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 January - December 2012

The objectives of this step were focused to the synthesis of organic compounds with different properties- bioactives and fluorescentes- by cycloaddition reactions, reactions which were conducted according to the "green chemistry" (reaction in the presence of the enzyme catalyst, in non-toxic solvents and activated with microwave (mw) or ultrasound(us)).

O.II.1 Synthesis of fluorescent molecules by cycloaddition reactions *was performed using enzymatic catalysis, by microwave and ultrasound activation and in non-toxic solvents (water, ionic liquids). The results of this phase consisted in the successful cyclization reactions using as* ***starting materials*** *different* ***nitrogen heterocycles,*** *various* ***halogenated derivatives*** *and compounds with* ***activated triple bond*** *bearing an electron-withdrawing group. Biocatalysed reactions were multiple because we varied the starting materials to obtain different functionalized compounds which can have different properties,* ***biologic actives, fluorescentes*** *due to the various substituents present in the molecule.*

Enzymatic reactions were conducted using both commercial enzymes and also enzymes from microbial biomass. Reactions were also activated using microwaves or ultrasound. The reactions were conducted and compared, both by the conventional method (heating, organic solvents), as well as alternative methods (biocatalysis, microwave, ultrasonic, aqueous medium, ionic liquids) to demonstrate the advantage of using biocatalysts. Reactions were carried out by the classical method in three stages (i, ii, iii).

Step (i) was carried out in acetonitrile, steps (ii), (iii) in N-methylpyrrolidone or benzene. Biocatalysed reactions carried out in an aqueous medium(at 25 ° C, 40 ° C or 50 ° C) were performed in a single step ("one-pot") simultaneously adding to the reaction mixture the N-heterocycle, the alkylating agent and the dipolarophile. Reactions were monitored by thin layer chromatography (TLC) and HPLC / MS. In the reactions carried out in the presence of commercial enzyme catalysts we have used: Candida Antarctica lipase CAL A (10 mg) - Ia sample, CAL B lipase from Candida Antarctica (10 mg) - Ib sample, lipase from Candida Rugoza (10 mg) - Ic sample, alcohol dehydrogenase from Sacharomyces cerevisiae (2.5mg) - Id sample, Deinococcus radiodurans recombinant alcohol dehydrogenase from E. coli (10μL) - Ie sample, pig pancreas lipase (10 mg) - sample If enzymefrom horseradish (Amoracia rusticana) - Ih sample.

Simultaneously, control reaction was also performed in aqueous medium at pH 7 in the absence of enzyme catalysts - Im. The progress of the reaction was monitored versus pure product (M), previously synthesized by us (R.Dinica, et all, Synlett, 1013, 2001), the presence of the indolizine product being highlighted by TLC (as a powerful green- blue fluorescent spot, Rf 0.85, Figure 1), by HPLC (retention time 3.45 -eluent CH_3CN) by UV-VIS (characteristic peaks at 231nm, 277 nm, 352 nm, 411 nm, respectively (Figure 2a, b) and in mass spectrum (APCI) where shows specific molecular ion peak. By analyzing the obtained chromatograms (Fig 1 a, b, c), lipase catalyzed reactions Cal A, Candida Rugoza and pig pancreas lipase, had the highest conversion to the desired product, while Cal B had a conversion lower. The reactions catalyzed by dehydrogenases went rapidly with almost total conversion to the desired product. It is noted also strong catalytic effect of horseradish enzymes (Amoracia rusticana), by carrying out the cycloaddition reaction relatively fast "one-pot", with the formation of the desired cycloadduct of a high purity, in the reaction medium seen the absence of other byproducts, occurring more or less in other enzymatic reactions, requiring purification of the products.

In nonenzymatically catalyzed reaction cycloadduct was obtained in low yield. Activation with ultrasound or microwave of biocatalysed reaction led to increased efficiency and the shortening of the reaction time. In

Figure 3 it can be seen that the reaction catalyzed by CAL A occurs with a high conversion in 2 hours when the reaction is activated with ultrasound as compared with the inactivated reaction, carried out in 48 hours.

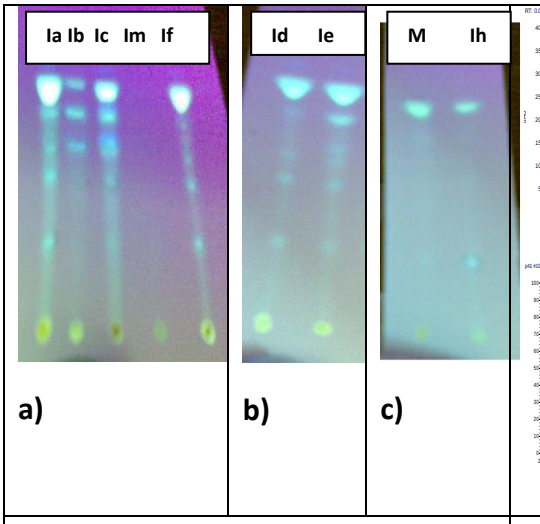


Figure 1. Thin layer chromatograms of chloroform extracts after 24 h

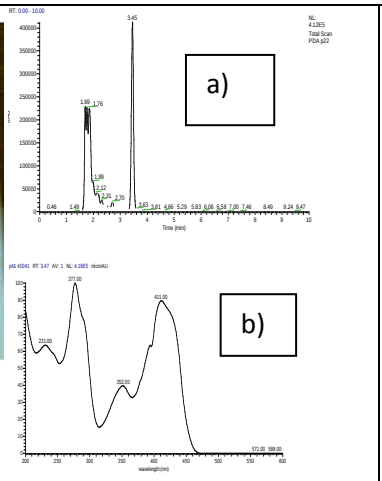
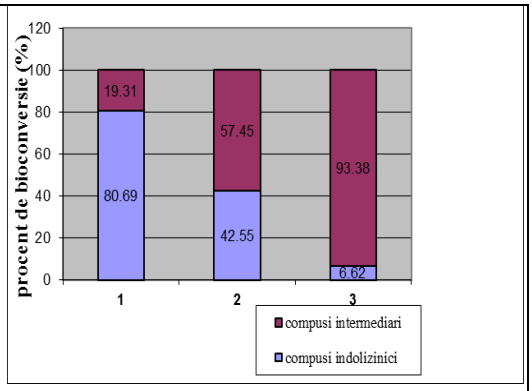


Figure 2 HPLC (a) and UV-Vis (b) of indolizine I1



1- CALA; 2-CAL B 3-reaction without enzyme

Figure 3. Performance of lipases in the reaction to produce the cycloadduct I_1

Preparation of new building blocks with the oxime function in the 4-position, such as the quaternary pyridinium salts, in order to obtaining the new bioactive compounds, is important , the oxime function being found in a number of important drugs used in the treatment of intoxication with organic phosphate (Acharya, Eur. J. Med. Chem. 46 3926, 2011) antibiotics (Lorke, Curr. Med. Chem., 15 743, 2008), or over-expressed with kinase inhibitory activity in neurodegenerative diseases such as Alzheimer's or Parkinson's disease. Acting as artificial nucleases may become useful tools in biotechnology, gene therapy or chemotherapy (Fernandes, Bioorg. Med. Chem. Lett., 18, 4499, 2008).

*Thus, starting from **4-pyridine-aldehyde** we obtained the oxime by two ways. Developed reactions in the presence of us or mw takes place in a short time (1-6h) and quantitative yield. Generating new activated alkyne with function amide, ester and thioester will lead to new potential bioactive indolizine. It is interesting to note that the reactions occurring only catalyzed by enzymes (CAL A, CAL B, porcine lipase type II), while the classical pathway does not. Some of these studies have been performed in external internship, 2012, at UJF, Grenoble, France and presented in international scientific meetings (Rencontres en Chimie Organique Biologique, Recob14 2012, Grenoble, France, Rhone-Alpes Journée SCF 2012); others are sent for publications. The same types of reactions were catalyzed by certain microorganisms selected in stage I of the project and evaluated by TLC and HPLC / MS. At this stage were also made reactions biocatalysed by extract microorganisms . TLC and HPLC chromatograms / MS showed that the first results appear after 24 hours of reaction. Reactions were catalyzed using cultures of microorganisms at 72, 96, 120 and 124h of cultivation over 6 days of reaction. All experiments were performed in triplicate. Evaluation of the results of the reactions biocatalysed by microbial biomass led to important conclusions such as that the used enzymatic extracts catalyze in different ways the cycloaddition reactions. The microbial culture PO1 and GC and liquid microbial culture RO13 obtained after 96 h of cultivation (4a) were the most active between the 4 strains used, 27D, P01, RO13 (Yarrowia lipolytica) and GC (Geotrichum candidum) . From the graphs 4b, 4c it can be observed that the GC liquid microbial culture has high biocatalytic capacity after 120h of cultivation and liquid microbial culture of strain RO13 after 144h of cultivation in submerged system. Figure 4d shows the best enzymatic extracts used in biosynthesis of compound I1. An important observation was also that although bioconversion yield is lower, we obtain by biomass catalyzed reactions products of the highest purity.*

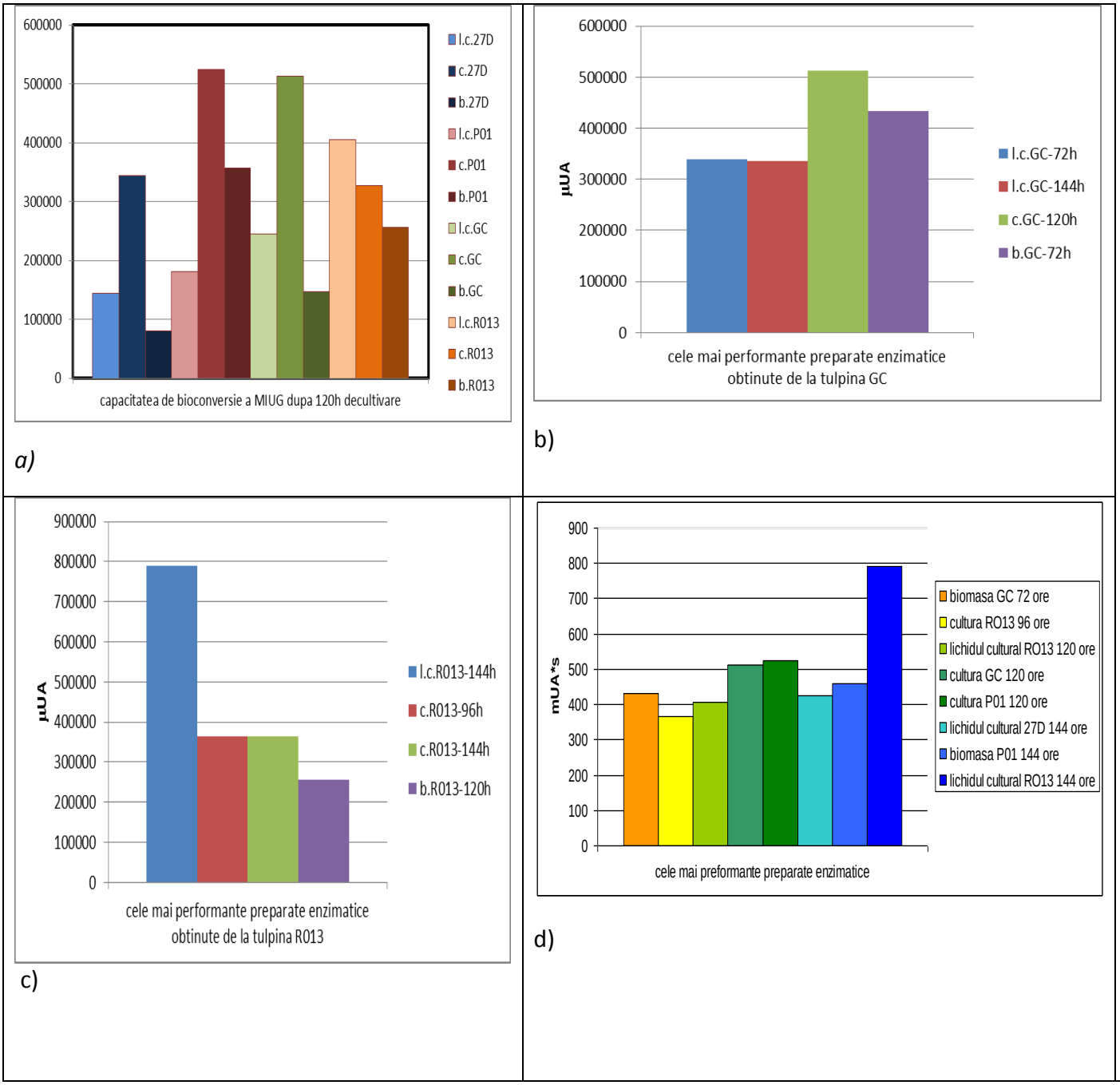


Figure 4 The results of the cycloaddition reactions by bioconversion with microorganisms from the MIUG collection

*The results show that the reactions carried out in the presence of biocatalysts are more advantageous for the following reasons: reactions were conducted in much shorter time, it eliminates toxic solvents, the obtained compounds have high purity and yields are comparable to those obtained by the classical pathway. Part of the results of this research were published in Tetrahedron, 68, 2012, 6164-6168, presented at international conferences (Septieme Colloque Franco-Roumain de Chimie Aplique-**COFrRoCA,** 27-29 iunie, 2012, Bacău, **13th Tetrahedron Symposium**, Challenges in Bioorganic & Organic Medicinal Chemistry, 27,-29 iunie, 2012, Amsterdam, Olanda,**Third Regional Symposium on Electrochemistry South-East Europe**, Bucuresti, 13-17 mai 2012, 63rd, **Annual Meeting of the International Society of Electrochemistry**, Electron transfer in proteins and enzymes, 19-24 August 2012, Praga, Cehia) or submitted for publication in Marine Drugs-FI 3,85.*

***O.II.2 Synthesis of fluorescent molecules by coupling reactions** consisted in carrying out the cyclisation reaction using as starting material 4-bromo-1,8-naftalinanhydride, amine compounds, and compounds with*

a triple bond activated by electron-withdrawing group to access to novel fluorescent triazole derivatives which can be used in the controlled synthesis of complex bioactive molecules. Enzyme-catalyzed reactions were performed in ionic liquids, less toxic solvents, which are selective, requires no other protection functions (amines, alcohols ...) present in synthons and generally give the the pure compounds (followed by TLC), who generate only little waste.

Conclusions *For the first time were obtained new compounds, with fluorescent properties, by "one-pot" reactions (which involves three components) using enzymatic catalysis. Many of the methods for obtaining of these compounds require metal catalysts, such as copper, platinum, silver, gold or palladium, long reaction toxic solvents (Singh, Eur.J.Med.Chem, 46, 5237, 2011, Liu, Org . Biomol. Chem., in August 2449, 2010, Wang, Chin. J. Chem., 24, 279, 2006). For the first time in cycloaddition reactions we used biocatalysts commercial enzymes, microorganisms from the UDJ collection (MIUG) or enzymes from plant sources (horseradish). The reactions took place in an aqueous medium or ionic liquids at temperatures of 25-50°C, reactions were performed by activation with us or mw. It was found that both enzymes and us or mw increase the rate of reaction and, in some cases increase the purity of the product. The results are promising since the reaction are made under mild conditions, with the enzymes that can be obtained easily and are renewable materials. The advantage of using biocatalysts are compared with the processes performed by classical reaction.*

The use of new biocatalytic process requires less energy consumption due to the mild conditions of work and fewer steps and the use of less toxic solvents, and can thus be regarded as a process that occurs within the concept of "green chemistry".

The research results obtained during this stage helps us to elucidate the mechanism of action of biocatalysts in cycloaddition reactions. The molecules used as substrate in our reactions are charged species. Although the active sites of enzymes exhibit hydrophobic areas, global electrostatic field produced by protein with all its polar and charged groups can lead to a electrostatic potential with a net charge in the active site region beeing possible that this potential target substrate in the active site of opposite charge , increasing the probability of producing contact. The ways in which the electric field can assist in the crosslinkage in our reactants may be illustrated as in Figure 5 and can be used even by a single enzyme.

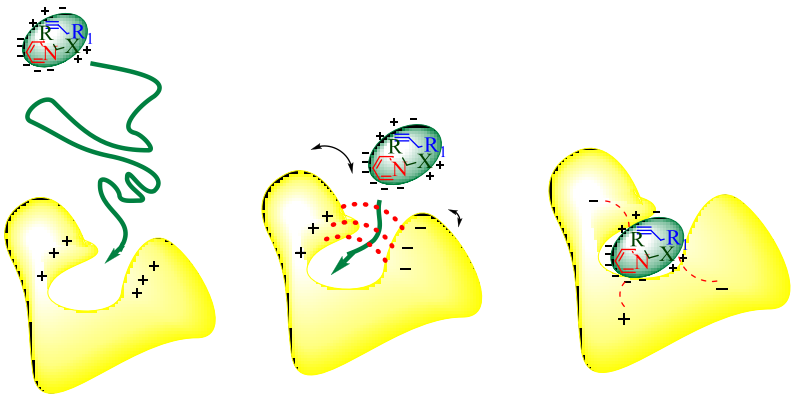


Figura 5

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