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Synthesis of a Functionalized GDP-Analogue Designed for Anti-GDP Antibody Production

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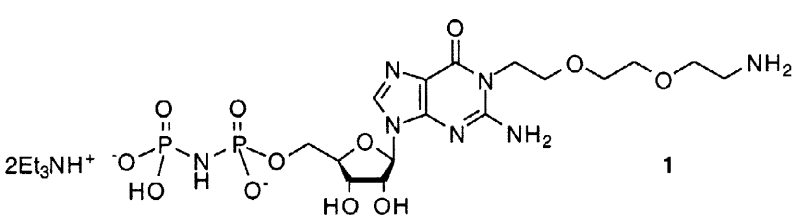
Abstract: An enzymatically stable analogue of GDP is prepared in 7 steps starting from guanosine. The guanine moiety is functionalized at the N^1 position so as to allow anchoring of the nucleotide onto a carrier protein for subsequent immunization of animals in order to raise antibodies against GDP.

Hundreds of cell-surface receptors employ G-proteins to initiate intracellular signalling chain reactions which guide a cell's action[1-5]. These heterotrimeric G-proteins ($\alpha\beta\gamma$) are under the control of both GDP and GTP : the GDP-bound form of α binds tightly to $\beta\gamma$ but is inactive, whereas the GTP-bound form of α dissociates from $\beta\gamma$ and serves as a regulator of effector proteins[6,7]. Thus one of the key points in the G-protein cascades is the equilibrium that exists between the GDP- and GTP-bound forms of the protein, that equilibrium itself being subjugated to the intracellular concentrations of GDP and GTP.

In the course of our work on G-proteins, we are interested in monitoring intracellular levels of GDP. Four types of methods emerge among those described to assay nucleotides : enzymatic methods[8,9], protein binding assay[10], radioimmunoassay (RIA)[11-14] and enzyme immunoassay (EIA)[15]. The last method was selected for its sensitivity and as it does not require HPLC purification steps[16-19] or radiolabelled compound manipulations[14,20]. This type of assay is performed in 96-well microtiter plates coated with anti-rabbit immunoglobulin antibodies and is based on the use of rabbit polyclonal antibodies raised against the substance to monitor and of a hapten/acetylcholinesterase conjugate as tracer[21].



1

- Figure 1 -

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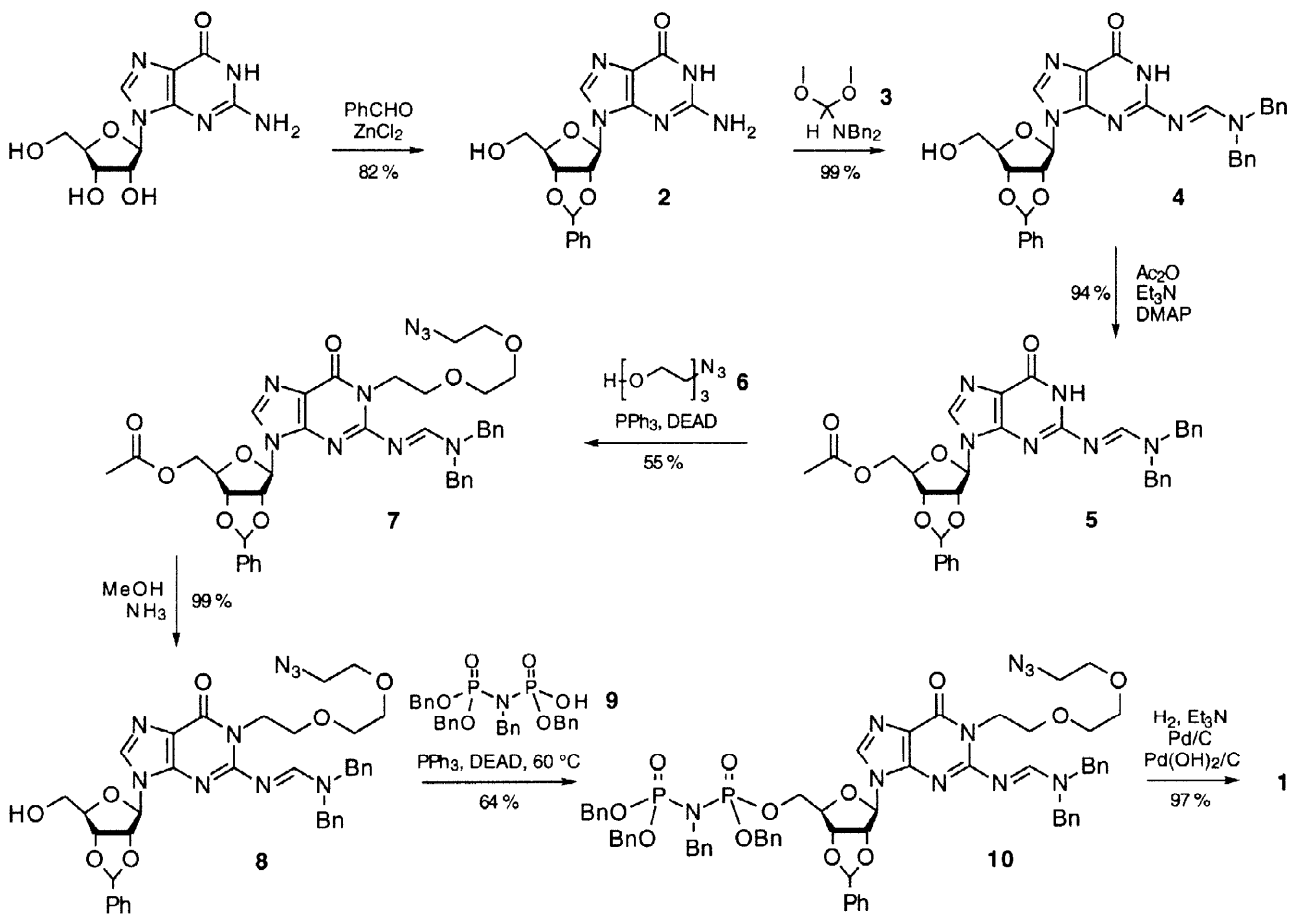
The production of antibodies directed against di- or triphosphate nucleosides is delicate to work out because of the sensitivity of the antigens towards enzymes in the recipient animals which effectively hydrolyze the pyrophosphate bridge on the hapten[22]. In order to overcome that difficulty, we synthesized a GDP analogue that is resistant towards enzymatic hydrolysis (Fig. 1).

The imidodiphosphate group has been widely used to mimic pyrophosphates in nucleotides[23-26]. The resulting nucleotide analogues are still recognized by the enzymes with nucleotidase activities, but they are generally not good substrates for them if any. As is the case for most small molecules, the hapten has to be coupled to an antigenic carrier (KLH, BSA...) that will amplify the immune response in the injected animal. This is to be achieved through the reaction of the amino spacer, introduced on position N^1 in the guanine moiety, with the carrier protein (using glutaraldehyde[27-29]).

The synthesis of the hapten has been realized according to the following scheme, starting from guanosine (Fig. 2).



- Figure 2 -

Guanosine was protected as its benzylidene acetal **2**[30,31] before reacting with *N,N*-dibenzyl formamide dimethyl acetal **3** to afford the corresponding *N,N*-dibenzyl formamidine **4**[32]. The 5'-hydroxyl group was acetylated in standard conditions and the N^1 position of **5** was regiospecifically alkylated with alcohol **6** using a Mitsunobu reaction[33]. The primary alcohol on the sugar moiety of **7** was then quantitatively restored in methanolic ammonia, and further phosphorylated with the acid compound **9**[34] in a modified Mitsunobu reaction. Interestingly, when the coupling reaction was conducted at room temperature, a major side-product was obtained that corresponds to the *N*-alkyl 1,2-dicarbethoxy hydrazine resulting from the displacement of the 5'-alkoxyphosphonium intermediate by disubstituted hydrazine produced in the course of the reaction[35-37]. Neither the use of other phosphine compounds (PBu_3, $(4\text{-Cl-Ph})_3P$[38]), nor other azo derivatives (DIAD, ADDP[39-41]) provided a higher yield than PPh_3 and DEAD at 60 °C. Final hydrogenolysis removed simultaneously all protective groups on **10** (*i.e.* the benzyl esters and *N*-benzylimide on the imidodiphosphate moiety, the benzylidene acetal on the sugar and the dibenzyl formamidine on the base) and reduced the azide group at the end of the spacer into primary amine to provide the target compound **1** in almost quantitative yield[42]. That compound proved to be chemically stable in solution at pH 8-10 in the presence of Mg_2^+ for more than 5 days.

In conclusion, we have synthesized an enzymatically stable analogue of GDP to be used to raise antibodies against the natural nucleoside diphosphate. The synthesis is worked out in such a manner that all intermediate compounds are soluble in common organic solvents (*i.e.* THF, CH_2Cl_2...). The final deprotection step consists in a simultaneous hydrogenolysis of 7 protective groups on the molecule and releases the pure title compound without chromatographic separation. Rabbits immunizations are currently underway.

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42. a) Selected spectral data for **7** (2 diastereomers): ^{1}H-NMR (200 MHz, $CDCl_3$) 8.91 and 8.88 (2s, 1H); 7.70 (s, 1H); 7.60-7.15 (m, 15H); 6.23 and 6.14 (2d, J = 2.3 Hz, 1H); 6.19 and 6.03 (2s, 1H); 5.47 and 5.42 (2dd, J = 2.3, 6.4 Hz, 1H); 5.03 (2dd, J = 3.2, 6.4 Hz, 1H); 4.71 (s, 2H); 4.60 (t, J = 6.3 Hz, 2H); 4.51-4.05 (m, 5H); 3.74 (t, J = 6.3 Hz, 2H); 3.65-3.45 (m, 6H); 3.28 (t, J = 5.2 Hz, 2H); 2.00 (s, 3H). IR (film) 2923; 2872; 2106, 1743; 1690; 1613; 1574; 1530; 1493; 1431; 1360; 1223; 1097; 1028; 760. b) Selected spectral data for **10** (4 diastereomers): ^{1}H-NMR (200 MHz, $CDCl_3$) 8.93 and 8.91 (2s, 1H); 7.80, 7.75, 7.72 and 7.68 (4s, 1H); 7.60-7.15 (m, 35H); 6.16 and 6.08 (2m, 1H); 6.10, 5.90 and 5.97 (3s, 1H); 5.17 (m, 1H); 5.02-4.35 (m, 16H); 4.25 and 3.95 (2m, 2H); 3.72 (t, J = 6.4 Hz, 2H); 3.65-3.45 (m, 6H); 3.27 (t, J = 5.0 Hz, 2H). IR (film) 3061; 3031; 2926; 2105; 1692; 1611; 1572; 1529; 1493; 1454; 1359; 1273; 1216; 1094; 1016. c) Selected spectral data for **1**: ^{1}H-NMR (300 MHz, D_2O) 8.04 (s, 1H); 5.83 (d, J = 6.6 Hz, 1H); 4.41 (m, 1H), 4.25-3.95 (m, 5H); 3.81 (t, J = 5.1 Hz, 2H); 3.65-3.45 (m, 6H); 3.11 (q, J = 7.3 Hz, 12H); 3.01 (t, J = 4.4 Hz, 2H); 1.19 (t, J = 7.3 Hz, 18H). ^{13}C-NMR (75 MHz, D_2O/CD_3OD : 9/1) 159.37; 155.00; 149.84; 138.15; 116.53; 86.93; 84.24; 73.63; 70.64; 70.62; 69.76; 68.06; 66.46; 64.46; 46.81; 42.37; 39.18; 8.37. ^{31}P-NMR (121 MHz, D_2O, [Et_3N] = 0.5 M) 3.73 (d, J = 2.38 Hz, 1P); -0.11 (d, J = 2.38 Hz, 1P). HRMS: calcd for $C_{16}H_{30}N_7O_{12}P_2$ 574.1428, found 574.1421 $[M+H]^+$; calcd for $C_{16}H_{29}N_7NaO_{12}P_2$ 596.1247 found 596.1244 $[M+Na]^+$.