Pergamon

Tetrahedron Letters 40 (1999) 3391–3394

TETRAHEDRON LETTERS

Synthesis of a non-Hydrolyzable AZT-Triphosphate Analogue Designed for the Production of Anti-AZT-TP Antibodies

Thierry Brossette[a], Alain Valleix[b], Laure Goujon[c], Christophe Créminon[c], Jacques Grassi[c], Charles Mioskowski[ab]* and Luc Lebeau[a]*

[a]Université Louis Pasteur de Strasbourg - Faculté de Pharmacie
Laboratoire de Synthèse Bioorganique associé au CNRS - 74, route du Rhin - BP 24 - 67 401 Illkirch - France.
[b]CEA - Service des Molécules Marquées - Département de Biologie Moléculaire et Cellulaire
Bât. 547 - CEA Saclay - 91 191 Gif sur Yvette - France.
[c]CEA - Service de Pharmacologie et d'Immunologie - Département de Recherche Médicale
Bât. 136 - CEA Saclay - 91 191 Gif sur Yvette - France.

Received 29 October 1998; accepted 3 March 1999

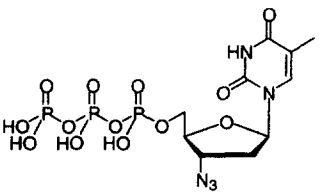
Abstract: An enzymatically stable analogue of AZT-triphosphate is prepared in 7 steps starting from thymidine. The thymine moiety is functionalized at N^3 to allow the further cross-linking to a carrier protein for subsequent immunization of rabbits in order to raise specific antibodies directed against AZT-TP.

AZT was the first dideoxynucleoside (ddN) to demonstrate useful antiviral activity in the treatment and prevention of AIDS. At present, five ddNs are approved by the US Food and Drug Administration and are currently used for treating HIV-infected patients (AZT, ddI, ddC, d4T and 3TC). These compounds have no intrinsic activity against the virus. They penetrate cells through a passive process[1], are metabolized into their respective 5'-triphosphate derivatives by intracellular kinases and nucleotidases, and can then inhibit competitively the reverse transcriptase of HIV and work as chain terminators (because they all lack a 3'-hydroxyl group). The rate of formation of dideoxynucleotides (ddNPs) being under the control of the intracellular metabolism[2-6], it is subject to large interindividual and longitudinal variations. Thus, it is not surprising that many studies indicate an absence of correlation between ddNs circulating levels and the efficacy of treatment[7-9]. When used in monotherapies, ddNs gave rather disappointing results due to the rapid appearance of resistance processes, which are mainly attributed to the selection of drug-resistant reverse transcriptase variants linked to the hypervariability of the virus. The recent promising results obtained in the framework of polytherapies[10,11] revived interest in this category of drugs and new classes of ddNs are now under preclinical evaluation[12,13]. This reinforces the necessity of studying precisely the intracellular metabolism of ddNs since metabolic interference is likely to occur. Clearly, the measurement of intracellular levels of ddNPs would allow optimization of ddN-based treatments both in terms of efficacy and toxicity. So far, the tools developed for the measuring of intracellular ddNPs[2,14-24] cannot be applied in clinical studies or involve indirect methods that are not ideally suited to routine monitoring.

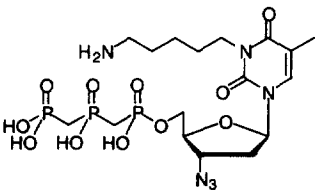
In order to develop a competitive enzyme immunoassay (EIA)[25] suitable for routine monitoring of intracellular levels of AZT 5'-triphosphate, we synthesized compound **1**. The EIA method was selected for its sensitivity and as it does not require any HPLC purification step[26-29] and/or radiolabeled compounds manipulation[30,31]. The assay is based on the use of rabbit polyclonal antibodies raised against the substance to monitor or an analogue and of a hapten/acetylcholinesterase conjugate as tracer[32].

* Fax: 33 (0)3 88 67 88 91. E-mail: mioskowski@aspirine.u-strasbg.fr / lebeau@aspirine.u-strasbg.fr

0040-4039/99/$ - see front matter
PII: S0040-4039(99)00497-9

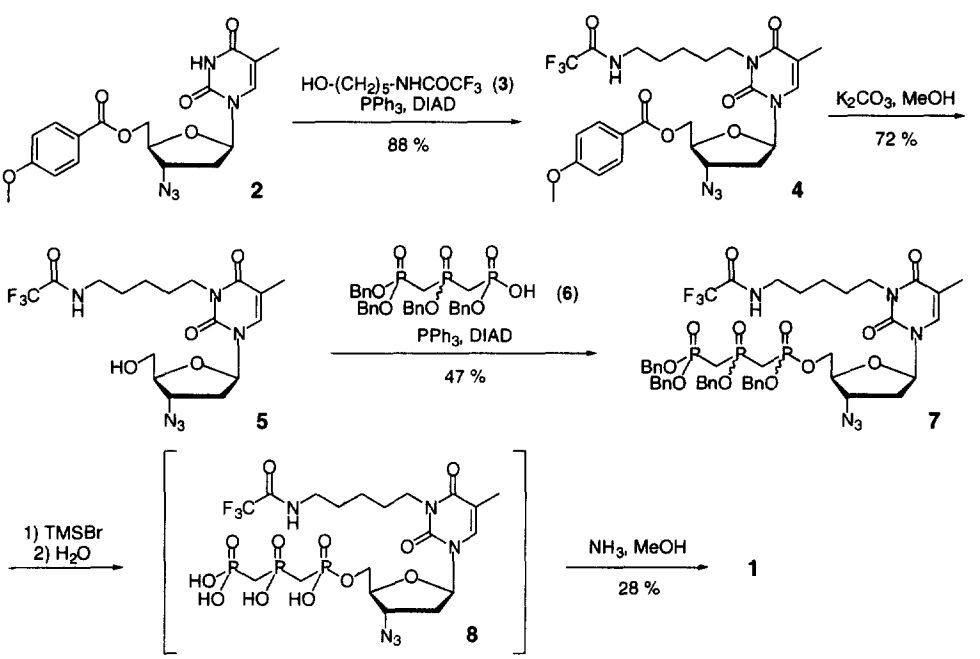


AZT-TP



1

The production of antibodies directed against polyphosphate nucleosides is delicate to achieve as the pyrophosphate bridges in the hapten are rapidly hydrolyzed by enzymes in the recipient animals[33]. The methylene-bis-phosphonate group has been widely used to mimic pyrophosphates in nucleotides[34,35]. The resulting nucleotide analogues are still recognized by many enzymes but are no longer substrates. Thus, the transposition of that structural modification to AZT-TP should yield a hapten highly resistant to hydrolytic enzymes. In order to elicit an immune response in the injected animal, the hapten has to be coupled to an antigenic carrier protein (KLH, BSA...). As the targeted antibodies are required to specifically recognize AZT-TP in the pool of intracellular nucleosides and nucleotides (including AZT, AZT-MP, AZT-DP and T-TP), the coupling to the carrier protein was planned through the base, so as to respect as much as possible the structural integrity of the polyphosphate mimicry and sugar moieties in the molecule. The linker arm is functionalized at the ω position with a primary amine to allow a further cross-coupling reaction with the carrier protein. The synthesis of hapten **1** has been realized according to the following scheme:



Compound **2** was prepared in two steps from thymidine according to a previously described procedure[36]. The masked precursor of the linker arm was regioselectively introduced at N^3 *via* a Mitsunobu reaction with the *N*-protected aminopentanol derivative **3**. The resulting compound **4** (Table 1, entry 1) was treated with potassium carbonate in methanol to restore the free 5'-hydroxyl group in **5** (Table 1, entry 2). The latter compound was coupled to the monofunctional triphosphate analogue building block **6**[37-39]. The coupling was realized using a Mitsunobu reaction which revealed to be very efficient for the esterification of monoesters of phosphonic acids[40,41]. Compound **7** (Table 1, entry 3) was obtained as a mixture of 4 diastereomers and could be adequately purified by chromatography over silica gel. The four benzyl protective groups were then removed by successive treatment of **7** with trimethylsilyl bromide and water[42,43] to yield compound **8**. This very polar compound was not purified and the crude reaction mixture was concentrated *in vacuo*. The removal of the trifluoroacetamide protective group at the linker arm was achieved in anhydrous methanolic ammonia according to a standard procedure[44]. The target compound **1** was purified to homogeneity by reverse-phase HPLC (Table 1, entry 4)[45].

Entry	Compound	Analyses
1	**4**	^{1}H-NMR (200 MHz, $CDCl_3$) 7.40 (AB syst., J = 8.8 Hz, 2H); 7.33 (m, 1H); 7.17 (s, 1H); 6.14 (t, J = 4.7 Hz, 1H); 4.53 (AB part of ABX syst., J_{AB} = 12.4 Hz, J_{AX} = 3.3 Hz, J_{BX} = 3.7 Hz, $\Delta\nu$ = 15.0 Hz, 1H); 4.32 (dt, J = 7.3, 4.7 Hz, 1H); 4.15 (dt, J = 4.7, 3.7 Hz, 1H); 3.84 (t, J = 6.9, 2H); 3.80 (s, 3H); 3.27 (dt, J = 6.6, 5.8 Hz, 1H); 2.49-2.28 (m, 2H); 1.63-1.50 (m, 7H); 1.36-1.25 (m, 2H). ^{13}C-NMR (50 MHz, $CDCl_3$) 167.6; 163.7; 163.1; 157.1 (q, J = 36.3 Hz); 150.4; 133.0; 131.4; 121.1; 115.7 (q, J = 285.9 Hz); 113.7; 110.2; 85.6; 81.8; 63.1; 60.3; 55.3; 40.5; 39.6; 37.6; 27.8; 26.7; 23.6; 12.7. MS (Cl/NH_3): 583 $[M+H]^+$.
2	**5**	^{1}H-NMR (200 MHz, $CDCl_3$) 7.49 (s, 1H); 7.20 (m, 1H); 6.08 (t, J = 6.6 Hz, 1H); 4.43-4.34 (m, 1H); 3.98-3.70 (m, 5H); 3.37-3.28 (m, 3H); 2.57-2.25 (m, 2H); 1.94 (s, 3H); 1.71-1.56 (m, 4H); 1.41-1.23 (m, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$) 163.5; 157.4 (q, J = 36.3 Hz); 150.8; 134.9; 115.8 (q, J = 287.7 Hz); 110.2; 86.9; 84.5; 61.8; 59.9; 40.6; 39.8; 37.4; 27.9; 26.9; 23.7; 13.1. MS (Cl/NH_3): 449 $[M+H]^+$; 466 $[M+NH_4]^+$.
3	**7** (mixture of 4 diastereomers)	^{1}H-NMR (300 MHz, $CDCl_3$) 7.40-7.28 (m, 21H); 6.90 (s, 1H); 6.20-6.08 (m, 1H); 5.20-4.91 (m, 8H); 4.36-4.14 (m, 4H); 3.93 (t, J = 7.0 Hz, 2H); 3.34 (dt, J = 6.4, 6.2 Hz, 2H); 3.15-2.67 (m, 4H); 2.45-2.15 (m, 2H); 1.94-1.84 (m, 3H); 1.68-1.61 (m, 4H); 1.45-1.30 (m, 2H).
4	**1**	^{1}H-NMR (200 MHz, D_2O) 7.66 (m, 1H); 6.20 (t, J = 6.1 Hz, 1H); 4.46 (m, 1H); 4.15-4.07 (m, 3H); 3.87 (t, J = 7.0 Hz, 2H); 2.92 (t, J = 7.0 Hz, 1H); 2.45 (m, 2H); 2.35-2.15 (m, 4H); 1.89 (s, 3H); 1.70-1.53 (m, 4H); 1.38-1.28 (m, 2H). ^{31}P-NMR (121 MHz, D_2O) 31.17 (m); 19.74 (m); 15.40 (m). HRMS: calcd for $C_{17}H_{31}N_6O_{11}P_3Na$ 611.1161, found 611.1122 $[M+Na]^+$; calcd for $C_{17}H_{30}N_6O_{11}P_3Na_2$ 633.0981, found 633.0973 $[M-H+2Na]^+$; calcd for $C_{17}H_{30}N_6O_{11}P_3NaK$ 649.0720, found 649.0687 $[M-H+Na+K]^+$.

- Table 1 -

In conclusion, we have prepared a stable analogue of AZT-TP to be used to raise specific antibodies against the antiviral triphosphate metabolite. The title compound has been further coupled to an antigenic carrier protein and rabbits have been immunized. The elaboration of a specific and sensitive enzyme immunoassay for AZT-TP using the polyclonal antibodies thereby produced is currently underway and results will be published elsewhere.

The authors are thankful to E. Le Gallo for complementary analytical work. This work was supported by a grant from ANRS (Agence Nationale de Recherches sur le SIDA, France).

References

1. Zimmerman, T.P.; Mahony, W.B.; Prus, K.L. *J. Biol. Chem.* **1987**, *262*, 5748-5754.
2. Balzarini, J.; Pauwells, R.; Baba, M.; Herdewijn, P.; De Clercq, E.; Broder, S.; John, D.G. *Biochem. Pharmacol.* **1988**, *37*, 897-903.
3. Balzarini, J.; Herdewijn, P.; De Clercq, E. *J. Biol. Chem.* **1989**, *264*, 6127-6133.
4. Gao, W.Y.; Shirasaka, T.; John, D.G.; Broder, S.; Mitsuya, H. *J. Clin. Invest.* **1993**, *91*, 2326-2333.
5. Gao, W.Y.; Agbaria, R.; Driscoll, J.S.; Mitsuya, H. *J. Biol. Chem.* **1994**, *259*, 12633-12638.
6. Sommadossi, J.P. *Clin. Infect. Dis.* **1993**, *16*, S7-S15, Suppl. 1.
7. Stretcher, B.N.; Pesce, A.J.; Frame, P.T.; Greenberg, K.A.; Stains, D.S. *AIDS* **1994**, *8*, 763-769.
8. Stretcher, B.N.; Pesce, A.J.; Hurtubise, P.E.; Frame, P.T. *Ther. Drug Monit.* **1992**, *14*, 281-285.
9. Stretcher, B.N.; Pesce, A.J.; Murzai, J.A.; Hurtubise, P.E.; Vine, W.H.; Frame, P.T. *Ther. Drug Monit.* **1991**, *13*, 325-331.
10. Gulick, R.M.; Mellors, J.W.; Havlir, D.; Eron, J.J.; Gonzalez, C.; McMahon, D.; Richman, D.D.; Valentine, F.T.; Jonas, L.; Meibohm, A.; Emini, E.A.; Chodakewitz, J.A. *New Engl. J. Med.* **1997**, *337*, 734-739.
11. Collier, A.C.; Coombs, R.W.; Schoenfeld, D.A.; Bassett, R.L.; Timpone, J.; Baruch, A.; Jones, M.; Facey, K.; Whitacre, C.; McAuliffe, V.J.; Merigan, R.C.; Reichman, R.C.; Hooper, C.; Corey, L. *New Engl. J. Med.* **1996**, *334*, 1011-1018.
12. Daluge, S.M.; Good, S.S.; Faletto, M.B.; Miller, W.H.; St.Clair, M.; Boone, L.R.; Tisdale, M.; Parry, N.R.; Reardon, J.E.; Dornsife, R.E.; Averett, D.R.; Kenitsky, T.A. *Antimicrob. Agents Chemother.* **1997**, *41*, 1082-1093.
13. Lefebvre, I.; Périgaud, C.; Pompom, A.; Aubertin, A.M.; Girardet, J.L.; Kirn, A.; Gosselin, G.; Imbach, J.L. *J. Med. Chem.* **1995**, *38*, 3941-3950.
14. Furman, P.A.; Fyfe, J.A.; St.Clair, M.H.; Weinhold, K.; Rideout, J.L.; Freeman, J.I.A.; Nusinhoff-Lehman, S.; Bolognesi, D.P.; Broder, S.; Mitsuya, H.; Barry, D. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 8333-8337.
15. Tornevik, Y.; Jacobson, B.; Britton, S.; Eriksson, S. *AIDS Res. Hum. Retrov.* **1991**, *7*, 751-759.
16. Toyoshima, T.; Kimura, S.; Maramatsu, S.; Takahagi, H.; Shimada, K. *Anal. Biochem.* **1991**, *196*, 302-307.
17. Molema, G.; Jansen, R.W.; Visser, J.; Meijer, D.K. *J. Chromatogr.* **1992**, *579*, 107-114.
18. Kuster, H.; Vogt, M.; Joos, B.; Nadai, V.; Luthy, R. *J. Infect. Dis.* **1991**, *164*, 773-776.
19. Stretcher, B.N.; Pesce, A.J.; Geisler, B.A.; Vine, W.H. *J. Liq. Chromatogr.* **1991**, *14*, 2261-2272.
20. Slusher, J.T.; Kuwahara, S.K.; Hamzeh, F.H.; Lewis, L.D.; Kornhauser, D.M.; Lietman, P.S. *Antimicrob. Agents Chemother.* **1992**, *36*, 2473-2477.
21. Barry, M.; Wild, M.; Veal, G.; Back, D.; Breckenridge, A.; Fox, R.; Beeching, N.; Nye, N.; Carey, P.; Timmins, D. *AIDS* **1994**, *8*, F1-F5.
22. Peter, K.; Lalezari, J.P.; Gambertoglio, J.G. *J. Pharm. Biomed. Anal.* **1996**, *14*, 491-499.
23. Robbins, B.L.; Waibel, B.H.; Fridland, A. *Antimicrob. Agents Chemother.* **1996**, *40*, 2651-2654.
24. Rodman, J.H.; Robbins, B.; Flynn, P.M.; Fridland, A. *J. Infect. Dis.* **1996**, *174*, 490-499.
25. Pradelles, P.; Grassi, J.; Chabardes, D.; Guiso, N. *Anal. Chem.* **1989**, *61*, 447-453.
26. Yamamoto, T.; Shimizu, H.; Kato, T.; Nagatsu, T. *Anal. Biochem.* **1984**, *142*, 395-399.
27. Maessen, J.G.; van der Vusse, G.J.; Vork, M.; Kootstra, G. *Clin. Chem.* **1988**, *34*, 1087-1090.
28. De Korte, D.; Haverkort, W.A.; Roos, D.; van Gennip, A.H. *Clin. Chim. Acta* **1985**, *148*, 185-196.
29. Di Pierro, D.; Tavazzi, B.; Perno, C.F.; Bartolini, M.; Balestra, E.; Calio, R.; Giardina, B.; Lazzarino, G. *Anal. Biochem.* **1995**, *231*, 407-412.
30. Cerpovicz, P.F.; Ochs, R.S. *Anal. Biochem.* **1991**, *192*, 197-202.
31. De Lean, A.; Rouleau, D.; Lefkowitz, R.J. *Life Sci.* **1983**, *33*, 943-954.
32. Goujon, L.; Brossette, T.; Dereudre-Bosquet, N.; Creminon, C.; Clayette, P.; Dormont, D.; Mioskowski, C.; Grassi, J. *J. Immunol. Methods* **in press.**
33. Bredehorst, R.; Ferro, A.; Hilz, H. *Eur. J. Biochem.* **1978**, *82*, 105-113.
34. Blackburn, G.M. *Chem. Ind.* **1981**, 134-138.
35. Engel, R. *Chem. Rev.* **1977**, *77*, 349-367.
36. Czernecki, S.; Valéry, J.M. *Synthesis* **1991**, 239-240.
37. Saady, M.; Lebeau, L.; Mioskowski, C. *J. Org. Chem.* **1995**, *60*, 2946-2947.
38. Saady, M.; Lebeau, L.; Mioskowski, C. *Helv. Chim. Acta* **1995**, *78*, 670-678.
39. Saady, M.; Lebeau, L.; Mioskowski, C. *Tetrahedron Lett.* **1995**, *36*, 5183-5186.
40. Saady, M.; Lebeau, L.; Mioskowski, C. *Tetrahedron Lett.* **1995**, *36*, 2239-2242.
41. Saady, M.; Lebeau, L.; Mioskowski, C. *Synlett* **1995**, 643-644.
42. McKenna, C.E.; Higa, M.T.; Cheung, N.H.; McKenna, M.C. *Tetrahedron Lett.* **1977**, 155-158.
43. McKenna, C.E.; Schmidhauser, J. *J.C.S. Chem. Commun.* **1979**, 739.
44. Greene, T.W.; Wuts, P.G.M. *Protective Groups in Organic Synthesis*, 2nd ed.; Wiley: New York, 1991.
45. Compound **1** was purified by HPLC (Zorbax SB C18; ammonium carbonate 0.5 M, acetonitrile 6%, pH 8.8; 1 ml/mn; retention time: 37 min).