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”

Analytical and preparative high pressure liquid chromatography (HPLC) as tools for monitoring chemical reactions (PBdZ 40)

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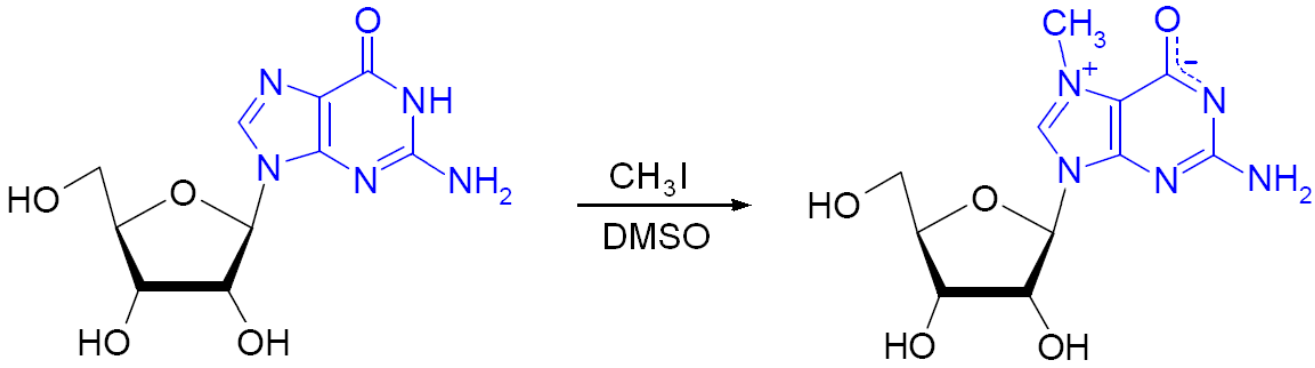
Introduction

Methyl derivatives of nucleic bases, nucleosides and nucleotides are often used in many biochemical and biophysical studies. Below the chemical synthesis of 7-methylguanosine from gauanosine by methylation with use of iodomethane is described. HPLC methods are used for monitoring the reaction and isolation of the product.



Scheme 1. Chemical synthesis of 7-methylguanosine (m^7Guo).

Experimental:

1. Synthesis.

Guanosine (200 mg) is magnetically stirred in round-bottom flask with DMSO (3 ml) and iodomethane (200 µl) at room temperature. Every one hour 5 µl of the mixture is taken out for monitoring the progress of the reaction by means of the high pressure liquid chromatography (HPLC). When the chromatographic analysis shows no content of the substrate (usually after 24 hours) water (30 ml) is added and, the resulted mixture is extracted twice with diethyl ether (10 ml each). Bottom layer is collected into round-bottom flask and condensed to 5 ml on a rotary evaporator. Next, the residue is transferred to Erlenmeyer flask (after pouring, an additional 1 ml amount of water is used to wash the rest of the material). The collected solution is adjusted to pH 8 with concentrated aqueous ammonia (using 50 µl portions) and diluted with acetone (60 ml). After chilling in a refrigerator the precipitate is filtered and washed with small amount of absolute alcohol. When dry, the product is weighted and analyzed by HPLC (see Section 2a).

The yield is usually about 60 %. More product my by isolated from mother liquor by means of preparative HPLC. To this aim the solution after filtration is condensed on a rotary evaporator to 5 ml and treated as described in Section 2b below.

2. Reverse phase high pressure liquid chromatography (RP HPLC).

a) Analytical HPLC.

Substrate (guanosine, Guo) and samples from the reaction mixture (Section 1) are analyzed by HPLC using an analytical reverse-phase (C_{18}, 4,6 mm x 25 cm with a 2 cm guard)

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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 1000 ml volumetric flask and 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.

5 µl samples from the reaction mixture are placed in small plastic (Eppendorf) vials and diluted with water (200 µl). 10 µl of the resulted mixtures are injected into column and at the end of each run the chromatographic profile is printed.

b) Preparative HPLC.

Crystallization (such as described in Section 1) as well as other classic preparation techniques have limited use. In case of troubles with isolation of any compound from mixtures chromatographic methods may be often very helpful. If we increase the scale of the chromatographic process we are able to separate during single run few or even significantly more miligrams of the purified substance. Below the isolation of 7-methylguanosine from the solution after filtration is described.

For preparative use a bigger column (21.2 mm diameter) is performed. As mobile phase aqueous ammonium acetate (0.05 M, pH 5.9) is used. This salt could be relatively easy removed from collected samples by decomposition. During chromatographic runs the linear gradient of methanol is used starting from 0% and ending with 50% (v/v) within 30 min. Flow rate is 5 ml/min, and spectrophotometric monitoring at 260 nm.

Single run is conducted with 200 µl of the mother liquor obtained in Section 1. During analysis, when peak of the product appears on the monitor, the flow is collected into a round-bottom flask. After as many as needed runs, the collected fractions are condensed on a rotary evaporator to remove methanol. Then the solution is frozen (– 80 °C) and lyophilized. The residue, dissolved in a small amount of water, is lyophilized again in small glass vial of known mass. Next, the yield is determined and test for homogeneity of this additional portion of the product is performed by the analytical HPLC (Section 2a).

3. Report

The report has to include:
1. List of the conducted chemical operations.
2. Profile (diagram) of the reaction progress (percent of the product in relation to time).
3. Printed all obtained chromatograms.
4. Yields calculations (first, additional, overall).
(MW Guo = 283 g/mol; MW m7Guo = 297 g/mol)

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.

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