridin-4-yl)phosphoramidate functionality into nucleosides and probably also into other biologically important compounds.

All AZT derived phosphoramidates revealed high anti-HIV potency, and among them, [*N*-(pyridinyl)]phosphoramidates (**4ad**−**af**) stood out with their very low cytotoxicity and very high antiviral activity. These features make phosphoramidates **4** attractive candidates for new anti-HIV pro-drugs. Although their mode of action is still unclear, the observed concentration-dependent anti-HIV activity of the ddU derivate (**4bf**) strongly points out that these compounds act as pronucleotides. Unfortunately, this was not confirmed in experiments with TK-deficient cells, in which none of examined phosphoramidates **4** was active against HIV. The case seems to be not simple and certainly requires careful conceptual and experimental verification and this is the subject of intensive studies in our laboratories. Finally, we would like to conclude that nucleoside (*N*-aryl)phosphoramidates **4** (particularly **4ae**−**af**) irrespective of their mode of action, might be considered as new attractive anti-HIV pro-drugs due to their exceptionally convenient pharmacokinetic parameters and very low cytotoxicity.

EXPERIMENTAL SECTION

General Methods and Materials. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on Varian Unity BB VT 300 MHz or Bruker Avance II 400 MHz machines. The ^{31}P NMR experiments were carried out in 5 mm tubes using 0.1 M solutions of the phosphorus-containing compounds. ^{31}P NMR chemical shifts are reported in ppm relative to 85% H_3PO_4 in water (external standard) and with the exception of compound **6** are listed in Table 1. Mass spectra were recorded with the ESI technique with negative ionization with accuracy below 5 ppm. HPLC analyses were performed on a C18 (5.0 μm) 4.6 mm × 150 mm column

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using solvent system A, 0.1 M triethylammonium acetate, and B, A/acetonitrile 1: 4 (v/v), and gradient B in A 0%−50% within 20 min with a flow rate of 1.5 mL/min, at 35 °C. For TLC analysis, the precoated plates (Merck silica gel F_{254}) were used, and for column chromatography silica gel Si 60, 35−70 mesh (Merck) was used.

DCM before use for reactions was dried over P_2O_5, distilled, and kept over molecular sieves 4 Å until the amount of water was less than 10 ppm. Commercial grade anhydrous pyridine was stored over molecular sieves of 4 Å until the amount of water was below 20 ppm. The amount of water in solvents was measured with Karl Fisher coulometric titration. Each evaporation of solvents was performed with a rotary evaporator under reduced pressure using water bath temperature not exceeding 40 °C. Nucleosid-5′-yl *H*-phosphonates were obtained as described earlier.[49]

As judged from ^{1}H, ^{13}C, and ^{31}P NMR spectra and HPLC analyses (Waters 1525; Waters 2487 UV detector, λ_{254}), the purity of the obtained compounds was as least 97% except for compounds **4bd**−**bf** whose purity was ca. 90%.

RPMI-1640 cell culture medium and heat non-inactivated fetal bovine serum (FBS) used for studies of stability of compounds were from Sigma (R7256 and F7524 respectively).

Cytotoxicity and Anti-HIV Activity. *CC_{50} and CC_{90} Parameters.* For the determination of compound cytotoxicity, CEM-T4 and CEM-A cells were cultured in standard conditions (37 °C, 5% CO_2) on 96 wells culture plates in media RPMI/FCS 10% (v/v). The experiments were carried out in media containing tested compounds in concentrations of the appropriate range. Cultures in neat medium (RPMI, 10% FCS) were used as a control. Viability of cells was determined after 7 days using the MTT test[50] in which to each well of a culture plate was added 10 μL of MTT solution (5 mg/mL), and cultures were incubated for 3 h at a temperature of 37 °C. After centrifugation, the supernatant was removed, and DMSO was added for lysis of the cells and to dissolve crystals of farmazan. Color intensity was measured with a plate reader ($\lambda_{560\ nm}$).

Anti-HIV Activity (EC_{50} and EC_{90}). To determine EC_{50} and EC_{90}, CEM-T4 cells were preincubated (96 flat bottom wells culture plates) for 24 h in standard conditions (37 °C, 5% CO_2) and in standard medium [RPMI, FCS 10% (v/v)] enriched with tested compounds in concentrations 0,001−20 μM. In each well, 20000 cells were suspended in the solution of the tested compound (200 μL). For each concentration, cultures were run in triplicate. As a reference, media containing AZT in concentrations of 1.25 μM, 2.5 μM, 5.0 μM, 10.0 μM, and 20 μM were used. As a reference of maximal replication of HIV-1, culture in neat standard medium [RPMI/FCS 10% (v/v)] was used. After 24 h of incubation in medium enriched with a tested compound and AZT, cells were inoculated with a known amount of HIV, and after 8 days, the amount of viral protein p24 was measured with the MTT method.[51] For each tested compound and for each concentration, the measurements of EC_{50} and EC_{90} were done in triplicate.

Inhibition of HIV RT. Recombinant HIV-1 reverse transcriptase (HIV-1 RT) was obtained with HIV-1 RT bacterial expression vector generously provided by Stephen H. Hughes from the National Cancer Institute, Frederick, MD, USA. A 50 nt DNA template (length of 50 nucleotide units) and primer (length of 21 nucleotide units) were purchased from IBA. T4 polynucleotide kinase was purchased from Promega and γ-[^{32}P]ATP from Amersham. All chemicals were from Sigma. Sequences of DNA molecules used in the experiments are presented below, and the complementary fragments are underlined. Template, 5′-TTTTAGTCAGTGTGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACTTG-3′; primer, 5′-CAAGTCCCTGTTCGGGCGCCA-3′. The inhibition HIV-1 RT potency with nucleoside (*N*-pyridyl)phosphoramidates **4ad**−**f** was tested *in vitro* in a primer extension reaction, involving a 50 nt DNA template a cDNA primer (length 21 nucleotide units, ^{32}P labeled at the 5′ end in a reaction involving T4 polynucleotide kinase and γ-[^{32}P]ATP). HIV-1 RT was preincubated in a reaction buffer (50 mM Tris-HCl at pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT) with increasing amounts of each compound (5 min at 45 °C), and after the DNA primer and template were added, the polymerizations were initiated by the addition of dNTPs to the final concentration of 50 μM. The above reaction mixtures were incubated for 60 min at 45 °C, and after that, the products were separated in a 12% denaturing polyacrylamide gel and visualized by autoradiography with PhosphorImager Typhoon (Molecular Dynamics). To determine the efficiency of polymerization, the amount of the fully extended product (FP) in each sample was quantified using the MultiGage program.

Syntheses. *General Procedure for the Synthesis of Nucleosid-5′-yl (N-Aryl)phosphoramidates **4** except for **4af** and **4bf**.* Nucleoside *H*-phosphonate **3** (1 mmol) and 2- or 3-aminopyridine (1.5 mmol) were rendered anhydrous by the evaporation of the added pyridine (3 × 10 mL) and then dissolved in 10 mL of DCM containing 10% (v/v) of pyridine. To this, DPCP (1.5 mmol) was added, and the reaction mixture was left for 5 min. To oxidize the produced nucleoside, *H*-phosphonate **3** iodine (2 mmol), dissolved in pyridine containing water (50 mmol), was added, and after 5−30 min (see Table 1), excess of iodine was decomposed with the added ethanethiol and the solvent evaporated. The remaining residue dissolved in water (10 mL/1 mmol of nucleoside) was washed with DCM (3 × 25 mL/1 mmol of nucleoside). The aqueous layer was evaporated, and the desired product was isolated with method **A** or **B** (*vide infra*).

*Purification by Method **A** (for Compounds **4aa**−**ac**).* The crude oily residue dissolved in a minimum volume of methanol was applied to a silica gel column (4 × 7 cm, equilibrated with 2-propanol) and washed with 2-propanol. Separation was carried out with a flash chromatography system using a gradient of water (0−20%) in 2-propanol containing 5% (v/v) of triethylamine. Fractions containing a pure compound were collected and evaporated, and the triethylammonium salt of nucleotide **4** was solidified by freeze-drying from benzene−methanol.

*Purification by Method **B** (for Compounds **4ad**−**af** and **4bd**−**bf**).* The crude residue was dissolved in a minimum volume of toluene/methanol 1:1 (v/v) and loaded on a silica gel column (loaded and equilibrated with toluene/methanol 5:1, v/v). A separation was run using gradient (20−50%, v/v) of methanol in toluene. Fractions containing pure compound **4** were collected and evaporated. After freeze-drying (from benzene−methanol), pure products **4ad**−**af** and **4bd**−**bf** were obtained as white dry powder.

*3-Azido-3′-deoxythymidyn-5′-yl (N-Phenyl)phosphoramidate, Triethylammonium Salt (**4aa**).* Yield, 0.22 g (71%). ^{1}H NMR (400 MHz) δ_H (D_2O) 1.28 (9H, t, J = 7.3 Hz), 1.87 (3H, s), 2.42 (2H, m), 3.18 (6H, q, J = 7.3 Hz), 4.01 (1H, m), 4.08 (2H, m), 4.32 (1H, m), 6.14 (1H, t, J = 6.6 Hz), 6.86 (1H, t, J = 7.8 Hz), 7.00 (2H, d, J = 7.8 Hz), 7.19 (2H, t, J = 7.8 Hz), 7.58 (1H, s). ^{13}C NMR (75 MHz) δ_C (D_2O) 7.8, 11.2, 35.6, 46.2, 60.0, 63.9 (d, J = 7.0 Hz), 82.6 (d, J = 10 Hz), 84.6, 110.9, 116.4 (d, J = 7.0 Hz), 119.8, 128.6, 137.0, 141.5, 151.0, 165.9. HRMS m/z 421.1045, calcd for $[C_{16}H_{18}N_6O_6P]^-$ 421.1031.

*3-Azido-3′-deoxythymidyn-5′-yl (N-4-Methylphenyl)phosphoramidate, Triethylammonium Salt (**4ab**).* Yield, 0.09 g (53%). ^{1}H NMR (400 MHz) δ_H (D_2O) 1.30 (9H, t, J = 7.6 Hz), 1.85 (3H, d, J = 0.8 Hz), 2.19 (3H, s), 3.19 (6H, q, J = 7.6 Hz), 3.97−4.01 (1H, br. m), 4.04−4.11 (2H, br. m), 4.30 (1H, m), 6.16 (1H, t, J = 6.8 Hz), 6.90 (2H, d, J = 8.4 Hz), 6.98 (2H, d, J = 8.4 Hz), 7.54 (1H, q, J = 0.8 Hz). ^{13}C NMR (100 MHz) δ_C (D_2O) 8.2, 11.6, 19.5, 35.9, 46.6, 52.1, 60.4, 64.2 (d, J = 4.7 Hz), 83.0 (d, J = 9.0 Hz), 84.9, 111.2, 117.1 (d, J = 7.0 Hz), 129.2, 129.7, 137.3, 139.2, 151.4, 166.1. HRMS m/z 435.1293, calcd for $[C_{17}H_{20}N_6O_6P]^-$ 435.1287.

*3-Azido-3′-deoxythymidyn-5′-yl (N-4-Chlorophenyl)phosphoramidate, Triethylammonium Salt (**4ac**).* Yield, 0.23 g (73%). ^{1}H NMR (400 MHz) δ_H (D_2O) 1.31 (9H, t, J = 7.2 Hz), 1.84 (3H, s), 2.48 (2H, t, J = 6.8 Hz), 3.21 (6H, q, J = 7.2 Hz), 4.01 (1H, m), 4.13 (2H, m), 4.36

(1H, m), 6.17 (1H, t, J = 6.8 Hz), 6.94 (2H, d, J = 8.8 Hz), 7.13 (2H, d, J = 8.8 Hz), 7.45 (1H, s). ^{13}C NMR (75 MHz) δ_C (D_2O), 7.8, 11.2, 35.4, 46.2, 59.8, 64.0 (d, J = 5.0 Hz), 82.5 (d, J = 10 Hz), 84.7, 110.9, 117.9 (d, J = 7.0 Hz), 123.8, 128.0, 137.0, 140.3, 151.0, 165.7. HRMS m/z 455.0631, calcd for $[C_{16}H_{17}ClN_6O_6P]^-$ 455.0641.

*3-Azido-3′-deoxythymidin-5′-yl [N-(Pyridin-2-yl)]phosphoramidate (**4ad**).* Yield, 0.13 g (84%). ^{1}H NMR (400 MHz) δ_H (D_2O) 1.84 (3H, s), 2.50 (2H, m), 4.04 (1H, m), 4.14 (2H, m), 4.40 (1H, m, J = 6.4 Hz), 6.16 (1H, t, J = 6.4 Hz), 6.87 (1H, m, J = 8.4 Hz, 6.4 Hz), 7.03 (1H, d, J = 8.4 Hz), 7.51 (1H, s), 7.60 (1H, m, J = 6.4 Hz, 1.6 Hz), 8.30 (1H, d, J = 6.4 Hz). ^{13}C NMR (100 Hz) δ_C (D_2O) 11.5, 35.8, 60.1, 64.6 (d, J = 4.0 Hz), 82.8 (d, J = 9.1 Hz), 85.0, 110.9, 111.2, 116.4, 137.3, 138.6, 147.0, 151.5, 154.0 (d, J = 5.0 Hz), 166.4. HRMS m/z 422.0912, calcd for $[C_{15}H_{17}N_7O_6P]^-$ 422.0983.

*3-Azido-3′-deoxythymidin-5′-yl [N-(Pyridin-3-yl)]phosphoramidate (**4ae**).* Yield, 0.14 g (92%), ^{1}H NMR (400 MHz) δ_H (D_2O), 1.82 (3H, s), 2.50 (2H, m), 4.04 (1H, m), 4.13 (2H, m), 4.34 (1H, dt, J = 6.4 Hz), 6.17 (1H, t, J = 6.4 Hz), 7. Twenty-two (1H, dd, J = 8.4, 4.8 Hz), 7.44 (1H, m), 7.46 (1H, s), 7.96 (1H, d, J = 4.8 Hz), 8.18 (1H, m). ^{13}C NMR (100 Hz) δ_C (D_2O) 11.4, 35.7, 60.0, 64.6 (d, J = 5.0 Hz), 82.6 (d, J = 9.1 Hz), 85.2, 111.1, 125.9, 129.5 (d, J = 8.0 Hz), 132.1 (d, J = 8.0 Hz), 134.9, 137.5, 141.5, 151.3, 166.1. HRMS m/z 422.0910, calcd for $[C_{15}H_{17}N_7O_6P]^-$ 422.0983.

*2,3′-Dideoxyuridin-5′-yl [N-(Pyridin-2-yl)]phosphoramidate (**4bd**).* Yield, 0.11 g (88%). ^{1}H NMR (400 MHz) δ_H (D_2O), 1.93 (1H, m), 2.13 (2H, m), 2.46 (1H, m), 4.00 (1H, m), 4.20 (1H, m), 4.36 (1H, m), 5.73 (1H, d, J = 8.0 Hz), 6.05 (1H, m), 7.10 (1H, dd, J = 6.6 and 7.2 Hz), 7.20 (1H, d, J = 8.8 Hz), 7.72 (1H, d, J = 8.0 Hz), 7.98 (1H, dd, J = 6.6 and 8.8 Hz), 8.06 (1H, d, J = 7.2 Hz). ^{13}C NMR (100 Hz) δ_C (D_2O) 24.4, 30.1, 66.1 (d, J = 5.6 Hz), 79.7 (d, J = 8.4 Hz), 85.8, 101.2, 113.6 (d, J = 5.3 Hz), 115.6, 139.0, 141.6 (d, J = 2.1 Hz), 143.2, 151.0, 151.7 (d, J = 2.6 Hz), 165.6. HRMS m/z 367.0776, calcd for $[C_{14}H_{16}N_4O_6P]^-$ 367.0813.

*2,3′-Dideoxyuridin-5′-yl [N-(Pyridin-3-yl)]phosphoramidate (**4be**).* Yield, 0.12 g (93%). ^{1}H NMR (400 MHz) δ_H (D_2O) 1.90 (1H, m), 2.12 (2H, m), 2.43 (1H, m), 3.93 (1H, m), 4.13 (1H, m), 4.36 (1H, m), 5.64 (1H, d, J = 8.0 Hz), 6.05 (1H, m), 7.37 (1H, dd, J = 5.1 and 8.4 Hz), 7.60 (1H, ddd, J = 1.2, 2.7, and 8.5 Hz), 7.68 (1H, d, J = 8.0 Hz), 8.03 (1H, d, J = 4.6 Hz), 8.22 (1H, d, J = 2.2 Hz). ^{13}C NMR (100 Hz) δ_C (D_2O) 24.5, 30.3, 65.7 (d, J = 5.2 Hz), 79.8 (d, J = 9.4 Hz), 86.0, 101.1, 124.4, 126.2 (d, J = 6.2 Hz), 135.2 (d, J = 8.1 Hz), 137.7, 139.7, 141.5, 151.0, 165.7. HRMS m/z 367.0778, calcd for $[C_{14}H_{16}N_4O_6P]^-$ 367.0813

*Synthesis of Di(1H-1,2,4-triazol-1-yl) [N-(Pyridin-4-yl)]phosphoramidate **6**.* To a solution of phosphoryl-tris-(1*H*-1,2,4-triazole) **5** in dioxane (1 mmol/5 mL), 4-aminopyridine (equimolar amount) dissolved in pyridine (1 mmol/1.25 mL) was added. After 10−20 min, di(1*H*-1,2,4-triazol-1-yl) [*N*-(pyridin-4-yl)]phosphoramidate **6** started to precipitate as a white solid. After 1 h, the solvent was decanted, and the remaining solid was washed with 1,4-dioxane (3× 5 mL), diethyl ether (1× 10 mL), and dried under vacuum. Yield, 91%. Mp 167−170 °C (not corrected). ^{1}H NMR (400 MHz) δ_H (DMSO-d_6), 6.95 (2H, d, J = 6.8 Hz), 8.13 (4H, m), 9.03 (2H, s). ^{13}C NMR (100 MHz) δ_C (DMSO-d_6), 158.0, 154.4 (d, J = 16.3 Hz), 149.8 (d, J = 8.2 Hz), 139.6, 118.5 (d, J = 23.0 Hz). ^{13}P NMR (121 MHz) δ_P (DMSO, ppm), −14.5 (s); HRMS (−) m/z 275.0786, calcd for $[C_9H_8N_8OP]^-$ 275.0559

*Procedure for the Synthesis of Nucleoside [N-(Pyridin-4-yl)]phosphoramidates **4af** and **4bf**.* To a suspension of di(1*H*-1,2,4-triazol-1-yl) [*N*-(pyridin-4-yl)]phosphoramidate **6** (2 molar equiv) in pyridine (1 mmol/12.5 mL) at 75 °C (oil bath), AZT or ddU (1 molar equiv) dissolved in pyridine (1 mmol/10 mL) was added in five equal portions in 1 min intervals with stirring (magnetic bar). The reaction was continued for ca. 10 min at 75 °C and cooled down to room temperature, and after a large excess of water was added (10% v/v), the whole mixture was left for 1 h. The solvent was evaporated (rotary evaporator), and the remaining oily residue dissolved in water (10 mL/1 mmol of nucleoside) was washed with methylene chloride (3 × 25 mL/1 mmol of nucleoside), and the aqueous layer was evaporated. Compounds **4af** and **4bf** were isolated with purification procedure **B** (see above).

*3-Azido-3′-deoxythymidin-5′-yl [N-(Pyridin-4-yl)]phosphoramidate (**4af**).* Yield, 0.50 g, 59%, ^{1}H NMR (300 MHz) δ_H (D_2O) 1.85 (3H, s), 2,51 (2H, m), 4.10 (3H, m), 4.41 (1H, dt, J = 6.0 Hz), 6.13 (1H, t, J = 6.6 Hz), 7.22 (2H, d, J = 6.3 Hz), 7.52 (1H, s), 8.19 (1H, d, J = 6.3 Hz). ^{13}C NMR (75 MHz) δ_C (D_2O), 11.1, 35.4, 59.6, 64.2 (d, J = 5.1 Hz), 82.6 (d, J = 9.1 Hz), 85.2, 111.2, 112.2 (d, J = 7.0 Hz), 137.5, 142.4, 151.3, 155.9, 166.1. HRMS m/z 422.1006, calcd for $[C_{15}H_{17}N_7O_6P]^-$ 422.0983.

*2,3′-Dideoxyuridin-5′-yl [N-(Pyridin-4-yl)]phosphoramidate (**4bf**).* Yield, 0.46 g, 62%. ^{1}H NMR (400 MHz) δ_H (D_2O) 1.90 (1H, m), 2.10 (2H, m), 2.45 (1H, m), 3.92 (1H, dt, J = 5.6 and 11.6 Hz), 4.13 (1H, dt, J = 3.2 and 11.6 Hz), 4.36 (1H, m), 5.68 (1H, d, J = 8.0 Hz), 6.05 (1H, dd, J = 2.8 and 7.2 Hz), 7.05 (2H, d, J = 6.4), 7.70 (1H, d, J = 8.0 Hz), 8.17 (2H, d, J = 6.4). ^{13}C NMR (75 MHz) δ_C (D_2O), 24.8, 30.7, 66.1 (d, J = 5.0 Hz), 80.1 (d, J = 9.1 Hz), 86.4, 101.5, 112.1 (d, J = 7.5 Hz), 141.9, 146.6, 151.8, 152.2, 166.6. HRMS m/z 367.0813, calcd for $[C_{14}H_{16}N_4O_6P]^-$ 367.0870.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. ^{1}H, ^{13}C, and ^{31}P NMR spectra of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

AZT, 3′-azido-3′-deoxythymidine; AZTMP, 3′-azido-3′-deoxythymidin-5′-yl monophosphate; AZTTP, 3′-azido-3′-deoxythymidin-5′-yl triphosphate; DCM, dichloromethane; ddU, 2′,3′-dideoxyuridine; DMSO, dimethyl sulfoxide; DPCP, diphenyl chlorophosphate; DTT, dithiothreitol; ESI, electron spray ionization; FBS, fetal bovine serum; HRMS, high-resolution mass spectrometry; NMP, nucleosid-5′-yl monophosphate; RPMI, Roswell Park Memorial Institute medium; SATE, *S*-acetyl-thioethyl; TK$^-$, thymidine kinase-deficient

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