Synopsis

In my thesis I describe the topic of development, synthesis, and application of molecular fluorescent nucleotide probes for real-time enzyme activity monitoring.

Nucleotides play a significant role in the cell metabolism. They are substrates in the nucleic acids synthesis (DNA, RNA), they transfer chemical energy necessary for enzymatic reactions, and they act as enzyme cofactors. Being involved in numerous biochemical reactions, and playing central role in the metabolism makes nucleotides interesting subjects for chemical modification. Synthesized analogues are useful either for enzyme activity monitoring, or even changing course of the mentioned processes.

Particularly interesting nucleotide degrading enzyme class are phosphohydrolases. These enzymes degrade oligophosphate chain of nucleotides. It is especially important in terms of controlling intracellular population of nucleosides mono-, di-, or triphosphates, which act as signal molecules crucial for proper functioning of cell metabolism. Dysfunctions of the phosphohydrolases were associated with various diseases, which makes these enzymes interesting therapeutic targets.

Until now, various methods for phosphohydrolases monitoring were developed. Unfortunately, these methods are usually complicated, time-absorbing, and expensive. In the project my aim was to develop a new method, in which tools necessary for the biological assays could be obtained during the simple chemical synthesis, and in which the enzymatic reaction could be monitored in real-time.

The first step of the project was to synthesize new class of nucleotide analogues – the ones with *C*-phosphonate moiety in the terminal position of oligophosphate chain. In addition, these compounds contain alkyne moiety attached to the *C*-phosphonate. The alkyne moiety enables them to react in the very efficient reaction called CuAAC (copper-catalysed alkyne-azide cycloaddition). Owing to this, I could subject the new nucleotide analogues to the reactions with various fluorescent compounds. Few of the compounds, were subjected to the CuAAC reaction with 5'-azidenucleosides. As a result, e.g. new cap analogues resistant to phosphohydrolases were obtained. Therefore, I proved that newly synthesized class of nucleotides reactive in CuAAC might serve as a tool for swift obtaining big libraries of new compounds, which may manifest interesting biological properties.

In the next step of the project I developed a new method for introducing two alkyne groups into the nucleotide analogue – one within ribose position, and the second attached to the terminal phosphate group. Therefore, it was possible to perform CuAAC reaction with two fluorescent dyes – pyrenes which exhibit excimer fluorescence. I synthesized series of nucleotide analogues, varying in nucleobase, and number of phosphate groups. All the compounds were studied spectroscopically, which proved that all exhibit excimer fluorescence. In the next step, all the probes were tested in reaction with PDE-I – nonspecific phosphodiesterase which cleaves oligophosphate chain. It turned out that fluorescent spectra of the probes changes during the enzymatic reaction, and all the compounds have completely different fluorescent spectra before and after enzymatic reaction. Therefore, they can be used for real-time enzyme activity monitoring. Some probes, with the highest difference in spectra

before and after enzymatic reaction, were directed to the *in vivo* studies. Pyrene groups was found to be able to transport nucleotide through the cell membrane, which is interesting observation, as polar nucleotides usually do not enter cells by themselves.

During pyrene probes synthesis it turned out, that two alkyne groups attached to the nucleotide have different reactivity. If the proper stoichiometry is kept, it is possible to make first alkyne moiety react in CuAAC with the azide containing fluorescent dye, and then, to make the second alkyne group react with the other azide fluorescent dye. Observed regioselectivity allowed for the synthesis of the new class of nucleotide probes – the ones that exhibit FRET effect. The new method is very interesting, as it does not require protecting groups and intermediate purification, which results in higher yields and easier synthesis. In enzymatic studies with PDE-I the probes fluorescent emission spectra changed significantly during enzymatic cleavage reaction. Hence, it makes them good candidates for enzyme monitoring activity probes. Tentative *in vivo* studies showed that the probes are able to penetrate the cell membrane and localize in the cytosol.

Methods developed in the project allow for obtaining various different nucleotide analogues useful in molecular biology studies. Technically, the method can be used for coupling nucleotides with any reactant containing azide moiety (fluorescent dyes, azide-nucleosides, nanoparticles, DNA, RNA, etc.) which opens way for huge libraries of many different compounds that exhibits interesting biological or biophysical properties.