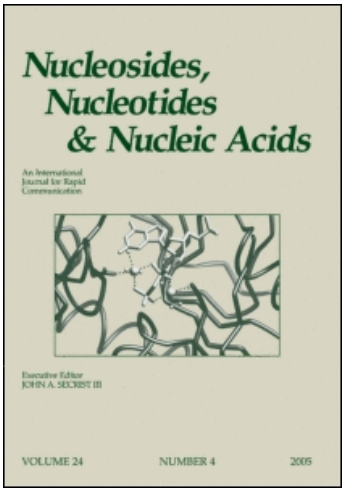

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Synthesis and Anti-Retroviral Activity of *O,O'-BIS*(3'-Azido-2',3'-Dideoxythymidin-5'-yl) Phosphoramidate Derivatives

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SYNTHESIS AND ANTI-RETROVIRAL ACTIVITY OF *O,O'-BIS*(3'-AZIDO-2',3'-DIDEOXYTHYMIDIN-5'-YL) PHOSPHORAMIDATE DERIVATIVES

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ABSTRACT: A simple and efficient protocol for the preparation of various symmetrical dinucleoside phosphoramidates derived from AZT, is presented. It consists of the phosphonylation of AZT with phosphonic acid in the presence of DCC to produce the symmetrical H-phosphonate diester, followed by its oxidative conversion to various phosphoramidate analogues. The synthesized compounds were evaluated for their anti-HIV activity in different cell cultures.

To be effective as anti-retroviral agents, 3'-azido-2',3'-dideoxythymidine (AZT, **1**) and other 2',3'-dideoxynucleoside analogues have to be phosphorylated intracellularly in three steps to their 5'-triphosphate form, which then act at the level of the viral reverse transcriptase as competitive inhibitors and/or alternate substrates.[1] Since it has been postulated that the nucleoside kinase activity (usually involved in the first phosphorylation step) may be lost upon a prolonged exposure to a drug,[2] *e.g.* AZT, in such kinase deficient cells only the intracellular delivery of the 5'-monophosphate itself can effect a viral inhibition. Unfortunately, the cell membrane poses a barrier to charged molecules. To circumvent this problem lipophilic prodrugs[3-8] have been developed and in this regard, the design of a successful prodrug must include an efficient mechanism for

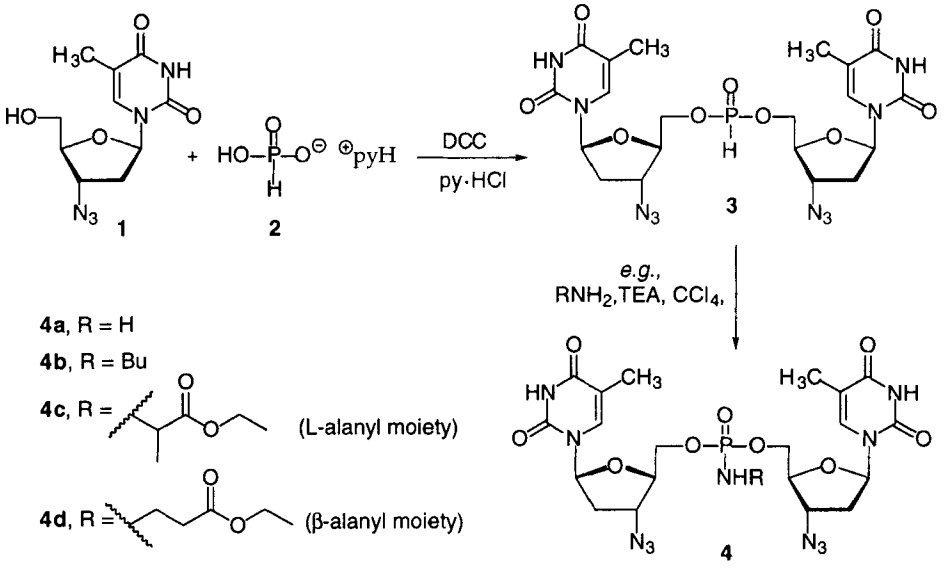
releasing a nucleoside 5'-phosphate inside the cell. Actually, most prodrugs currently designed to deliver a 5'-nucleoside monophosphate intracellularly are based on neutral mono- and dinucleoside phosphate derivatives.[8] Of potential interest are dinucleoside phosphotriesters,[9-18] dinucleoside phosphonates[19-26] and phosphonamidates[23] bearing as nucleoside moiety compounds with established antiviral properties. They, in principle, may enhance (i) the therapeutic potential of the parent drugs by changing their pharmacokinetic behaviour in organisms due to the presence of different functional groups attached to the phosphorus center, and (ii) deliver non-phosphorylated and 5'-phosphorylated nucleosides to a cell. In this regard, it has been shown that several 4-acyloxybenzyl *bis*(3'-azido-2',3'-dideoxythymidin-5'-yl) phosphates were significantly more active than AZT against HIV in thymidine kinase deficient cells.[15]

With these in mind we embarked on investigation of a new class of prodrugs for both AZT and its 5'-phosphorylated derivative, namely symmetrical dinucleoside phosphoramidates **4** (see the SCHEME).

The 5',5'-dinucleoside phosphoramidates **4** contain a P–N bond of potential different lability *in vivo*, and the rate of releasing AZT or/and its monophosphate can be additionally modified by the presence or absence of chiral or achiral substituents on the nitrogen atom in **4**. The synthetic strategy for the preparation of symmetrical phosphoramidates **4** consisted of two steps: (i) the formation of the symmetrical H-phosphonate diester of AZT and (ii) its conversion into the desired phosphoramidates *via* oxidative coupling with the corresponding amine.

The symmetrical H-phosphonate diester derivative **3** of AZT has earlier been prepared from phosphonic acid using pivaloyl chloride (Pv-Cl) as a condensing agent.[22] This approach, however, may have some experimental disadvantages. To avoid acylation of the nucleosidic component and other possible side reactions, the condensing agent has to be added slowly, during an extended period of time to a stirred solution of a nucleosidic component and phosphonic acid. Since this may result in variable yields of the H-phosphonate diesters produced, we wished to develop a more convenient, general procedure for the synthesis of symmetrical dinucleoside H-phosphonate diesters. We chose to study the dicyclohexylcarbodiimide (DCC) mediated condensation of phosphonic acid with a nucleoside, first using 3'-O-dimethoxytritylated thymidine as a model compound. We found that symmetrical H-phosphonate diesters could be produced from pyridinium phosphonate **2** (1 equiv.) and the suitable nucleoside (2 equiv.) with DCC (3 equiv.) as a condensing agent in pyridine, in practically quantitative yields (^{31}P NMR). The presence of pyridinium hydrochloride (2 equiv.) was necessary to secure

SCHEME



reproducible results. The condensation was usually complete within one hour. These reaction conditions turned out to be also suitable for the preparation of symmetrical H-phosphonate diester **3** derived from AZT. The produced **3** was of purity >95% (^{31}P NMR) and could be used for the subsequent oxidative transformations without purification.

The formation of symmetrical H-phosphonate diester **3** proceeded (according to ^{31}P NMR spectroscopy studies) *via* an initial formation of H-pyrophosphonate, followed by its reaction with AZT to produce the corresponding 5'-H-phosphonate monoester. This, reacted further with another molecule of AZT to give the desired symmetrical H-phosphonate diester **3**. Recently, a similar approach to synthesis of diesters of phosphonic acid has been reported.[27]

For the preparation of the unsubstituted phosphoramidate diester **4a**, H-phosphonate diester **3** was treated with saturated ammonia in a CCl_4/dioxane mixture (4/1, v/v).[28] Compounds **4b-d** were prepared using a slightly different method. The oxidative coupling was achieved by the Atherton-Todd reaction [CCl_4, pyridine, triethylamine (TEA) and an appropriate primary amine (3 equiv.)][29,30] using butylamine, L-alanine ethyl ester hydrochloride and β-alanine ethyl ester hydrochloride as N-nucleophiles. The presence of TEA was necessary to produce non-protonated amine nucleophile under the reaction conditions and to facilitate oxidative transformation of

symmetrical H-phosphonate diester **3** into a chlorophosphate intermediate. For all amines investigated, the reaction of **3** under the Atherton-Todd oxidation conditions proceeded smoothly to produce dinucleoside phosphoramidates **4a-d** in good yields (78 - 89%, after silica gel column chromatography).

Compounds **4a-d** were evaluated for their anti-HIV activity in different cell cultures, and the results are reported in the TABLE below.

TABLE. Antiviral activity of AZT phosphoramidate dimers compared to AZT in several cell types infected with HIV-1*

	CEM-SS		CEM/TK⁻ (thymidine kinase deficient cells)		MT-4	
Compound	EC_{50} (μM)†	CC_{50} (μM)‡	EC_{50} (μM)†	CC_{50} (μM)‡	EC_{50} (μM)†	CC_{50} (μM)‡
4a	$1.4\ 10^{-2}$	>100	>100	>100	0.39	>100
4b	0.34	>100	70	>100	3.0	>100
4c	$2.7\ 10^{-2}$	>100	89	>100	0.35	>100
4d	$1.6\ 10^{-2}$	>100	64	89	0.79	>100
AZT	$6.0\ 10^{-3}$	>100	>100	>100	$1.7\ 10^{-2}$	75

*Values are expressed as mean ± SD obtained from at least three separate experiments.
†EC_{50} : concentration required to inhibit the replication of HIV-1 by 50%.
‡CC_{50} : concentration required to reduce the viability of uninfected cells by 50%.

The studied phosphoramidate dimers **4a-d** significantly inhibited the multiplication of HIV-1 in human T4-lymphoblastoid cells, CEM-SS, and MT-4. Striking differences were found in the antiviral activities of the test compounds in HIV-1-infected CEM/TK⁻ cells. This cell line is highly deficient in cytosolic thymidine kinase and should be considered as an ideal target cell line to investigate the potential antiviral activities of AZT nucleotide prodrugs. Indeed, AZT proved to be completely inactive against HIV-1 replication in CEM/TK⁻ cells at concentrations up to 100 μM. In this cell line, the phosphoramidate dimer **4a** is also inactive, and the other phosphoramidate derivatives **4c-d** exhibited a low anti-HIV activity. This seems to demonstrate that dinucleoside phosphoramidate diesters cannot be considered as pronucleotides, as they are unable (or with a very low efficiency) to deliver the corresponding 5'-mononucleotide inside the cells. The anti-HIV-1 activity observed for these compounds in TK expressing cell lines

may be related to the release of the parent nucleoside in the extracellular medium. Such results could be tentatively explained in regard to the chemical and enzymatic stability of the studied compounds, and raise the importance of the affinity of mononucleotide prodrugs (and their corresponding expected metabolites) for intracellular target enzymes. In this respect, we have recently demonstrated that the use of *S*-acyl-2-thioethyl (SATE) biolabile phosphate protection may overcome some limitations encountered in the decomposition pathways of mononucleoside aryl phosphoramidate diesters.[32,33] In light of the present results more detailed studies are necessary to prove the suitability of dinucleoside phosphoramidates in antiviral chemotherapy.

EXPERIMENTAL SECTION

Chemistry: ^{31}P and ^{1}H NMR spectra were recorded on a Jeol GSX-270 FT spectrometer. The ^{31}P NMR experiments were carried out in 10-mm NMR tubes and 2% H_3PO_4 in D_2O was used as an external standard (coaxial inner tube). ^{1}H chemical shifts are given relative to tetramethylsilane. The assignment of proton resonances was done using ^{1}H - ^{1}H correlated NMR spectroscopy. Multiplicity of some signals in ^{1}H NMR spectra of **4** (marked in the spectra with subscripts "a" and "b") is due to diastereotopic character of both AZT moieties, which appeared as chemical shifts non-equivalent groups. High resolution FAB mass spectra were recorded on a Jeol SX-102 instrument.

Pyridine was dried by refluxing with CaH_2 over night followed by distillation and storage over molecular sieves (4 Å). 1,3-Dicyclohexylcarbodiimide was vacuum distilled and butyl amine and triethyl amine were distilled from and stored over CaH_2. L-alanine ethyl ester hydrochloride and β-alanine ethyl ester hydrochloride were commercial grade (Fluka). 2 M stock solution of phosphonic acid (commercial grade, Aldrich) was prepared by evaporation of added pyridine to the appropriate amount of H_3PO_3 and dissolving the residue in anhydrous pyridine.

Anti-HIV evaluation: The origin of the viruses and the techniques used for measuring inhibition of virus replication have previously been described.[31]

***O*, *O'*-*Bis*(3'-azido-2',3'-dideoxythymidin-5'-yl) hydrogenphosphonate (3).** 3'-Azido-2',3'-dideoxythymidine **1** (108 mg, 0.4 mmol) was dried by evaporation of added pyridine and dissolved in the same solvent (4 mL). To this mixture, pyridinium phosphonate (100 mL 2 M stock solution in pyridine), pyridinium

hydrochloride (46 mg, 0.4 mmol) and 1,3-dicyclohexylcarbodiimide (124 mg, 0.6 mmol) were added. After 1 hour was the formed precipitate removed *via* filtration and washed with toluene. The solvents were evaporated under vacuum followed by evaporation of added pyridine. The desired compound **3** could be used in the next step without further purification.

***O, O'-Bis*(3'-azido-2',3'-dideoxythymidin-5'-yl) phosphoramidate (4a).**

H-Phosphonate **3** (116 mg, 0.2 mmol) was treated with a saturated solution of ammonia in carbon tetrachloride/dioxane (10 mL, 4:1, v/v) for 1 hour at 0°C. The reaction mixture was evaporated under vacuum. The residue was chromatographed on a silica-gel column using a stepwise gradient of methanol (0-8%) in methylene chloride to give the desired compound **4a**. Yield, 95 mg (80%).

^{1}H NMR (δ in ppm, DMSO-d_6) 11.3 (s, 2H, 2 x NH), 7.51 (m, 2H, 2 x H-6), 6.11 (t, 2H, 2 x H-1', J= 6.5 Hz), 4.94 (d, 2H, NH_2, J=6.3 Hz), 4.44 (m, 2H, 2 x H-3'), 4.08 (m, 4H, 2 x H-5' & H-5'') 3.99 (m, 2H, 2 x H-4'), 2.37 (m, 4H, 2 x H-2' & H-2''), 1.77 (d, 6H, 2 x C5-CH_3, J=1.1 Hz); **^{31}P NMR** (DMSO-d_6) δ_P = 13.1 ppm; **MS** FAB>0: 596 $(M+H)^+$; FAB<0: 594 $(M-H)^-$.

***O, O'-Bis*(3'-azido-2',3'-dideoxythymidin-5'-yl) *N*-butylphosphoramidate (4b).**

To H-phosphonate **3** (0.2 mmol) in pyridine were butylamine (59.3 mL, 0.6 mmol), triethylamine (0.4 mL) and carbon tetrachloride (1.0 mL) added. The reaction mixture was after 10 min concentrated under vacuum and the residue was purified on a silica gel column using chloroform - 2-propanol (10:1, v/v) containing 0.5 % triethylamine as eluent to afford the title compound in 113 mg yield (83%). **1c** and **1d** was also prepared according to this procedure, using the same amounts of the corresponding amine (L-alanine ethyl ester hydrochloride or β-alanine ethyl ester hydrochloride, respectively).

^{1}H NMR (δ in ppm, $CDCl_3$) 9.55 (s, 2H, 2 x NH), 7.33 (d, 1H, H_a-6, J=1.1 Hz), 7.33 (d, 1H, H_b-6, J=1.1 Hz), 6.08 (t, 1H, H_a-1', J=6.4 Hz), 6.01 (t, 1H, H_b-1', J=6.4 Hz), 4.40 (m, 2H, 2 x H-3'), 4.26 (m, 4H, 2 x H-5' & H-5''), 4.04 (m, 2H, 2 x H-4'), 3.28 (m, 1H, NH), 2.92 (m, 2H, CH_2-1), 2.43 (m, 4H, 2 x H2' & H2''), 1.92 (s, 6H, 2 x C5-CH_3), 1.48 (m, 2H, CH_2-2), 1.33 (m, 2H, CH_2-3), 0.90 (t, 3H, CH_3-4). **^{31}P NMR** (pyridine) δ_P = 13.1 ppm **HRMS** Found $[M-H]^-$, 650.2214. $C_{24}H_{34}N_{11}O_9P$ requires M-H, 650.2200.

***O, O'-Bis*(3'-azido-2',3'-dideoxythymidin-5'-yl) *N*-(1-(*S*)-ethoxycarbonyl-ethyl)phosphoramidate (4c) :** Yield: 108 mg (78%).

^{1}H NMR (δ in ppm, $CDCl_3$) 9.07 (s, 2H, 2 x NH), 7.30 (d, 1H, H_a-6, J=1.1 Hz), 7.23 (d, 1H, H_b-6, J=1.1 Hz), 6.04 (m, 2H, 2 x H-1'), 4.10-4.45 (m, 8H, 2 x H-3', 2 x H-5' & H-5", $\underline{CH_2}$-CH_3, according to the order in the multiplet), 3.77-4.08 (m, 4H, 2 x H-4', $\underline{CH}$-CH_3, NH), 2.45 (m, 4H, 2 x H-2'), 1.92 (s, 6H, 2 x C5-CH_3), 1.41 (d, 3H, CH-$\underline{CH_3}$, J=6.6 Hz), 1.26 (t, 3H, CH_2-$\underline{CH_3}$, J=7.1 Hz). **^{31}P NMR** (pyridine) δ_P = 9.6 ppm. **HRMS** Found $[M-H]^-$, 694.2128. $C_{25}H_{34}N_{11}O_{11}P$ requires M-H, 694.2098.

***O, O'-Bis*(3'-azido-2',3'-dideoxythymidin-5'-yl) *N*-(2-ethoxycarbonyl-ethyl)phosphoramidate (4d) :** Yield: 124 mg (89%).

^{1}H NMR (δ in ppm, $CDCl_3$) 8.79 (s, 2H, 2 x NH), 7.30 (d, 1H, H_a-6, J=1.1 Hz), 7.24 (d, 1H, H_b-6, J=1.1 Hz), 6.03 (q, 2H, 2 x H-1', J=6.6 Hz), 4.39 (m, 2 x H-3'), 4.24 (m, 4H, 2 x H-5' & H-5"), 4.15 (q, 2H, $\underline{CH_2}$-CH_3, J=7.1 Hz), 4.02 (m, 2H, 2 x H-4'), 3.22 (m, 1H, NH), 3.21 (m, 2H, $CH_2\underline{CH_2}NH$), 2.47 (m, 6H, $\underline{CH_2}CH_2NH$, 2 x H-2' & H-2"), 1.93 (s, 6H, 2 x C5-CH_3), 1.27 (m, 3H, $CH_2\underline{CH_3}$). **^{31}P NMR** (pyridine) δ_P = 10.9 ppm. **HRMS** Found $[M-H]^-$, 694.2147. $C_{25}H_{34}N_{11}O_{11}P$ requires M-H, 694.2098.

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