Proteases, also known as peptidases are proteolytic enzymes that degrade proteins into smaller fragments by hydrolysis of peptide bonds. They play a very important role in the proper functioning of all cells and organisms. Research conducted over the past 50 years has shown that proteases are not only nonspecific digestive enzymes, but are involved in almost all biological processes in living organisms including digestion, growth, maturation, aging and death. In addition, they take part in many events of tumor cell biology. However, their activity must be tightly regulated to avoid excessive and dangerous proteolysis in cells or tissues. Therefore, many research groups around the world are involved in the exploration of specific tools for the study of this class of enzymes. One of the major obstacles to the development of chemical tools is a lack of selectivity for the target enzyme, which is caused by overlapping substrate specificity of these enzymes. This means that enzymes belonging to one group recognize similar substrates, and inhibitors designed based on the peptide sequence of these substrates inhibit not only one enzyme, but the all group of enzymes. This cross-specificity significantly impedes enzymes study in biological systems, where several active enzymes are present.

Attachment of low molecular weight protein - ubiquitin to the target substrate, is one of the most important post-translational modification that affects activity, protein-protein interactions, location and stability of the target protein. Covalent attachment of ubiquitin to protein substrates is a reversible process, carried out by deubiquitinating enzymes (DUBs). These enzymes catalyze the reaction involving the release of ubiquitin from its conjugates or polyubiquitin chain. An increasing number of reports in the literature indicate deubiquitinating enzymes as potential therapeutic target for the treatment of viral diseases, neurodegenerative diseases and cancer.

Fluorogenic substrates are commonly used in biochemical studies. They provide easy to interpret photochemical signal generated upon peptide bond hydrolysis. These substrates contain an appropriate peptide sequence on the left of the cleaved bond and a fluorescent tag. Commercially available fluorogenic substrates for study DUBs activity are based on the C-terminal motif of ubiquitin or contain ubiquitin coupled to a fluorophore (Z-LRGG-AMC, Ub-AMC). Furthermore, biochemical analysis of fluorogenic substrates revealed that they are not efficiently hydrolyzed by DUBs. Further analysis of substrate preference of DUBs using fluorogenic substrates library containing only natural amino acids (biogenic amino acids) showed that DUBs can recognized other amino acids not only Leu and Arg. These results suggest that dissection of binding pockets in the active site of enzymes can lead to the development of small molecule substrates or leading structure to the synthesis of inhibitors that selectively inhibit the DUBs. This analysis is possible by using unnatural amino acids (produced by organic synthesis), the differential structure of the side chains is an ideal tool for the exploration of the enzyme-substrate interaction. The use of substrates library containing the natural, and wide variety of unnatural amino acids will enable a precise examination of size, shape and amino acids preferences of the binding pockets of DUBs. **As a result new and more active fluorogenic substrates, as well as the specific peptide sequence for the target enzyme will be found, which is the main goal of this project.**

Implementation of the research aim will be divided into two sections: the first will involve the synthesis of substrate libraries, as well as individual fluorogenic substrates, while the second biochemical analysis. The synthesis of the library and substrates will be carried out according to standard method of solid peptide synthesis. This method involves attaching a amino acids to the insoluble polymer possessing appropriate chemical moiety.Then the peptide chain will be elongated by coupling another amino acids. The library will be composed of two sublibraries (P3 and P4). Each of sublibraries will contain the fluorescent tag ACC with glycine. The next position will also be occupied by glycine, because that structure of library will ensure that the library will be recognized by the DUBs. However, in the two remaining positions it will include the 19 natural and about 90 unnatural amino acids, or an equimolar mixture of natural amino acids. The next step of research will involve the use of a library to determine substrate specificity matrix of DUBs. **Based on the results new active fluorogenic substrates, as well as the specific peptide sequences for DUBs investigation will be designed and synthesized, which is the main goal of this project.** In the last stage of the research the catalytic parameters (k_{cat}, K_M, k_{cat}/K_M) for these substrates will be determined.

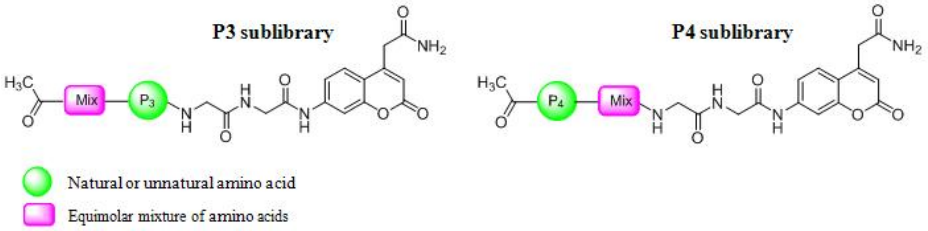

Fig. 1. The structure of substrate library for DUBs investigation.

Determination of full substrate specificity of deubiquitinating enzymes will allow to identify new chemical tools for measuring their activity. New, more active, and the specific fluorogenic substrates can be used to study biological function of these enzymes. In addition, the library can be utilized to profiling the substrate specificity of each enzyme exhibiting deubiquitinating activity.