Scientific report

concerning 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
during October 2011 – October 2013

All of the objectives assumed for the 2011-2013 period have been achieved. The collaboration between the partners involved in the project has produced scientific papers, published or pending publication, that present various activities related to the project. In this report the objectives are presented briefly, accentuating the scientific data that has not yet been published.

*The aim of the project was to **synthesize** organic compounds with various properties – **bioactive, fluorescent** – through **cycloaddition** reactions, performed within the guidelines of green chemistry (**enzyme catalysts**, non-toxic solvents, reactions activated by micowaves – **mw** or ultrasounds – **us**) and the analysis of said compounds from the point of view of their structure, purity, physico-chemical properties, as well as the evaluation of their biological properties.*
The results obtained during the stages included in the evaluation period are presented below.

Stage I

O.I.1. Biocatalyst selection

*The microorganism **selection** process was based on the eveluation of the degree of toxicity of the reagents of a model-reaction (4,4'-bipyridyl, 2-bromacetophenone, ethyl propiolate) upon s**ix bacterial** strains, **nine yeast** strains and **six mold** strains, included in the microorganism collection of the Bioaliment Platform of Dunarea de Jos University of Galati, encoded MIUG. During the selection process, we have monitored samples resulting from the incubation of 21 microorganisms for 5 days in the presence of four different concentrations of the reagents. The process revealed three yeast strains, one mold strain and one bacteria strain to be the most resistant. The strains Yarrowia lipolytica RD13 MIUG, Yarrowia lipolytica RD14 MIUG, Yarrowia lipolytica RD15 MIUG, Geothricum candidum MIUG 27 and Pseudomonas fluorescens MIUG MP11 were selected for the further evaluation of their biocatalytic potential on reactions that yield heterocyclic compounds, in neutral aqueous medium.*

O.I.2. The synthesis of the starting materials for the cyloaddition reactions

*We have established faster and cheaper methods to obtain and purify numerous compounds, that have been starting material for cyclisation reactions, compounds such as rea**ctive halogenated compounds**, **quaternary pyridinium salts**, with or without an oxime function (dipoles in the cylisation reactions) and **alkynes**, compounds with a triple bond, that bear a propiolamide group derived from alifatic amines (dipolarophiles in the cylisation reactions). Obtaining new synthons functionalised with an oxime group, such as quaternary pyridinium salts was aimed at improving the bioactivity of our compounds, as the oxime function plays a key role in numerous pharmaceuticals used to treat organophosphate poisoning (Acharya, Eur. J. Med. Chem. 46 3926, 2011,) antibiotics (Lorke, Curr. Med. Chem., 15 743, 2008), or molecules that can inhibit kinase, overexpressed in neurodegenerative disorders such as Alzheimer or Parkinson. Acting as artificial nucleases, they could become effective tools in biotechology, gene therapy or chemotherapy (Fernandes, Bioorg. Med. Chem. Lett., 18, 4499, 2008).*
Randamentele reacțiilor au fost foarte bune, cuprinse între 85 și 96%. Utilizând activarea cu ultrasunete acești compuși se obțin mai rapid, în solvent mai puțin nociv, păstrându-se sau îmbunătățindu-se randamentul obținut prin metodele descrise anterior în literatură.

In this stage we obtained and purified twelve dipolar synthons and two precursors and three dipolarophile synthons through biocatalysed reactions. The 12 quaternary ammonium salts were also obtained via reactions activated by ultrasounds. The yields of the reactions were very good, between 85 and 96%. Using ultrasound activation of these compounds are obtained faster, using a less harmful solvent, while maintaining or improving the yield obtained using the methods described previously in the literature.

Stage II

O.II.1. The synthesis of fluorescent molecules via cycloaddition reactions *was performed using enzymatic catalysis, microwaves, ultrasound and non-toxic solvents. The results of this stage consisted in performing successful cyclization reactions using as* ***starting materials*** *different* ***nitrogen heterocycles****, various reactive* ***halogenated compounds****,* ***quaternary ammonium salts*** *and compounds bearing a* ***triple bond*** *activated by an electron-withdrawing group (compounds that were synthesized in the first stage of the project, scheme 2). There were multiple biocatalysed reactions because we varied both biocatalysts and starting compounds to obtain differently functionalized compounds which can have different properties,* ***bioactive****,* ***fluorescent,*** *due to the substituents present in the molecule. The enzymatic reactions were carried out using both commercial enzymes (Candida antarctica lipases CAL A and CAL B, Candida Rugoza lipase, Sacharomyces cerevisiae alcohol-dehydrogenase, Deinococcus radiodurans recombinant from E. Coli alcohol-dehydrogenase, porcine pancreatic lipase, horseradish peroxydase) as well as microbial biomass enzymes (from the microorganisms selected in the first stage) and of plant origin (Amoracia rusticana, Alliacee). From the strains grown on more days cultures were obtained total biomass and cultural liquids that served as enzyme biocatalysts. Reactions were activated using microwaves or ultrasound. From the strains grown on more days we obtained total cultures, biomass and cultural liquids that served as enzyme biocatalysts. Reactions were also activated using microwaves or ultrasound. Scheme 1 shows the acces route to differently functionalized fluorescent compounds, from the indolizine class (I 1-24). The reactions were conducted comparing the conventional method (heating, organic solvents) with alternative methods (biocatalysis, microwaves, ultrasounds, aqueous medium) to demonstrate the advantage of using biocatalysts.*
The reactions carried out using the conventional method take place in three phases (i, ii, iii), according to Scheme 1. Step (i) was carried out in acetonitrile, steps (ii), (iii) in N-methylpyrrolidone or benzene. Biocatalysed reactions were carried out in an aqueous medium at 25 °C and 40 °C or 50 °C in a single step ("one-pot") simultaneously adding the N-heterocycle, the alkylating agent and the dipolarophile to the reaction mixture. Reactions were monitored by thin layer chromatography (TLC) and HPLC/MS. The results show the catalytic activity of these enzymes, the reactions catalyzed by the lipases Cal A, Candida rugosa, the dehydrogenases and porcine pancreatic lipase have shown the highest conversion rate. The strong catalytic effect of horseradish (Amoracia rusticana) is observed, by carrying out the "one-pot" cycloaddition reaction relatively fast, with the formation of the desired cycloadduct of a high purity in the reaction medium, in the absence of other byproducts, which occur in more or less amount in the other enzymatic reactions requiring purification of products.

Following the completion of reactions biocatalysed with enzyme preparations derived from MIUG microorganisms, some biocatalysts which led to significant biotransformation yields were identified: the cultural liquid of Yarrowia lipolytica strain RD14 obtained from its cultivation for six days, which led to the best biotransformation yield, Yarrowia lipolytica RD15 total culture after five days of cultivation, the biomass produced by Geotrichum candidum MIUG 27 after three days of

cultivation (which yielded the most pure products) and the total culture of Pseudomonas fluorescens strain MP11 MIUG obtained after 72 hours of cultivation at 25 ° C, resulting in yields between 56 and 68%.
In the case of the reaction without enzyme catalysts, the cycloadducts were obtained with lower yields. Activation using ultrasound or microwave on the biocatalysed reaction led to increased efficiency and shorten the reaction time time. A reaction catalyzed by CAL A, for example, took place with a high conversion in 2 hours when the reaction was activated with ultrasound as compared with the inactivated reaction, carried out in 48 hours.

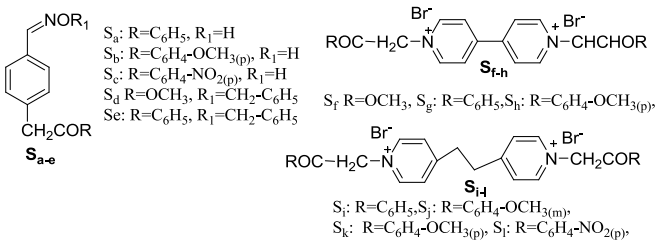
O.II.2. The synthesis of fluorescent molecules by coupling reactions *consisted in carrying out the cyclisation reaction using as starting material 4-bromo-1,8-naphtalenanhydride, amine compounds, and compounds with a triple bond activated by electron-withdrawing group in order to acess* ***novel*** *fluorescent triazole derivatives (scheme 3), which could be used in the controlled synthesis of complex bioactive molecules.*
The enzyme-catalyzed reactions were performed in ionic liquids, less toxic solvents, are selective, require no protection of the functions (amines, alcohols ...) present in synthons and generally yield pure compounds (followed by TLC) who generate only a few waste.
The results of this stage show that the reactions carried out in the presence of biocatalysts are more advantageous for the following reasons: reactions are carried out in less time, toxic solvents are not involved, the compounds obtained have high purity and yields are comparable to those obtained using the classical pathway. Part of these results have been published in ISI Journals such as ***Tetrahedron,*** *68 , 6164-6168, 2012-IF- 3,25,* ***Tetrahedron****, 69, 5495-5500, 2013- IF- 3,25,* ***Marine Drugs,*** *11(2), 431-439, 2013- IF- 3,84 or presented at international conferences (REncontres en Chimie Organique Biologique,* ***Recob14****, 2012, Grenoble, France; Journée SCF Rhone-Alpes, martie 2012; Septieme Colloque Franco-Roumain de Chimie Aplique-****COFrRoCA,*** *iunie, 2012, Bacău;* ***13th Tetrahedron Symposium****, Challenges in Bioorganic & Organic Medicinal Chemistry, 27,- iunie, 2012, Amsterdam, Olanda;* ***Third Regional Symposium on Electrochemistry South-East Europe****, Bucuresti, mai 2012; 63rd,* ***Annual Meeting of the International Society of Electrochemistry****, Electron transfer in proteins and enzymes, August 2012, Praga, Cehia).*

Stage III
O.III.1. Physico-chemical characterization of the synthesized compounds. Characterization of compounds by spectral analysis, IR, NMR, MS
All new compounds obtained for the first time in our laboratory through bioconversion reactions were characterized by spectral methods, IR, NMR and MS to prove their structure. The compounds which have been obtained both by conventional and biotransformation reactions were characterized in order to prove their structure, by the methods mentioned above.

O.III.2 ***The biological characterization of the synthesized compounds. Microbiological activity by disk diffusion method. The antioxidant activity of the compounds obtained***
The analyzes that were carried out to determine the biological activity of the compounds obtained were the following: Determination of the antimicrobial activity - agar diffusion method (disks); Determination of antioxidant activity - DPPH method; Determination of antioxidant activity - β-carotene bleaching method; Determination of anti-acetylcholinesterase activity - the Ellman method. Measurements were performed with a Microplate reader Nanoquant Tecan Infinite Pro 200 purchased in phase II of the project.

For the antimicrobial analysis, first we selected the intermediates, quaternary ammonium salts (scheme 4). Antibacterial and antifungal properties of similar compounds are known in the

industrial, medical and cosmetic industries. Inhibition tests have used the method of agar diffusion and were conducted on nine microbial strains from the MIUG collection. In general, the quaternary ammonium salts showed moderate antimicrobial activity, those derived from bipyridyl leading to the poorest results. Two of the compounds, Sb and Se, pyridine derivatives, have shown to strongly inhibit the growth of bacteria. Two other salts, Sg and Sh, have demonstrated a broad spectrum of action, but moderate inhibition (Figures 2-4).



Scheme 4 Quaternary ammonium salts analysed as antimicrobial agents

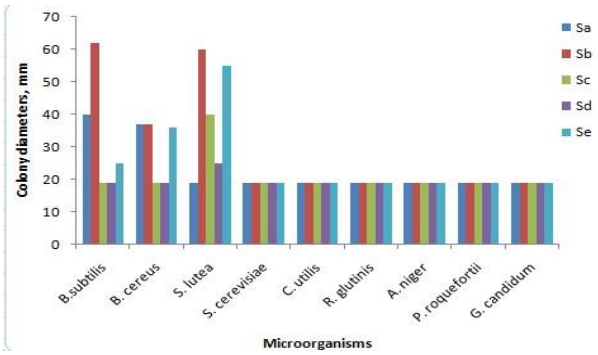


Figure 2 Bacterial inhibition

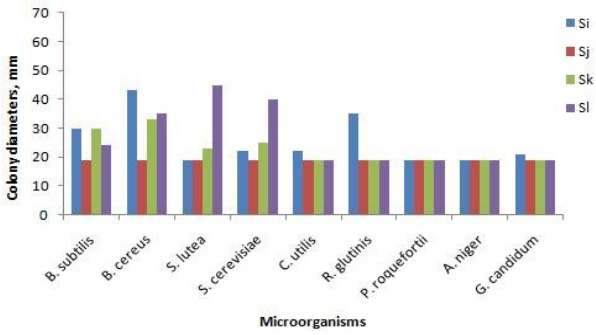


Figure 4 Antimicrobial activity of salts Si-Sl

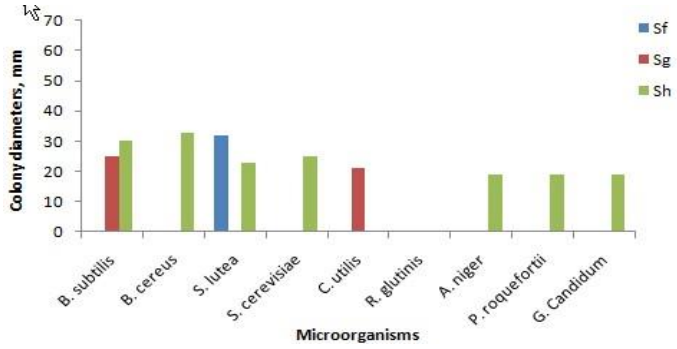


Figure 3 Fungal inhibition

*The **antioxidant activity**, measured by two methods and the anti-acetylcholinesterase activity were carried out for 28 organic compounds obtained in the previous stages. The results are compared with the antioxidant respectively anti-AChE activity of some known reference compounds for the biological activity analyzed (rutin, BHA and vitamin C for the antioxidant activity and galantamine, donepezil and rivastigmine for anti-acetylcholinesterase activity).*

*The **determination of antioxidant activity using the DPPH free radical scavenging method** required the processing of 744 samples, six concentrations of each compound were analyzed at four time intervals.*
During the analysis of antioxidant activity of the quaternary ammonium salts prepared by non-conventional activation methods, two compounds have shown very good antioxidant activities, with IC 50 under 20 mg / mL (Sg, Si), and a compound of IC 50 slightly over 20 mg / mL (Sh) but with a very good reduction speed (Figures 5-7).

After analyzing the antioxidant activity of the indolizine compounds using the same method, we determined that indolizine Ik has and IC 50 below 20 mg / mL and a high reduction speed, at 40 mg / mL showing antioxidant activity of about 52% after only 5 minutes of reaction.

The determination of antioxidant activity using the β-carotene bleaching method required the processing of 1860 samples, six concentrations of each compound were analyzed at 10 time intervals.
From the quaternary ammonium salts, the best antioxidant activity determined using the β-carotene bleaching method was that of a salt derived from pyridine, Se, that of 85% after 180 minutes, at 40 mg / mL.
After analyzing the antioxidant activity of indolizine compounds through the β-carotene bleaching method, two compounds stood out with high antioxidant activity compared to the reference compounds analyzed (rutin, BHA and vitamin C). Thus, indolizine Im and Ip had a very good stability over time, with values of the antioxidant activity of 91% and 89%, at the minimum concentration analyzed, after 180 minutes (Figures 9-10).
Part of the results obtained in this stage have been published in international journals or presented at international conferencessuch as ***Current opinion Biotechnology,*** *24, 2013, (International Conference of Applied Sciences Chemistry and Chemical Engineering* ***CISA****, Bacău, Romania 2013,* ***European Biotechnological Congres,*** *Bratislava 2013; 2nd International Symposium on Green Chemistry: Renewable Carbon and Eco-efficient Processes* ***ISGC2,*** *La Rochelle, France, 2013; World Chemistry* ***IUPAC Congres****, Istanbul, Turkey, 2013;* ***XIIth International Conference on Molecular Spectroscopy*** *- From Molecules to Nano- and Biomaterials, Wroclaw, Poland, 2013;* ***Euroaliment,*** *Galati, Romania, 2013) and other results are pending publishing or being analysed.*

Conclusions *For the first time novel compounds have been obtained, from different classes of organic compounds such as quaternary ammonium salts (compounds known in the literature to have important antimicrobial properties), alkynes activated by electron-withdrawing groups and compounds with fluorescent properties, from the class of heterocyclic nitrogen compounds (indolizine, triazole). The latter were obtained by "one-pot" reactions (one step in which three components are involved) using enzymatic catalysis. Many of the methods for obtaining these compounds require metal catalysts, such as copper, platinum, silver, gold or palladium, long reaction time, toxic solvents. For the first time, we used as biocatalysts commercial enzymes, microorganisms from the collection of UDJ Platform Bioaliment enzymes from plant sources (horseradish) in cycloaddition reactions. The reactions took place in aqueous medium, or non-toxic solvent (ionic liquids) at temperatures of 25-50°C, reactions performed also after activation using US and mw. It was found that enzymes, US and mw increase the reaction speed and, in some cases increase the purity of the product. The results are promising since the reactions take place under mild conditions, with enzymes that can be purchased or obtained easily and are renewable materials. The biological studies conducted proved remarkable biological activity of some compounds synthesized by enzymatic reactions. A quaternary salt derived from pyridine, Se, had the best antioxidant activity of all the compounds assayed, determined with the β-carotene bleaching method. Among the salts derived from bipyridyl two compounds have emerged, Sg and Sh, with very good results against the DPPH free radical and also a high inhibition of AChE. The compound Si showed very good DPPH antioxidant activity and antimicrobial assays have shown it to be a compound with a broad spectrum of action. Three indolizine derivatives led to significant results, being the most active compounds against β-carotene bleaching.*

The biocatalytic processes used by us for the first time in obtaining said heterocyclic compounds require ***lower power consumption*** *due to the mild reaction conditions (cheap biocatalysts, low T),* ***fewer steps*** *and the use of* ***less toxic solvents (water)*** *can thus be considered a process that takes place in the spirit of the concept of "****green chemistry****". These latter findings are substantiated by the results obtained from the application of the "green matrix", comparing the enzymatic process*

to the chemical cycloaddition reaction model, which is shown in the table below. For the biocatalyzed reaction all analyzed factors have values close to those given in literature [David JC Constable, et all, Green Chemistry, 2002] for a process to be considered as conducted in the concept of sustainable chemistry.

The objectives proposed so far in the PCCE- project have been completely achieved. The collaboration between the partners involved in the project had as a result the development of papers published, submitted for publication, or that are being drafted, presented the results of the interdisciplinary research performed for this project. The results obtained in this period were disseminated as follows: four papers published in ISI journals; 15 papers presented at international conferences, a doctoral thesis completed, plus a significant number of manuscripts submitted for publication.
The project has supported and continues to support three projects of bilateral cooperation, two French and one with China involving several members of this project. Also, research conducted within the project led to the development of other Idei projects that have resulted in a project proposal in the competition Parteneriate 2013.

During this period, using financial resources from the amount allocated to this project, a research laboratory was equipped with modern instruments and equipment, this being the location in which the project activities take place.the amount of financial resources allocated to this project was equipment laboratory research now in progress project activities.
To obtain and process these results, together with experienced researchers, young researchers contributed significantly in the framework of the project, thus reaching the objective of human resource training.

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