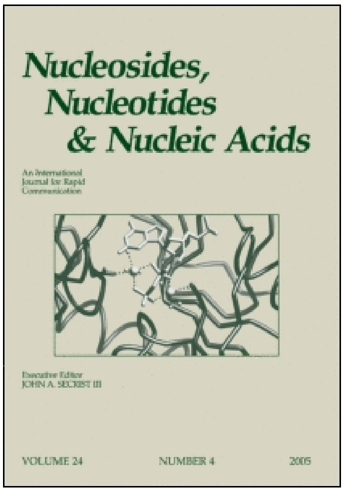

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SYNTHESIS AND BIOLOGICAL APPLICATIONS OF 2',3'-DIDEOXYNUCLEOSIDE-5'-O-(α-*THIO*)TRIPHOSPHATES

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ABSTRACT

Fast and efficient syntheses of 2',3'-dideoxynucleoside-5'-O-(α-*thio*)triphosphates (α-S-ddNTP's) starting from 2'-deoxynucleosides are described. They have been used in a modified DNA-sequencing method and were tested as potential inhibitors of HIV-1 reverse transcriptase. An X-ray structure of 3'-deoxythymidine is presented and compared to other known 2',3'-dideoxynucleoside structures.

1. Synthesis of 2',3'-dideoxynucleosides: 2',3'-Dideoxynucleoside precursors were synthesized by a 4-step procedure starting from 2'-deoxyadenosine, thymidine, 4-N-acetyl-2'-deoxycytidine or 2-N-isobutyryl-2'-deoxyguanosine. After direct selective 5'-benzoylation with a small excess of benzoyl chloride in pyridine[1] and work-up by silica gel flash chromatography (80-90% yield), 4-dimethylaminopyridine (DMAP; 8-10 equivalents) was used to catalyse the reaction of 1.2 equiv. of phenyl chlorothionocarbonate[2] (PTC-Cl) with the 3'-hydroxyl group in dry actetonitrile (18 hr., 30^{0}C, 60-80% yield). Smooth homolytic deoxygenation[3] of the alcoholic thionoester using 8-10 equiv. of tri-n-butyltin hydride and 0.2 equiv. of 2,2'-azobis(2-methyl-propanitrile) in refluxing toluene (2 hr.) followed by mild ammonolysis and fast medium pressure chromatography (MPLC) gave the desired 2',3'-dideoxynucleosides in almost quantitative yield.

Since the introduction of PTC-Cl for the regio and stereoselective conversion of ribonucleosides to 2'-deoxynucleosides by M.J.Robins et al.[2] in 1983, the homolytic hydrogenolysis of phenoxythiocarbonyl esters has become a standard method for the deoxygenation of secondary hydroxyl functionalities in nucleoside chemistry. Although phenoxythiocarbonyl chloride has been used previously to activate the 3'-position[4], our reaction sequence is, to the best of our knowledge, the only one in which PTC-Cl has been used directly in combination with base-labile protection of the 5'-hydroxyl group. In a series of analytical experiments, the amount of 5'-O-benzoyl-3'-phenoxythiocarbonyl-2'-deoxynucleoside was shown to correlate mainly with the amount of DMAP used for catalysis. Thus the 4 stage reaction sequence described here is a useful and interesting variation of Samukov and Ofitserov's[5] and Prisbe and Martin's[6] dideoxynucleoside synthesis.

2. Synthesis of 2',3',-dideoxynucleoside-5'-O-(α–*thio*)triphosphates: Starting from dideoxynucleoside precursors, a rapid one flask synthesis of the corresponding α–thiotriphosphates is possible[7,8]. Thiophosphoryl chloride (1.8 equiv.) in 2.5 ml/mmol triethylphosphate (2.6 ml/mmol of lutidine for basic catalysis), 60-120min at 0°, were used for the first reaction step, followed by addition of 5 equiv. of tri-n-butylamine- and tri-n-octylamine complexed pyrophosphate in dimethylformamide. After 1-2 min at 25° under vacuum, the reaction was stopped by addition of 5 ml/mMol 2M triethylammonium bicarbonate, pH 7.6. QAE-Sephadex ion-exchange purification led to the α–thiotriphosphates of the 2',3'-dideoxynucleosides in 15-20% yield over all 5 steps starting from the 2'-deoxynucleosides. The products were characterized by: a) enzymatic degradation to α–thiodiphosphates by myosin and to monothiophosphates by alkaline phosphatase monitored by HPLC; b) ^{31}P-NMR; c) demonstration of their DNA chain-terminating properties in DNA-polymerase reactions.

3. Crystal structure of 3'-deoxythymidine (ddT): Diffusion crystallization (diethyl ether/methanol) of 3'-deoxythymidine gave excellent crystals which were well suited for X-ray crystallography. Substances discovered to be potent selective inhibitors of HIV-1 replication in recent years are almost exclusively 2',3'-dideoxynucleosides. In order to recognize a common mode of action of this class of substances on their most probable target enzyme, the viral reverse transcriptase, details of their structures are required. Thus, it is of interest to compare the structure of 3'-deoxythymidine to the known structures of 3'-azido-3'-deoxythymidine (AZT)[9,10], 2',3'-dideoxycytidine (ddC)[11,12], 2',3'-dideoxyribavirine (ddR)[13] and 2',3'-dideoxyadenosine.[12]

ddT crystallized in the monoclinic space group $P2_1$ with unit cell dimensions a = 1.1045(2), b = 0.6016(1), c = 1.5925(3)nm, ß = 91.65(1)°. The crystal structure was solved by direct methods (MULTAN). All hydrogen atoms could be located in a difference Fourier map, with the exception of the hydroxyl H-O5' in one of the two molecules in the asymetric unit. Refinement was by full matrix least squares; R = 0.049.[14]

Like AZT[9,10], ddT contains two conformationally different molecules in the asymetric unit. The glycosidic torsion angle (χ_{CN} [C6-N1-C1'-O4']) of molecule A (49.6°) is, as in AZT, in the range seen for 2'-deoxynucleosides. The furanose pucker is C2'-endo (pseudorotation phase angle P = 165°). In molecule B, χ_{CN} is 7.3°. Thus, in contrast to most 2'-deoxynucleosides but like the one molecule in the asymetric unit of ddC and molecule B in AZT and ddR, molecule B of ddT adopts an unusually small glycosidic torsion angle leading to a longer than usual glycosidic bond (A: 0.1468 nm; B: 0.1501 nm). However, the low value of P for molecule B of ddT (10.8°, corresponding to a C3'-endo/C2'-exo ring pucker) stands in contrast to the extremely large values of P in AZT (213°) and ddC (208°) corresponding to C3'-exo/C4'-endo puckering. The small value of P for ddT is more similar to that seen for some purine nucleosides and nucleotides. It has been discussed in the literature that the decrease of the torsion angle in pyrimidine nucleosides is caused by the unusual C3'-exo/C4'-endo pucker of the furanose ring because of possible steric interaction between H5' and H6. The conformation seen in ddT makes this assumption questionable, since the ring pucker is between ^{3}E and C3'-endo/C2'-exo and steric hindrance between the heterocyclic base and the C5'-OH function should be less significant.

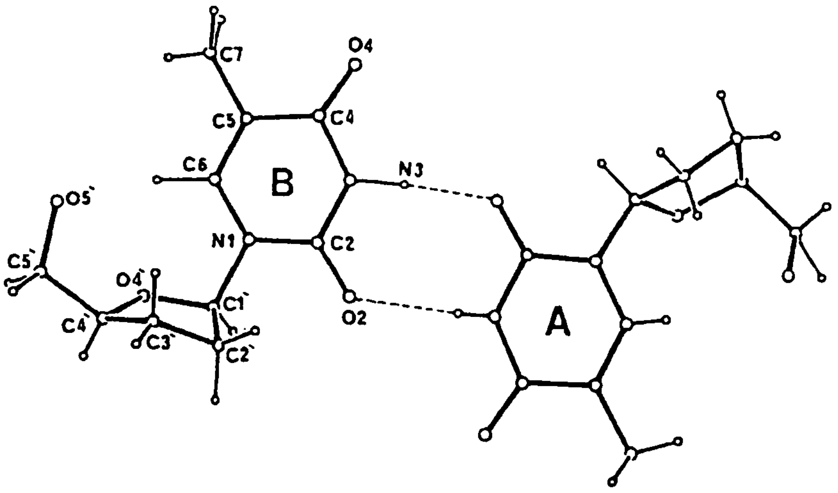

Fig. 1

Crystal structure of 2',3'-dideoxythymidine showing the two non-identical molecules in the asymetric unit.

Complete protection of MT4-lymphocytes against HIV-1 has been seen at concentrations of 0.02 μM (AZT), 0.2 μM (ddC) and 1 μM (ddT).[15,16] It may well be that the rare conformation found in AZT (B) and ddC fits very well into the "bound nucleotide state" of the catalytic cleft of one or more of the metabolically involved enzymes, making these two to the best known inhibitors of HIV-1 found so far. With a furanose pucker lying in the range of most ribo- and 2'-deoxyribonucleosides but with the unusually small χ, ddT lies between AZT/ddC and thymidine in structural deviation as well as in potency of inhibition of the virus.

4 α-S-ddNTP's and ddNTP's as inhibitors of HIV reverse transcriptase and of HIV replication: To test whether the α-thiophosphate group has an additional effect, α-S-ddTTP was investigated in a standard HIV-1 reverse transcriptase assay.[17] Determination of IC$_{50}$ at a substrate (dTTP) concentration of 20 μM gave values of 44 nM for ddTTP and 65 nM for α-S-ddTTP, indicating only a slight negative effect of the thiophosphate group on the inhibitory properties. The maximal amount of substrate which could be incorporated after allowing the polymerisation reactions to go to completion was reduced in the presence of ddTTP or α-S-ddTTP, which could be a result of chain termination. Classical Michaelis-Menten investigations indicated, not surprisingly, but in complete contrast to published data, that the inhibition was neither purely competitive nor purely non-competitive.

All four α-S-ddNTP's were tested against HIV-1 in cell culture and compared to the ddNTP's. Antiviral activity of the compounds was assessed by the inhibition of virus induced cytopathic effect in MT4 cells as described by Baba et al..[16] More than 99% pure (HPLC) triphosphates were used in concentrations up to 100 µM (200 µM for dTTP analogs). The ddNTP's gave IC_{50} values of ca. 200 µM (ddTTP), 75 µM (ddATP), 8 µM (ddGTP) and 4 µM (ddCTP) with little or no cytopathic effect with the exception of ddATP where there were no surviving cells at a concentration of 100 µM in a control assay. These effects may occur via dephosphorylation to the nucleoside. Since thiophosphate residues are stable to hydrolysis by phosphohydrolases, it is not surprising that no protective effect was seen up to 100 µM with the exception of α–S-ddCTP, which gave an IC_{50} of about that concentration.

5. DNA sequencing: The α–S-ddNTP's combine the chemical features of two classes of nucleotide analogs which have been used for DNA sequencing, namely the 2',3'-dideoxy structure used to induce DNA chain termination in the "dideoxy" method[18], and the thiophosphate residue used to introduce resistance to an exonuclease in newly synthesized DNA in the "thiophosphate" method.[19,20] This has been exploited in a new modification which appears to incorporate the advantages of both methods and avoid the main disadvantages (briefly, the production of wrong stops in the dideoxy method and weak bands in the thiophosphate method). α-S-ddNTP's are used in a standard Sanger sequencing protocoll, leading to fragments which have exclusively thiophosphate residues at their 3'ends. Since these are resistant to exonuclease III, wrong stops which arise as in the standard method can be removed by treatment with the nuclease without degrading fragments which have genuine base-specific stops. In practice, it was found better to perform the polymerization and degradation reactions simultaneously. This appears to lead to the establishment of a steady state, in which wrong stops are eliminated and only genuine stops survive. We have shown that with a protocoll using T7 polymerase and exonuclease III during the primer extension reaction, clones can be sequenced which were intractable to the Sanger method and even to the thiophosphate method.

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