Neighboring Group Catalysis in the Design of Nucleotide Prodrugs†

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Received January 25, 1996⊗

An approach is described for potential application to the delivery of polar nucleosides and nucleotides across lipophilic membranes, namely, nucleotide prodrugs based on salicyl phosphate. 3′-Azido-3′-deoxythymidine (AZT) and 3′-deoxythymidine (ddT) were chosen as models. For the synthesis of prototype compounds **1** and **2**, the approach was first to react either methyl salicylate (for **1**) or phenyl salicylate (for **2**) with phosphorus oxychloride in dry methylene chloride at 0 °C with the addition of triethylamine as acid scavenger. The resulting intermediate phosphorodichloridate was reacted immediately with excess nucleoside under the same conditions. The control model compound **3** was prepared by reaction of phenyl phosphorodichloridate and excess nucleoside in pyridine/methylene chloride at 0 °C to give **3** in 82% yield. The synthesis of triester **7** involved reaction of α-(chloroacetyl)salicyl chloride with 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose to give [[(2,3,4,6-tetra-*O*-benzyl-D-glucopyranosyl)-oxy]carbonyl]-2-(1-chloroacetoxy)benzene (**4**) which was dechloroacetylated to **5**, 2,3,4,6-tetra-*O*-benzyl-D-glucopyranosyl salicylate. Phosphorylation of **5** with phosphorus oxychloride provided the phosphorodichloridate which was directly converted to **6** by reaction with dideoxythymidine. Removal of benzyl groups by catalytic hydrogenation gave compound **7**, bis(2′,3′-dideoxythymidin-5′-yl) D-glucopyranosyl phosphate. The AZT prodrug triesters, **1** and **2**, underwent much more rapid hydrolysis than the triester **3**, most probably due to the formation of an acyl phosphate complex from the attack on phosphorus of the salicylate carboxylate. The hydrolysis of the less lipophilic **7** was significantly slower than that of **1** or **2**. Both pig liver esterase and rat brain cytosol were able to effect the cleavage to dinucleotide or mononucleotide of prodrug forms **2** and **7**, much more rapidly than either **3** or **1**, suggesting that the esterase-like enzymatic activity of rat brain was similar to that of pig liver esterase. This study suggests the possibility of use of salicylic acid-based prodrugs for nucleotides, subject to specific refinements in the choice of carboxylate- and phosphoric acid ester-protecting groups.

Introduction

Attainment of effective concentrations of antiviral or antitumor nucleoside and nucleotide analogues in the brain requires penetration of the blood−brain barrier by polar agents. For instance, the anti-HIV drug DDC (2′,3′-dideoxycytidine) does not accumulate in the brain in sufficient concentrations useful in the blockade of HIV replication.[1] In addition, the development of resistance to antiviral or antitumor nucleosides often results from the emergence of thymidine kinase deficient cell populations which are no longer sensitive to the nucleoside since it is the phosphorylated forms which display inhibitory activity.[2] Finally, since 5′-phosphorylation of certain anti-human immunodeficiency virus nucleosides by kinases may be a limiting step in the accumulation of inhibitory nucleotides, a means to bypass the cellular kinase could enhance the antiviral potency of such nucleosides.[3,4]

A host of chemical solutions has been explored to circumvent these problems. For instance, several groups of investigators have applied the dihydropyridine−pyridinium redox chemical delivery system to 3′-azido-3′-deoxythymidine (AZT) or DDC.[5−10] A series of aryl bis(3′-*O*-acetylthymidin-5′-yl) phosphate derivatives were prepared by Farrow et al.[11] to find which one might hydrolyze to the dinucleotide under physiological conditions. An intramolecular cyclization−elimination released 5-bromo-2′-deoxyuridine from 3′- or 5′-[[(alkylamino)ethyl]glycyl] esters under mild conditions.[12] Puech and co-workers[13] synthesized bis[*S*-[(2-hydroxyethyl)sulfidyl]-2-thioethyl] esters of 2′,3′-dideoxyuridine 5′-monophosphate, 3′-azido-3′-deoxythymidine 5′-monophosphate, and 9-[(phosphonylmethoxy)ethyl]adenine (PMEA) and reported their conversion into appropriate 5′-monophosphates through a reductase-mediated activation process. A glycosyl phosphotriester prodrug of 3′-azido-3′-deoxythymidine was reported to give good delivery of AZT and AZT 5′-monophosphate to the central nervous system (CNS).[14] Other investigators have explored the potential of 5′-hydrogen phosphonates of anti-HIV nucleosides but concluded that their antiviral activity is due to the release of parent nucleoside.[15,16] On the other hand, McGuigan and co-workers reported that the 5′-bis(trichloroethyl) phosphate derivatives of several 3′-*O*-acylthymidines possessed significant antiviral activity possibly as a result of "kinase bypass".[17]

More than 4 decades ago, the rate acceleration associated with the hydrolysis of salicyl phosphate to salicylic acid and orthophosphate was described.[18] Subsequently, Bromilow et al.,[19] Khan et al.,[20] and Abell and Kirby[21] described the intramolecular catalysis of phosphate triester catalysis through the neighboring group participation of the ortho carboxyl group in dialkyl 2-carboxyphenyl phosphates. On the basis of

† Preliminary account of this work was presented at the 1994 National Medicine Chemistry Symposium, Salt Lake City, UT, June 20−24, 1994.

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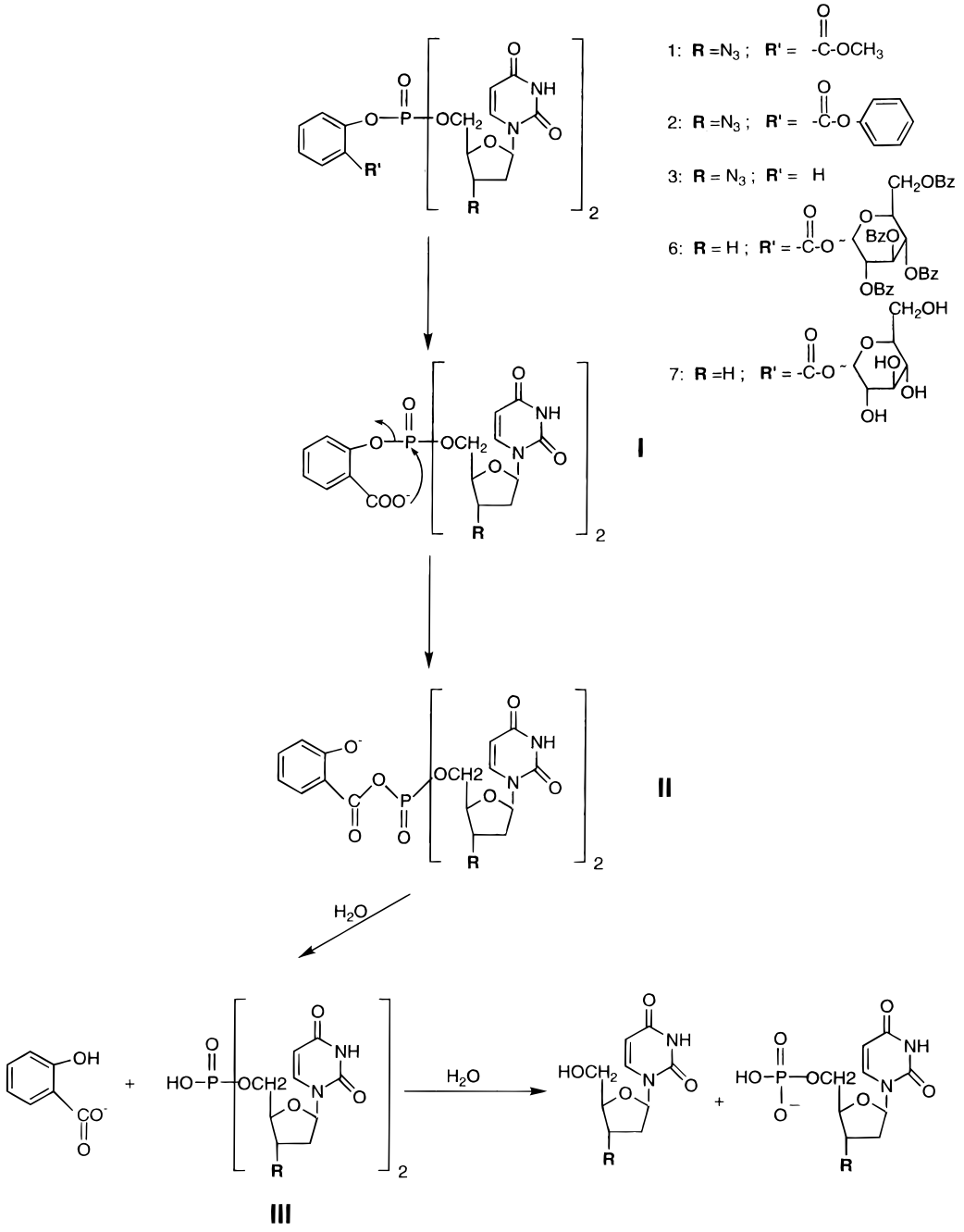
⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1996.

S0022-2623(96)00075-1

Scheme 1. Mechanism for the Release of Nucleotide and Nucleoside from a Salicylate-Based Prodrug



this background, we speculated that phosphate triesters of the general formulation of Scheme 1 might possess properties appropriate to prodrug forms of biologically active nucleosides. Such a triester might undergo a protease- or esterase-catalyzed hydrolysis of the carboxyl ester function to provide an intermediate (**I** of Scheme 1) which then could mount an intramolecular attack on the phosphotriester. Because of the likely generation of a phenolate anion as a leaving group, a dinucleotide could be released as the initial hydrolysis product through the intermediate formation of an acyl phosphate (**II** of Scheme 1). This dinucleotide (**III** of Scheme 1) could then be processed to free nucleoside and nucleoside 5′-monophosphate which could be further metabolized to nucleoside (Scheme 1).

Results

Chemistry. As model nucleosides for investigation of the chemical aspects of this study, we chose AZT and 3′-deoxythymidine (ddT). For the synthesis of compounds **1** and **2**, the approach was first to react either methyl salicylate (for **1**) or phenyl salicylate (for **2**) with phosphorus oxychloride in dry methylene chloride at 0 °C with the addition of triethylamine as acid scavenger. The resulting reactive intermediate phosphorodichloridate was not isolated, but rather it was reacted immediately with excess nucleoside under the same conditions. After purification by silica gel chromatography, the triesters **1** and **2** were obtained in yields of 68% for **1** and 22% for **2**. To prepare compound **3**, commercially available phenyl phosphorodichloridate was reacted with excess nucleoside in pyridine/methylene chloride at 0 °C to give **3** in 82% yield.

The synthesis of triester **7** included a total of five steps. The susceptibility of the salicylate ester bond to basic hydrolysis restricted the choice of protecting groups for the salicylate phenolic hydroxyl. Reasonable

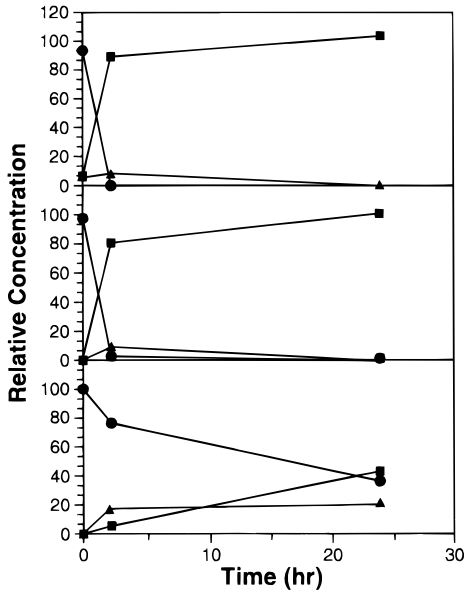


Figure 1. Hydrolysis of triester prodrugs with 5 mM NaOH at 37 °C. C_0 for each starting compound was 0.5 mM. Aliquots of 50 μL were removed at appropriate intervals from the initial 1.0 mL reaction volume for immediate neutralization and analysis by HPLC. Upper panel: triester **1** (●), intermediate of general formula **II** with R = N_3 (▲), and hydrolysis product dinucleotide of general formula **III** with R = N_3 (■). Middle panel: triester **2** (●), intermediate of general formula **II** with R = N_3 (▲), and hydrolysis product dinucleotide of general formula **III** with R = N_3 (■). Bottom panel: triester **7** (○), intermediate of general formula **II** with R = H (▲), and hydrolysis product dinucleotide of general formula **III** with R = H (■).

success was obtained through use of the chloroacetyl group which can be removed under mild conditions with thiourea.[22] Thus, α-(chloroacetyl)salicylic acid, prepared by a known procedure,[23] was converted to the corresponding acid chloride by thionyl chloride. This was used to acylate 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose to give [[(2,3,4,6-tetra-*O*-benzyl-D-glucopyranosyl)oxy]-carbonyl]-2-(1-chloroacetoxy)benzene (**4**). Dechloroacetylation was accomplished with excess thiourea in MeOH to give compound **5**, 2,3,4,6-tetra-*O*-benzyl-D-glucopyranosyl salicylate. Phosphorylation of **5** with phosphorus oxychloride provided the intermediate phosphorodichloridate which was directly converted, without isolation, to compound **6** by reaction with dideoxythymidine. Benzyl groups were removed by catalytic hydrogenation to give compound **7**, bis(2′,3′-dideoxythymidin-5′-yl) glucopyranosyl phosphate.

Enzymatic and Nonenzymatic Hydrolysis Studies. 1. Base-Catalyzed Hydrolysis. To determine if the presence of the carboxylate ester functionality in triesters **1** and **2** would cause a hydrolysis enhancement compared to the non-carboxy-functionalized triester **3**, hydrolytic half-lives ($t_{1/2}$) were determined in aqueous 5×10^{-3} M NaOH at 37 °C (Figure 1). HPLC analysis permitted determination of the following $t_{1/2}$'s: triester **1**, 1.1 h; triester **2**, 1.1 h; triester **3**, 17 h. The first two prodrug forms of AZT showed a very similar course in terms of rates of hydrolysis, transient intermediate formation, and products. No hydrolysis occurred in the absence of base. In both cases, as starting triester disappeared, an intermediate formed which gradually dissipated as product formed. In the case of both compounds **1** and **2**, the intermediate had an identical retention time, 13.7 min, thereby implying the existence of a common intermediate in both hydrolyses. In addition, for both **1** and **2**, two of the hydrolytic final products were identical by HPLC. One product was salicylic acid. The other was identified by a combination of enzyme digestion and mass spectrometry (*vide infra*) and corresponded to the dinucleotide bis(3′-azido-3′-deoxythymidin-5′-yl) phosphate. The third product, identified in the case of the hydrolysis of **2**, was phenol. The corresponding product for **1** would be expected to be methanol which was not detected by our analytical procedure.

In order to confirm the structure of the suspected dinucleotide final product from the hydrolysis of **1** and **2**, sufficient amounts were collected from the HPLC separations so that after concentration, the peak could be subjected to enzymatic and mass spectral analysis. Digestion of the hydrolysis product with snake venom phosphodiesterase produced equimolar amounts of AZT and AZT 5′-monophosphate. The implied structure, bis-(3′-azido-3′-deoxythymidin-5′-yl) phosphate, also was confirmed by high-resolution mass spectrometry (HRMS).

Base hydrolysis of triester **7** proceeded much more slowly than that of **1** and **2** (Figure 1, bottom). The $t_{1/2}$ for **7** was approximately 17 h. However, in this case, as for triesters **1** and **2**, a labile intermediate formed as **7** disappeared. This intermediate had a different retention time (15.6 min) than that formed in the case of compounds **1** and **2**, but, in accord with the behavior of the previously described intermediates, it also disappeared as product was formed. The different retention time for the hydrolytic intermediate would be expected since it would be based on the dideoxythymidine nucleosides instead of AZT. As with derivatives **1** and **2**, the ultimate product of hydrolysis under these conditions was the dinucleotide bis(2′,3′-dideoxythymidin-5′-yl) phosphate, as ascertained by HRMS.

The triester **3** was hydrolyzed under identical conditions as employed for **1**, **2**, and **7**. Its $t_{1/2}$ was 17 h (data not shown). However, the course of its hydrolysis was significantly different from that of the other examined triesters. It was hydrolyzed directly to the dinucleotide bis(3′-azido-3′-deoxythymidin-5′-yl) phosphate without any detectable intervention of an intermediate as for **1**, **2**, and **7**.

2. Pig Liver Esterase-Catalyzed Hydrolysis. When triesters **1**−**3** and **7** individually were incubated in a Tris (pH 7.8) buffer at a concentration of 2×10^{-4} M with 2.2 units/mL pig liver esterase, major differences in their behavior were observed (Figure 2). Compound **3** was unchanged even after 24 h incubation (data not shown). The $t_{1/2}$ for triester **1** was 20 h, whereas the $t_{1/2}$ for compound **2** was just 1 h. The glycosylated triester **7** was hydrolyzed also, albeit at a considerably slower rate with a $t_{1/2}$ of more than 48 h. No hydrolysis of **1**−**3** or **7** occurred in the absence of enzyme. Aside from the differences in kinetics, the hydrolytic course catalyzed by pig liver esterase was quite similar to that witnessed with hydroxide ion-catalyzed hydrolysis, namely, triesters **1**, **2**, and **7** all gave rise to a labile intermediate (the same for **1** and **2**), and all gave dinucleotides (same as with hydroxide) as final products. Specifically, compounds **1** and **2** gave bis(3′-azido-3′-deoxythymidin-5′-yl) phosphate, and compound **7** gave bis(2′,3′-dideoxythymidin-5′-yl) phosphate.

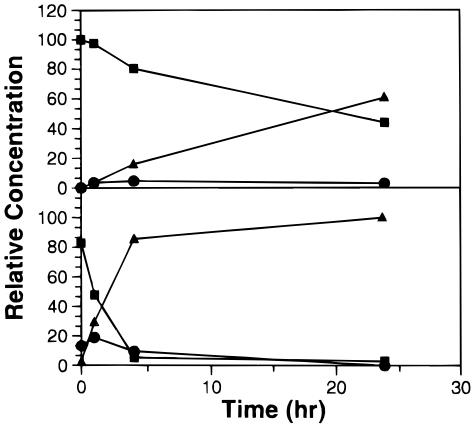


Figure 2. Pig liver esterase-catalyzed hydrolysis of triester prodrugs at 37 °C. These reactions were followed in a volume of 1.0 mL which was 0.01 M in Tris (pH 7.8), with a C_0 for the triester of 1 mM and containing 2.17 units of enzyme (108.5 units/mg). Upper panel: triester **1** (■), intermediate of general formula **II** (Scheme 1) with R = N_3 (●), and hydrolysis product dinucleotide of general formula **III** with R = N_3 (▲). Bottom panel: triester **2** (■), intermediate of general formula **II** with R = N_3 (●), and hydrolysis product dinucleotide of general formula **III** with R = N_3 (▲).

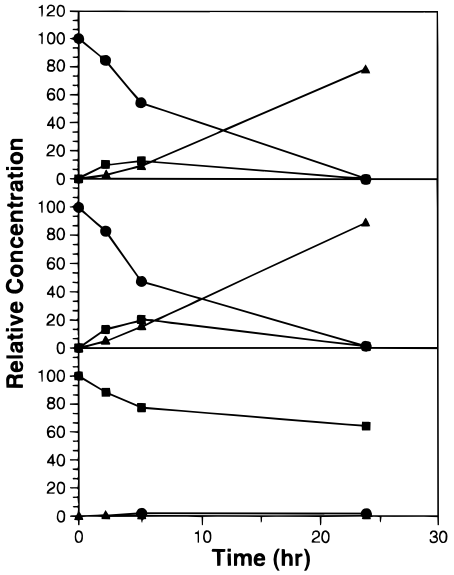


Figure 3. Human serum-catalyzed hydrolysis of triester prodrugs at 37 °C. Reactions were carried out in 0.1 M phosphate buffer (pH 7.4) containing 1% DMSO and 50% human serum. C_0 for the starting triester was 0.1 mM. Aliquots of 50 μL were removed immediately to acetonitrile for precipitation, centrifugation, and analysis of the supernatant by HPLC. Upper panel: triester **1** (●), intermediate of general formula **II** with R = N_3 (■), and hydrolysis product dinucleotide of general formula **III** (Scheme 1) with R = N_3 (▲). Middle panel: triester **2** (●), intermediate of general formula **II** with R = N_3 (■), and hydrolysis product dinucleotide of general formula **III** with R = N_3 (▲). Bottom panel: triester **7** (■), intermediate of general formula **II** with R = H (▲), and hydrolysis product dinucleotide of general formula **III** with R = H (●).

3. Hydrolysis Caused by Human Serum. Hydrolysis of the triesters by human serum was observed when the triesters were incubated at 37 °C in a 1:1 mixture of human serum and 0.1 M phosphate buffer (pH 7.4) containing 1% DMSO (Figure 3). In the absence of serum, no measurable hydrolysis was noted. In this case, triesters **1** and **2** possessed similar $t_{1/2}$'s of 4 h. Compound **3** showed only slight hydrolysis after 72 h of incubation under these conditions. Prodrug form **7** possessed a $t_{1/2}$ significantly longer than 48 h. As before, compounds **1**, **2**, and **7** all hydrolyzed *via* the same labile intermediates observed with hydroxide ion and pig liver esterase (data not illustrated). As products of the action of human serum, all triesters gave rise to the previously observed 5′,5′-dinucleotides. Prolonged incubation led to formation of the hydrolysis product of the dinucleotide, namely, the nucleoside AZT. Presumably, because of the phosphatase normally found in human serum, any generated 5′-mononucleotide would be immediately dephosphorylated. Specifically, after a total of 72 h of incubation, about 50% of the dinucleotide was split to nucleoside product. Compound **3** was extremely stable, showing only a trace of breakdown to dinucleotide after a total of 150 h of incubation (data not shown).

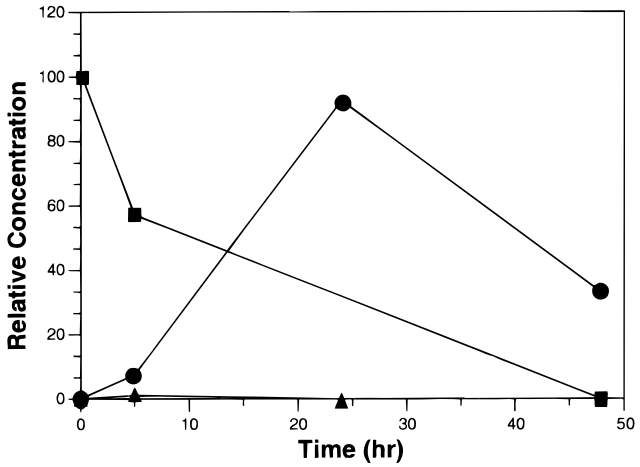


Figure 4. Hydrolysis of triester prodrug **2** caused by rat brain cytoplasmic extract. The triester **2** (■) disappeared with the concomitant formation of the transient intermediate of the general formula **II** (Scheme 1) (▲) and the formation of the dinucleotide of general formula **III** (●). This latter dinucleotide itself eventually also was hydrolyzed over a longer time course and gave rise to the nucleoside 3′-azido-3′-deoxythymidine (not shown).

4. Hydrolysis Catalyzed by Brain Extract. Incubation of the various triesters with brain cytosol in phosphate buffer (pH 7.4) at 37 °C provided a result somewhat similar to that seen when the pig liver esterase-catalyzed hydrolysis was examined (Figure 4). Triester **1** was virtually unchanged after 24 h incubation. The same result was obtained for compound **3**. On the other hand, triester **2** underwent hydrolysis with a $t_{1/2}$ of 5 h to yield the 5,5′-dinucleotide bis(3′-azido-3′-deoxythymidin-5′-yl) phosphate through the same labile intermediate involved in hydroxide ion and pig liver esterase hydrolysis. Compound **7** also was hydrolyzed by rat brain extract with a $t_{1/2}$ of more than 72 h. However, as before, the same labile intermediate was observed to form and give rise to the product dinucleotide bis(2′,3′-dideoxythymidin-5′-yl) phosphate.

Discussion

As would be predicted based on earlier work with neighboring group participation in 2-carboxyphenyl phosphates, the AZT prodrug triesters **1** and **2** underwent considerably more rapid hydrolysis than the triester **3**. Compounds **1** and **2** each possessed a $t_{1/2}$ of 1.1 h. This similarity in rate of hydrolysis of the methyl

carboxylic ester **1** and the phenyl carboxylic ester **2** likely is related to the formation and subsequent hydrolysis of a common intermediate: most probably, the acyl phosphate complex (see Scheme 1) formed by the addition of the salicylate carboxylate to the phosphorus−oxygen double bond. In accord with this, triester **3**, which has no 2-carboxyl function to participate in a neighboring group reaction, had a much longer $t_{1/2}$ (17 h) under the same conditions. Triester **7**, although it bore the requisite 2-carboxyl functionality for facilitated hydrolysis by means of neighboring group participation, possessed a $t_{1/2}$ of approximately 17 h also. Insofar, however, as a similar intermediate was detected with **7** as in the case of the base-catalyzed hydrolysis of triesters **1** and **2**, we hypothesize a similar neighboring group participation in the hydrolysis of compound **7**. In this case, it is possible that the initial carboxylate ester hydrolysis to yield the obligatory acyl phosphate intermediate was slowed by the sterically hindered nature of the carboxylate ester which was roughly equivalent to an α-substituted secondary ester. In any case, it was clear from the hydroxide-catalyzed reactions of compounds **1**−**3** and **7** that salicyl phosphate prodrug forms of AZT and ddT could undergo a neighboring carboxylate-facilitated release of the desired dinucleotide form of the drug.

With pig liver esterase and rat brain cytosol, in contrast to base-catalyzed hydrolysis, a different behavior of triesters **1** and **2** was noted. Although both compounds **1** and **2** showed an increased rate of hydrolysis compared to the noncarboxylated triester **3**, the overall rate of triester hydrolysis depended on the enzymatic cleavage of the carboxylate ester, and this depended upon substrate structure.

The behavior of the prodrug forms toward hydrolysis by human serum was significantly different. Hydrolysis of derivatives **1** and **2** in human serum showed a different dependence on the nature of the alcohol moiety of the carboxylic ester compared to the pig liver esterase and brain extract results; nonetheless, compounds **1**, **2**, and **7** still displayed an accelerated hydrolysis rate due to the presence of the esterase-generated neighboring carboxyl group.

The hydrolytic behavior of phosphotriesters **1** and **2** in a variety of systems, including hydroxide, pig liver esterase, human serum, and brain cytosol, has demonstrated the essential requirement for potential salicylate-based nucleotide prodrugs: specifically, the generation of a carboxylate at a neighboring position that facilitates phosphotriester hydrolysis to dinucleotide through the formation of a unstable acyl phosphate intermediate. The carboxylate esters **1** and **2** showed a dependence of hydrolytic rate on the nature of the alcohol of the carboxylic ester. This expected behavior would be an advantage in prodrug design since it would permit adjustment of the rate of release of the drug in question. Nonetheless, prototype prodrug derivatives **1** and **2** displayed undesirable solubility characteristics in aqueous media. To address this problem, the glycosyl congener **7** was synthesized. This derivative did possess improved solubility properties; however, its rate of hydrolysis to give the requisite dinucleotide was very slow, and its lipophilicity was drastically reduced. Future studies in this area will have to address consideration of the effect of the alcohol domain of the salicylate on both prodrug solubility and its ability to function as an esterase substrate.

In preliminary assays of compounds **1** and **3** against HIV-1 in CEM cells, it was found that the EC_{50} for **1** was 0.07 μM and the EC_{50} for **3** was 0.65 μM, while that for AZT was 0.002 μM. In CEM/TK$^-$ cells, all of these compounds were devoid of antiviral activity ($EC_{50} > 50$ μM). These findings imply that compounds **1** and **3** may be hydrolyzed extracellularly to free nucleoside before being taken up by the cells.

Experimental Section

Reagents. 3′-Azido-3′-deoxythymidine, 3′-deoxythymidine, and 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose were obtained from Sigma Chemical Co. (St. Louis, MO). Fluka Chemie AG (Switzerland) was the source for pig liver esterase, phosphorus oxychloride, and chloroacetyl chloride. Other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI).

HPLC Analysis. The HPLC was System Gold from Beckman Instruments (Berkeley, CA). It consisted of two Model 110B solvent delivery modules, an analogue interface Module 406, and a scanning detector module 167. The column was a μ-Bondapak C-18 reverse-phase (3.9 × 300 mm) (Waters-Millipore Corp., Milford, MA) with elution using the following conditions: solvent A, 50 mM ammonium acetate; solvent B, CH_3CN/H_2O (9:1); flow rate, 2.0 mL/min. The elution program was 15% solvent B in buffer A for 5 min followed by linear gradient for 15 min to 70% solvent B in buffer A. The retention times (min) were as follows: **1**, 20.4; **2**, 20.1; **3**, 22.7; AZT, 9.9; AZTMP, 2.8; phenol, 13.77; bis(3′-azido-3′-deoxythymidin-5′-yl) phosphate, 11.7; intermediate in hydrolysis of **1** and **2**, 13.7; **7**, 18.2; bis(2′,3′-dideoxythymidin-5′-yl) phosphate, 13.4; intermediate in hydrolysis of **7**, 15.6.

Spectral Measurements. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra were recorded on either Varian XL-300 or GE GN-300 instruments using the solvents indicated below. Chemical shifts (δ) are reported in ppm (TMS as an internal standard). Multiplicity is abbreviated as s (singlet), d (doublet), t (triplet), and m (multiplet). Coupling constants are expressed in hertz (Hz). UV absorption spectra were recorded on a Varian DMS-200 spectrophotometer and mass spectra on a Finnigan/Extrel 1015 instrument (chemical ionization instrument).

TLC Analysis. Analytical TLC used precoated silica gel GHLF plates (250 μm, F254) from Analtech (Newark, DE). The solvent system used was $CHCl_3$/EtOAc/MeOH (7:2.7:0.3) for AZT and its triester analogues. The TLC R_f's were AZT, 0.28; **1**, 0.1; **3**, 0.13; **2**, 0.16. The system $CHCl_3$/MeOH (9.5:0.5) was used for ddT analogues. The TLC R_f's were ddT, 0.23; GBzSP-$(ddT)_2$, 0.3; **7**, 0.05.

Hydrolysis Studies. (a) Hydrolysis of Triesters with 5 mM NaOH at 37 °C. Hydroxide ion-catalyzed triester hydrolysis was carried out in water containing a final concentration of 0.5 mM triester and 5 mM NaOH in a total volume of 1.0 mL. Incubation was at 37 °C. Aliquots (50 μL) were removed at the indicated times for immediate analysis by HPLC.

(b) Hydrolysis of Triesters with Pig Liver Esterase at 37 °C. Enzyme-catalyzed hydrolysis was followed in a total volume of 1.0 mL of water which was 0.01 M in Tris buffer (pH 7.8) and 1 mM in triester and contained 2 μL (2.17 units) of pig liver esterase (EC 3.1.1.1; 10 mg/mL, 108.5 units/mg). Incubation was at 37 °C. Again, 50 μL aliquots were removed for HPLC analysis.

(c) Hydrolysis of Triesters with Human Serum. Human blood (postclotting, 20 mL) was centrifuged at 750*g* for 10 min to give 2.5 mL of serum. Triester stock solutions were dissolved in DMSO. For hydrolysis studies, each reaction mixture contained 0.1 mM triester in 0.1 M phosphate buffer (pH 7.4), 1% DMSO, and a final concentration of 50% human serum. The samples were incubated at 37 °C, and 50 μL aliquots were removed at different times. The plasma proteins

were precipitated with CH_3CN (50 μL) and centrifuged down at 1000*g*, and 50 μL of supernatant was injected for HPLC analysis.

(d) Hydrolysis of Triesters with Rat Brain Extract. Two rat brains (total weight 3.3 g) were washed in phosphate buffer (0.1 M, pH 7.4) and homogenized in ice-cold buffer (13.2 mL), and the resulting suspension was centrifuged at 10000*g* for 20 min at 4 °C. The supernatant was removed and kept on ice. A solution of NADP (36 mg), glucose-6-phosphate (163.3 mg), $MgCl_2 \cdot 6H_2O$ (101.6 mg), and glucose-6-phosphate dehydrogenase (80 U) was freshly prepared in phosphate buffer (10 mL, 0.1 M, pH 7.4) and stored on ice. This solution (200 μL) and 500 μL of brain supernate were added to a solution (5 μL) of triester in phosphate buffer containing 0.5% DMSO at 37 °C. Samples (50 μL) were removed at different time points and added to centrifuge tubes containing acetonitrile (50 μL), the protein precipitate was spun down, and 50 μL of the supernate was injected directly to the HPLC.

***O,O′*-Bis(3′-azido-3′deoxythymidin-5′-yl)-*O″*-[2-[(methyloxy)carbonyl]phenyl]phosphate (1).** Methyl salicylate (0.165 g, 1.1 mmol) was dissolved in dry methylene chloride (4 mL), and distilled triethylamine (500 μL, 0.363 g, 3.5 mmol) was added to this solution. The reaction mixture was stirred at 0 °C for 5 min, and then freshly distilled $POCl_3$ was added (100 μL, 0.167 g, 1 mmol) dropwise. The reaction proceeded rapidly. After 30 min, silica gel TLC in $CHCl_3$ showed the methyl salicylate had disappeared. Due to the high reactivity of methylsalicyl phosphorodichloridate, it was not isolated or purified. Instead, 3′-azido-3′-deoxythymidine (0.75 g, 2.8 mmol) in dry pyridine (2 mL) was added dropwise to the mixture. After 30 min at 0 °C, the solution was allowed to warm to room temperature, and stirring was continued for another 5 h. The solvents were evaporated *in vacuo*. The residue was applied to a silica gel column which was eluted with $CHCl_3$/ETOH (7:3). Appropriate fractions (as determined by TLC on silica gel plates using $CHCl_3$/EtOAc/MeOH, 7:2.7:0.3) were combined to give, after evaporation, compound **1** (0.5 g, 68%) as a colorless amorphous glass: UV λ_{max} (MeOH) 267 nm ($\epsilon = 20\,000$); ^{1}H NMR ($CDCl_3$) δ 1.836 (s, 3, thymine CH_3), 1.880 (s, 3, thymine CH_3), 2.3−2.4 (m, 4, 2′-H′s), 3.8 (s, 3, salicylate-CH_3), 4.02−4.07 (m, 2, 4′-H′s), 4.32−4.4 (m, 4, 5′-H′s), 4.48−4.5 (m, 2, 3′-H′s), 6.06−6.15 (m, 2, 1′-H′s), 7.2−7.5 (m, 6, 3 salicylate aromatic protons, 2 thymines 6-H′s), 7.93 (d, 1, $J = 7.8$ Hz, salicylate), 9.06 (s, 1, thymine 3-NH), 9.09 (s, 1, thymine 3-NH); ^{31}P NMR ($CDCl_3$) δ −6.35; HRMS (FAB) calcd for 731.1939 (M^+), found 731.1945.

***O,O′*-Bis(3′-azido-3′-deoxythymidin-5′-yl)-*O″*-[2-[(phenyloxy)carbonyl]phenyl]phosphate (2).** Phenyl salicylate (0.214 g, 1 mmol) in dry methylene chloride (2.5 mL) and dry triethylamine (500 μL, 0.363 g, 3.5 mmol) were combined and stirred at 0 °C. Freshly distilled $POCl_3$ (100 μL, 0.167 g, 1 mmol) was added dropwise to this mixture. TLC using $CHCl_3$ showed no phenyl salicylate was present in the reaction mixture after 30 min reaction time. Dry 3′-azido-3′-deoxythymidine (0.75 g, 2.8 mmol) in dry pyridine (2 mL) was added dropwise to this mixture. The reaction mixture was kept for 30 min at 0 °C and then for an additional 3−4 h at room temperature. The solvents were evaporated *in vacuo*, and the residue was applied to a silica gel column which then was eluted with $CHCl_3$/EtOH (100:4). Appropriate fractions (as determined by silica gel TLC as above) were combined to give, after evaporation, *O,O′*-bis(3′-azido-3′-deoxythymidin-5′-yl)-*O″*-[2-[(phenyloxy)carbonyl]phenyl]phosphate (**2**; 0.175 g, 22%): UV λ_{max} (MeOH) 266 nm ($\epsilon = 20\,000$); ^{1}H NMR ($CDCl_3$) δ 1.78 (s, 3, thymine CH_3), 1.86 (s, 3, thymine CH_3), 2.3−2.4 (m, 4, 2′-H′s), 3.9−4 (m, 2, 4′-H′s), 4.2−4.5 (m, 6, 5′,3′-H′s), 6.04−6.15 (m, 2, 1′-H′s), 7.16−7.62 (m, 10, 5 phenyl, 3 salicylate, 2 thymine 6-H′s), 8.16 (d, 1, $J = 7.7$ Hz, salicylate), 8.78 (s, 1, thymine 3-NH), 8.82 (s, 1, thymine 3-NH); ^{31}P NMR ($CDCl_3$) δ −6.5; HRMS (FAB) calcd for 793.2095 (M^+), found 793.2115.

***O,O′*-Bis(3′-azido-3′deoxythymidin-5′-yl)-*O″*-phenylphosphate (3).** Phenyl phosphorodichloridate (150 μL, 0.21 g, 1 mmol) was added dropwise to a solution of 3′-azido-3′-deoxythymidine (75 mg, 2.8 mmol) in a mixture of dry pyridine (2 mL) and methylene chloride (1 mL) at 0 °C. After overnight reaction at room temperature, solvents were removed by evaporation *in vacuo*, and the residue was applied to a silica gel column which was eluted with $CHCl_3$/MeOH (20:1). Compound **3** was obtained as a colorless amorphous glass in a yield of 0.55 g (82%): UV λ_{max} (MeOH) 266 nm ($\epsilon = 18\,000$); ^{1}H NMR ($CDCl_3$) δ 1.82 (s, 3, thymine CH_3), 1.85 (s, 3, thymine CH_3), 2.34−2.40 (m, 4, 2′-H′s), 3.9 (m, 2, 4′-H′s), 4.2−4.4 (m, 6, 3′,5′-H′s), 5.99−6.08 (m, 2, 1′-H′s), 7.15−7.23 (m, 5, 3 phenyl, 2 thymine 6-H′s), 7.3 (t, 2, $J = 8$ Hz, phenyl), 9.39 (s, H, thymine 3-NH), 9.43 (s, H, thymine 3-NH); ^{31}P NMR ($CDCl_3$) δ −6.35; HRMS (FAB) calcd for 673.1884 (M^+), found 673.1857.

(Chloroacetyl)salicylic Acid. Monochloroacetyl chloride (12 mL, 0.3 mol) in benzene (40 mL) was added to a suspension of salicylic acid (dried over P_2O_5, 28 g, 0.2 mol) in benzene (120 mL). The mixture was heated on an oil bath and refluxed for 5 h or until evolution of HCl ceased. The solvent and excess acid halide were distilled under reduced pressure. The resulting crystals were crystallized twice from benzene:[6] EI-MS *m/z* 214 (M); ^{1}H NMR (CD_3OD) δ 4.5 (s, 2H, $-OCOCH_2Cl$), 7.1 (d, 1H, $J = 8.3$ Hz, phenyl), 7.3 (t, 1H, $J = 7.8$ Hz, phenyl), 7.6 (t, 1H, $J = 8.3$ Hz, phenyl), 8 (d, 1H, $J = 7.8$ Hz, phenyl); ^{13}C NMR (CD_3OD) δ 171.3, 170.5, 155, 138.3, 136.2, 130.7, 127.7, 112, 44.9.

[[(2,3,4,6-Tetra-*O*-benzyl-D-glucopyranosyl)oxy]carbonyl]-2-(1-chloroacetoxy)benzene (4) and [[(2,3,4,6-Tetra-*O*-benzyl-D-glucopyranosyl)oxy]carbonyl]phenol (5). (Chloroacetyl)salicylic acid (7.3 g, 34 mmol, dried over P_2O_5) was mixed with thionyl chloride (40 mL, 65.6 g, 556 mmol), and the solution was refluxed until evolution of hydrogen chloride had practically ceased (4 h). The excess of thionyl chloride was evaporated *in vacuo*, and the final traces were removed from the residue by azeotroping[7] with benzene. The product was dissolved in dry methylene chloride (25 mL), and the resultant solution was added dropwise to a cold solution of 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (10 g, 18 mmol) in triethylamine (5.6 mL, 4 g, 40 mmol) and methylene chloride (60 mL). The reaction mixture was kept at room temperature for 4 h. After the reaction was completed, an excess of thiourea (10.6 g, 140 mmol) in MeOH was added directly to this mixture.[5] The mixture was stirred at 50 °C for 48 h until the reaction was complete as determined by TLC [silica gel TLC in hexane/EtOAc (9:1)]. Appropriate fractions were combined to give, after evaporation, [[(2,3,4,6-tetra-*O*-benzyl-D-glucopyranosyl)oxy]carbonyl]phenol (**5**) (5 g, 7.5 mmol): CI-MS *m/z* 678 ($M + NH_4$), 156 (base); ^{1}H NMR ($CDCl_3$) δ 3.66−3.8 (m, 6, 2−6 glucose-H's), 4.47−4.94 (m, 8, benzyl $-CH_2-$), 5.89 (d, 1, $J = 7$ Hz, glucose H-1), 6.88 (t, 1, $J = 7.6$ Hz, salicylate), 6.99 (d, 1, $J = 8.3$ Hz, salicylate), 7.16−7.31 (m, 20, benzyl H's), 7.49 (t, 1, $J = 7.8$ Hz, salicylate), 7.8 (d, 1, $J = 8$ Hz, salicylate), 10.5 (s, 1, salicylate hydroxyl).

***O,O′*-Bis(2′,3′-dideoxythymidin-5′-yl)-*O″*-[[[(2,3,4,6-tetra-*O*-benzyl-D-glucopyranosyl)oxy]carbonyl]phenyl]-phosphate (6).** $POCl_3$ (100 μL, 0.167 g, 1.1 mmol) was added to a cold solution of [[(2,3,4,6-tetra-*O*-benzyl-D-glucopyranosyl)-oxy]carbonyl]phenol (**5**) (0.726 g, 1.1 mmol) in methylene chloride (4 mL) and triethylamine (500 μL, 0.363 g, 3.5 mmol). The mixture was stirred and kept at 0 °C until there was no sign of starting material by TLC analysis ($CHCl_3$). Dry dideoxythymidine (0.61 g, 2.7 mmol) in dry pyridine (2.7 mL) was added to this mixture at 0 °C. The reaction mixture was stirred and kept at 4 °C for 30 min and then for another 3 h at room temperature. The solvent was evaporated, and the residue was applied to a silica gel column which was eluted with $CHCl_3$/MeOH (9:1). Appropriate [as determined by silica gel TLC in $CHCl_3$/MeOH (19:1)] fractions were combined to give, after evaporation, compound **6** as a colorless glass in 47% yield (600 mg, 0.52 mmol): ^{1}H NMR ($CDCl_3$) δ 1.83−2.3 (m, 14, thymine CH_3's, 2′,3′-H's), 3.6−3.7 (m, 6, 2−6 glucose-H's), 4.2−4.9 (m, 14, benzyl-CH_2-, 4′,5′-H's), 5.82 (d, 1, $J = 7$ Hz, glucose H-1), 5.99 (dd, 1, $J = 6$, 4.6 Hz, 1′-H's), 6.07 (dd, 1, $J = 6.1$, 4.1 Hz, 1′-H's), 7.16−7.32 (m, 22, salicylate, benzyl H's), 7.46−7.49 (m, 3, salicylate, thymine 6-H's), 7.89 (d, 1, $J = 7.6$ Hz, salicylate), 9.01 (s, 1, thymine-NH), 9.07 (s, 1, thymine-NH); ^{31}P NMR ($CDCl_3$) δ −6.58.

Bis(2′,3′-dideoxythymidin-5′-yl) D-Glucopyranosyl Phosphate (7). Compound **5** (0.4 g, 0.35 mmol) was dissolved in acetone/methanol (1:1, 100 mL). To this mixture was added

10% palladium-on-carbon (0.3 g) in methanol (40 mL).[8] Hydrogenolysis was repeated twice at 50 psi overnight. After one cycle of reduction, the used catalyst was filtered, fresh catalyst (0.3 g) was added, and hydrogenation was continued for an additional 2.4 h. The mixture was filtered, and the solvent was evaporated. The residue was dissolved in MeOH, dried over silica gel, and directly applied to a silica gel column [$CHCl_3$/MeOH (7:3)]. Appropriate fractions were combined to give bis(2′,3′-dideoxythymidin-5′-yl) glucopyranosyl phosphate (**7**) (56 mg, 0.07 mmol) in 20% yield: ^{1}H NMR (D_2O) δ 1.57 (s, 1, thymine CH_3), 1.59 (s, 1, thymine CH_3), 1.75−2.3 (m, 8, 2′,3′-H's), 3.37−3.8 (m, 6, 2−6 glucose-H's), 4.2−4.5 (m, 6, 4′,5′-H's), 5.6−5.62 (m, 1, glucose H-1), 5.92 (dd, 1, J = 6.7, 4 Hz, 1′-H's), 5.98 (dd, 1, J = 6.7, 3 Hz, 1′-H's), 7.18−7.22 (m, 3, salicylates, thymine 6-H's), 7.3 (s, 1, thymine 6-H's), 7.43−7.49 (m, 1, salicylate), 7.89 (d, 1, J = 7.8 Hz, salicylate); HRMS (FAB) calcd for 797.2283 (M^+), found 797.2262.

Acknowledgment. This research was supported in part by the NIH Director's Intramural AIDS-Targeted Antiviral Research Program. We are indebted to Dr. Paul Kovac (NIH) for valuable discussions and to Dr. Jan Balzarini (Rega Institute, Leuven, Belgium) for HIV assays.

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JM9600757