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SYNTHESIS AND HIV-1 REVERSE TRANSCRIPTASE INHIBITION PROPERTIES OF TWO NEW METHYLENEPHOSPHONATE ANALOGUES OF 3'-AZIDO-3'-DEOXYTHYMIDINE-5'-TRIPHOSPHATE

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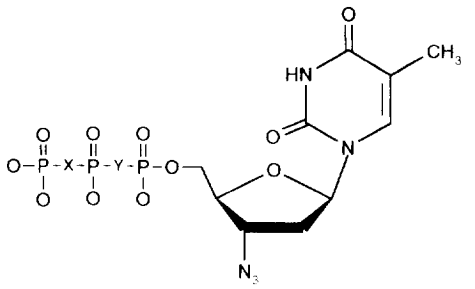
Abstract : Two new 5'-phosphorylated derivatives of 3'-azido-3'-deoxythymidine (AZT), namely α,β;β,γ-bis (methylene) AZT-5'-triphosphate **1** and α,β-propylene AZT 5'-diphosphate **2**, were synthesized. When evaluated for their inhibitory effects on human immunodeficiency virus (HIV) reverse transcriptase, these compounds were about 1000-fold less active than AZT-5'-triphosphate (AZTTP) as competitive inhibitors of this enzyme.

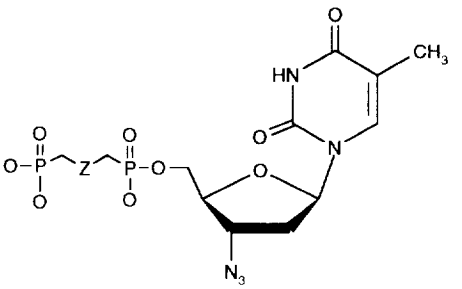
AZT, which was first synthesized in 1964 as a potential anti-cancer agent,[1] has been shown, twenty years later, to be a potent *in vitro* inhibitor of HIV, [2,3] the etiologic agent of the acquired immunodeficiency syndrome (AIDS). Despite some associated haematological toxicity, this compound was quickly approved by the Food and Drug Administration for the treatment of HIV infection, and to date remains the recommended initial therapy for the treatment of AIDS patients. As with other 2', 3'-dideoxynucleosides possessing antiretroviral activity, AZT has no intrinsic activity. After entering mammalian cells by passive diffusion, AZT is first phosphorylated by thymidine kinase, then transformed to its diphosphate form by thymidylate kinase, and finally further phosphorylated by a diphosphate kinase to yield AZTTP [4] This 5'-triphosphate derivative has selective affinity for HIV reverse transcriptase, acting as a competitive inhibitor and is preferentially incorporated into the viral DNA transcript in place of thymidine. This incorporation leads to premature termination of elongating viral DNA, since the lack of a 3'-hydroxyl group prevents the formation of a 3', 5'-phosphodiester linkage with an incoming 2'-deoxynucleoside-5'-triphosphate.[5]

Although AZTTP represents the main active metabolite of the administered drug, its utility as a chemotherapeutic agent is limited. Firstly it is charged at physiological pH, and as with all anionic nucleotides it cannot penetrate the cell [6,7] Secondly, it is susceptible to rapid degradation to AZT due to the chemical instability of the phosphoric acid anhydride bonds and due to the action of nonspecific phosphohydrolases.[8,9] To circumvent this latter problem, efforts have already been made to substitute the bridging oxygens in the polyphosphate chain of AZTTP by stable groups [10-12] The previous work in this area has been mainly involved with the replacement of either the α,β or the β,γ bridging oxygen by CH_2,[10] CF_2[11] and NH[12] (Fig. 1). We report here the synthesis and enzymatic evaluation of the hitherto unknown α,β,β,γ-bis(methylene) AZTTP **1** and α,β-propylene AZTDP **2** (Fig. 1). We reasoned that if all the bridging oxygen atoms were replaced with such methylene and propylene groups, the modified analogues would : i) be very stable, ii) still be recognized by

HIV reverse transcriptase, iii) act as potent and typical competitive inhibitors since they could not be incorporated as chain terminators into the proviral DNA owing to their non-hydrolyzable α,β junctions. If the compounds fulfill all these three requirements, further masking their charges by bioreversible phosphate protecting functions, as we have already and successfully implemented in the case of AZT-5'-monophosphate[13] and other anti-HIV dideoxynucleotides,[14-17] could lead to new kinds of prodrugs which eliminate the need of rate-limiting di- and triphosphorylation by cellular nucleotide kinases.



X = O, Y = O (AZTTP)
X = O, Y = CH_2 (ref. 10)
X = CH_2, Y = O (ref. 10)
X = CF_2, Y = O (ref. 11)
X = NH, Y = O (ref. 12)
X = O, Y = NH (ref. 12)



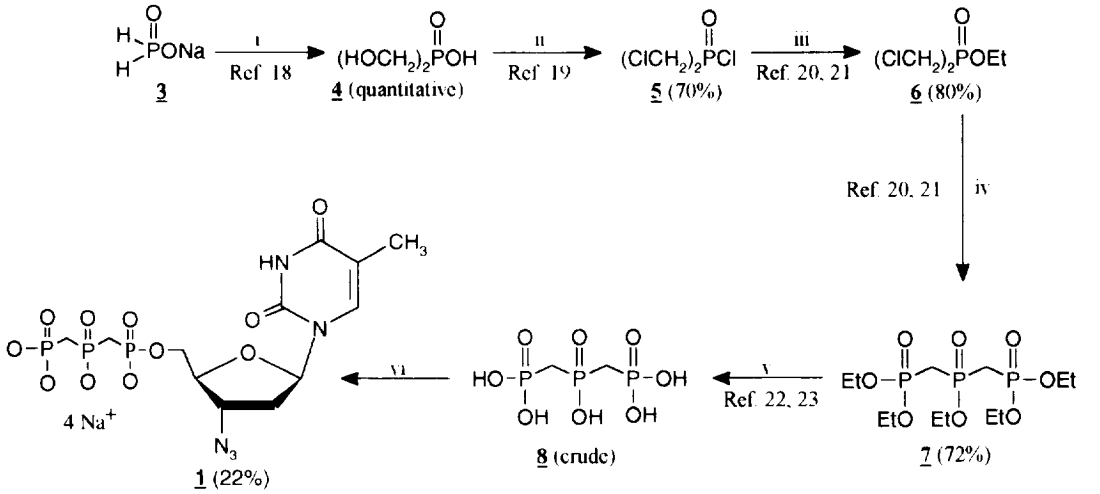
Z = $P(O)O^-$, **1** (this work)

Z = CH_2, **2** (this work)

Figure 1. Structural formulae of AZTTP and its analogues modified in the polyphosphate chain.

Synthesis of α,β;β,γ-bis (methylene) AZTTP 1 (Scheme 1)

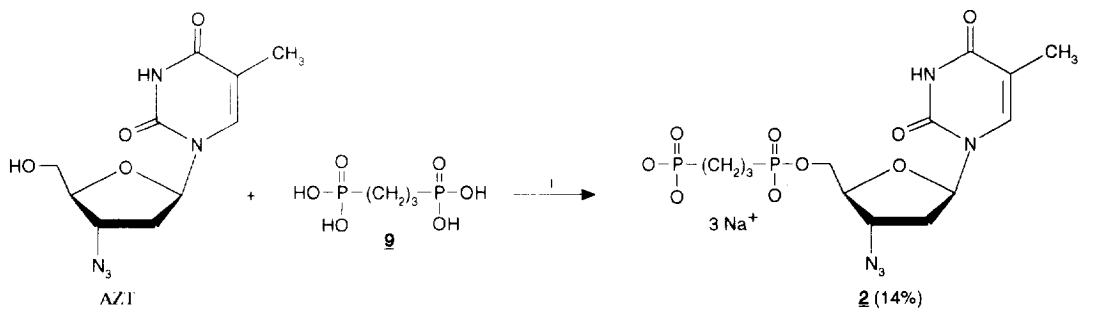
The synthesis of **1** was accomplished by coupling bis(dihydroxyphosphinylmethyl)phosphinic acid **8** with 3'-azido-5'-*O*-(*p*-tolylsulfonyl)-3'-deoxythymidine (5'-*O*-tosylAZT). The reagent **8** was prepared from sodium hypophosphite by a multistep reaction sequence, using in each step the best approach previously described in the literature. Reaction of **8** with 5'-*O*-tosylAZT was carried out in dimethylformamide (DMF) in the presence of tri-*n*-butylamine following a similar procedure to that implemented by J.A. Stock in the case of thymidine.[25] The solvent was evaporated under vacuum, the residue was dissolved in a small amount of water and applied to a DEAE-Sephadex A-25 column which was subsequently eluted with a linear triethylammonium bicarbonate (TEAB) buffer (pH 8.5) gradient (0 to 0.5 M). The appropriate fractions were collected and further purified by HPLC on a Hypersil BDS C_{18} (150×4.6 mm, 3 μm) column, using isocratic elution with 6% acetonitrile containing triethylammonium acetate (0.05 M, pH 6.9) and a flow rate of 1 mL/mn (retention time : 11.5 mn). Cation exchange (Dowex 50W X 2 column, Na^+ form) and subsequent lyophilization from water gave the colorless tetrasodium salt **1** of α,β,β,γ-bis(methylene) AZTTP in 22% yield. Evidence for the given structure and purity of **1** was based on analysis of its HPLC, UV, ^1H NMR, ^{31}P NMR and mass spectra.[26]



Scheme 1. *Reagents and conditions* i) $(CH_2O)_n$/aqueous HCl; ii) $SOCl_2$; iii) EtOH, Et_3N/diethylether; iv) $(EtO)_3P$, v) aqueous 48% HBr; vi) 5'-*O*-tosylAZT,[24] nBu_3N/DMF, 120 °C, 12 h, then DEAE-Sephadex A-25 (HCO_3^- form) column chromatography using a linear gradient of aqueous TEAB as eluent, then conversion to the Na^+ form by passage through an ion exchange Dowex 50W X 2 column.

Synthesis of α,β-propylene AZTDP 2 (Scheme 2)

Following a similar procedure previously reported for the preparation of adenosine-5'-methylenediphophonate,[27] the synthesis of **2** was accomplished by the reaction of commercially available propylenediphosphonic acid **9** (Alpha, Johnson Matthey GmbH, ref. 10387) with AZT in the presence of dicyclohexylcarbodiimide (DCC) and excess tri-*n*-butylamine in anhydrous pyridine solution.



Scheme 2. *Reagents and conditions* (n-Bu)$_3$N, DCC/pyridine reflux, then repetitive DEAE-Sephadex A-25 (HCO_3^- form) column chromatography, then cation exchange (Dowex 50W X 2, Na^+ form) column chromatography.

The reaction was carried out under reflux for 16 h. After the removal of *N,N'*-dicyclohexylurea and pyridine, the residue was purified 3 times by column chromatography on DEAE-Sephadex A-25 using as eluent a linear TEAB buffer (pH 8.5) gradient of 0-0.35 M. Cation exchange (Dowex 50W X 2 column, Na^+ form) and subsequent lyophilization from water gave the pure colorless trisodium salt **2** of α,β-propylene AZTDP in 14% yield.[28]

Inhibition studies of HIV reverse transcriptase

The analogues **1** and **2** of AZTTP were evaluated for their inhibitory effect on the heterodimer (p66/p51) reverse transcriptase[29] of HIV-1 and were compared with AZTTP. The reaction mixtures (100 µl) contained 50 mM Tris-HCl (pH 8.0), 1mM dithiothreitol, 80 mM KCl, 8 mM $MgCl_2$, poly(rA)-oligo(dT)$_{14}$ (A/T base ratio 5/1, primer concentration 1µM), 20 µM [^{3}H]dTTP (specific activity 0.25 Ci/mmol), 4 nM reverse transcriptase, and varying concentrations of **1** or **2**. The reaction mixtures were incubated at 37°C for 10 min, at which time the reaction was stopped by adding 5% trichloroacetic acid. Acid insoluble materials were collected on nitrocellulose filters and the radioactivity retained on the filters was measured by scintillation counting.

In this assay, AZTTP was a strong inhibitor of HIV-1 reverse transcriptase, with a 50% inhibitory concentration (IC_{50}) of 0.043 µM which was in the range of previously reported values.[10,11] On the contrary, the new analogues **1** and **2** of AZTTP proved to be much less inhibitory to this viral enzyme, having IC_{50} values that were about 1000-fold higher (IC_{50} = 48 and 39 µM, respectively). These results can be related to those previously obtained with other analogues of AZTTP (Fig 1) in which either the α,β or the β,γ bridging oxygen was replaced by CH_2,[10] CF_2,[11] and NH,[12] and which also demonstrated a markedly decreased affinity for HIV-1 reverse transcriptase as compared to AZTTP. Among the several hypotheses that could explain why such modified polyphosphate chain analogues show little affinity for HIV-1 reverse transcriptase, the significant difference in the P-O-P (128.7°) and P-CH_2-P (117°) bond angle,[30,31] as well as the difference in the dissociation constant (pK_a) of the phosphate acid function,[31] can be proposed. Furthermore, assuming that these analogues bind to reverse transcriptase at the natural substrate (dNTP) site, other explanations for their loss of affinity for the enzyme may be found in the consideration of a recently proposed hypothetical mechanism for the polymerase reaction of reverse transcriptase.[32] According to this model, binding and reaction of dNTP could be controlled by magnesium ions interacting with the triphosphate group and aspartic acid residues 185, 186 and 100 of the reverse transcriptase α-aminoacid sequence.[33] Therefore, replacement of phosphoryl groups or internal oxygen atoms by methylene groups should decrease the affinity of the analogues for reverse transcriptase compared to natural dNTP. Finally, from the present work and previously reported results,[10-12] it can be concluted that the unmodified 5'-triphosphate chain should be a conserved feature in the structure of any nucleotide for potent inhibiton of reverse transcriptase

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References and Notes

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1. Horwitz, J.P., Chua, J., Noel, M. *J. Org. Chem.* **1964**, *29*, 2076
2. Mitsuya, H., Weinhold, K.J., Furman, P.A., St. Clair, M.H., Lehrman, S.N., Gallo, R.C., Bolognesi, D., Barry, D.W., Broder, S. *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 7096.
3. Mitsuya, H., Broder, S. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 1911.
4. Parker, W.B., White, E.L., Shaddix, S.C., Ross, L.J., Buckheit, R.W., Germany, J.M., Secrist, J.A., Vince, R., Shannon, W.M. *J. Biol. Chem.* **1991**, *266*, 1754
5. For a recent review about AZT and its metabolites, see Gosselin, G., Imbach, J.-L., Sommadossi, J.-P. *Bull. Inst. Pasteur* **1994**, *92*, 181
6. Liebman, K.C., Heidelberger, C. *J. Biol. Chem.* **1955**, *216*, 823
7. Roll, P.M., Weinfeld, H., Carroll, E., Brown, G.B. *J. Biol. Chem.* **1956**, *220*, 439.
8. Hampton, A., Hampton, E.G., Eidinoff, M.L. *Biochem. Pharmacol.* **1962**, *11*, 155.
9. Holmsen, H., Rosenberg, M.C. *Biochim. Biophys. Acta* **1968**, *155*, 326.
10. Balzarini, J., Herdewijn, P., Pauwels, R., Broder, S., De Clercq, E. *Biochem. Pharmacol.* **1988**, *37*, 2395.
11. Hebel, D., Kirk, K.L., Kinjo, J., Kovacs, T., Lesiak, K., Balzarini, J., De Clercq, E., Torrence, P.F. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 357
12. Ma, Q.-F., Bathurst, I.C., Barr, P.J., Kenyon, G.L. *J. Med. Chem.* **1992**, *35*, 1938.
13. Puech, F., Gosselin, G., Lefebvre, I., Pompon, A., Aubertin, A.-M., Kirn, A., Imbach, J.-L. *Antiviral Res.* **1993**, *22*, 155
14. Gosselin, G., Imbach, J.-L. *International Antiviral News*, **1993**, *1*, 100.
15. Perigaud, C., Gosselin, G., Lefebvre, I., Girardet, J.-L., Benzaria, S., Barber, I., Imbach, J.-L. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 2521
16. Perigaud, C., Aubertin, A.-M., Benzaria, S., Pelicano, H., Girardet, J.-L., Maury, G., Gosselin, G., Kirn, A., Imbach, J.-L. *Biochem. Pharmacol.* **1994**, *48*, 11
17. Benzaria, S., Girardet, J.-L., Perigaud, C., Aubertin, A.-M., Pelicano, H., Maury, G., Gosselin, G., Kirn, A., Imbach, J.-L. *Nucleic Acids Symposium Series*, **1994**, *31*, 129.

18. Ivanov, B.E.; Panteleeva, A.R.; Shagidullin, R.R.; Shermergorn, I.M. *Zhur. Obs. Khim.* **1967**, *37*, 1856. [Chem. Abstr. **1968**, *68*, 29797e].

19. Frank, A.W.; Gordon, I. *Can. J. Chem.* **1966**, *44*, 2593.

20. Maier, L. *Angew. Chem. Int. Ed. Engl.* **1968**, *7*, 384.

21. Maier, L. *Helv. Chim. Acta* **1969**, *52*, 827.

22. Trowbridge, D.B.; Kenyon, G.L. *J. Am. Chem. Soc.* **1970**, *92*, 2181.

23. Trowbridge, D.B.; Yamamoto, D.M.; Kenyon, G.L. *J. Am. Chem. Soc.* **1972**, *94*, 3816.

24. Lin, T.S.; Shen, Z.-Y.; August, E.M.; Brankovan, V.; Yang, H.; Ghazzouli, I.; Prusoff, W. *J. Med. Chem.* **1989**, *32*, 1891.

25. Stock, J.A. *J. Org. Chem.* **1979**, *44*, 3997.

26. Selected physicochemical data for **1** : U.V. (HPLC buffer) : λ_{max} = 267 nm; ^1H NMR (D_2O) δ ppm : 1.99 (s, 3H, CH_3), 2.31 and 2.36 (2 t, 2H each, 2 P(CH_2P; $J_{H,P}$ 18 Hz each), 2.5-2.6 (m, 2H, $H_{2'}$ and $H_{2''}$), 4.1-4.2 (m, 2H, $H_{5'}$ and $H_{5''}$), 4.3 (brs, 1H, $H_{4'}$), 4.6-4.7 (m, 1H, $H_{3'}$), 6.33 (t, 1H, $H_{1'}$; $J_{H,H}$ 6.9 Hz), 7.82 (s, 1H, H_6); ^{31}P NMR (D_2O) δ ppm : 16.5 (d, P_γ; $J_{P,P}$ 8.4 Hz), 18.7 (d, P_α; $J_{P,P}$ 8.4 Hz), 28.3 (t, P_β; $J_{P,P}$ 8.4 Hz); mass spectrum (FAB < 0, matrix = glycerol-thioglycerol, 1:1, v/v) : 568 $(M - Na)^-$, 546 $(M - 2Na + H)^-$, 524 $(M - 3Na + 2H)^-$, 125 $(B)^-$.

27. Myers, T.C.; Nakamura, K.; Danielzadeh, A.B. *J. Org. Chem.* **1965**, *30*, 1517.

28. Selected physicochemical data for **2** : U.V. (H_2O) : λ_{max} = 267 nm (ε = 9500), ^1H NMR (D_2O) δ ppm : 1.6-1.9 (m, 6H, 3 CH_2), 1.98 (s, 3H, CH_3), 2.55 (t, 2H, $H_{2'}$ and $H_{2''}$; $J_{H,H}$ 6.2 H_z), 4.10-4.20 (m, 2H, $H_{5'}$ and $H_{5''}$), 4.25-4.30 (m, 1H, $H_{4'}$), 4.56 (dd, 1H, $H_{3'}$), 6.31 (t, 1H, $H_{1'}$; $J_{H,H}$ 6.6 H_z), 7.78 (s, 1H, H_6); ^{31}P NMR (D_2O) δ ppm : 25.5 (s, P_β), 28.6 (s, P_α); mass spectrum (FAB < 0, matrix = glycerol-thioglycerol, 1:1, v/v) : 496 $(M - Na)^-$, 474 $(M - 2Na + H)^-$, 452 $(M - 3Na + 2H)^-$, 125 $(B)^-$.

29. Buller, B.; Restle, T.; Weiss, S.; Gautel, M.; Sczakiel, G.; Goody, R.S. *J. Biol. Chem.* **1989**, *264*, 13975.

30. Scheit, K.H. In *Nucleotide Analogs. Synthesis and Biological Function, John Wiley ed.*, New York, **1980**; pp 96-141.

31. Young, R.G.; Babcock, D.; Ballantyne, W.; Ojala, D. Biochemistry, **1971**, *10*, 2484.

32. Steiz, T.A. *Current Opinion in Structural Biology*, **1993**, *3*, 31.

33. Tantillo, C.; Ding, J.; Jacobo-Molina, A.; Nanni, R.G.; Boyer, P.L.; Hughes, S.H.; Pauwels, R.; Andries, K.; Janssen, P.A.J.; Arnold, E. *J. Mol. Biol.* **1994**, *243*, 369.

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