Scientific report

regarding the implementation of the PCE_ PNII-ID-PCE-2011-3-0226 project

ACCES TO NEW BIOACTIVE MOLECULES BY DEVELOPING ORIGINAL BIOCATALYSTS FOR CLICK CHEMISTRY REACTIONS

Between October – December 2011

The project aims to identify, analyze and use of new biocatalysts from Romanian biodiversity, microorganisms and vegetables, which will be used to catalyze important reactions of organic chemistry.
The project aims to synthesize functionalized N-heterocyclic compounds with bioactive properties (Girija S. Singh, Edward E. Mmatli, Recent progress in synthesis and bioactivity studies of indolizine, European Journal of Medicinal Chemistry 46, 2011, 5237-525), fluorescent, by conventional and unconventional methods using biocatalysts, microwave activation, ultrasounds, methods known as belonging to "green chemistry".
The general scheme for preparation is outlined in Figure 1:

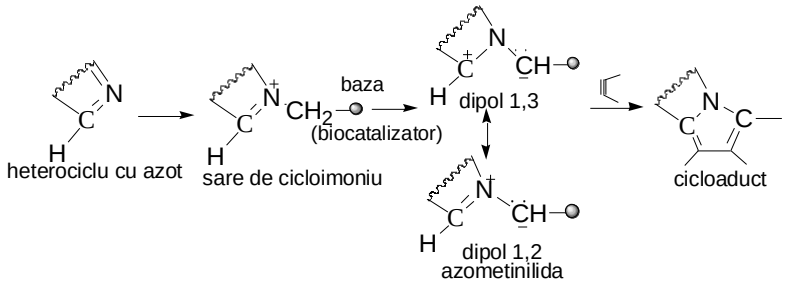


Figure 1. The general scheme underlying the synthesis of cycloadducts

*The objectives of this phase are **biocatalysts selection** and **synthesis** of organic compounds which are **starting materials** in cycloaddition reactions.*
Enzymes in organic synthesis are now widely accepted and increasingly more synthesis papers show that enzymes are an alternative source of catalysis being as robust as other chemical catalysts, their use continues to gain wide expansion (Benjamin G. Davis and Viviane Boyer, Biocatalysis and enzymes in organic synthesis, Nat. Prod. Rep., 2001, 18, 618–640).

Scientific progress shows that enzymes from different species may exhibit different catalytic characteristics including substrate or stereochemical selectivity. For biocatalysts, the advantages of selective synthesis (chemo- , regio- and stereoselective) are associated with the benefits of environmentally friendly strategy. Compared to classical chemical synthesis methods, methods of chemoenzymatic synthesis of drug substances obtained by enzymatic reactions association with modern chemical reactions, usually consist of fewer reaction steps, leading to a smaller amount of waste and presents greater efficiency both in the biotransformation yield as well as the enantiomers and / or diastereoselectivity (Ramesh N. Patel, Synthesis of chiral pharmaceutical intermediates by biocatalysis, Coordination Chemistry Reviews 252, 2008, 659–701)

Data analysis on mainstream publications and preliminary experiments performed in the first stage of the project clearly show the applicability of enzyme catalysis in the synthesis of

intermediates which in turn can lead to molecules with bioactive properties (Margaret M. Kayser, "Designer reagents' recombinant microorganisms: new and powerful tools for organic synthesis, Tetrahedron 65, 2009, 947–974).

The project focused on first the selection of biocatalysts to be used in the catalysis of reactions for the synthesis of N - heterocycles. We took into account commercial enzymes, or natural sources of enzymes (microorganisms and plant tissues). The cells of microorganisms (bacteria, yeasts and molds) were tested both in terms of the potential to produce various extracellular enzymes acting in biocatalysis and the use of biomass as complex biocatalysts. Whole cell enzyme complex is much more useful than pure enzyme complex due to the existence of complex enzymes that can catalyze different reactions. Moreover, the plant cells will be used throughout the whole cellular system, live cell systems or systems doesn't requiring cofactor regeneration of cofactors. Compared with microbial biocatalysts, plants have more complex metabolic pathways that are much less understood, so may have unknown and unique enzymes. Since most of the genomic DNA sequences from fruits and vegetables are not available, is of great importance the plant cell exploitation, with extremely good catalytic activities. (B. Xie et al., Enantioselective reduction of fluorenones in surfactant-aqueous solutionby fruits and vegetables / Journal of Molecular Catalysis B: Enzymatic 61, 2009, 284–288). Biocatalysts such as microorganisms' biomass or plant tissues are effective to be used in organic synthesis because they are readily available on the market and easy to handle (Emre M. Isin, F. Peter Guengerich, Complex reactions catalyzed by cytochrome P450 enzymes, Biochimica et Biophysica Acta 1770, 2007, 314–329).
Preliminary studies carried out have pursued the use of microbial cells and enzymes synthesized by them, plant tissues or commercial enzymes as biocatalysts in the cycloaddition reactions in order to obtain compounds with bioactive properties, fluorescent antioxidant. 4,4'-bipyridyne (bpy), ω-bromacetophenone (ωBr), dibromure of N,N'-di(p-methoxiphenacyl)-4,4'-bipyridinium and ethyl propiolate (PE) were chosen in this step as model substrates for the selection of biocatalysts.

The microorganisms tested as biocatalysts were strains of: bacteria, Pseudomonas spp., filamentous bacteria, Streptomyces spp. (Strains isolated from polar soils); yeasts, Yarrowia lipolytica, Saccharomyces cerevisiae, Candida utilis, Candida tropicalis, Candida arborea, molds, Geotrichum candidum, Aspergillus niger, Penicillium roquefortii, Epicoccum nigrum; plant cells (Armoracia rusticana, Daucus carota, Petroselinum hortense - sativum, Raphanus sativus, Allium porrum, Fagopyrum sagittatum) and commercial enzymes (lipases from Candida).

For microorganisms was tested the biocatalytic potential of biomass (whole cells), of cultures of different ages and of released extracellular crude enzyme mixtures. Biocatalysts' selection was made based on their ability to produce transformations of substrates to form fluorescent compounds, property that has been emphasized by thin layer chromatography and UV fluorescence visualization.

Selection of active cultures was achieved by inoculation and cultivation system in stationary point on YPD medium (yeast extract peptone dextrose) with agar, supplemented with

solutions of % concentrations of the starting compounds: bpy, ωBr, PE. After the colony developing was monitored the formation fluorescent compounds in the neighbouring colonies.

Studies have continued with experiments in which the yeast Yarrowia lypolitica was used as biocatalyst. Were obtained yeast cultures aged 24, 48 and 72 hours by submerged cultivation in YPD liquid medium. A volume of 5 mL culture was mixed with bpy (0,05 mmol), ωBr (0,15 mmol), PE (0,15 mmol). Reactions were conducted under very mild conditions, in solution pH 7,2, at 25°C temperature, with shaking at 200 rpm for 72 h. The reaction products were detected by thin layer chromatography (TLC). The appearance of a fluorescent spot in the chromatogram, visualized with UV light, highlights the effectiveness of biocatalysts (figure 2).

Plant cell biocatalysis reactions were carried out with the plant material cut into small pieces under sterile conditions in the same very mild conditions in phosphate buffer pH 7.2, at a temperature of 25 °C, with stirring at 200rpm for 72 hours.

All the biocatalyzed synthesis reactions were monitored by TLC (figure 2).

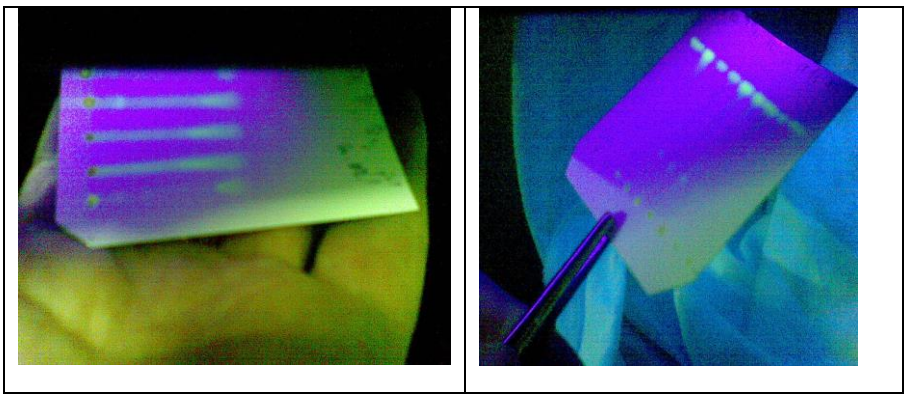


Figure 2 Biocatalysed reaction monitoring chromatograms

Biocatalysed synthesis reactions which lead to formation of fluorescent N - heterocycles have been made by two ways.

The first way was performed in two stages, with the same synthetic route that is used in the synthesis through the classical method, without biocatalysts (figure 3):

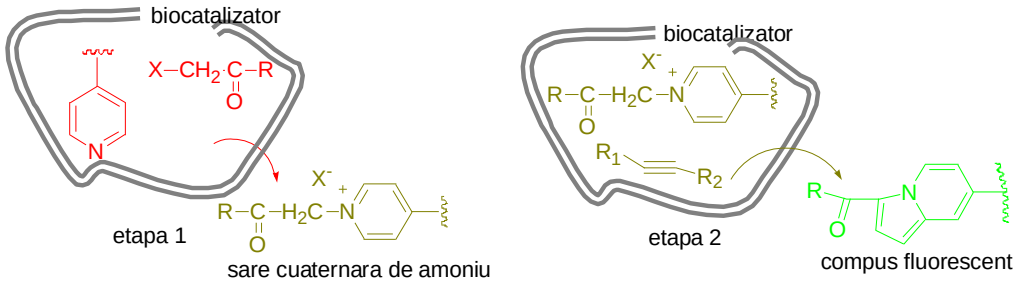


Figure 3. Biocatalysis carried out in two stages: 1- quaternary ammonium salt preparation; 2- the reaction of salts with dipolarophile to give the fluorescent indolizine cycle

2) The second way are done by synthesis "one-pot" in one step, (figure 4), method being more advantageous (shorter time, overall yield higher).

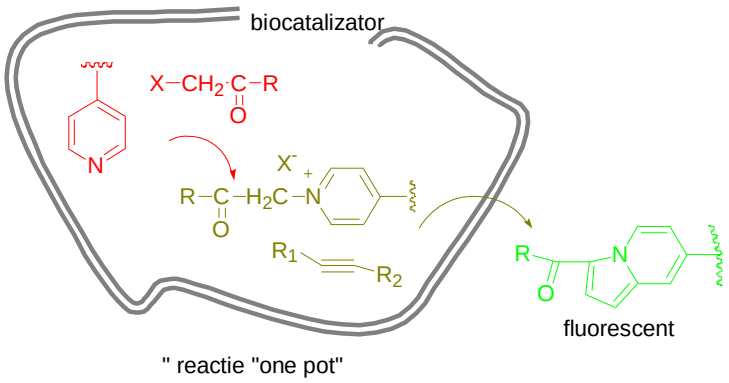
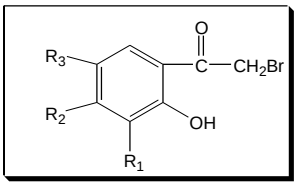


Figure 4. The one stage biocatalyzed synthesis

*In order to obtain fluorescent cycloadducts the necessary precursors are the **activated halogenated reagents** which are then used in the synthesis of new quaternary ammonium salts. The synthesis of these compounds was another accomplished objective of this stage.*

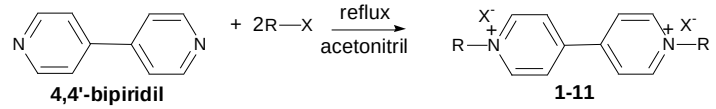
These compounds were obtained by the obtaining methods from the literature (R. Dinica, B. Furdui, G. Bahrim, M. Demeunynck, Rev. Roum. Chim, 2008, 53(1), 21)

General procedure for obtaining of halogenated o-hidroxyketones - reactive halogen derivatives

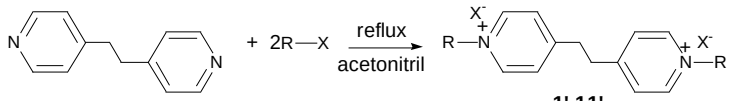


To a solution of o- hydroxyketone in glacial acetic acid is added bromine over 30 minutes and maintained under stirring. The reaction mixture was refluxed until the bleaching solution. After cooling , the mixture was poured into cold water, leaved a few hours at room temperature and the obtained solid was filtered and recrystallized from suitable solvent.

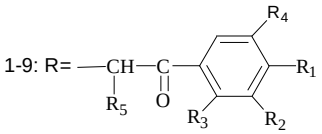
Subsequently, halogenated compounds were used to obtain the quaternary ammonium salts, precursors to obtaining the indolizines. The compounds were obtained according to the reaction scheme below:



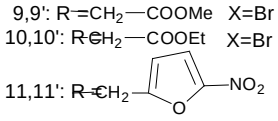
4,4'-bipiridil **1-11**



1,2-bis(4-piridil)-etan **1'-11'**



1,1': R_1=H, R_2=H, R_3= H, R_4 =H, R_5=H, X=Br
2,2': R_1=Br, R_2=H, R_3=H, R_4 =H, R_5=H, X=Br
3,3': R_1=OCH_3, R_2=H, R_3=H, R_4 =H, R_5=H, X=Br
4,4': R_1=NO_2, R_2=H, R_3=H, R_4 =H, R_5=H, X=Br
5,5': R_1=Cl, R_2=H, R_3=H, R_4 =H, R_5=H, X=Br
6,6': R_1=H, R_2=OCH_3, R_3=H, R_4 =H, R_5=H, X=Br
7.7': R_1=OH, R_2=OH, R_4 =H, R_5=H, X=Cl
8,8': R_1=H, R_2=H, R_3=OH, R_4 =Br, R_5=CH_3, X=Br



Preliminary studies demonstrated that the tested biocatalysts have the ability of bioconversion of compounds, with formation of fluorescent products, the biotransformation process being influenced by the reaction time, pH, temperature, the catalytic properties of the biocatalyst. In the case of microorganisms, bioconversion evolve differently depending on the type, energy and metabolism specificities of cells and also their age and growing conditions. In the case of plant cells used so far, the reaction was carried at higher speed, compared to the use of microbial cultures as biocatalysts.

Project manager,

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