LABORATORY OF BIOPHYSICS

FOR ADVANCED

Experimental exercises for III year of the First cycle studies

Field: “Applications of physics in biology and medicine”

Specialization: “Molecular Biophysics”

Column ion-exchange and reverse phase high pressure liquid chromatography in chemical synthesis of nucleoside 5’-diphosphates (PBdZ 17)

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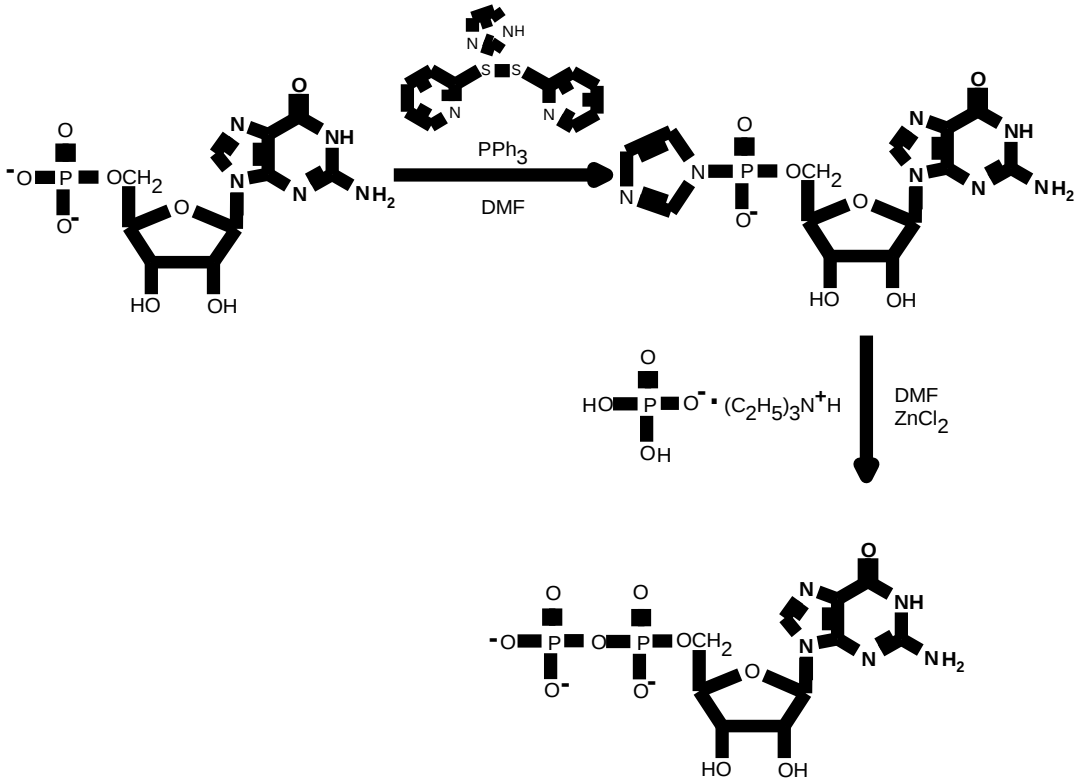
Chemical background

One of the key processes in living cells is the pyrophosphate bond formation. The pyrophosphate is an anhydride type connection and is known as „energy rich" bond. An important biochemical example is ADP↔ATP reaction, which is basic for energy transfer within living cells.

Nucleoside 5'-di- and triphosphates can be prepared on a chemical way. Since Mn^{2+} cations are able to catalyze enzymatic reactions of pyrophosphate bond formation the chemical methods that mimics this reaction have been established. The synthesis proceeds in aqueous condition and one of the phosphate substrate should be activated for example as imidazolide derivative. This process of pyrophosphate bond formation is effective to some extent only (yield: 20 – 30%). Further experiments proved that manganese cations may by replaced by zinc salts. However, in this case anhydrous conditions should be performed because zinc ions in water solution are not effective as catalysts of this particular reaction. Below, the Scheme 1 shows one of the possible examples of such reaction, which gives much higher yield up to 80 %.



Scheme 1. Chemical synthesis of GDP (guanosine 5'-diphosphate).

Experimental:

1. Ion exchange procedure to obtain so called organic salt of nucleotide.

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Preparation of 5'-GMP as triethylammonium salt.

Most often GMP is handled in form of sodium salt. However, this form is useless if any reaction proceeds in organic medium. In that case most convenient form of GMP is triethylammonium salt.

Ion exchange is conducted with use of cation-exchange resin (cationite) i.e. chemically modified organic polymer for example polymer-COOH or polymer-SO_3H. In the presence of any positively charged ions (cations) the acidic groups bound cations by electrostatic interactions (for instance the following reaction is performed: polymer-$SO_3H + Na^+ \longrightarrow$ polymer-$SO_3Na + H^+$). Usually the resins (cationites, anionites) have multifold uses and can be regenerated.

Below the procedure for conversion of 5 g GMP/Na^+ into triethylammonium salt is described.

At first, to the preparation, cation-exchange resin in triethylammonium form is needed [for example: polymer-$SO_3^-N^+H(C_2H_5)_3$]. The experiment must be conducted under well-working hood. A column á 150 mL equipped with stopcock plug and glass-pore (or cotton-roll) bottom is filled up with a water slurry of a cation-exchange resin (for example Dowex 50W, 50-100 mesh), and hydrochloric acid (10 %) is passed through the column (500 mL). Next, the column is washed with deionized water until pH of the eluent becomes neutral. When the top level of last portion of water is on the level of top layer of resin, triethylamine (20 mL) is added, and after permeation of triethylamine, the continuous addition of water is continued until pH of the eluent is neutral. At this stage the column should be refilled. It means the whole content of the column is transferred to the auxiliary beaker, and the column is filled up with the water slurry of the prepared resin again.

GMP (commercial sodium salt, 5 g) dissolved in water (50 mL) is applied to the column and the column is washed with water to yield about 500 mL . The obtained solution is evaporated to dryness on a rotary evaporator under diminished pressure (≤15 hPa). The residue (thick syrup) is treated with absolute ethanol (30 mL) and evaporated again. Next, the flask is placed in a vacuum desiccator over P_4O_{10} to obtain monotriethylammonium salt of GMP (GMP/TEA) in form of white powder (if necessary the dry mass of the product could be grounded with a spatula).

Optical density of the product is determined (see Section 4).

2. Ion-exchange liquid chromatography

2a) $P^{5'}$-imidazolido-GMP.

In a round-bottom flask á 500 mL placed on a magnetic stirrer and equipped with a stirring bar GMP/TEA prepared in Section 1 (0.9 – 1.0 g, mass measured with an accuracy of 0.01 g, **M1** – see Section 4b), imidazole (0.68 g), 2,2'-ditiodipyridine (0.88 g), anhydrous dimethylformamide (DMF, 10 mL) and triethylamine (TEA, 0.3 mL) is added and the stirring is started. Then triphenylphosphine (1.1 g) is added and the stirring is continued for at least 6 hours. After that time GMP should be completely dissolved and the solution becomes yellow or pale-brown. The reaction is quenched by addition of anhydrous acetone solution containing 1 g of $NaClO_4$. Then more acetone is added (20 mL) and after intensive stirring the solution is chilled for 2 hr in a refrigerator. The precipitation is filtered off on a Schott funnel and washed 3 – 4 times with anhydrous acetone. Each new portion of acetone should be well mixed with the precipitation. The last washing should be colorless. Finally, the precipitation is dried in a vacuum desiccator over P_4O_{10}**.**

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2b) Synthesis of 5'-GDP (pyrophosphate bond formation).

The product from Section 2a is placed in round-bottom flask (250 mL) equipped with stirring bar and triethylammonium salt of ortho-phosphoric acid* (1.6 g) is added. Then DMF (12 mL) and anhydrous zinc chloride (1 g) is added and the mixture is stirred during 4 hrs. The resulted mixture is poured in to a beaker containing EDTA (2 g) and water (200 mL). With intensive mixing the solution is adjusted to pH 6 by portion wise addition of $NaHCO_3$ and finally filtered through 0.45 μm filter. The resulted solution is analyzed by HPLC (see Section 3.)

** Triethylammonium phasphate is prepared by other procedure – it will be delivered by a mentor. About 10-molar excess of phosphate group donor is important due to prevention of Gp_2G formation. Gp_2G is a side product which is difficult to separate from GDP by ion-exchange chromatography.*

2c) Chromatographic isolation of 5'-GDP.

*The mixture from Section 2b contains besides GDP few other organic compounds as side products (for example: unused GMP, Gp_2G and Gp_3G) and many inorganic salts. The best method of nucleotides separation from complicated mixtures is ion-exchange column chromatography over DEAE-Sphadex-A25 (bicarbonate form) using increasing linear gradient of triethylammonium bicarbonate (TEAB)** aqueous solution as mobile phase.*

The solution from Section 2b is applied by means of peristaltic pump on the top of column ($\Phi \cong 4$ cm, length 1.5 m) containing DEAE-Sephadex-A25 (HCO_3^- form). The column is washed with water (at least 500 mL). After that, chromatography is started using linear gradient of TEAB (0 – 1 M) and collecting (in automatic fraction collector) fractions every 6 min with flow rate of 2 – 2.5 mL/min. Total volume of used mobile phase is about 4.6 L.

Using spectrophotometer UV-Vis, absorption in every fifth fraction is measured at 260 nm to be able to prepare the chromatography profile. The contents of tubes within the main chromatographic peak are analyzed by means of HPLC method (see Section 3). Fractions containing GDP over 95 % purity are pooled and evaporated to dryness on a rotary evaporator under diminished pressure (≤15 hPa). During evaporation ethanol is added from time to time to facilitate the decomposition of TEAB. Finally, when there is no TEA left in the flask, the residue (thick syrup) is treated with absolute ethanol (30 mL) and evaporated again. Next, the flask is placed in a vacuum desiccator over P_4O_{10} to obtain GDP (triethylammonium salt) in form of white powder. Mass of the dry product is weighted*** (value **M2**) and optical density is determined (see Section 4).

*** TEAB buffer is prepared by other procedure – it will be delivered by a mentor.*
****Since GDP/TEA is slightly hygroscopic the operations with the substance should be made as quick as possible.*

3. Reverse Phase High Pressure (Performance) Liquid Chromatography (RP HPLC).

Substrate (GMP from Section 1), product from Section 2a (GMP imidazolide) and designated fractions within GDP peak in Section 2c are analyzed by HPLC using an analytical reverse-phase (C_{18}, length 25-30 cm) column and 0.1 M KH_2PO_4/K_2HPO_4 water-methanol solution as mobile phase. Solvents are prepared as follows: (A) KH_2PO_4 (11.0 g, HPLC grade) and K_2HPO_4 (2 g, HPLC grade) are dissolved in water (HPLC grade) in 1L volumetric flask and

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filtered; (B) 300 mL of the just prepared solution A is mixed with 300 mL of methanol (HPLC grade). During chromatographic analysis linear gradient of methanol is used starting from 100 % A (0 % B) and ending with 50%A/50%B after 15 minutes; flow rate 1.0 mL/min. Monitoring is spectrophotometric at 260 nm.

4. Evaluation of the reaction effectiveness

Since the contents of triethylammonium counter ions as well as traces of water in GMP/TEA and GDP/TEA are slightly variable from batch to batch, determination of the yield by measuring mass is not reliable. Since base ring in GMP and in GDP is the same, and there are no other UV chromophores in these molecules, spectrophotometric determination of the yield is more accurate.

Follow the procedure described below:
a) weigh about 5 mg (mass measured with an accuracy of 0.1 mg) of GMP/TEA prepared in Section 1, dissolve it in water in 10 mL volumetric flask; take by a micropipette 100 μL of the solution and drop it into absorption cuvette (10 mm) containing 2 mL of buffer A (prepared in Section 3); before that, spectrophotometer should be balanced to "0"; measure absorption at 260 nm; make at least 3 independent measurements and calculate average value;
b) calculate the number **N1** of the so called optical units (o.u.) for the substrate (GMP/TEA) used in Section 2a according to the equation:
N1 = **O.D.1** (optical density in o.u./mg) x **M1** (mass of GMP/TEA used in Section 2a in mg), where **O.D.1** is calculated as follows:
(average value of UV absorption from point a) x [(21 x 10) / (mass of the sample from point a)]
c) weigh about 5 mg (mass measured with an accuracy of 0.1 mg) of GDP/TEA*** prepared in Section 2c, dissolve it in water in 10 mL volumetric flask; take by a micropipette 100 μL of the solution and drop it into absorption cuvette (10 mm) containing 2 mL of buffer A (prepared in Section 3); before that, spectrophotometer should be balanced to "0"; measure absorption at 260 nm; make at least 3 independent measurements and calculate average value;
d) calculate the number **N2** of the optical units for the product (GDP/TEA) according to the equation:
N2 = **O.D.2** (optical density in o.u./mg) x (mass of GDP/TEA obtained in Section 2c in mg), where **O.D.2** is calculated as follows:
(average value of UV absorption from point c) x [(21 x 10) / (mass of the sample from point c)]
e) calculate the final yield of the GDP preparation:
N2 / N1 x 100 % =

Literature (examples):

1. „High Resolution Chromatography. A practical Approach", P.A. Millner Ed., OXFORD University Press 1999.
2. „HPLC of Macromolecules. A practical Approach", R.W.A. Oliver Ed., OXFORD University Press 1998.

Report

The report has to include:

1. List of the conducted chemical operations.
2. All calculations made in Section 4.

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3. Chromatographic profile from Section 2c (it could be hand written or on-computer generated, for example using Excel).
4. Printed chromatograms from Section 1 (profile of GMP/TEA), Section 2a (imidazole derivative of GMP) and from Section 2b (crude solution).
5. Printed chromatograms of selected fractions from Section 2c.
5. Printed chromatogram of the final product (GDP/TEA).
6. Conclusions (for example including analysis of yield and purity of the obtained GDP).

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