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Bis(benzoyloxybenzyl)-Di*PP*ro Nucleoside Diphosphates of Anti-HIV Active Nucleoside Analogues

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Nucleoside analogues are extensively used as antiviral and anticancer agents. Their efficiency is dependent on their metabolism into the ultimately active nucleoside triphosphates. Often one step or even more in the metabolism of the nucleoside to the triphosphate is inefficient. To overcome this hurdle, prodrugs of the nucleotides are needed. Bis(acyloxybenzyl)nucleoside diphosphates have been reported by us as a first example of an efficient nucleoside *dip*hosphate *pro*drug (Di*PP*ro nucleotides). Here, the synthesis and the properties of bis(benzoyloxybenzyl)nucleoside diphosphates of the nucleoside analogues d4T and AZT are disclosed. The synthesis was achieved by using a phosphoramidite/oxidation route. In chemical hydrolysis studies, most of the compounds formed a nucleoside diphosphate. This was confirmed in CEM cell extracts, although the prodrug stability in extracts was lower than in phosphate buffer. Furthermore, the stability and the amount of nucleoside diphosphate formed were dependent on the substituent in the benzoyl moiety. Some of the compounds were more active against HIV in thymidine kinase-deficient CEM/TK$^-$ cells than were d4T or AZT.

Introduction

Nucleoside analogues are widely used as anticancer and antiviral agents. However, their activity depends on their efficient phosphorylation by kinases. The metabolism leads via the nucleoside monophosphate (NMP) and the nucleoside diphosphate (NDP) to the ultimately antivirally active nucleoside triphosphate (NTP). Former studies have shown that cellular kinases often catalyze this biotransformation insufficiently, and this results in a loss in antiviral activity or adverse effects can occur.[1] This hurdle can be overcome by using lipophilic prodrugs of the phosphorylated parent nucleosides (pronucleotides), which are able to bypass the rate-limiting, kinase-catalyzed conversion steps. This aim has been successfully achieved in the past for NMP with prodrugs such as the *cyclo*Sal, bisSate, bisPOM, and phosphoramidate nucleotides.[2]

In the case of the anti-HIV-active 3′-azido-3′-deoxythymidine (AZT), the rate-limiting step is the conversion of AZT monophosphate (AZTMP) to AZT diphosphate (AZTDP).[3] The result is high intracellular concentrations of AZTMP, which lead to several side effects.[4] Therefore, the use of an AZTDP prodrug could overcome this limitation. However, reports on such NDP delivery systems have been rare.[5] In the case of NDPs, complete lipophilic modification of the highly polar diphosphate group in particular can lead to a marked decrease in the chemical stability of the pyrophosphate moiety, and this results in a cleavage of this part of the molecule. As a consequence, for chemical reasons, the design of NDP prodrugs is more demanding than the development of NMP prodrugs.

Hostetler et al. synthesized different NDP diglycerides as potential NDP prodrugs.[6] However, the hydrolysis of these compounds delivered the monophosphates instead of the diphosphates due to cleavage of the diphosphate moiety. Another approach was reported by Huynh-Dinh et al., who acylated the β-phosphate with fatty acids to give a mixed anhydride bond.[7] In chemical hydrolysis studies, the NDP was indeed formed; however, in cell extracts, an undefined decomposition of the compounds was observed.

Recently, we reported a first successful NDP prodrug approach. In our approach, the β-phosphate of the NDP was esterified with two acyloxybenzyl moieties; this neutralized the two charges on this phosphate group (Di*PP*ro approach).[8,9] Because this approach is based on an enzymatically triggered delivery mechanism, the diphosphate is released preferentially inside cells, and the released NDP is trapped due to the highly charged pyrophosphate. The chemical stability at physiological pH was found to be high. The general principle of the hydrolysis of these Di*PP*ro nucleotides is summarized in Scheme 1. Enzymatic cleavage of the acyl group attached to the phenol residue destabilizes the benzyl phosphate ester bond. As a result, a spontaneous 1,6-elimination gives the mono-masked NDP intermediate **3**. Repetition of this process eventually delivers the nucleoside diphosphate.

We have reported Di*PP*ro compounds that bear short-chain carboxylic acids and fatty acids of different lengths in the acyloxybenzyl masking unit.[8,9] In that report, 3′-deoxy-2′,3′-didehydrothymidine (d4T) was used as a model nucleoside. The corresponding Di*PP*ro compounds were synthesized and ana-

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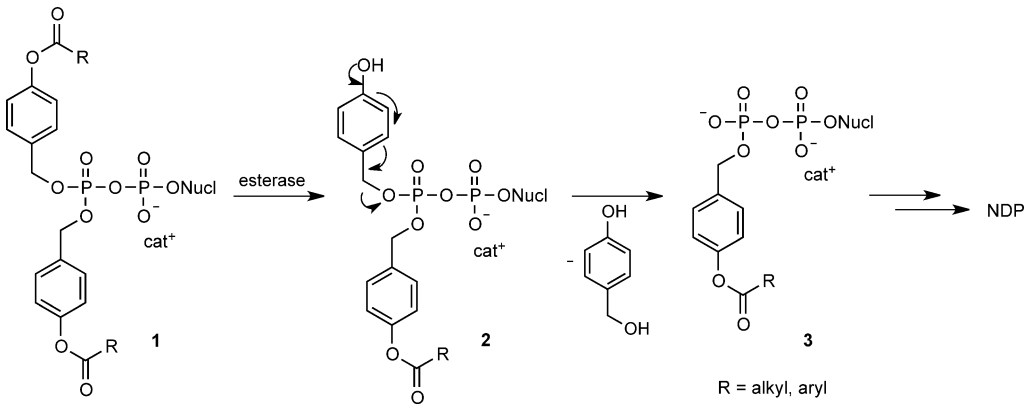
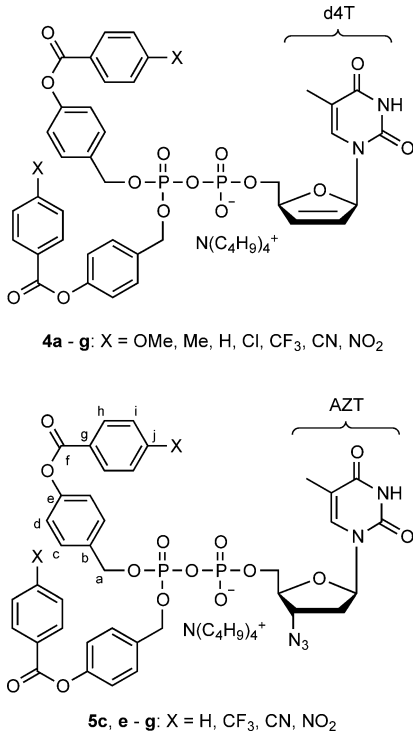


Scheme 1. General principle of the hydrolysis of Di*PP*ro nucleotides **1**.

lyzed to investigate the relationship between chain length, hydrolysis behavior, and antiviral activity. It was proven that the stability of these compounds increased with increasing chain length. Up to an alkyl chain length of C_9 (Scheme 1, R = C_9H_{19}), the nucleoside diphosphate was preferentially released in phosphate buffer as well as in cell extract. A further increase in the chain length was associated with a further increase in chemical stability and, at the same time, led to a side reaction: nucleophilic attack of, for example, water at the β-phosphorus atom favored cleavage of the diphosphate moiety and thus formation of unwanted NMP in addition to the NDP.

Among the Di*PP*ro compounds published earlier were the benzoyl-protected derivatives bis(benzoyloxybenzyl)-d4TDP (**4c**; X = H; Scheme 2) and the corresponding AZTDP derivative **5c**.[8] The benzoyl modification offers the possibility to fine-tune the hydrolysis behavior by adding different substituents without varying the lipophilicity to any considerable degree. This was not possible with the previously described acyloxybenzyl counterparts. In this report, we disclose a series of Di*PP*ro nucleoside diphosphates bearing lipophilic and enzymatically cleavable benzoyloxybenzyl moieties. Di*PP*ro-d4TDP derivatives **4** with different donor and acceptor substituents in the 4-position of the benzoyl moiety were synthesized (Scheme 2). The hydrolysis behavior was investigated with regard to chemical stability in phosphate buffer. Incubation with T-lymphocyte CEM cell extracts provided information about the properties in biological media. A second series of acceptor-substituted bis(benzoyloxybenzyl)-AZTDP prodrugs **5** of the nucleoside analogue AZT was also synthesized.



4a - **g**: X = OMe, Me, H, Cl, CF_3, CN, NO_2

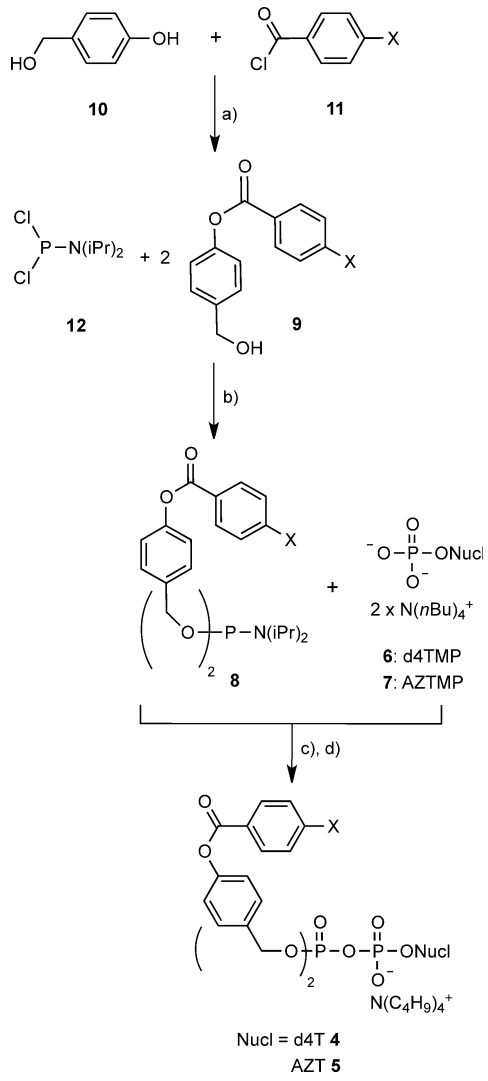
5c, **e** - **g**: X = H, CF_3, CN, NO_2

Scheme 2. Synthesized bis(benzoyloxybenzyl)-NDP prodrugs **4** and **5** (lettering shown in compounds **5** is used for NMR assignment in the Experimental Section).

Results and Discussion

The synthesis route towards Di*PP*ro-NDPs **4** and **5**, based on phosphoramidite chemistry, is summarized in Scheme 3. Corresponding phosphoramidites **8** bearing two masking units were coupled with d4TMP (**6**) or AZTMP (**7**) to form the target pronucleotide after oxidation. This convergent approach offered high flexibility for combining different bis(benzoyloxybenzyl)-phosphoramidites with nucleotides. Briefly, different 4-benzoylbenzyl alcohols (**9**) were obtained in good yield by selective benzoylation of 4-hydroxybenzyl alcohol (**10**) by using benzoylchlorides **11** at low temperatures (up to 75% yield). Subsequent reaction with (*N*,*N*-diisopropyl)dichlorophosphine (**12**) gave phosphoramidites **9** in yields of up to 90%. Nucleotides **6** and **7** were synthesized in yields of 83 and 61%, respectively, by following the Sowa–Ouchi protocol.[10] The final synthesis of Di*PP*ro nucleoside diphosphates **4** and **5** was successfully achieved by dicyanoimidazole-mediated coupling and subsequent oxidation with *tert*-butylhydroperoxide, which gave the target products in yields of 30–66% over the two final steps.

The X substituents in the benzoyl moiety of d4TDP prodrugs **4a**, **b**, **e**–**g** ranged from strong donors to strong acceptor groups. The masks that gave the highest selectivity for the delivery of d4TDP in the hydrolysis studies were also applied to AZTMP **7**; this led to compounds **5e**–**g**.



Scheme 3. Synthesis of benzoyl ester-bearing Di*PP*ro-NDPs **4** and **5**. a) 1 equiv benzoylchloride **11**, 1.1 equiv 4-hydroxybenzylalcohol **10**, 1 equiv TEA, THF, 2 h, 0 °C; b) 1 equiv P^{III}-reagent **12**, 2.2 equiv benzyl ester **9** 2.3 equiv TEA, THF, 16–20 h, RT; c) 1 equiv $[N(nBu)_4]_2NMP$, 1.5–1.8 equiv phosphoramidite **8**, 1.5–1.75 equiv 4,5-dicyanoimidazole, 16–20 h, RT; d) *t*BuOOH, RT, 20 min.

Next, Di*PP*ro compounds **4** and **5** were studied with regard to their chemical stabilities in aqueous phosphate buffered saline (PBS, pH 7.3) and in CEM cell extracts. The latter was used to simulate the biological environment and to study the contribution of enzymes present in the extracts to the hydrolysis process. In both cases, the studies were monitored by analytical RP-HPLC. Generally, the half-lives—measured by the decrease in the corresponding peak of the pronucleotide **4** or **5**—in PBS were significantly higher than those in cell extracts (Table 1), and the half-lives increased with the increasing electron-donating effect of the substituent.

Detailed studies showed that from acceptor-substituted compounds **4e**–**g,** d4TDP was formed in phosphate buffer as well as in cell extracts either exclusively (**4g**) or at least predominantly (**4e**, **f**). However, when the electron-withdrawing effect was reduced by introducing donor substituents, the corresponding NMP **6** was also formed.

Table 1. Hydrolysis half-lives ($t_{1/2}$) of bis(benzoyloxylbenzyl)-Di*PP*ro-NDPs in PBS (pH 7.3) and CEM cell extracts as well as retention times.

Compound **4** or **5**	X	$t_{1/2}$ [h] PBS (7.3)	CEM/0	t_R [min] (RP-HPLC)
4a	OMe	480	9	14.8[a]
4b	Me	80	3	15.8[a]
4c	H[8]	82	7	9.41
4d	Cl	13	7	16.1[a]
4e	CF_3	20	5.5	15.7[a]
4f	CN	7	4	13.2[a]
4g	NO_2	4	1.25	14.8[a]
5c	H[8]	82	0.5	10.32
5e	CF_3	12	3.5	18.9[b]
5f	CN	6.5	4	15.9[b]
5g	NO_2	4.5	1.5	16.7[b]

[a] Gradient A with $CH_3CN/[N(C_4H_9)_4]$ phosphate (0.55 mM). [b] Gradient B with $CH_3CN/[N(C_4H_9)_4]$ acetate (2 mM).

In the case of the strongest donor substituent (X = OCH_3), the stability increased markedly; at the same time, almost no d4TDP could be detected. Surprisingly, Me-Ph-Di*PP*ro-d4TDP **4b** showed relatively low stability in the cell extracts. For some unknown reason, this compound seemed to be a good substrate for the esterases present in the cell extracts.

The studies on Di*PP*ro-d4TDPs **4** revealed a direct correlation between the stability of the compounds and the amount of d4TDP released, also when using CEM cell extracts. In the case of the strong acceptor-substituted Di*PP*ro-d4TDPs **4e**–**g**, mainly d4TDP, but also small amounts d4TMP (**6**) were detected. However, the formation of the d4TMP can also be a result of dephosphorylation of d4TDP by phosphatases present in the cell extracts. In contrast, the enzymatic hydrolysis of MeO-Ph-Di*PP*ro-d4TDP (**4a**) did not deliver d4TDP, only d4TMP **6** was detected. In addition, only very small amounts of intermediate **3** were observed in the chromatograms. In this case, it is most probable that d4TMP (**6**) was formed as a result of anhydride bond cleavage.

In Scheme 4 the different possible hydrolysis routes are summarized. In addition to the benzoyl ester hydrolysis (pathway A), cleavage of the phosphate anhydride occurred as a side reaction (pathway B). The latter led to the formation of d4TMP, as described above.

On the other hand, the faster the first hydrolysis of the prodrug to the mono-masked NDP intermediate **3**, the higher the amount of nucleoside diphosphate formed. It was observed that only very small amounts—if any—of NMP were formed in the hydrolysis of the intermediate **3**. Therefore, the phosphate anhydride bond was most likely cleaved by nucleophilic attack of water or hydroxide at the β-phosphorus atom of the doubly masked starting material **4** or **5**. The second charge at the β-phosphorus atom present in the mono-masked NDP intermediate **3** prevented a nucleophilic attack at the pyrophosphate moiety. This explains why d4TDP was formed predominantly in both media from Di*PP*ro compounds bearing acceptor-substituted masking units (X = CF_3, CN, NO_2; Figure 1).

Because of the favorable properties of the masks bearing strong electron-withdrawing groups in the benzoyl moiety,

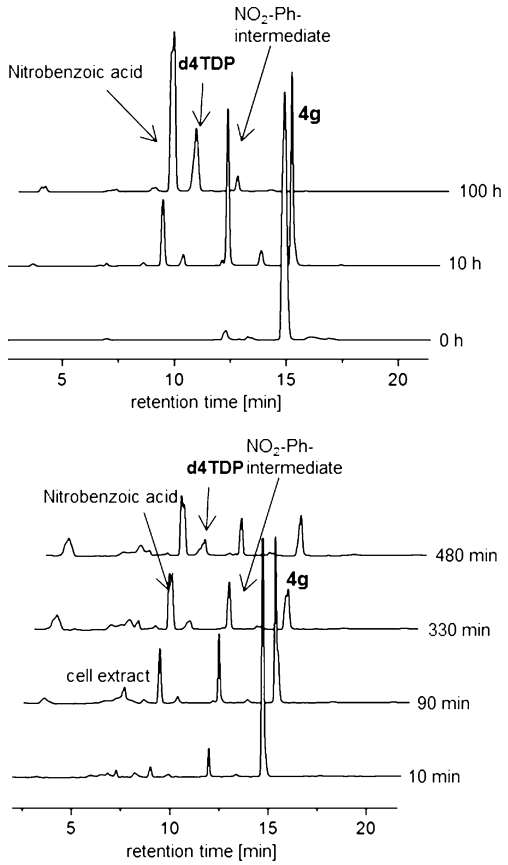
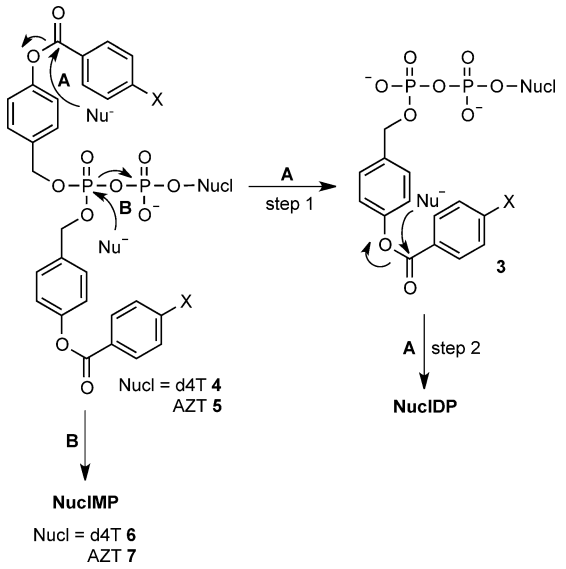


Figure 1. HPL chromatograms of the hydrolysis of NO_2-Ph-Di*PP*ro-d4TDP (**4g**) in phosphate buffer at pH 7.3 (top) and in CEM cell extracts (bottom).



Scheme 4. Two different hydrolysis pathways of bis(benzoyloxybenzyl)-Di*PP*ro-nucleoside diphosphates **4** and **5**.

these were also applied to achieve AZTDP delivery. Interestingly, the stabilities and the product distribution in PBS as well as in cell extracts from Di*PP*ro-AZTDPs **5e–g** were comparable with the observations made with the corresponding Di*PP*ro-d4TDP compounds. However, CF_3-Ph-Di*PP*ro-AZTDP **5e** was found to be more labile than the corresponding d4TDP derivative **4e** (Table 1).

As compared to bis(acyloxybenzyl)-Di*PP*ro-d4TDPs, the chemical stability is markedly lower for all acceptor-substituted bis(benzoyloxybenzyl)-Di*PP*ro-NDPs, except for R = CH_3 ($t_{1/2}$ = 10 h).[8,9] As a consequence, the amount of d4TDP formed from the prodrug was also higher. Additionally, it was observed that Di*PP*ro compounds with short aliphatic chains seemed to be better substrates for the enzymes present in cell extracts (R = CH_3, $t_{1/2}$ = 0.05 h; R = C_4H_9, $t_{1/2}$ = 0.6 h).[9]

The lipophilicity of the bis(benzoyloxybenzyl)-Di*PP*ro compounds was estimated from the retention times detected by RP-HPLC. As seen in Table 1, the values were all in the same range, so changing the substituent X had only a small influence on the lipophilicity of the pronucleotide. However, as can also be seen in Table 1, the substituents had a significant effect on the stability, for example, CF_3-Ph-Di*PP*ro-d4TDP **5e** was beyond the most lipophilic compounds, it showed a low stability in PBS as well as in the cell extract. This behavior contrasted with that of the recently reported bis(acyl)-Di*PP*ro compounds.[9]

Antiviral activity

All compounds were evaluated for their potency in inhibiting HIV replication in HIV-1- and -2-infected wild-type CEM/0 cells and in HIV-2-infected mutant thymidine kinase-deficient CEM/TK$^-$ cells. Table 2 summarizes the antiviral and cytotoxic data of the potential prodrugs, with the parent nucleoside analogues d4T and AZT as reference compounds.

Table 2. Anti-HIV activity of Di*PP*ro-NDPs in wild-type and mutant CEM cells.

Compd	EC_{50}[a] [μM]			CC_{50}[b] [μM]
	CEM/0		CEM/TK$^-$	CEM/0
	HIV-1	HIV-2	HIV-2	
4a	0.69 ± 0.41	1.1 ± 0.29	3.9 ± 2.3	98 ± 5.7
4b	0.80 ± 0.23	0.91 ± 0.28	≥ 2	41 ± 0.0
4c[8]	0.40	0.30	0.85	36 ± 5
4d	0.71 ± 0.24	1.0 ± 0.51	2.9 ± 2.3	51 ± 2.8
4e	0.97 ± 0.49	4.8 ± 2.5	38 ± 20	193 ± 45
4f	0.24 ± 0.16	1.4 ± 0.61	14 ± 7.9	108 ± 6.4
4g	1.0 ± 0.64	2.0 ± 1.2	13 ± 5.2	101 ± 3.5
5e	0.042 ± 0.015	0.052 ± 0.0057	7.7 ± 2.1	108 ± 6
5f	0.021 ± 0.0046	0.071 ± 0.052	≥ 10	95 ± 7
5g	0.008 ± 0.003	0.28 ± 0.20	≥ 10	110 ± 8
d4T	0.86 ± 0.45	2.3 ± 2.4	173 ± 70	> 250
AZT	0.0066 ± 0.0048	0.021 ± 0.018	> 250	> 250

[a] Antiviral activity in T-lymphocytes: 50% effective concentration. [b] Cytotoxic activity: 50% cytotoxic concentration to decrease cell viability.

All compounds showed antiviral activity in CEM/0 cells. The antiviral activity against HIV-1 and HIV-2 were in all cases in the same range as for the corresponding reference compounds d4T and AZT. Furthermore, in thymidine kinase-deficient CEM/TK$^-$ cells, the activity of the donor-benzoyl-Di*PP*ro compounds was up to 60 times higher than the activity of the parent nu-

cleosides. This is an indication that the intact pronucleotides were taken up by the cells and, once inside the cells, delivered a phosphorylated compound.

In comparison to the parent nucleosides, the most active prodrugs, **4b–d**, showed a slightly increased cytotoxicity. The reason for this remains unclear. It has often been observed, that in the case of highly active nucleotide prodrugs, such as the phosphoramidate compounds or the *cyclo*Sal-phosphate triesters, the antiviral activity is always associated with an increase in the CC_{50} values. However, in the case of the *cyclo*Sal compounds, we have clearly proven that, in comparison to 3-methyl-*cyclo*Sal-d4TMP, which also showed an increase in the toxicity value, the corresponding dTMP derivative did not show an increase in CC_{50} value, even though the same *o*-quinonemethide or salicylalcohol was formed during the delivery of the nucleoside monophosphate. In compounds **4** and **5**, a *p*-quinonemethide or 4-hydroxybenzylalcohol is formed. Further studies have to be performed to answer this question.

Somewhat surprising was the loss of antiviral activity in case of almost all acceptor-substituted Di*PP*ro pronucleotides **4** and **5** because, as shown in the hydrolysis studies, they released NDPs almost selectively. This might be the result of a too rapid hydrolysis preventing the efficient uptake of the intact pronucleotides. As a consequence, the NDP was most likely formed before membrane passage took place. The phosphorylated hydrolysis product is then extracellularly dephosphorylated to give the parent nucleoside, which is then taken up by the cells and proved to be active in the wild-type cell line but not in the TK-deficient cell line. Another reason for the partial loss of antiviral activity might be insufficient membrane permeation as a result of insufficient lipophilicity. Alternatively, it can be a mix of both. However, it should be noted that in all cases, the prodrugs were endowed with a markedly higher anti-HIV efficacy in the CEM/TK^- cell cultures than the parental d4T or AZT.

Conclusions

Bis(benzoyloxybenzyl)-Di*PP*ro-NDPs have been studied as a new class of nucleoside diphosphate prodrug. Their synthesis by phosphoramidite chemistry was straightforward and led to high purities and good chemical yields. Their stability could be adjusted by the use of different donor or acceptor substituents at the 4-position of the benzoyl moiety. We were able to show that, unlike the previously reported bis(acyloxybenzyl)-NDPs, the chemical or enzymatic stability of our compounds was no longer dependent on the lipophilicity of the prodrugs. Di*PP*ro-NDPs with acceptor substituents at the 4-position of the benzoyl group hydrolyzed preferentially or even selectively to give the nucleoside diphosphate in phosphate buffer as well as in CEM cell extracts. Although we have shown that the basic idea of using bis(benzoyloxybenzyl) moieties as biolabile masking groups worked in principle, the results of the antiviral activity assay were somewhat unexpected. The observed significant loss in anti-HIV activity in TK-deficient cells might be due to the fact that the acceptor-substituted bis(benzoyloxybenzyl)-NDPs were too unstable and/or too low in lipophilicity to achieve a rapid uptake of intact Di*PP*ro compounds into the cells.

Experimental Section

Synthesis

General: All experiments involving water-sensitive compounds were conducted under absolutely anhydride conditions and under nitrogen. All solvents were dried by using standard procedures. Et_3N and CH_3CN were dried by being heated under reflux over calcium hydride for several days followed by distillation. CH_3CN was stored over 3 Å molecular sieves. THF was dried by being heated under reflux over potassium followed by distillation. Ethyl acetate, petroleum ether 50–70, CH_2Cl_2, and CH_3OH for chromatography were distilled before use. Solvents were evaporated on a rotary evaporator under reduced pressure or by using a high-vacuum pump. Column chromatography was performed by using Merck silica gel 60, 230–400 mesh. Analytical thin-layer chromatography was performed on Merck precoated aluminum plates $60\,F_{254}$ with a 0.2 mm layer of silica gel containing a fluorescent indicator; sugar-containing compounds were visualized with a spray reagent (0.5 mL 4-methoxybenzaldehyde, 9 mL EtOH, 0.1 mL glacial acetic acid, 0.5 mL concentrated sulfuric acid) by heating with a fan. For some separations, a chromatotron (Harrison Research 7924T) with glass plates coated with 1-, 2-, or 4-mm layers of VWR $60\,PF_{254}$ silica gel containing a fluorescent indicator (VWR no. 7749) was used.

Instrumentation: ^1H NMR spectroscopy was carried out on a Bruker AMX 400 at 400 MHz, a Bruker DMX 500 at 500 MHz or a Bruker AV 400 at 400 MHz with $CDCl_3$, [D_6]DMSO or CD_3OD as internal standards. ^{13}C NMR spectra were recorded on a Bruker AMX 400 at 101 MHz or a Bruker AV 400 at 101 MHz ($CDCl_3$ [D_6]DMSO or CD_3OD as internal standard). ^{19}F NMR spectra were recorded on a Bruker AV 600 MHz. ^{31}P NMR spectra were recorded on a Bruker AMX 400 at 162 MHz (H_3PO_4 as internal standard). All ^{13}C and ^{31}P NMR spectra were recorded in the proton-decoupled mode. Mass spectra were obtained on a VG Analytical VG/70-250F FAB spectrometer (xenon, matrix was *m*-nitrobenzyl alcohol) or a VG70S EI spectrometer. High-resolution ESI mass spectra were recorded on an Agilent 6224 ESI-TOF spectrometer in positive or negative mode.

Analytical HPLC: LiChroCART 125-3 with LiChrospher 100 RP-18 (5 μm) standard gradient. Method A: 5–100% CH_3CN in [$N(C_4H_9)_4$] phosphate buffer (0.55 mM; 0–27 min), flow rate 0.1 mL min^{-1}. UV detection at 265 nm. Method B: 5–80% CH_3CN in [$N(C_4H_9)_4$] acetate buffer (2 mM; 0–24 min), flow rate 0.1 mL min^{-1}. UV detection at 265 nm. The purity of the Di*PP*ro nucleotides was evaluated by HPLC and was in all cases $\geq 95\%$.

General procedure A: Preparation of phenylbenzoates: Benzoylchloride (1.1 equiv) dissolved in THF was added dropwise to a weak solution of 4-hydroxybenzylalcohol (1 equiv) and Et_3N (1 equiv) in THF at 0 °C. After the mixture had been stirred for 1.5–2.5 h at 0 °C and filtration of triethylammonium chloride, the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and washed twice with saturated $NaHCO_3$ solution and once with water. The combined organic layers were dried over sodium sulfate and concentrated under reduced pressure. The byproduct was removed by filtration over silica gel in CH_2Cl_2 or by crystallization from the product in CH_2Cl_2.

General procedure B: Preparation of bis(4-benzoyloxybenzyl)-*N,N*-diisopropylaminophosphoramidites: A solution of phenyl-

benzoate (2.2 equiv) and Et_3N (2.2 equiv) in THF was added dropwise to dichloro-*N*,*N*,diisopropylaminophosphoramidite (1 equiv), also dissolved in THF. After the mixture had been stirred for 1 to 2 days at RT and filtration of triethylammonium chloride, the solvent was removed under reduced pressure. The crude product was purified by radial preparative TLC (chromatotron) with petroleum ether/ethyl acetate and 10% Et_3N.

General procedure C: Preparation of Di*PP*ro nucleoside diphosphate: Phosphoramidite (1.8 equiv) was added to a solution of nucleoside monophosphate (1 equiv) in dry CH_3CN. The reaction was started by the addition of 4,5-dicyanoimidazole (1.6 equiv) at RT. After being stirred for 2 h, the mixture was oxidized by the addition of *tert*-butylhydroperoxide (1.8 equiv, 5.5 molar solution in *n*-decane). The solvents were removed under reduced pressure. The compounds were purified by RP-18 chromatography (CH_3CN/water). In some cases the tetra-*n*-butylammonia ions were exchanged against ammonia by elution over DOWEX 50WX8 (NH_4^+).

General procedure D: Preparation of Di*PP*ro nucleoside diphosphate: Phosphoramidite (1.5 equiv) was added to a solution of nucleoside monophosphate (1 equiv) in CH_3CN. The reaction was started by the addition of 4,5-dicyanoimidazole (0.5 equiv, 0.25 M solution in CH_3CN) at RT. Every 5 min a further 0.25 equiv of 4,5-dicyanoimidazole were added up to a total of 1.5 or 1.75 equiv. After being stirred for 5 min after the last addition, the mixture was oxidized by the addition of *tert*-butylhydroperoxide (1.5 equiv, 5.5 molar solution in *n*-decane). The solvents were removed under reduced pressure. The compounds were isolated by automatic flash RP-18 chromatography (CH_3CN/water). In some cases the tetra-*n*-butylammonia ions were exchanged against ammonia by elution over DOWEX 50WX8 (NH_4^+).

4-(Hydroxymethyl)phenyl-4′-methoxybenzoate (9a): General procedure A with 4-hydroxybenzyl alcohol (4.00 g, 32.2 mmol) and Et_3N (4.53 mL, 32.2 mmol) dissolved in THF (30 mL), and 4-methoxybenzoyl chloride (4.62 mL, 35.2 mmol) dissolved in THF (15 mL). The product (4.85 g, 18.8 mmol, 59%) was obtained as a colorless solid. $R_f = 0.34$ in CH_2Cl_2/CH_3OH (19:1); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.09–8.07$ (m, 2H; H-2′), 7.39–7.38 (m, 2H; H-3), 7.20–7.19 (m, 2H; H-2), 7.13–7.11 (m, 2H; H-3′), 5.24 (t, $^3J_{HH} = 5.4$ Hz, 1H; -OH), 4.52 (d, $^4J_{HH} = 5.3$ Hz, 2H; Ph-CH_2), 3.87 ppm (s, 3H; -OCH_3);^{13}C NMR (101 MHz, [D$_6$]DMSO): $\delta = 164.3$ (C=O), 163.7 (C-4′), 149.4 (C-1), 140.1 (C-4), 131.9 (C-2′), 127.5 (C-3), 121.5 (C-2), 121.0 (C-1′), 114.2 (C-3′), 62.4 (Ph-CH_2), 55.6 ppm (CH_3); IR: $\tilde{\nu} = 3536$, 3324, 3010, 2973, 2938, 2844, 1723, 1703, 1604, 1507, 1254, 1162, 1078, 1002, 842, 761 cm^{-1}; MS (FAB): *m/z* 259.1 $[M+H^+]^+$.

4-(Hydroxymethyl)phenyl-4′-methylbenzoate (9b): General procedure A with 4-hydroxybenzylalcohol (4.00 g, 32.2 mmol) and Et_3N (4.53 mL, 32.2 mmol) dissolved in THF (30 mL), and 4-methylbenzoyl chloride (4.66 mL, 35.2 mmol) dissolved in THF (15 mL). The product (5.46 g, 22.5 mmol, 70%) was obtained as a colorless solid. $R_f = 0.53$ in CH_2Cl_2/CH_3OH (19:1); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.03–8.01$ (m, 2H; H-2′), 7.42–7.38 (m, 4H; H-3′, H-3), 7.22–7.20 (m, 2H; H-2), 5.24 (t, $^3J_{HH} = 5.7$ Hz, 1H; -OH), 4.52 (d, $^3J_{HH} = 5.7$ Hz, 2H; Ph-CH_2), 2.43 ppm (s, 3H; -CH_3); ^{13}C NMR (101 MHz, [D$_6$]DMSO): $\delta = 163.9$ (C=O), 149.4 (C-1), 144.1 (C-4′), 140.2 (C-4), 129.6 (C-2′), 127.5 (C-3′), 126.2 (C-3), 121.5 (C-2), 120.3 (C-1′), 62.3 (Ph-CH_2), 21.2 ppm (CH_3); IR: $\tilde{\nu} = 3398$, 2930, 2877, 1726, 1610, 1506, 1269, 1175, 1073, 1003, 882, 747, 482 cm^{-1}; MS (FAB): *m/z* 243.1 $[M+H^+]^+$.

4-(Hydroxymethyl)phenyl-4′-chlorobenzoate (9d): General procedure A with 4-hydroxybenzylalcohol (3.00 g, 24.2 mmol) and Et_3N (3.40 mL, 24.2 mmol) dissolved in THF (30 mL), and 4-chlorobenzoyl chloride (3.40 mL, 26.6 mmol), dissolved in THF (30 mL). The product (3.96 g, 15.1 mmol, 62%) was obtained as colorless crystals. $R_f = 0.54$ in CH_2Cl_2/CH_3OH (19:1); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.13–8.12$ (m, 2H; H-2′), 7.69–7.67 (m, 2H; H-3′), 7.41–7.39 (m, 2H; H-2), 7.24–7.22 (m, 2H; H-3), 5.25 (s, 1H; -OH), 4.53 ppm (s, 2H; Ph-CH_2); ^{13}C NMR (101 MHz, [D$_6$]DMSO): $\delta = 163.8$ (C=O), 149.1 (C-1), 140.4 (C-4′), 138.9 (C-4), 131.6 (C-2′), 129.1 (C-3′), 127.9 (C-1′), 127.5 (C-3), 121.3 (C-2), 62.3 ppm (Ph-CH_2); IR: $\tilde{\nu} = 3312$, 3211, 3188, 2926, 2872, 1732, 1593, 1509, 1293, 1277, 1075, 850, 752, 682, 581, 504 cm^{-1}; MS (FAB): *m/z* 263.0 $[M+H^+]^+$.

4-(Hydroxymethyl)phenyl-4′-trifluoromethylbenzoate (9e): General procedure A with 4-hydroxybenzylalcohol (1.50 g, 12.1 mmol) and Et_3N (1.70 mL, 12.1 mmol) dissolved in THF (30 mL), and 4-trifluoromethylbenzoyl chloride (1.54 mL, 13.3 mmol) dissolved in THF (15 mL). The product (2.69 g, 9.07 mmol, 75%) was obtained as a colorless solid. $R_f = 0.85$ in CH_2Cl_2/CH_3OH (19:1); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.33–8.32$ (m, 2H; H-2′), 7.99–7.97 (m, 2H; H-3′), 7.42–7.41 (m, 2H; H-3), 7.28–7.26 (m, 2H; H-2), 5.26 (t, $^3J_{HH} = 5.7$ Hz, 1H; -OH), 4.53 ppm (d, $^3J_{HH} = 5.7$ Hz, 2H; Ph-CH_2); ^{13}C NMR (101 MHz, [D$_6$]DMSO): $\delta = 163.7$ (C=O), 149.1 (C-1), 140.6 (C-4), 133.3 (d, $^2J_{CF} = 32$ Hz, C-4′), 132.8 (C-1′), 130.7 (C-2′), 127.6 (C-3), 125.9 (d, $^3J_{CF} = 3.7$ Hz, C-3′), 125.9 (CF_3), 121.7 (C-2), 64.9 ppm (Ph-CH_2); ^{19}F NMR (101 MHz, [D$_6$]DMSO): $\delta = 61.68$ ppm; IR: $\tilde{\nu} = 3345$, 2926, 2857, 1736, 1509, 1411, 1375, 1167, 1128, 879, 771, 699 cm^{-1}; HRMS (EI): *m/z* calcd for $[C_{15}H_{11}F_3O_3]^+$ 296.07 $[M]^+$, 173.02 $[C_8H_4F_3O]^+$, found: 295.9, 172.9.

4-(Hydroxymethyl)phenyl-4′-cyanobenzoate (9f): General procedure A with 4-hydroxybenzylalcohol (1.37 g, 11.0 mmol) and Et_3N (1.55 mL, 11.0 mmol) dissolved in THF (20 mL), and 4-cyanobenzoyl chloride (2.00 g, 12.1 mmol) dissolved in THF (10 mL). The product (1.85 g, 7.31 mmol, 66%) was obtained as a colorless solid. $R_f = 0.45$ in petroleum ether/ethyl acetate (1:1); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.29–8.27$ (m, 2H; H-2′), 8.10–8.08 (m, 2H; H-3′), 7.42–7.40 (m, 2H; H-3), 7.28–7.26 (m, 2H; H-2), 5.25 (t, $^3J_{HH} = 5.7$ Hz, 1H; -OH), 4.53 ppm (d, $^3J_{HH} = 5.8$ Hz, 2H; Ph-CH_2); ^{13}C NMR (101 MHz, [D$_6$]DMSO): $\delta = 163.2$ (C=O), 149.2 (C-1), 140.4 (C-4), 133.6 (C-1′), 132.7 (C-3′), 130.7 (C-2′), 128.4 (C-3), 121.7 (C-2), 118.0 (CN), 115.4 (C-4′), 62.3 ppm (Ph-CH_2); IR: $\tilde{\nu} = 3380$, 3096, 2874, 2231, 1732, 1605, 1508, 1407, 1269, 1212, 1161, 1077, 1036, 1015, 995, 862, 807, 760, 685, 545, 504 cm^{-1}; MS (FAB): *m/z* 253.1 $[M]$.

4-(Hydroxymethyl)phenyl-4′-nitrobenzoate (9g): General procedure A with 4-hydroxybenzylalcohol (4.00 g, 32.2 mmol) and Et_3N (4.53 mL, 32.2 mmol) dissolved in THF (30 mL), and 4-nitrobenzoyl chloride (6.58 g, 35.4 mmol) dissolved in THF (30 mL). The product (5.27 g, 19.3 mmol, 60%) was obtained as yellow crystals. $R_f = 0.41$ in CH_2Cl_2/CH_3OH (19:1); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.38–8.37$ (m, 4H; H-2′, H-3′), 7.48–7.46 (m 2H; H-3), 7.24–7.22 (m, 2H; H-2), 4.75 (s, 2H; Ph-CH_2), 1.62 ppm (s, 1H; -OH); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 163.5$ (C=O), 150.1 (C-4′), 149.9 (C-1) 139.1 (C-4), 134.9 (C-1′), 131.3 (C-2′), 128.2 (C-3), 123.7 (C-3′), 121.5 (C-2), 64.7 ppm (Ph-CH_2); IR: $\tilde{\nu} = 3675$, 3392, 3109, 3075, 2988, 2901, 1731, 1608, 1522, 1507, 1270, 1195, 852, 713, 500 cm^{-1}; MS (FAB): *m/z* 273.1 $[M]$.

Bis[4-(4′-methoxybenzoyloxy)benzyl]-*N*,*N*-diisopropylaminophosphoramidite (8a): General procedure B with 4-(hydroxymethyl)-phenyl-4′-methoxybenzoate (**7a**; 2.36 g, 9.12 mmol) and Et_3N (1.34 mL, 9.54 mmol) dissolved in THF (25 mL), and dichloro-*N*,*N*-diisopropylaminophosphoramidite (838 mg, 4.15 mmol) dissolved in THF (25 mL). The product (1.60 g, 2.48 mmol, 60%) was obtained as a beige solid. $R_f = 0.58$ in petroleum ether/ethyl acetate (3:1) with 10% Et_3N; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.09–8.07$ (m, 4H; H-2′), 7.43–7.41 (m, 4H; H-3), 7.25–7.23 (m, 4H; H-2), 7.13–7.11

(m, 4H; H-1′), 4.79–4.68 (m, 4H; Ph-CH_2), 3.87 (s, 6H; OCH_3), 3.72–3.63 (m, 2H; NC-H), 1.19 ppm (d, $^3J_{HH}$ = 6.8 Hz, 12H; *i*Pr); ^{13}C NMR (101 MHz, [D_6]DMSO): δ = 164.5 (C=O), 158.3 (C-4′), 150.5 (C-1), 137.5 (C-4), 132.0 (C-3′), 128.0 (C-3), 121.8 (C-2), 121.1 (C-1′), 114.3 (C-2′), 64.3 (Ph-CH_2), 55.4 (OCH_3), 42.7 (NC), 24.2 ppm (*i*Pr); ^{31}P NMR (162 MHz, [D_6]DMSO, decoupled): δ = 147.4 ppm; IR: $\tilde{\nu}$ = 3044, 2966, 2927, 2867, 2845, 1728, 1606, 1508, 1451, 1252, 1186, 1026, 964, 840, 759, 511 cm^{-1}; MS (FAB): *m/z* 646.3 [*M*+H$^+$]$^+$.

Bis[4-(4′-methylbenzoyloxy)benzyl]-*N*,*N*-diisopropylaminophosphoramidite (8b): General procedure B with 4-(hydroxymethyl)-phenyl-4′-methylbenzoate (**7b**; 2.16 g, 8.90 mmol) and Et_3N (1.31 mL, 9.29 mmol), dissolved in THF (20 mL), and dichloro-*N*,*N*-diisopropylaminophosphoramidite (817 mg, 4.04 mmol) dissolved in THF (25 mL). The product (2.38 g, 3.88 mmol, 44%) was obtained as a beige solid. R_f = 0.79 in petroleum ether/ethyl acetate (4:1) with 10% Et_3N; ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.03–8.01 (m, 4H; H-3′), 7.44–7.40 (m, 8H; H-2′, H-3), 7.26–7.24 (m, 4H; H-2), 4.80–4.68 (m, 4H; Ph-CH_2), 3.72–3.63 (m, 2H; NC-H), 2.43 (s, 6H; CH_3), 1.19 ppm (d, $^3J_{HH}$ = 6.8 Hz, 12H; *i*Pr); ^{13}C NMR (101 MHz, [D_6]DMSO): δ = 165.1 (C=O), 150.1 (C-1), 145.0 (C-4′), 137.5 (C-4), 130.5 (C-2′), 129.8 (C-3′), 128.3 (C-3), 126.4 (C-1′), 122.3 (C-2), 64.9 ($PhCH_2$), 43.1 (NC), 25.2 (*i*Pr), 22.4 ppm (CH_3); ^{31}P NMR (162 MHz, [D_6]DMSO, decoupled): δ = 147.5 ppm; IR: $\tilde{\nu}$ = 2966, 2927, 2862, 1738, 1614, 1508, 1269, 1200, 1079, 979, 737, 500 cm^{-1}; MS (FAB): *m/z* 614.3 [*M*+H$^+$]$^+$.

Bis[4-(4′-chlorobenzoyloxy)benzyl]-*N*,*N*-diisopropylamino-phosphoramidite (8d): General procedure B with 4-(hydroxymethyl)-phenyl-4′-chlorobenzoate (**7d**; 2.00 g, 7.61 mmol) and Et_3N (1.12 mL, 7.95 mmol), dissolved in THF (15 mL), and dichloro-*N*,*N*-diisopropylaminophosphoramidite (699 mg, 3.46 mmol) dissolved in THF (15 mL). The product (1.30 g, 1.99 mmol, 58%) was obtained as a colorless solid. R_f = 0.70 in petroleum ether/ethyl acetate (4:1) with 10% Et_3N; ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.14–8.12 (m, 4H; H-2′), 7.69–7.67 (m, 4H; H-3′), 7.44–7.43 (m, 4H; H-2), 7.29–7.27 (m, 4H; H-3), 4.80–4.69 (m, 4H; Ph-CH_2), 3.72–3.63 (m, 2H; NC-H), 1.19 ppm (d, $^3J_{HH}$ = 6.8 Hz, 12H; *i*Pr); ^{13}C NMR (101 MHz, [D_6]DMSO): δ = 164.5 (C=O), 150.1 (C-1), 139.5 (C-4′), 137.8 (C-4), 132.1 (C-2′), 132.0 (C-1′), 129.6 (C-3′), 128.5 (C-2), 122.2 (C-3), 64.6 (Ph-CH_2), 42.8 (NC), 24.2 ppm (*i*Pr); ^{31}P NMR (162 MHz, [D_6]DMSO, decoupled): δ = 147.6 ppm; IR: $\tilde{\nu}$ = 3675, 3659, 2968, 2931, 2872, 1731, 1592, 1506, 1363, 1092, 875, 750, 520, 507 cm^{-1}; MS (FAB): *m/z* 654.4 [*M*].

Bis[4-(4′-trifluoromethylbenzoyloxy)benzyl]-*N*,*N*-diisopropyl-aminophosphoramidite (8e): General procedure B with 4-(hydroxymethyl)phenyl-4′-trifluoromethylbenzoate (**7e**; 808 mg, 2.73 mmol) and Et_3N (0.40 mL, 2.9 mmol) dissolved in THF (10 mL), and dichloro-*N*,*N*-diisopropylaminophosphoramidite (250 mg, 1.24 mmol) dissolved in THF (10 mL). The product (553 mg, 62%) was obtained as a colorless solid. T_m = 142 °C; R_f = 0.85 in petroleum ether/ethyl acetate (4:1) with 10% Et_3N; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.33–8.30 (m, 4H; H-2′), 7.79–7.77 (m, 4H; H-3′), 7.44–7.42 (m 4H; H-3), 7.20–7.18 (m, 4H; H-2), 4.84–4.70 (m, 4H; Ph-CH_2), 3.77–3.68 (m, 2H; NC-H), 1.23 ppm (d, $^3J_{HH}$ = 6.8 Hz, 12H; *i*Pr); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 164.0 (C=O), 150.0 (C-1), 137.7 (d, $^3J_{CP}$ = 7.5 Hz, C-4), 135.0 (d, $^2J_{CP}$ = 32 Hz C-4′), 133.0 (C-1′), 130.7 (C-2′), 128.4 (C-3), 125.8 (d, $^3J_{CP}$ = 7.5 Hz, C-3′), 122.2 (CF_3), 121.5 (C-2), 64.9 (d, $^2J_{CP}$ = 18.5 Hz, Ph-CH_2), 43.2 (d, $^1J_{CN}$ = 12.4 Hz, NC), 24.8 ppm (d, $^2J_{CN}$ = 7.3 Hz, *i*Pr); ^{31}P NMR (162 MHz, $CDCl_3$, decoupled): δ = 148.2 ppm; ^{19}F NMR (203 MHz, $CDCl_3$): δ = 67.12 ppm; IR: $\tilde{\nu}$ = 2972, 2936, 2874, 1736, 1506, 1411, 1324, 1267, 1123, 1082, 1028, 971, 877, 829, 769, 698, 515 cm^{-1}; MS (FAB): *m/z* 722.2 [*M*+H$^+$]$^+$.

Bis[4-(4′-cyanobenzoyloxy)benzyl]-*N*,*N*-diisopropylaminophosphoramidite (8f): General procedure B with 4-(hydroxymethyl)-phenyl-4′-cyanolbenzoate (**7f**; 1.00 g, 3.95 mmol) and Et_3N (0.58 mL, 4.1 mmol) dissolved in THF (20 mL), and dichloro-*N*,*N*-diisopropylaminophosphoramidite (363 mg, 1.80 mmol) dissolved in THF (15 mL). The product (1.02 g, 1.60 mmol, 89%) was obtained as a colorless solid. T_m = 154 °C; R_f = 0.48 in petroleum ether/ethyl acetate (4:1) with 10% Et_3N; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.31–8.29 (m, 4H; H-2′), 7.83–7.81 (m, 4H; H-3′), 7.44–7.42 (m, 4H; H-3), 7.20–7.17 (m, 4H; H-2), 4.83–4.70 (m, 4H; Ph-CH_2), 3.77–3.67 (m, 2H; NC-H), 1.23 ppm (d, $^3J_{HH}$ = 6.8 Hz, 12H; *i*Pr); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 164.6 (C=O), 149.7 (C-1), 137.7 (C-4), 133.5 (C-1′), 132.4 (C-3′), 130.6 (C-2′), 128.2 (C-3), 121.2 (C-2), 117.8 (CN), 117.0 (C-4′), 64.8 (d, $^2J_{CP}$ = 18.4 Hz, Ph-CH_2), 43.3 (d, $^2J_{CP}$ = 12.3 Hz, NC), 24.7 ppm (*i*Pr); ^{31}P NMR (162 MHz, $CDCl_3$, decoupled): δ = 148.2 ppm; IR: $\tilde{\nu}$ = 3056, 2968, 2929, 2868, 2228, 1740, 1507, 1365, 1260, 1189, 1172, 1072, 1028, 1013, 975, 876, 776, 685, 506, 485 cm^{-1}; MS (FAB): *m/z* 636.3 [*M*+H$^+$]$^+$.

Bis[4-(4′-nitrobenzoyloxy)benzyl]-*N*,*N*-diisopropylaminophosphoramidite (8g): General procedure B with 4-(hydroxymethyl)-phenyl-4′-nitrobenzoate (**7g**; 1.88 g, 6.89 mmol) and Et_3N (1.01 mL, 7.20 mmol), dissolved in THF (25 mL), and dichloro-*N*,*N*-diisopropylaminophosphoramidite (633 mg, 3.13 mmol) dissolved in THF (25 mL). The product (1.91 g, 2.83, 90%) was obtained as a colorless solid. R_f = 0.79 in petroleum ether/ethyl acetate (4:1) with 10% Et_3N; ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.37–8.37 (m, 8H; H-2′, H-3′), 7.46–7.43 (m, 4H; H-3), 7.22–7.19 (m, 4H; H-2), 4.84–4.71 (m, 4H; Ph-CH_2), 3.77–3.68 (m, 2H; NC-H), 1.24 ppm (d, $^3J_{HH}$ = 6.8 Hz, 12H; *i*Pr); ^{13}C NMR (101 MHz, [D_6]DMSO): δ = 163.3 (C=O), 150.1 (C-4′), 149.7 (C-1), 137.7 (C-4), 135.0 (C-1′), 131.3 (C-2′), 128.8 (C-3), 128.2 (C-3′), 123.7 (C-2), 66.6 (Ph-CH_2), 43.2 (d, $^2J_{CP}$ = 12.3 Hz, NC) 24.7, 24.6 ppm (*i*Pr); ^{31}P NMR (162 MHz, [D_6]DMSO, decoupled): δ = 147.6 ppm; IR: $\tilde{\nu}$ = 3109, 2968, 2930, 2864, 1738, 1603.35, 1519, 1346, 1260, 1186, 1072, 713, 500 cm^{-1}; MS (FAB): *m/z* 676.2 [*M*+H$^+$]$^+$.

(N[*n*Bu]$_4$)$_2$-d4TMP (6) and **(N[*n*Bu]$_4$)$_2$-AZTMP (7)** were synthesized according to the protocol of Sowa and Ouchi.[10] Compound **6** was obtained in 83% yield, and compound **7** was obtained in 61% yield. The analytical data agreed with the literature.[8,9]

Ammonium-OMe-Ph-Di*PP*ro-d4TDP (4a): General procedure C with phosphoramidite **8a** (376 mg, 0.583 mmol), d4T monophosphate (216 mg, 0.324 mmol), and 4,5-dicyanoimidazole (61.2 mg, 0.518 mmol) in CH_3CN (7 mL). Oxidation by the addition of *tert*-butylhydroperoxide (5.5 molar solution in *n*-decane, 106 μL, 0.583 mmol). The product (76 mg, 0.086 mmol, 27%) was obtained as a colorless solid. ^{1}H NMR (400 MHz, [D_4]MeOH): δ = 8.10–8.08 (m, 4H; H-h), 7.69 (s, 1H; H-6), 7.44–7.41 (m, 4H; H-c), 7.18–7.16 (m, 4H; H-d), 7.04–7.02 (m, 4H; H-i), 6.97–6.96 (m, 1H; H-1′), 6.42 (d, $^3J_{HH}$ = 6.0 Hz, 1H; H-3′), 5.86 (d, $^3J_{HH}$ = 5.9 Hz, 1H; H-2′), 5.16–5.13 (m, 4H; H-a), 4.99–4.97 (m, 1H; H-4′), 4.27–4.18 (m, 2H; H-5′), 3.89 (s, 6H; OCH_3), 1.92 ppm (s, 3H; H7); ^{13}C NMR (101 MHz, [D_4]MeOH): δ = 165.0 (C-4), 164.3 (C-f), 152.0 (C-e), 137.4 (C-6), 133.3 (C-3′), 133.2 (C-h), 130.4 (C-i), 127.3 (C-2′), 123.1 (C-c), 122.7 (C-2), 115.1 (C-d), 110.7 (C-5), 90.1 (C-1′), 85.5 (C-4′), 70.0 (C-a), 68.1 (d, $^2J_{CP}$ = 5.4 Hz, C5′), 56.1 (OCH_3), 11.1 ppm (C-7); ^{31}P NMR (162 MHz, [D_4]MeOH, decoupled): δ = −12.0 (d, $^2J_{PP}$ = 21.7 Hz, P_β), −12.9 ppm (d, $^2J_{PP}$ = 21.9 Hz, P_α); HPLC: t_R = 14.8 min, method A; HRMS (ESI$^-$): *m/z* [*M*−H$^+$]$^-$ calcd for [$C_{40}H_{37}N_2O_{16}P_2$]$^-$: 863.1624, found: 863.1616; NMR assignment: Scheme 2.

(N[*n*Bu]$_4$)$_2$-Me-Ph-Di*PP*ro-d4TDP (4b): General procedure C with phosphoramidite **8b** (265 mg, 0.432 mmol), d4T monophosphate

(160 mg, 0.240 mmol), and 4,5-dicyanoimidazole (45.4 mg, 0.384 mmol) in CH_3CN (5 mL). Oxidation by the addition of *tert*-butylhydroperoxide (5.5 molar solution in *n*-decane, 79 μL, 0.432 mmol). The product (98 mg, 0.091 mmol, 38%) was obtained as a colorless solid. ^{1}H NMR (400 MHz, [D$_4$]MeOH): δ = 8.02–8.00 (m, 4H; H-h), 7.68 (s, $^4J_{HH}$ = 1.2 Hz, 1H; H-6), 7.46–7.42 (m, 4H; H-c), 7.33–7.31 (m, 4H; H-i), 7.20–7.18 (m, 4H; H-d), 6.97 (ddd, $^3J_{HH}$ = 3.5 Hz, $^4J_{HH}$ = 1.6 Hz, $^4J_{HH}$ = 1.6 Hz, 1H; H-1′), 6.41 (dd, $^3J_{HH}$ = 6.0 Hz, $^4J_{HH}$ = 1.6 Hz, 1H; H-3′), 5.85 (d, $^3J_{HH}$ = 5.4 Hz, $^4J_{HH}$ = 1.9 Hz, 1H; H-2′), 5.18–5.14 (m, 4H; H-a), 4.98–4.97 (m, 1H; H-4′), 4.28–4.18 (m 2H; H-5′), 3.21 (t, $^3J_{HH}$ = 8.4 Hz, 16H; H-A), 2.42 (s, 6H; CH_3), 1.92 (d, $^4J_{HH}$ = 0.9 Hz, 3H; H-7), 1.67–1.58 (m, 16H; H-B), 1.43–1.34 (m, 16H; H-C), 1.01 ppm (t, $^3J_{HH}$ = 7.3 Hz, 24H; H-D); ^{13}C NMR (101 MHz, [D$_4$]MeOH): δ = 166.5 (C-f), 166.5 (C-4), 152.8 (C-2), 152.5 (C-e), 146.2 (C-9), 138.2 (C-6), 135.3 (C-b), 135.0 (d, $^4J_{CP}$ = 7.1 Hz, C-3′), 131.1 (C-h), 130.4 (C-i), 130.4 (C-c), 127.9 (C-g), 127.5 (C-2′), 123.0 (C-d), 112.1 (C-5), 90.1 (C-1′), 87.0 (d, $^3J_{CP}$ = 9.2 Hz, C-4′), 70.3 (C-a), 68.1 (d, $^2J_{CP}$ = 6.2 Hz, C-5′), 59.5 (C-A), 24.8 (C-B), 21.7 (CH_3), 20.7 (C-C), 13.9 (C-D), 12.5 ppm (C-7); ^{31}P NMR (162 MHz, [D$_4$]MeOH, decoupled): δ = −12.1 (d, $^2J_{PP}$ = 21.4 Hz, P$_\beta$), −12.9 ppm (d, $^2J_{PP}$ = 21.4 Hz, P$_\alpha$); HPLC: t_R = 15.8 min, method A; HRMS (ESI$^-$): *m/z* [*M*−H$^+$]$^-$ calcd for [$C_{40}H_{37}N_2O_{14}P_2$]$^-$: 831.1726, found: 831.1714; NMR assignment: Scheme 2.

(N[*n*Bu]$_4$)$_2$- Cl-Ph-Di*PP*ro-d4TDP (4d): General procedure C with phosphoramidite **8d** (280 mg, 0.428 mmol), d4T monophosphate (158 mg, 0.238 mmol), and 4,5-dicyanoimidazole (45 mg, 0.381 mmol) in CH_3CN (5 mL). Oxidation by the addition of *tert*-butylhydroperoxide (5.5 molar solution in *n*-decane, 78 μL, 0.428 mmol). The product (112 mg, 0.101 mmol, 42%) was obtained as a colorless solid. ^{1}H NMR (400 MHz, [D$_4$]MeOH): δ = 8.12–8.10 (m, 4H; H-h), 7.69 (d, $^4J_{HH}$ = 1.2 Hz, 1H; H-6), 7.54–7.53 (m, 4H; H-i), 7.46–7.41 (m, 4H; H-c), 7.20–7.18 (m, 4H; H-d), 6.97 (ddd, $^3J_{HH}$ = 3.4 Hz, $^4J_{HH}$ = 1.8 Hz, $^4J_{HH}$ = 1.7 Hz, 1H; H-1′), 6.42 (dd, $^3J_{HH}$ = 6.0 Hz, $^4J_{HH}$ = 1.7 Hz, 1H; H-3′), 5.86 (dd, $^3J_{HH}$ = 5.2 Hz, $^4J_{HH}$ = 2.2 Hz, 1H; H-2′), 5.17–5.13 (m, 4H; H-a), 4.99–4.97 (m, 1H; H-4′), 4.28–4.18 (m, 2H; H-5′), 3.21 (m, 8H; H-A), 1.91 (d, ^4J = 1.2 Hz, 3H; H-7), 1.74–1.62 (m, 8H; H-B), 1.47–1.37 (m, 8H; H-C), 1.04 ppm (t, $^3J_{HH}$ = 7.3 Hz, 12H; H-D); ^{13}C NMR (101 MHz, [D$_4$]MeOH): δ = 164.9 (C-4), 163.8 (C-f), 152.4 (C-e), 151.4 (C-2), 141.2 (C-g), 138.7 (C-6), 135.4 (C-3′), 132.7 (C-h), 130.5 (C-i), 130.1 (C-c), 129.7 (C-b), 127.6 (C-2′), 122.9 (C-d), 122.6 (C-5), 90.9 (C-1′), 87.0 (d, $^3J_{CP}$ = 9.2 Hz, C-4′), 70.3 (d, $^2J_{CP}$ = 3.8 Hz, C-a), 68.1 (d, $^2J_{CP}$ = 4.8 Hz, C-5′), 59.6 (C-A), 24.8 (C-B), 20.7 (C-C), 13.9 (C-D), 12.5 ppm (C-7); ^{31}P NMR (162 MHz, [D$_4$]MeOH, decoupled): δ = −12.0 (d, $^2J_{PP}$ = 1 Hz, P$_\beta$), −12.9 ppm (d, $^2J_{PP}$ = 6 Hz, P$_\alpha$); HPLC: t_R = 16.1 min, method A; HRMS (ESI$^-$): *m/z* [*M*−H$^+$]$^-$ calcd for [$C_{38}H_{31}Cl_2N_2O_{14}P_2$]$^-$: 871.0633, found: 871.0621; NMR assignment: Scheme 2.

(N[*n*Bu]$_4$)$_2$-CF$_3$-Ph-Di*PP*ro-d4TDP (4e): General procedure D with phosphoramidite **8e** (110 mg, 0.16 mmol), d4T monophosphate (64 mg, 0.10 mmol), and 4,5-dicyanoimidazole activator solution (624 μL, 0.16 mmol) in CH_3CN (2 mL). Oxidation by the addition of *tert*-butylhydroperoxide (5.5 molar solution in *n*-decane, 28 μL, 0.16 mmol). The product (69 mg, 58%) was obtained as a colorless solid. ^{1}H NMR (500 MHz, [D$_4$]MeOH): δ = 8.32–8.31 (m, 4H; H-h), 7.8–7.83 (m, 4H; H-i), 7.69 (s, 1H; H-6), 7.47–7.44 (m, 4H; H-c), 7.23–7.22 (m, 4H; H-d), 6.97 (br s, 1H; H-1′), 6.43–6.42 (m, 1H; H-3′), 5.87–5.86 (m, 1H; H-2′), 5.18–5.15 (m 4H; H-a), 4.99 (m, 1H; H-4′), 4.28–4.22 (m, 2H; H-5′), 3.25–3.22 (m, 9H; H-A), 1.91 (s, 3H; H-7), 1.69–1.64 (m, 9H; H-B), 1.45–1.39 (m, 9H; H-C), 1.03 ppm (t, ^3J = 7.3 Hz, 13.5H; H-D); ^{13}C NMR (126 MHz, [D$_4$]MeOH): δ = 166.6 (C-4), 165.2 (C-f), 152.3 (C-2), 151.2 (C-e), 138.7 (C-6), 135.5 (C-j), 135.4 (C-3′), 134.4 (C-g), 131.8 (C-h), 130.6 (C-h), 130.6 (d, $^4J_{CP}$ = 5.3 Hz, C-c), 127.6 (C-2′), 126.8 (d, $^4J_{CF}$ = 3.4 Hz, C-i), 124.3 (CF_3), 122.9 (C-d), 112.1 (C-5), 90.8 (C-1′), 87.0 (d, $^3J_{CP}$ = 9.4 Hz, C-4′), 70.2 (dd, $^2J_{CP}$ = 4.3 Hz, $^4J_{CP}$ = 3.6 Hz, C-a), 68.1 (d, $^2J_{CP}$ = 6.0 Hz, C-5′), 59.5 (t, $^4J_{CN}$ = 6.0 Hz, C-A), 24.8 (C-B), 20.7 (C-C), 13.9 (C-D), 12.5 ppm (C-7); ^{19}F NMR (203 MHz, [D$_4$]MeOH): δ = 64.71 ppm; ^{31}P NMR (203 MHz, [D$_4$]MeOH, decoupled): δ = −12.0 (d, $^2J_{PP}$ = 20.7 Hz, P$_\beta$), −12.9 ppm (d, $^2J_{PP}$ = 20.6 Hz, P$_\alpha$); HPLC: t_R = 15.7 min, method A; HRMS (ESI$^-$): *m/z* [*M*−H$^+$]$^-$ calcd for [$C_{40}H_{31}F_6N_2O_{14}P_2$]$^-$: 939.1160, found: 939.1163; NMR assignment: Scheme 2.

Ammonium-CN-Ph-Di*PP*ro-d4TDP (4f): General procedure D with phosphoramidite **8f** (66 mg, 0.104 mmol), d4T monophosphate (43 mg, 0.07 mmol), and 4,5-dicyanoimidazole activator solution (416 μL, 0.104 mmol) in CH_3CN (2 mL). Oxidation by the addition of *tert*-butylhydroperoxide (5.5 molar solution in *n*-decane, 19 μL, 0.104 mmol). The product (38 mg, 0.044 mmol, 63%) was obtained as a colorless solid. ^{1}H NMR (400 MHz, [D$_4$]MeOH): δ = 8.29–8.28 (m, 4H; H-h), 7.91–7.90 (m, 4H; H-i), 7.73 (s, 1H; H-6) 7.49–7.47 (m, 4H; H-c), 7.24–7.23 (m, 4H; H-d), 6.22 (d, $^3J_{HH}$ = 6.8 Hz, $^3J_{HH}$ = 6.8 Hz, 1H; H-1′), 5.20–5.17 (m, 4H; H-a), 4.44–4.42 (m, 1H; H-3′), 4.24–4.22 (m, 1H; H-5′a), 4.17–4.14 (m, 1H; H-5′b), 4.06 (br s, 1H; H-4′), 2.37–2.27 (m, 2H; H-2′), 1.90 ppm (s, 3H; H-7); ^{13}C NMR (101 MHz, [D$_4$]MeOH): δ = 166.4 (C-4), 164.9 (C-f), 152.3 (C-e), 137.8 (C-6), 134.7 (C-b), 133.7 (C-i), 131.7 (C-h), 130.6 (C-g), 130.6 (C-c), 122.9 (C-d), 118.8 (C-j), 118.1 (CN), 112.2 (C-5), 85.8 (C-1′), 84.4 (d, ^3J = 9.3 Hz, C-4′), 70.3 (d, ^2J = 5.4 Hz, C-a), 67.3 (d, ^2J = 6.5 Hz, C-5′), 62.5 (C-3′), 38.1 (C-2′), 12.6 ppm (C7); ^{31}P NMR (162 MHz, [D$_4$]MeOH, decoupled): δ = −12.1–12.1 (m, P$_\beta$), −12.7 ppm (d, $^2J_{PP}$ = 19.6 Hz, P$_\alpha$); HPLC: t_R = 13.2 min, method A; HRMS (ESI$^+$): *m/z* [*M*+Na$^+$]$^+$ calcd for [$C_{40}H_{32}N_4NaO_{14}P_2$]$^+$: 877.1282, found: 877.1228; NMR assignment: Scheme 2.

Ammonium-NO$_2$-Ph-Di*PP*ro-d4TDP (4g): General procedure C with phosphoramidite **8g** (584 mg, 0.864 mmol), d4T monophosphate (320 mg, 0.480 mmol), and 4,5-dicyanoimidazole (90.7 mg, 0.768 mmol) in CH_3CN (10 mL). Oxidation by the addition of *tert*-butylhydroperoxide (5.5 molar solution in *n*-decane, 157 μL, 0.864 mmol). The product (137 g, 0.150 mmol, 31%) was obtained as a colorless solid. ^{1}H NMR (400 MHz, [D$_4$]MeOH): δ = 8.36 (br s, 8H; H-h, H-i), 7.68 (m, 1H; H-6), 7.48–7.50 (m, 4H; H-c), 7.25–7.23 (m, 4H; H-d), 6.96 (ddd, $^3J_{HH}$ = 3.5 Hz, $^4J_{HH}$ = 1.8 Hz, $^4J_{HH}$ = 1.7 Hz, 1H; H-1′), 6.42 (ddd, $^3J_{HH}$ = 6.0 Hz, $^3J_{HH}$ = 6.0 Hz, $^4J_{HH}$ = 1.6 Hz, 1H; H-3′), 5.86 (ddd, $^3J_{HH}$ = 5.9 Hz, $^3J_{HH}$ = 5.2 Hz, $^4J_{HH}$ = 2.2 Hz, 1H; H-2′), 5.18–5.15 (m, 4H; H-a), 5.00–4.96 (m, 1H; H-4′), 4.28–4.18 (m, 2H; H-5′), 1.91 ppm (d, $^4J_{HH}$ = 1.2 Hz, 3H; H-7); ^{13}C NMR (101 MHz, [D$_4$]MeOH): δ = 165.1 (C-4), 164.7 (C-f), 152.4 (C-2), 152.2 (C-j), 150.6 (C-e), 138.7 (C-6), 136.2 (C-3′), 135.3 (C-9), 132.4 (C-h), 130.6 (d, $^4J_{CP}$ = 3.8 Hz, C-c), 127.7 (C-2′), 124.8 (C-i), 122.8 (C-d), 112.1 (C-5), 90.9 (C-1′), 86.5 (d, $^3J_{CP}$ = 9.5 Hz, C-4′), 70.3 (C-a), 67.7 (d, $^2J_{CP}$ = 6.4 Hz, C-5′), 12.5 ppm (C-7); ^{31}P NMR (162 MHz, [D$_4$]MeOH, decoupled) δ = −12.0 (d, $^2J_{PP}$ = 20.6 Hz, P$_\beta$), −12.9 ppm (d, $^2J_{PP}$ = 20.2 Hz, P$_\alpha$); HPLC: t_R = 14.8 min, method A; HRMS (ESI$^-$): *m/z* [*M*−H$^+$]$^-$ calcd for [$C_{38}H_{31}N_4O_{18}P_2$]$^-$: 893.1114, found: 893.1100; NMR assignment: Scheme 2.

Ammonium-CF$_3$-Ph-Di*PP*ro-AZTDP (5e): General procedure D with phosphoramidite **8e** (238 mg, 0.33 mmol), AZT monophosphate (140 mg, 0.22 mmol), and 4,5-dicyanoimidazole activator solution (1320 μL, 0.33 mmol) in CH_3CN (6 mL). Oxidation by the addition of *tert*-butylhydroperoxide (5.5 molar solution in *n*-decane, 60 μL, 0.33 mmol). The product (117 mg, 53%) was obtained as a colorless solid after ion exchange. ^{1}H NMR (400 MHz, [D$_4$]MeOH): δ = 8.32–8.31 (m, 4H; H-h), 7.85–7.83 (m, 4H; H-i), 7.74 (s, 1H; H-6), 7.49–7.47 (m, 4H; H-c), 7.25–7.23 (m, 4H; H-d), 6.22 (dd, $^3J_{HH}$ = 6.8 Hz, $^3J_{HH}$ = 6.8 Hz, 1H; H-1′), 5.19–5.18 (m, 4H; C-a), 4.45 (dt, $^3J_{HH}$ =

6.7 Hz, $^{3}J_{HH}$ = 3.5 Hz, 1H; H-3′), 4.26–4.23 (m, 1H; H-5′a), 4.19–4.15 (m, 1H; H-5′b), 4.08–4.07 (m, 1H; H-4′), 2.39–2.29 (m, 2H; H-2′), 1.91 ppm (s, 3H; H-7); ^{13}C NMR (101 MHz, [D$_4$]MeOH): δ = 166.6 (C-4), 165.2 (C-f), 152.4 (C-2), 152.3 (C-e), 137.8 (C-6), 136.0 (C-j), 134.4 (C-g), 131.8 (C-h), 130.6 (d, $^{4}J_{CP}$ = 3.1 Hz, C-c), 126.8 (d, $^{4}J_{CF}$ = 3.4 Hz, C-i), 124.4 (CF_3), 122.9 (C-d), 112.3 (C-5), 85.8 (C-1′), 84.2 (d, $^{3}J_{CP}$ = 9.2 Hz, C-4′), 70.4 (d, $^{2}J_{CP}$ = 5.4 Hz, C-a), 67.1 (d, $^{2}J_{CP}$ = 6.2 Hz, C-5′), 62.5 (C3′), 38.1 (C2′), 12.6 ppm (C7); ^{31}P NMR (162 MHz, [D$_4$]MeOH, decoupled): δ = −12.1 (d, $^{2}J_{PP}$ = 21.3 Hz, P$_\beta$), −12.7 ppm (d, $^{2}J_{PP}$ = 21.4 Hz, P$_\alpha$); HPLC: t_R = 18.9 min, method B; HRMS (ESI$^+$): *m/z* [*M*+H$^+$]$^+$ calcd for [$C_{40}H_{34}F_6N_5O_{14}P_2$]$^+$: 984.1487, found: 984.1457; NMR assignment: Scheme 2.

Ammonium-CN-Ph-Di*PP*ro-AZTDP (5 f): General procedure D with phosphoramidite **8 f** (38 mg, 0.06 mmol), AZT monophosphate (31 mg, 0.05 mmol), and 4,5-dicyanoimidazole activator solution (240 μL, 0.06 mmol) in CH_3CN (2 mL). Oxidation by the addition of *tert*-butylhydroperoxide (5.5 molar solution in *n*-decane, 11 μL, 0.06 mmol). The product (21 mg, 0.023 mml, 46%) was obtained as a colorless solid. ^{1}H NMR (400 MHz, [D$_4$]MeOH): δ = 8.29–8.28 (m, 4H; H-h), 7.91–7.90 (m, 4H; H-i), 7.73 (s, 1H; H-6) 7.49–7.47 (m, 4H; H-c), 7.24–7.23 (m, 4H; H-d), 6.22 (d, $^{3}J_{HH}$ = 6.8 Hz, $^{3}J_{HH}$ = 6.8 Hz, 1H; H-1′), 5.20–5.17 (m, 4H; H-a), 4.44–4.42 (m, 1H; H-3′), 4.24–4.22 (m, 1H; H-5′a), 4.17–4.14 (m, 1H; H-5′b), 4.06 (brs, 1H; H-4′), 2.37–2.27 (m, 2H; H-2′), 1.90 ppm (s, 3H; H-7); ^{13}C NMR (101 MHz, [D$_4$]MeOH): δ = 166.4 (C-4), 164.9 (C-f), 152.3 (C-e), 137.8 (C-6), 134.7 (C-b), 133.7 (C-i), 131.7 (C-h), 130.6 (C-g), 130.6 (C-c), 122.9 (C-d), 118.8 (C-j), 118.1 (CN), 112.2 (C-5), 85.8 (C-1′), 84.4 (d, ^{3}J = 9.3 Hz, C-4′), 70.3 (d, ^{2}J = 5.4 Hz, C-a), 67.3 (d, ^{2}J = 6.5 Hz, C-5′), 62.5 (C-3′), 38.1 (C-2′), 12.6 ppm (C7); ^{31}P NMR (162 MHz, [D$_4$]MeOH, decoupled): δ = −12.1–12.1 (m, P$_\beta$), −12.7 ppm (d, $^{2}J_{PP}$ = 19.6 Hz, P$_\alpha$); HPLC: t_R = 18.9 min, method B; HRMS (ESI$^+$): *m/z* [*M*+H$^+$]$^+$ calcd for [$C_{40}H_{34}N_7O_{14}P_2$]$^+$: 898.1639, found: 898.1640; NMR assignment: Scheme 2.

Ammonium-NO$_2$-Ph-Di*PP*ro-AZTDP (5 g): General procedure D with phosphoramidite **8 g** (160 mg, 0.24 mmol), AZT monophosphate (100 mg, 0.16 mmol), and 4,5-dicyanoimidazole activator solution (1120 μL, 0.28 mmol) in CH_3CN (4 mL). Oxidation by the addition of *tert*-butylhydroperoxide (5.5 molar solution in *n*-decane, 44 μL, 0.24 mmol). The product (100 mg, 0.105 mmol, 66%) was obtained as a colorless solid after ion exchange. ^{1}H NMR (400 MHz, [D$_4$]MeOH): δ = 8.29 (brs, 8H; H-h, H-i), 7.74 (s, 1H; H-6) 7.50–7.48 (4H; H-c), 7.26–7.24 (m 4H; H-d), 6.23 (dd, $^{3}J_{HH}$ = 6.8 Hz, $^{3}J_{HH}$ = 6.8 Hz, 1H; H-1′), 5.20–5.18 (m, 4H; H-a), 4.45–4.43 (m, 1H; H-3′), 4.26–4.22 (m, 1H; H-5′a), 4.18–4.15 (m, 1H; H-5′b), 4.07–4.06 (m, 1H; H-4′), 2.38–2.29 (m, 2H; H-2′), 1.91 ppm (s 3H; H-7); ^{13}C NMR (101 MHz, [D$_4$]MeOH): δ = 166.5 (C-4), 164.7 (C-f), 152.4 (C-j), 152.3 (C-2), 137.7 (C-6), 136.3 (C-g), 135.5 (C-b), 132.4 (C-h), 130.6 (d, $^{4}J_{CP}$ = 3.8 Hz, C-c), 124.8 (C-i), 122.9 (C-d), 112.3 (C-5), 85.8 (C-1′), 84.4 (d, ^{3}J = 8.9 Hz, C-4′), 70.3 (d, $^{2}J_{CP}$ = 5.7 Hz, C-a), 67.3 (d, $^{2}J_{CP}$ = 5.7 Hz, C-5′), 62.5 (C-3′), 38.1 (C-2′), 12.6 ppm (C-7); ^{31}P NMR (162 MHz, [D$_4$]MeOH, decoupled): δ = −12.1 (d, $^{2}J_{PP}$ = 20.5 Hz, P$_\beta$), −12.7 ppm (d, $^{2}J_{PP}$ = 20.5 Hz, P$_\alpha$); HPLC: t_R = 16.7 min, method B; HRMS (ESI$^+$): *m/z* [*M*+H$^+$]$^+$ calcd for [$C_{38}H_{34}N_7O_{18}P_2$]$^+$: 938.1441, found: 938.1428; NMR assignment: Scheme 2.

Compound evaluation methods

Hydrolysis studies in phosphate buffer at pH 7.3: Hydrolysis was started by the addition of PBS (300 μL 50 mM, pH 7.3) to a solution of the Di*PP*ro-NDP (300 μL, 1.9 mM) in DMSO. The mixture incubated at 37 °C. Aliquots were taken at certain points of time and frozen in liquid nitrogen. Directly after unfreezing, samples were analyzed by RP-HPLC.

Hydrolysis studies in CEM cell extracts: A magnesium chloride solution (20 μL, 70 mM) was mixed with a solution of the Di*PP*ro-NDP (20 μL, 6 mM) in DMSO. Hydrolysis was started by the addition of wild-type CEM cell extract (100 μL). Six to eight of these samples were prepared and incubated at 37 °C for different time periods. Hydrolysis was stopped by quenching with CH_3OH (300 μL). After 5 min on ice and centrifugation at 13000 rpm (Heraeus, Biofuge Pico) the samples were filtered and frozen in liquid nitrogen until required. Samples (10–40 μL) were analyzed by RP-HPLC. Hydrolysis was monitored for 10 h.

Preparation of CEM cell extracts: Human CD4$^+$ T-lymphocyte CEM cells were grown in RPMI-1640-based cell culture medium to a final density of about 3×10^6 cells mL^{-1}. After centrifugation for 10 min at 1250 rpm (Heraeus, Megafuge 3.0R) and 4 °C, the pellet was washed twice with cold PBS, resuspended at 10^8 cells mL^{-1}, and sonicated (3×10 s). After a second centrifugation of this suspension at 10000 rpm (Heraeus, Megafuge 3.0R), the supernatant was divided into aliquots and frozen to −80 °C until used.

Antiviral assay: Inhibition of HIV-1(III$_B$) and HIV-2(ROD)-induced cytopathicity in wild-type CEM/0 or thymidine kinase-deficient CEM/TK$^-$ cell cultures was measured in microtiter 96-well plates containing $\approx3\times10^5$ CEM cells mL^{-1} infected with 100 $CCID_{50}$ of HIV mL^{-1} and containing appropriate dilutions of the test compounds. After 4–5 days of incubation at 37 °C under a CO_2-controlled humidified atmosphere, CEM giant (syncytium) cell formation was examined microscopically. EC_{50} (50% effective concentration) was defined as the compound concentration required to inhibit HIV-induced giant cell formation by 50%.

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Keywords: anti-HIV · antivirals · bioreversible protection · drug delivery · nucleoside analogues · pronucleotides

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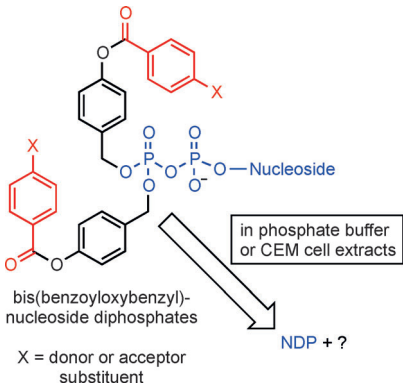
Fine-tuning stability: Bis(benzoyloxybenzyl) groups were used to mask two of the negative charges of nucleoside diphosphates to form Di*PP*ro compounds. Modification by different substituents in the 4-positions had a strong impact on the chemical and enzymatic stability. With strong acceptor substituents, the NDPs were released almost exclusively in PBS or in cell extracts.



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Bis(benzoyloxybenzyl)-Di*PP*ro Nucleoside Diphosphates of Anti-HIV Active Nucleoside Analogues

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