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SYNTHESIS OF A DIFLUOROMETHYLENEPHOSPHONATE ANALOGUE OF AZT 5'-TRIPHOSPHATE AND ITS INHIBITION OF HIV-1 REVERSE TRANSCRIPTASE

D. Hebel, K. L. Kirk,* J. Kinjo, T. Kovács, K. Lesiak,
J. Balzarini,# E. De Clercq,# and P. F. Torrence*

Laboratories of Bioorganic Chemistry and Medicinal Chemistry,
National Institute of Diabetes and Digestive and Kidney Diseases,
National Institutes of Health, Bethesda MD 20892
and #Rega Institute for Medical Research, Minderbroedersstraat 10,
B-3000 Leuven, Belgium

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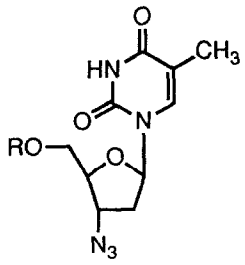
Abstract: Difluoromethylenebisphosphonic acid was prepared by acetyl hypofluorite-mediated fluorination of tetraisopropyl methylenebisphosphonate and ester hydrolysis. Coupling to 3'-azido-3'-deoxythymidine 5'-monophosphate gave the title compound. The difluoromethylene-phosphonate was 30-fold less effective than AZT-triphosphate as a competitive inhibitor of HIV-1 reverse transcriptase but 10-fold more effective than the methylenephosphonate analogue.

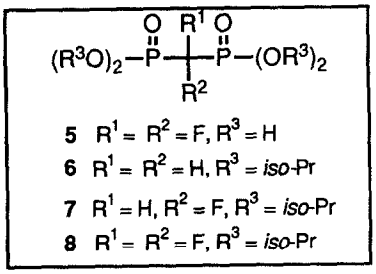
The replacement of oxygen in phosphate esters with CF_2 has been an effective strategy in the development of a number of non-hydrolyzable difluoromethylenephosphonate analogues of critical biomolecules. Examples include difluoromethylenephosphonate analogues of glycolytic intermediates, of isoprenoid pyrophosphates,[2] and of nucleoside phosphates.[3] In their pioneering work in the latter area, Blackburn et al.[3c] prepared fluorophosphonate analogues of several nucleotides, including β,γ-difluoromethylene-bridged analogues of ATP and GTP. Noteworthy in this work was the good substrate activity of the ATP-analogue with several ATP-utilizing enzymes, including DNA-dependent RNA polymerase, adenylate kinase, and (2'-5')-oligoadenylate synthetase.

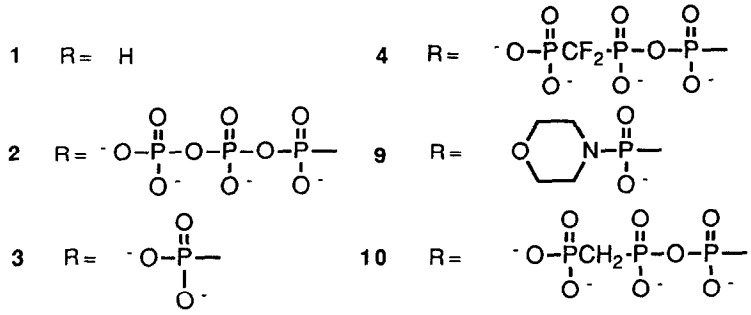
3'-Azido-3'-deoxythymidine (AZT, **1**), used extensively as an approach to the management of human immunodeficiency virus (HIV) infection, is converted in vivo to the 5'-triphosphate (AZTTP), the proximate inhibitor of HIV reverse transcriptase.[4] In the conversion of AZT to its triphosphate (**2**), the phosphorylation of AZT-5'-monophosphate (AZTMP, **3**) to AZT-diphosphate is thought to be the rate limiting step. Blackburn et al.[3c] had shown earlier that β,γ-difluoromethylene-bridged nucleoside triphosphate are often good mimics of parent nucleoside triphosphates. We report here the preparation of the β,γ-difluoromethylene-bridged analogue (β,γ-CF_2-AZTTP, **4**) of AZTTP. This compound, which has a stable β-γ linkage, obviates the need for enzymatic phosphorylation.

The synthesis of **4** was accomplished by the coupling of difluoromethane diphosphonic acid (**5**) to AZT 5'-monophosphate, activated as the morpholidate.[5] In this work, we have

developed a new synthesis of **5**, based on electrophilic fluorination of tetraisopropyl methylenebisphosphonate (**6**) sodium salt with acetyl hypofluorite (AcOF),[6] a conversion previously carried out with the more sensitive reagent, perchloryl fluoride.[7] Thus, **6** (1.72 g, 5 mmol) was converted to the sodium salt using NaH in 10 mL of dry THF at 0 °C for 30 min. This was added to 10 mmol of AcOF in $CFCl_3$ at -78 °C. After 5 min the solution was added to 400 ml of saturated sodium thiosulfate, the organic phase was separated, washed with sodium bicarbonate, dried and evaporated. Chromatography on silica gel (80% ethyl acetate in petroleum ether) gave 1.2 g of fluoromethylenebisphosphonate **7**: ms $(M + 1)^+$ 363; ^{19}F-NMR ($CDCl_3$) δ(ppm) -222[8] (dt, J_{FP} = 65 Hz, J_{FH} = 44Hz). Anion formation and fluorination of 1.2 g of **7** using identical conditions gave 500 mg (75% conversion, 52% yield) of crude tetraisopropyl difluoromethylene bisphosphonate (**8**) and 300 mg of **7**: ms $(M + 1)^+$ 383; ^{19}F NMR ($CDCl_3$) δ(ppm) -121.5[9] (t, J_{FP} = 85 Hz). Ester cleavage of **8** (250 mg, 0.66 mmol) was effected by addition of trimethylsilyl iodide in 5 mL of methylene chloride at -40 °C, followed by stirring for 2 h at room temperature. The solvent was then evaporated, water was added, and the aqueous solution was washed with ether. Evaporation of the aqueous solution and thorough drying gave 95 mg of **5** as a white solid (68 %). ^{19}F NMR (D_2O) δ(ppm) -122.7[8] (t, J_{FP} = 87 Hz; ^{31}P NMR[9] (D_2O) δ(ppm) 5.2 (broad multiplet).







AZT-5'-monophosphate (**3**) was prepared using a similar approach to that described previously.[10] Conversion to the corresponding 5'-phosphoromorpholidate was accomplished by suspension of 174 mg (Na salt, 445 μmol) of **3** in 10 mL of pyridine containing morpholine (250 μL, 1 mmol) and N-(dimethylaminopropyl)-N'-ethylcarbodiimide (DEC) hydrochloride (958.5 mg, 5 mmol). The mixture was stirred for 30 h at ambient temperature. After evaporation of the orange solution, an aqueous solution of the residue was applied to a preparative scale C_{18} column which was eluted with a stepwise gradient of 0%, 10%, 20%, and 30% methanol in water. The 10% fraction contained the desired AZT 5'-phosphoromorpholidate (**9**) (95 mg, 220 μmol, 49%) as the free acid. IR(KBr) $\nu_{MAX}cm^{-1}$: 2100 (N_3).

Reaction of **9** with the bis(tri-n-butylammonium) salt of **5** was carried out using earlier reported procedures.[10] The yield of triphosphate analogue was quantitative. β,γ-CF_2-AZTTP (**4**) was further purified on a Beckman C_{18} semipreparative (5 μ) column, using isocratic elution with 10% methanol containing triethylammonium acetate (0.1 M, pH 7.0) and a flow rate of 4.5 mL/min (Retention time of β,γ-CF_2-AZTTP: 22 min). The sample obtained was 99.9% pure as assayed by analytical HPLC. ^{1}H NMR (D_2O) δ(ppm): 7.74 (s, 1H, H-6); 6.27 (t, 1H, H-1'); 4.57 (m, 1H, H-3'); 4.21 (m, 3H, H-4',5',5''), 2.47 (q, 2H, H-2,2''); 1.91 (s, 3H, 5-CH_3): ^{31}P NMR (D_2O) δ(ppm): 3.1 (m, 1, βP); 1.9 (m, J_{POP} = 29.7 Hz), J_{PF} = 88.2 Hz), J_{PCP} = 59.6 Hz, γP); -11.28 (d, J = 29.7 Hz, αP). ^{19}F NMR[11] δ(ppm)(D_2O); -43.10 (t, J = 84) Hz, CF_2)

β,γ-CF_2-AZTTP (**4**) was evaluated for its inhibitory effect on the recombinant reverse transcriptase (p66) (RT) of HIV-1 [generously supplied by P. J. Barr (Chiron)] and compared with AZTTP and β,γ-CH_2AZTPP (**10**) that had been the subjects of earlier studies.[12] The reaction mixture (50 μL) contained 50 mM of Tris-HCl (pH 7.8), 5 mM of dithiothreitol, 500 mM of EDTA, 150 mM of KCl, 5 mM of $MgCl_2$, 1.25 μg of bovine serum albumin, exogenous poly(rA)-oligo$(dT)_{12-18}$, 2 μCi of [^{3}H]dTTP (specific activity 30 Ci/mmol), 0.03% Triton X-100, 10 μL of varying concentrations of β,γ-CF_2-AZTTP, AZTTP, or β,γ-CH_2-AZTTP, and 1 μL of the RT preparation. The reaction mixtures were incubated at 37 °C for 20 min, at which time the reaction was stopped and acid-insoluble material was analyzed for radioactivity. The initial concentration of radiolabelled dTTP in the reaction mixture was 1 μM.

AZTTP was strongly inhibitory to HIV-RT. The 50% inhibitory concentration (IC_{50}) was 0.022 μM which corresponds to the earlier reported value when tested against HIV-1 peptide-derived RT obtained from HIV-1-infected H9 cell cultures.[12] β,γ-CF_2-AZTTP and β,γ-CH_2-AZTTP proved to be less inhibitory to HIV-1 RT, having IC_{50} values that were 30-fold and 300-fold higher, respectively (IC_{50} = 0.62 and 6.95 μM, respectively). In the HIV-1 RT assays in which the [^{3}H]dTTP concentration was varied (ie. 40, 20, 10, 6, and 4 μM) and the inhibitor concentration (β,γ-CF_2-AZTTP) was kept constant at 0, 2, and 5 μM, a K_i of 2.23 μM

was determined (K_i/K_m = 0.47). Lineweaver-Burk plots revealed competitive inhibition of β,γ-CF_2-AZTTP with respect to dTTP as the natural substrate.

These results show that the CH_2 and CF_2-phosphonate derivatives of AZT have decreased affinities for HIV-1 RT as compared to AZTTP. However, β,γ-CF_2-AZTTP had much greater anti-HIV-RT activity than did β,γ-CH_2-AZTTP, being only 30-fold less effective than AZTTP. Thus, as shown in previous work, the CF_2 moiety appears to mimic O more effectively than does CH_2. The diminished interaction of β,γ-AZTTP suggests that the ß,γ-phosphoanhydride oxygen plays, directly or indirectly, a significant role in binding of AZTTP to HIV-1 reverse transcriptase. Based on these results, continued synthetic efforts towards β,γ- (and α,β) difluorophosphonate analogues of other anti-HIV-RT nucleotides, such as 2'3'-dideoxythymidine (DDT), 2',3'-dideoxycytidine (DDC) and 2'3'-dideoxyadenosine (DDA), may be warranted.

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