Nucleoside Diphosphate Prodrugs

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Bioreversible Protection of Nucleoside Diphosphates**

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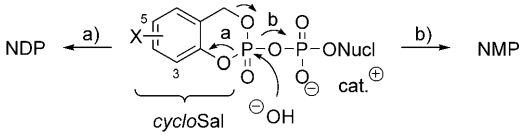
Nucleoside analogues are applied widely in antiviral and antitumor therapy. A severe limitation of these compounds is that they must undergo biotransformation into the corresponding active nucleoside triphosphates (NTPs) by the stepwise addition of phosphate groups by kinases.[1] If this activation proceeds insufficiently, the antiviral or antitumor activity of the nucleoside analogues can possibly be enhanced by using prodrugs of the phosphorylated metabolites. This approach bypasses nucleoside kinases, which are responsible for the often inefficient activation of the analogues, and hence leads to an increase in the intracellular levels of active metabolites. Drug resistance can also be overcome in some cases, and the spectrum of application of a nucleoside may be broadened further to cover multiple viruses.[2] Moreoever, masked nucleotides are able to penetrate cell membranes in their intact form as a result of their high lipophilicity and are therefore not prone to degradation by nonspecific plasma phosphatases. For these reasons, various prodrugs for nucleoside monophosphates (NMPs) have been designed, for example, in *cyclo*Sal, phosphoramidate, bis(*S*-acylthioethyl), or bis(pivaloxymethyl) approaches.[3] However, the design of nucleoside diphosphate (NDP) or triphosphate (NTP) prodrugs has only been addressed very rarely. The lack of research towards the development of such prodrugs is remarkable, because it is known, for example, that 3′-azido-3′-deoxythymidine (AZT), the first approved nucleosidic anti-HIV drug, is only phosphorylated very slowly to the diphosphate AZTDP by thymidylate kinase.[4] The resulting accumulation of AZT monophosphate (AZTMP) provokes severe side effects.[5]

The reason for the difficulty in masking NDPs lipophilically lies in the inherent instability of the phosphate anhydride bond. This bond is only stable kinetically as a result of the negative charges, which prevent nucleophilic attack at the phosphate moieties. Several nucleoside pyrophosphate diesters based on glycerides were reported by Hostetler and co-workers.[6] However, these compounds do not serve as NDP prodrugs, but instead release the corresponding NMPs through cleavage of the pyrophosphate group. Another approach was described by Huynh-Dinh and co-workers,[7] who attached different acyl moieties to the β phosphate group of the pyrophosphate unit. This method relies on a faster cleavage of the mixed anhydride bond than the cleavage of the phosphate anhydride bond. The concept was proven in hydrolysis studies in an aqueous buffer. However, undesired decomposition occurred in biological media (RPMI culture medium).[8]

We first attempted to apply the *cyclo*Sal approach to the lipophilic modification of NDPs. It had been shown in extensive studies that the *cyclo*Sal system enhances the antiviral activity of some nucleoside analogues considerably when used to mask NMPs.[3b] After the preparation of several *cyclo*Sal nucleoside diphosphates (*cyclo*Sal-NDPs), we analyzed the hydrolysis pathways by ^{31}P NMR spectroscopy (Scheme 1; paths a and b). We found that the compounds did not release NDPs effectively, as a result of the initial chemical activation step. The hydrolytic cleavage of the pyrophosphate bond dominated (Scheme 1, path b), with the predominant release of the corresponding NMP and *cyclo*Sal phosphate. Only small amounts of the NDP were detected.



Scheme 1. Hydrolysis of *cyclo*Sal NDP prodrugs by paths a and b. ONucl = nucleoside.

To circumvent these problems, we investigated an enzymatically activated type of prodrug:[9] bis(4-acyloxybenzyl)-nucleoside diphosphates (BAB-NDPs, **1**). The general structure of these prodrugs and the proposed mechanism of hydrolysis are shown in Scheme 2. To gain insight into the behavior of these potential NDP prodrugs, we prepared different derivatives by varying the nucleoside and the acyl moiety. In this context, we were interested in modulating their stability and polarity. The removal of the masking units should be initialized by hydrolysis of the acyl ester bond, either by pH-dependent chemical hydrolysis or by enzyme-catalyzed hydrolysis.

This initial cleavage results in an inversion of the polarity of the substituent (an acceptor is transformed into a donor) and thus to the destabilization of the benzyl phosphate ester bond. Through 1,4-elimination and hydrolysis, the prodrug decomposes to give 4-hydroxybenzylalcohol and the mono-

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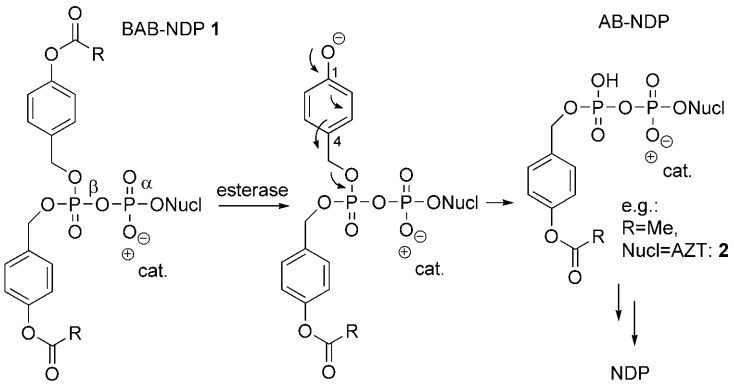


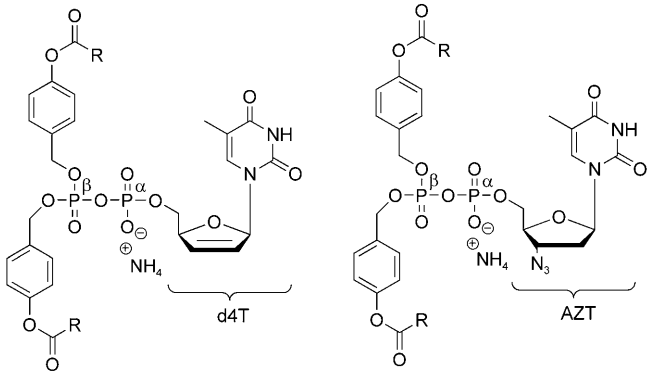
Scheme 2. Structure of BAB-NDP prodrugs and proposed mechanism of enzymatic hydrolysis.

substituted NDP (AB-NDP; e.g. **2**, Scheme 2). Repetition of this cleavage mechanism should then lead to formation of the NDP.

In the mechanism shown in Scheme 2, no reaction takes place at the phosphate anhydride bond. Thus, possible side reactions are minimized. Moreover, we showed in preliminary studies that the α-phosphate atom has to remain unmasked to prevent rapid cleavage of the anhydride bond (data not shown).

The compounds were synthesized by dicyanoimidazole-mediated coupling of *para*-acyloxybenzylphosphoramidites with bis(tetra-*n*-butylammonium)nucleoside monophosphates and subsequent oxidation with *tert*-butyl hydroperoxide (TBHP; see the Supporting Information). The products were purified by chromatography on RP-18 silica gel by gradient elution with water/methanol mixtures. First, 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) and AZT were chosen as antivirally active nucleoside analogues. The derivatives synthesized and nonoptimized yields are summarized in Scheme 3. During the course of the project, the yields were increased considerably, in particular through optimization of the purification procedures, so that compounds could be prepared in yields ranging from 46 to 65%.

Next, we determined the stability of compounds **1a–h** in different media (phosphate buffer (PBS, pH 7.3), citrate/HCl buffer (pH 2.0), T-lymphocyte (CEM/0) cell extracts, 20% human plasma, and RPMI culture medium). We also investigated the mechanism of hydrolysis by ion-pair reversed-phase HPLC analysis and ^{31}P NMR spectroscopic studies. The obtained data confirmed the mechanism postulated in Scheme 2. For example, BAB-AZTDP (**1e**) underwent hydrolysis via the singly masked intermediate AB-AZTDP (**2**), which was isolated and characterized, to give AZTDP almost exclusively at physiological pH (PBS, pH 7.3; see the Supporting Information). The determined half-lives of BAB-AZTDP (**1e**) in PBS for this process based entirely on chemical hydrolysis were 17 h for the degradation of the first masking group to give AB-AZTDP (**2**) and 110 h for the cleavage of the second masking group to give AZTDP. Only a very small amount (≈5%) of AZT monophosphate, which results from the cleavage of the anhydride bond of BAB-AZTDP (**1e**), was observed. The incubation of AB-AZTDP (**2**) in PBS did not lead to the formation of AZT monophosphate; thus, the anhydride bond in this compound is not prone to hydrolysis. In line with our expectation, the chemical stability of the compounds increased if branched alkyl residues were used in the acyl moiety. For example, the half-life of BIB-AZTDP (**1f**) in PBS is considerably higher (39 h) than that of **1e** (Table 1). Similarly high stability was observed in citrate/HCl buffer (pH 2.0). In contrast, the incubation of BAB-AZTDP (**1e**) in CEM/0 cell extracts led to a dramatic acceleration of the cleavage reaction, as the phenyl ester moiety was now hydrolyzed enzymatically. The first hydrolysis to form AB-AZTDP (**2**) was 500 times faster ($t_{1/2}$ = 2 min) than the corresponding chemical hydrolysis. A half-life of 3 min was observed for the second hydrolysis step to give AZTDP (>95%), which corresponds to a 2500-fold acceleration with respect to the chemical hydrolysis. This result is quite astonishing, as the enzymatic cleavage of the second masking unit in BAB-nucleotide prodrugs was sig-



R=Me; BAB-d4TDP **1a**: 10%
R=*t*Bu; BPB-d4TDP **1b**: 46%
R=heptyl; BOB-d4TDP **1c**: 65%
R=phenyl; BBB-d4TDP **1d**: 33%

R=Me; BAB-AZTDP **1e**: 29%
R=*i*Pr; BIB-AZTDP **1f**: 48%
R=*t*Bu; BPB-AZTDP **1g**: 20%
R=phenyl; BBB-AZTDP **1h**: 49%

Scheme 3. Combination of the nucleosides and acyl moieties in prodrugs **1a–h**. BAB = bis(4-acetoxybenzyl), BPB = bis(4-pivaloyloxybenzyl), BIB = bis(4-isobutyryloxybenzyl), BOB = bis(4-octanoyloxybenzyl), BBB = bis(4-benzoyloxybenzyl).

Table 1: Half-lives ($t_{1/2}$ in h) of **1a** and **1c–h** in different media.

	pH 7.3[a]	CEM/0[b]	plasma[c]	RPMI/FCS[d]
1a	10	0.05	n.d.[e]	n.d.
1c	63	1	12	3
1d	82	7	13	n.d.
1e	17	0.02	5	2
1f	39	0.3	10	2.5
1g	75	1	24	3
1h	82	0.5	4	1

[a] Phosphate buffer (25 mM). [b] Human T-lymphocyte cell extract (pH 6.9). [c] Human plasma (20%) diluted with PBS (80%, 50 mM, pH 6.8). [d] RPMI incubation medium with 10% heat-inactivated fetal calf serum (FCS). [e] Not determined.

nificantly slower than the first step.[9c] The HPLC traces in Figure 1 show the selective cleavage of compound **1e** after incubation in CEM/0 cell extracts. Identical results were obtained for BAB-d4TDP (**1a**). To the best of our knowledge, these compounds are the first NDP prodrugs to be described that exhibit high chemical stability and undergo fast and highly selective enzymatic cleavage in a cell extract to deliver NDPs.

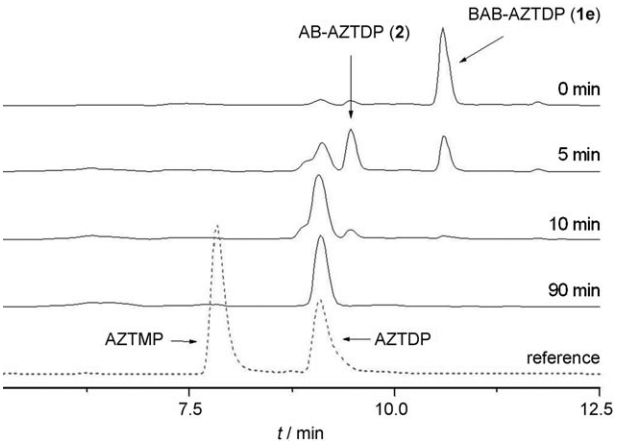


Figure 1. HPLC traces for BAB-AZTDP (**1e**) after incubation in CEM/0 cell extracts for the time shown (0–90 min).

Extremely rapid and highly selective degradation in cell extracts is crucial to the success of this method, as undesired decomposition of the phosphate anhydride bond is thus suppressed. In contrast to the design of NMP prodrugs (e.g. the *cyclo*Sal approach), we believe that only concepts based on fast enzyme-catalyzed cleavage at least in the first activating step without the involvement of the phosphate anhydride bond will be successful for the design of NDP prodrugs. As already mentioned, we were able to increase the chemical stability of the compounds by introducing branching in the acyl moiety. However, this modification also enhanced the enzymatic stability of the compounds (compare data for **1e**–**g**, Table 1).

Strikingly, an increase in enzymatic stability led to a decrease in the amount of NDP released. In the case of BIB-AZTDP (**1f**; $t_{1/2} = 20$ min, first hydrolysis), the amount of AZTDP released dropped to 60% in the CEM/0 cell extract. BOB-d4TDP (**1c**; $t_{1/2} = 60$ min, first hydrolysis) only released about 35% d4TDP in the CEM/0 cell extract. Derivative **1g**, which contains pivaloyl ester groups, released no AZTDP, as the intermediate PB-AZTDP (analogous to **2**) was not degraded by the esterase. In this case, we observed complete cleavage of the phosphate anhydride bond with the formation of various products. The following requirements for the development of an efficient diphosphate prodrug can be derived from these results: Despite the desired high chemical stability, the masks must be removed in biological media as fast as possible; otherwise, hydrolysis of the pyrophosphate competes effectively. Half-lives of 1–30 min enable the formation of large amounts of NDP. Although the compounds lose some stability in human plasma, they are much more stable in human plasma than in cell extracts, in which the esterase activity is significantly higher.

We determined the half-lives of the compounds in the incubation medium used in anti-HIV tests (RPMI/FCS). We found a destabilization of all compounds in this medium relative to their stability in phosphate buffer. We then evaluated compounds **1a**–**h** for their ability to inhibit the replication of HIV in T-lymphocyte cells. For this purpose, a suspension of wild-type CEM cells was infected with HIV-1 or HIV-2, and a mutant thymidine kinase deficient CEM cell culture was infected only with HIV-2. The infected cell suspensions (100 μL per well) were transferred to a 96-well microtiter plate and mixed with solutions of the test compounds at the appropriate dilution (100 μL). After 4–5 days, giant-cell formation was recorded microscopically in the HIV-infected cell cultures. Although d4T derivatives **1a**–**d** were found to be less stable in RPMI/FCS culture medium than in phosphate buffer, compounds **1c** and **1d** retained anti-HIV activity in the thymidine kinase deficient cell line (CEM/TK$^-$; Table 2). This result is a first proof of the ability of these compounds to diffuse across cell membranes and release biologically active metabolites intracellularly. We think that compounds **1a** and **1b** failed to bypass TK for different reasons. Although **1a** released d4TDP efficiently in the other experiments described, it may be too polar to penetrate the cell membrane efficiently and is thus degraded in RPMI/FCS. The lack of activity of compound **1b** may result from an inability to be fully degraded to unmasked d4TDP, as only the first masking unit was cleaved in CEM/0 extracts. None of the compounds examined displayed marked cytotoxicity.

Table 2: Antiviral activity of **1a**–**d** against HIV-1 and HIV-2.

	EC_{50} [μM][a] CEM/0[b] HIV-1	EC_{50} [μM] CEM/0 HIV-2	EC_{50} [μM] CEM/TK$^-$[c] HIV-2	CC_{50} [μM][d] CEM/0
1a	1.05	1.5	21.0	>125
1b	0.78	0.90	14	66±1
1c	0.90	1.30	2.6	81±1
1d	0.20	0.30	0.85	36±5
d4T	0.40	0.3	70	>100

[a] Antiviral activity in T lymphocytes: 50% effective concentration (values shown are the mean of two or three independent experiments). [b] Wild-type T lymphocytes. [c] Thymidine kinase deficient T lymphocytes. [d] Cytostatic concentration: CC_{50} is the concentration of the compound required to inhibit CEM/0 cell proliferation by 50%.

In summary, our study has revealed the requirements for the bioreversible protection of nucleoside diphosphates as prodrugs. In principle, the BAB concept presented herein should be applicable to other nucleoside analogues. We are currently optimizing of the properties of different target structures with respect to hydrolysis and lipophilicity.

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