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Synthesis of Haptens for the Development of a Solid-Phase Immobilized Epitope-Immunoassay (SPIE-IA) of AZT-TP

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Abstract: The synthesis of the reduced form of AZT-triphosphate, 3'-amino-3'-deoxythymidine-5'-triphosphate, and of its stable analog, 3'-amino-3'-deoxythymidine-5'-(α,β:β,γ-bis-methylene)triphosphate, is described. AMT-TP and AMT-PCPCP were prepared to develop a specific immunometric assay for AZT-TP.

Till the discovery in the mid-1980s that AIDS results from infection by a retrovirus, human immunodeficiency virus (HIV), therapy has focused on the design of inhibitors targeted to HIV-reverse transcriptase (RT) and protease (PR), two main enzymes of the virus. Despite several classes of promising new anti-HIV agents, the clinical emergence of drug resistant variants of HIV has severely limited the long term effectiveness of these drugs[1-4]. Combination of anti-HIV agents is now being explored as therapeutic modalities to prevent or delay emergence of virus-drug resistance[5-11]. In these conditions however the emergence of other causes leading to therapy failure are observed. In 1998 a treatment escape mechanism related to the metabolism of nucleoside RT inhibitors (NRTIs) has been reported[12,13], supported by previous observations indicating negative metabolic interferences between antiviral nucleosides[14-16]. In order to precociously detect the emergence of such resistance phenomena, clinicians need efficient tools to monitor the intracellular concentration of NRTIs and of their metabolites (NRTI-MPs, NRTI-DPs, and NRTI-TPs).

Herein we describe our strategy for the production of specific antibodies directed against AZT-triphosphate (AZT-TP) and the elaboration of an enzyme immunometric assay called SPIE-IA (solid-phase immobilized epitope-immunoassay)[17-21]. In the SPIE-IA technique, a specific monoclonal antibody is immobilized on a 96-well titerplate to ensure full capture of hapten during the immunological reaction (Sch. 1). The trapped molecule is then covalently linked to the plate by means of either a bifunctional cross-linking

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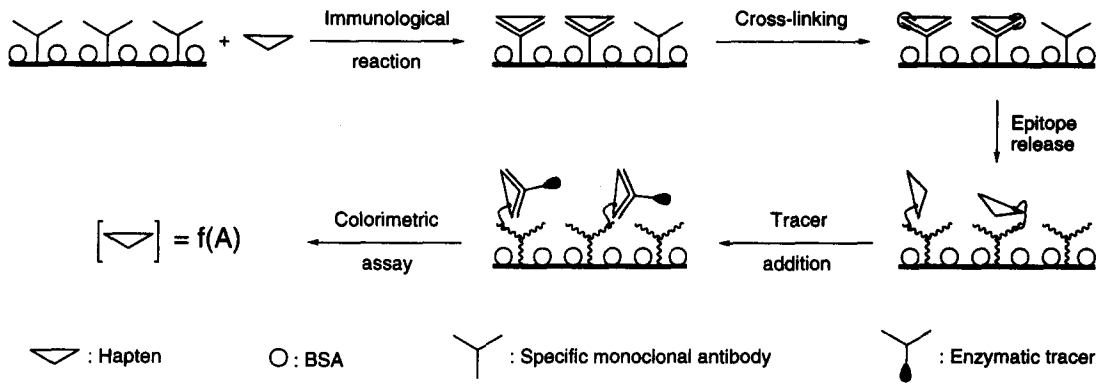
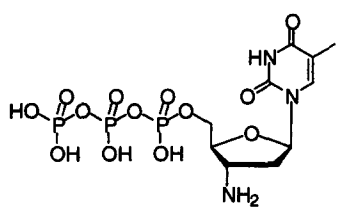


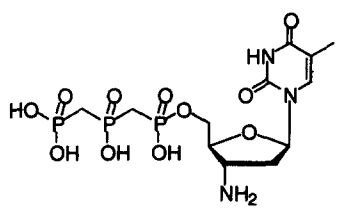
- Scheme 1 -

reagent able to react with both the hapten and the plate, or by long wave UV irradiation (photo SPIE-IA)[22]. After denaturing treatment, the hapten covalently bound to the solid phase can react again with the same acetylcholinesterase-labeled monoclonal antibody used as tracer[23]. Due to the use of excess reagent, SPIE-IA is 70 to 200 times more sensitive than conventional competitive EIA using the same monoclonal antibody and tracer.

In the case of AZT-TP monitoring, the key points for the elaboration of a SPIE-IA are the production of a specific monoclonal antibody and the cross-linking procedure of the hapten to the plate. Our strategy required the synthesis of the reduced form of AZT-TP, AMT-TP **1**, and its stable analog AMT-PCPCP **2**.



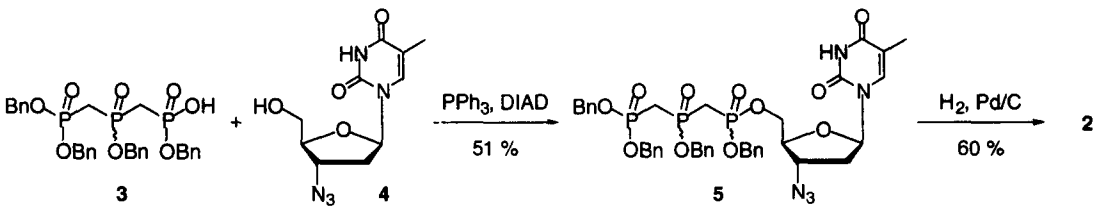
1 : AMT-TP



2 : AMT-PCPCP

These nucleotide analogs were designed to be coupled to an antigenic carrier protein through the 3'-amino group using glutaraldehyde as cross-linking reagent prior to immunizations. It is important to note that AZT-TP only differs from thymidine triphosphate (T-TP) by its 3'-azido group. As this function is directly modified to achieve the coupling to the carrier protein, it is likely that the resulting antibodies will not be able to distinguish AZT-TP from AMT-TP and T-TP. This is of no importance as prior to performing the ELISA test, the samples have to be treated with tris(2-carboxyethyl)phosphine (TCEP)[24] in order to reduce the 3'-azide into secondary amine. The reduced samples are then put in contact with the plate and treatment with glutaraldehyde or dimethyl suberimidate covalently links AMT-TP to the matrix. Addition of methanol provokes antibody denaturation and epitope release. The tracer is then added and incubated for a short period of time before addition of Ellman's reagent. Absorbance (A) is read, allowing the calculation of initial hapten concentration in the samples.

AMT-TP has been prepared by direct careful hydrogenolysis of AZT-TP to avoid thymine reduction[25]. That procedure proved to be more convenient than that described in two earlier reports and involving the azide reduction by triphenylphosphine[26,27]. Immunizations of mice by injection after coupling to keyhole limpet hemocyanin (KLH) however did not trigger the synthesis of specific antibodies in the recipient animals. This is likely due to the rapid hydrolysis of the pyrophosphate bridges of the hapten *in vivo*[28]. In order to alleviate the chemical and enzymatic instability of the pyrophosphate, the stable analog AMT-PCPCP **2** has been synthesized. It was prepared *via* direct Mitsunobu coupling of phosphonic acid **3**[29] and AZT **4**[30] to produce the intermediate compound **5**[31]. Careful hydrogenolysis of the four benzyl esters and of the azido group yielded AMT-PCPCP[32].



- Scheme 2 -

Compound **2** has been coupled to KLH and the conjugate has been injected into mice. The production and selection of monoclonal antibodies are currently underway and results will be reported in due course.

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25. Preparation of **1**. AZT-TP (14 mg, 27.6 µmol) and Pd/C 10% (7 mg) are stirred under 1 atm H_2 in methanol/water 1:1 (0.6 ml) for 2.5 h at room temperature. The catalyst is removed by filtration, the filtrate is reduced under vacuum and lyophilized to yield a white hygroscopic powder (10 mg, 74 %). ^{1}H-NMR (D_2O, 300 MHz) δ 7.52 (s, H6); 6.16 (t, J = 6.0 Hz, H1'); 4.27-4.23 (m, H3', H4'); 4.15-4.11 (m, 2H5'); 2.52-2.46 (m, 2H2'); 1.76 (s, 3H5).
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31. Preparation of **5**. Compound **3** (170 mg, 0.28 mmol), **4** (74 mg, 0.28 mmol), and DIAD (220 µl, 1.10 mmol) in anhydrous THF (3 ml) are treated dropwise at room temperature with PPh_3 (250 mg, 0.95 mmol). The reaction mixture is stirred for 30 min before the solvent is removed under vacuum. The residue is purified by chromatography over silica gel (AcOEt/EtOH 100:0 to 85:15) to yield **5** as a glassy solid (mixture of 4 diastereomers, 122 mg, 51 %). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 7.36-7.27 (m, 20HAr, H6); 6.21-6.01 (m, H1'); 5.11-4.95 (m, 8HCAr); 4.40-3.83 (m, H3', H4', 2H5'); 3.17-2.65 (m, 4PCHP); 2.28-2.18 (m, 2H2'); 2.07 (s, 3H5). ^{31}P-NMR ($CDCl_3$, 80 MHz) δ 40.9-39.1 (m, Pβ); 22.7-20.4 (m, Pα,Pγ). IR (neat, cm^{-1}) ν 3173; 3066; 2955; 2107; 1699; 1456; 1374; 1251; 998.
32. Preparation of **2**. Compound **5** (82 mg, 95 µmol) and Pd/C 10% (7 mg) in EtOH/THF 7:3 (10 ml) are stirred for 1 h at room temperature under 1 atm H_2. The suspension is half reduced under vacuum and water (5 ml) is added before the resulting mixture is stirred under hydrogen for an additional 2 h period. The catalyst is removed by filtration and the filtrate is washed twice with AcOEt. The aqueous layer is lyophilized to yield **2** as a white hygroscopic powder (27 mg, 60 %). ^{1}H-NMR (D_2O, 200 MHz) δ 7.43 (s, H6); 6.09 (m, H1'); 4.24-4.14 (m, H4'); 4.07-3.90 (m, 2H5'); 4.87-3.76 (m, H3'); 2.65-2.27 (m, 2H2', 4PCHP); 1.69 (s, 3H5). ^{31}P-NMR (D_2O, 80 MHz) δ 38.2 (m, Pβ); 17.1 (m, Pα, Pγ). IR (neat, cm^{-1}) ν 2962(b); 1696; 1475; 1373; 1270; 1202. Anal HPLC (Zorbax SB C18, 4.6x250 mm, 5µ, Et_3NH^+ $HCO3^-$: 0.062 M, CH_3CN: 0.1 %, pH 7.63) t_R 45.1 min.