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 2011 – October 2016***

The project aims was 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 (Singh G. S. et al., 2011), fluorescent, by conventional and unconventional methods using biocatalysts, microwave activation, ultrasounds, methods known as belonging to "green chemistry".
The project objectives have been met and are summarized below.

O.I.1. Biocatalysts selection

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., 2009). 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 (E. M. Isin et al., 2007).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, ω-Br, PE. After the colony developing was monitored the formation fluorescent compounds in the neighbouring colonies.

Reactions were conducted under very mild conditions, in solution pH 7,2, at 25^{o}C temperature, with shaking at 200 rpm for 72 h. The reaction products were detected by TLC and HPLC. The appearance of a fluorescent spot in the chromatogram, visualized with UV light, highlights the effectiveness of biocatalysts.

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.

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 biocatalyst (published results in Curr.Opinion Biotech, 2013, IF 8,4).

O.I.2 Obtaining of starting materials reactives derivatives halides and new quaternary salts of ammonia

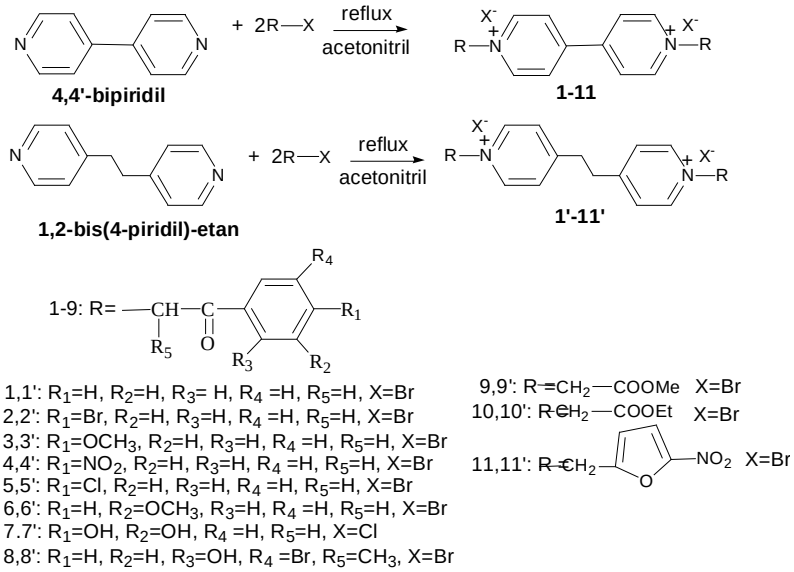
*In order to obtain fluorescent cycloadducts the necessary precursors are the **activated halogenated reagents** which are then used in the synthesis of new quaternary ammonium salts. The synthesis of these compounds was another accomplished objective of this stage.*

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.

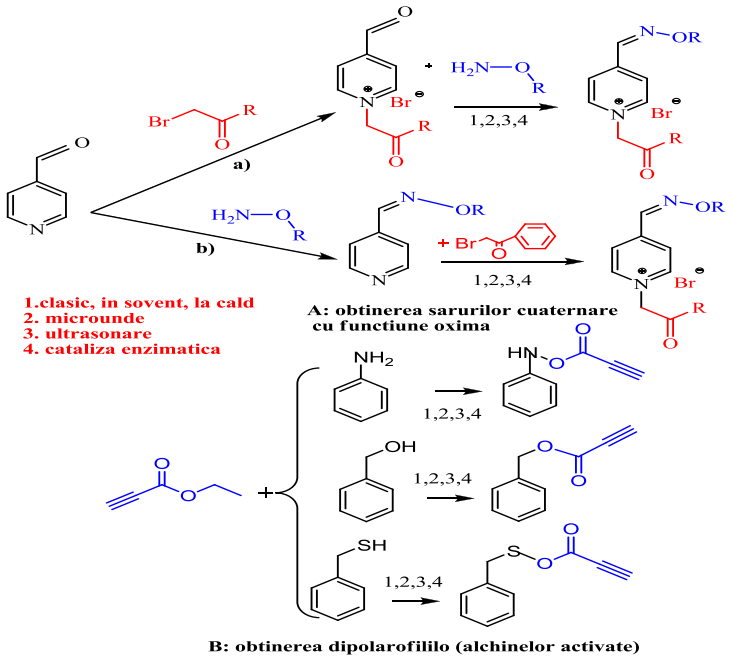
Subsequently, halogenated compounds were used to obtain the quaternary ammonium salts, precursors to obtaining the indolizines (published results in Tetrahedron, 2012, I.F. 2,621). The compounds were obtained according to the reaction scheme below:



Schema 1. The synthesis of quaternary ammonium salts

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, 2011) antibiotics (Lorke, 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, 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 (published resultsin în Tetrahedron, 2013, I.F. 2,621).*



Schema 2. Obtaining of intermediates used in cycloaddition reactions

O.II.1 Synthesis of fluorescent molecules by cicloaddition reactions

Tis objective 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.*

"Cick" chemistry is a concept proposed by Sharpless in 2001 describing the coupling reactions between two chemical reactive subunits that can easily generate new compounds (Kolb, H. C., 2004). By definition, these reactions should be very fast, to be flexible and use very wide, and lead to stereospecific formation of a single product with high efficiency. In addition, "click" reactions must be carried out under mild reaction (ideal in water at room temperature) and does not generate toxic byproducts. Finally, the product formed would be easily purified and stable under physiological conditions.

The most popular type is the reaction of the 1,3-dipolar cycloaddition reaction between azide and alkyne groups which results in the formation of a 1,2,3-triazole ring. Original Huisgen reaction temperature is high and leads to a mixture of the two regioisomers 1,4 and 1,5-triazoles (Figure 1).

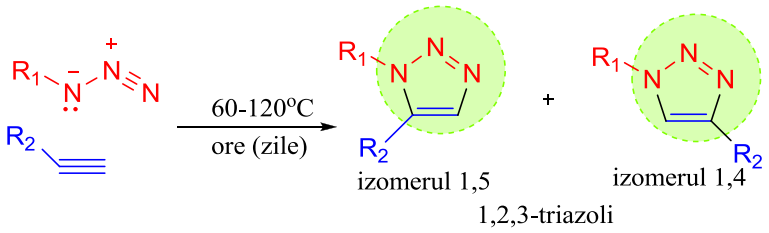
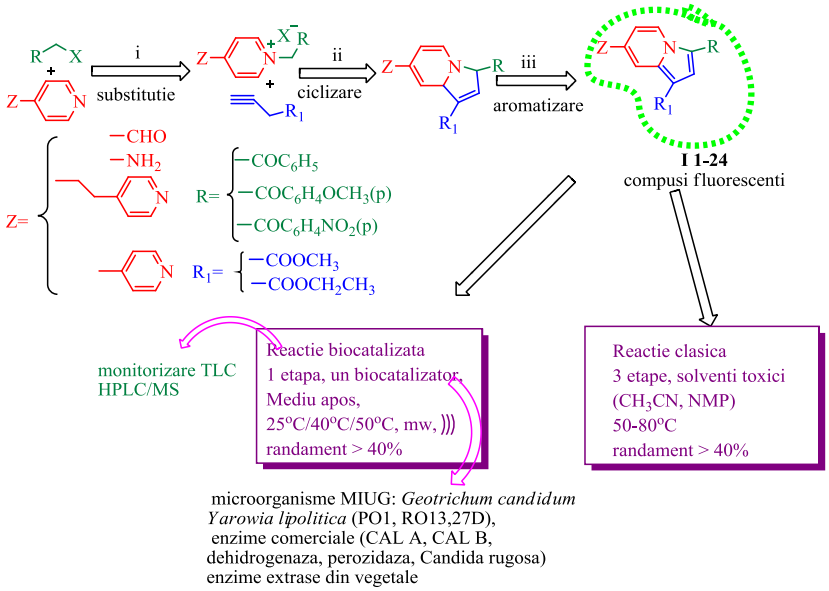


Figure 1. Original Huisgen reaction

Considering the various "click" methods we continued to study in detail cycloaddition reactions to obtain new molecules, bioactives (antibacterial, antioxidant and fluorescent) and explore their use in chemistry "click".

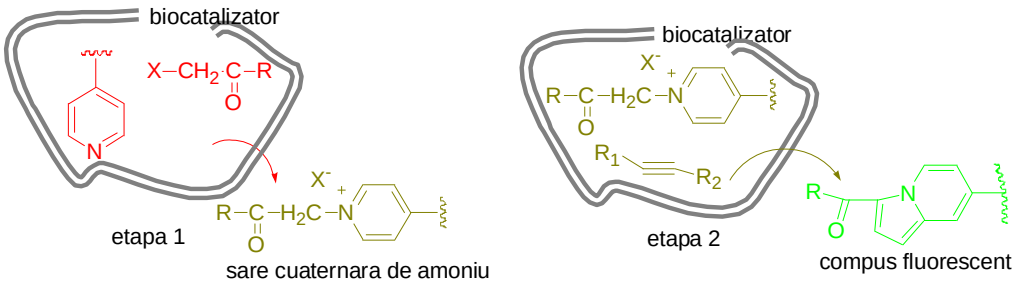
Enzymatic reactions were conducted using both commercial enzymes and also enzymes from microbial biomass. Reactions were also activated using microwaves or ultrasound. The reactions were conducted and compared, both by the conventional method (heating, organic solvents), as well as alternative methods (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), schema 3:



Schema 3.Cycloadducts synthesis

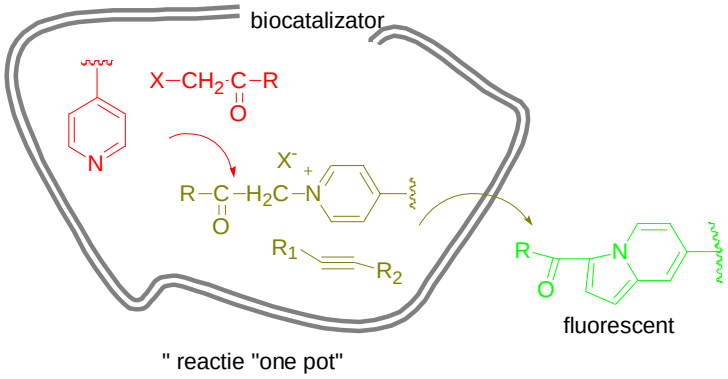
Step (i) was carried out in acetonitrile, steps (ii), (iii) in N-methylpyrrolidone or benzene. Biocatalysed synthesis reactions which lead to formation of fluorescent N - heterocycles have been made by two ways.

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:



Schema 4. Biocatalysis carried out in two stages: 1- quaternary ammonium salt preparation; 2- the reaction of salts with dipolarophile to give the fluorescent indolizine cycle

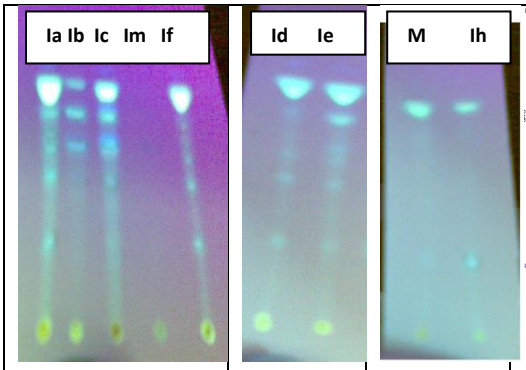
2) The second way are done by synthesis "one-pot" in one step, (figure 4), method being more advantageous (shorter time, overall yield higher).

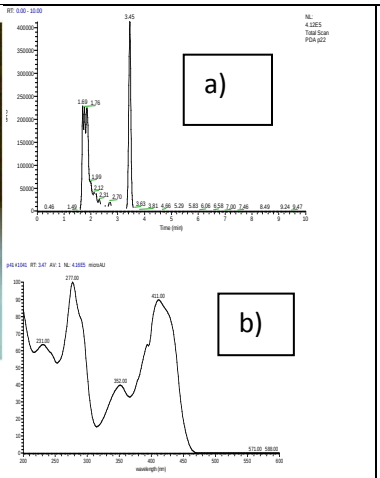


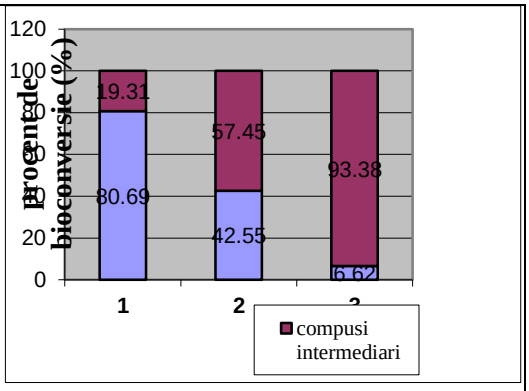
Schema 5. The one stage biocatalyzed synthesis

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, CAL B, lipase from Candida Rugoza alcohol dehydrogenase from Sacharomyces cerevisiae, , pig pancreas lipase enzyme from horseradish (Amoracia rusticana).
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 by UV-VIS and in mass spectrum (APCI) where shows specific molecular ion peak(figure 2) (published results in Marine drugs, 2013, IF 4.031 şi Molecules, 2016, IF 2,749).







1- CALA; 2-CAL B 3-reacţia fără enzime

Thin layer chromatograms of the extracts in chloroform after 24 h

The HPLC chromatogram (a) and the UV-Vis spectrum (b) of the product I_1

Cromatograma HPLC (a) şi spectrul UV-Vis (b) al produsului Performance of lipases in the reaction of producing the cycloadduct I1

Figure 2. The results obtained from catalyzed reactions

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 2 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.

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 (figure 3) 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.

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.

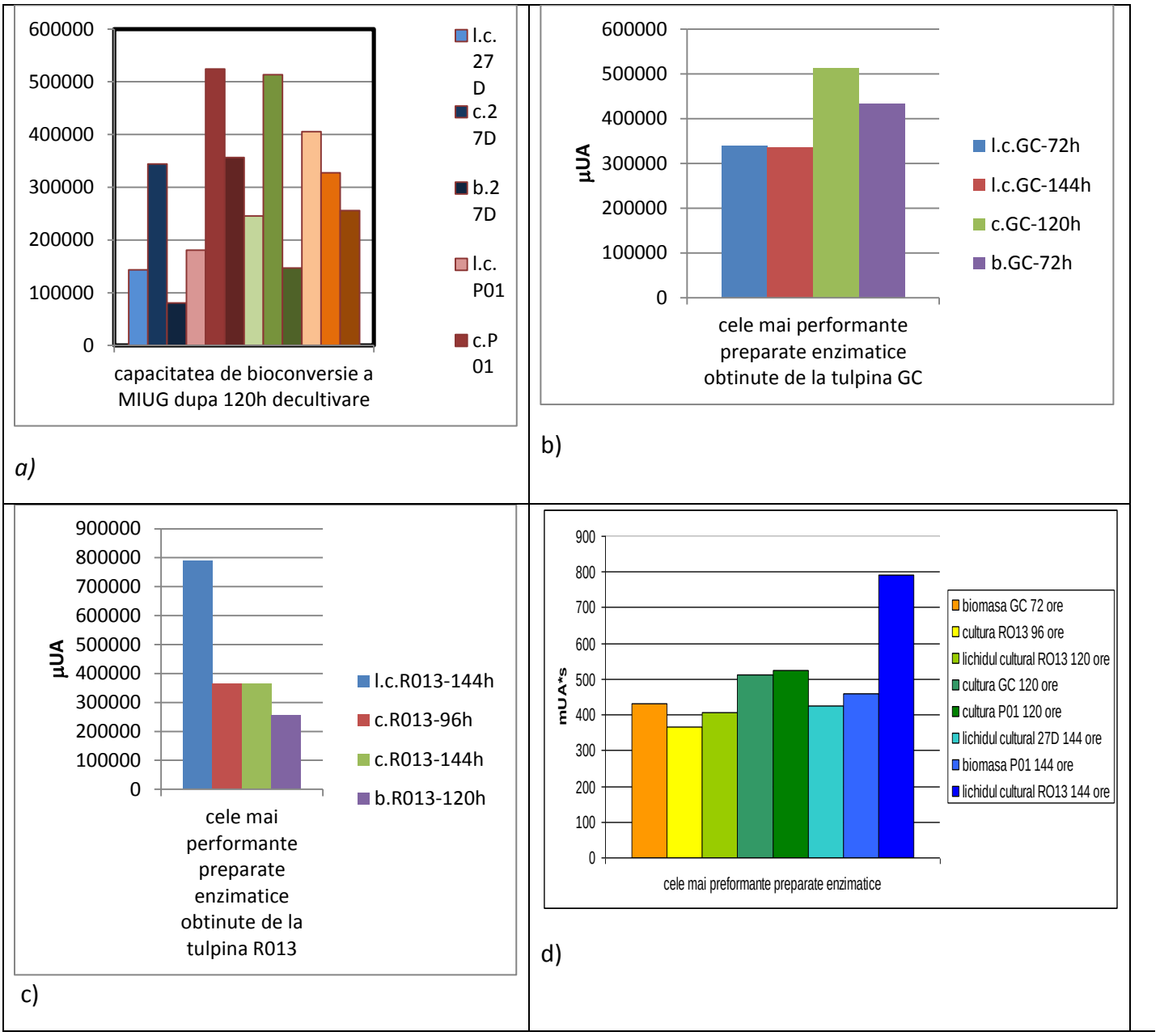


Figura 3. Results obtained by "one pot" bioconversion reactions with microorganisms from the collection MIUG

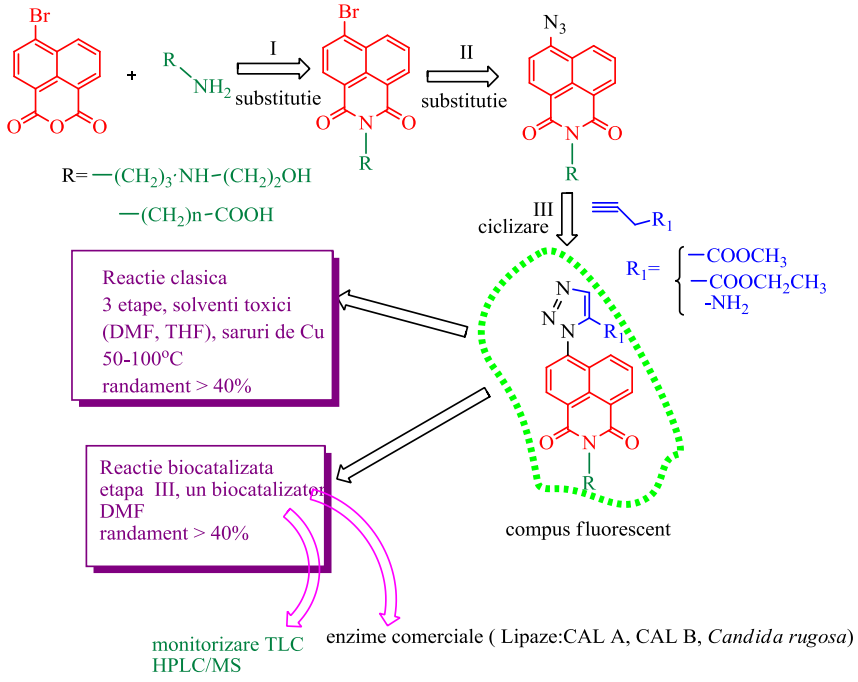
O.II.2. The synthesis of fluorescent molecules by coupling reactions

This objective 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 novelfluorescent triazole derivatives (scheme 3), which could be used in the controlled synthesis of complex bioactive molecules (schema 6).

The enzyme-catalyzed reactions were performed in 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 purityand yieldsare comparable to those obtained using the classical pathway.

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.



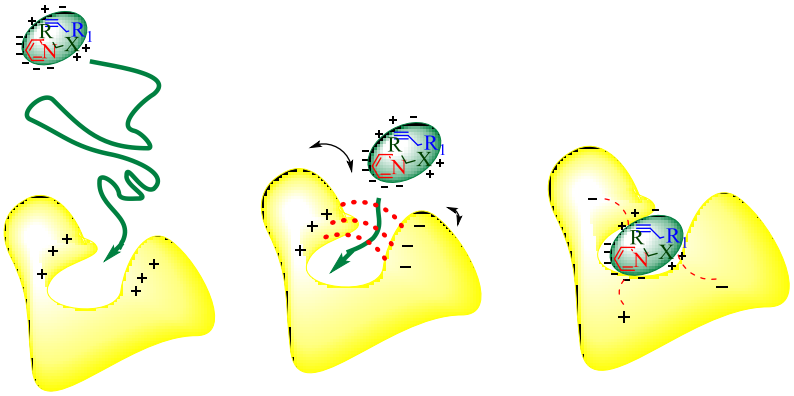
Schena 6.Triazole derivatives by coupling reactions

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 speedand, in some cases increase the purityof 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 advantage of using biocatalysts originates in Table I are compared two possible processes that can achieve reaction cicloadiţie- classic or enzymatic (results published in ICA, 2016 IF 1.78).

Table 1. Media used in chemical reaction process versus biocatalysis

Classical chemical process	*Biocatalysis "Green Chemistry"*
acetonitril	*Tampon phosphate pH=7*
NMP/ Benzen	*H_2O, enzyme*
methanol	*Chloroform*
chloroform	



Scheme 7. Possible mechanism of biocatalysts action

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.

O.III.1. Physico-chemical characterization of synthesized compounds

All new compounds obtained for the first time in our laboratory through bioconversion reactions were characterized by spectral methods, UV-Vis, fluorescence 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.

The study of fluorescence spectra

Literature shows that fluorescence spectroscopy is generally used in life sciences as a non-destructive way of tracking or analyzing biological molecules.

The use of fluorophores marking has many applications in molecular biology and medicine, such as for the investigation of conformational and dynamic chemical changes of and quantitative determination of the various elements, ions and molecules present in the cells, body fluids or other complex biological systems (J.Chan et all, 2011) with the purpose of analyzing the intracellular composition of different microorganisms as well as investigating the course of action of drugs in vivo or in vitro ((B. Mandal, et all, 2012). The number of fluorescent probes is great and there are also several new systems are available, therefore, it is now possible to choose a fluorophore which is optimal for the characteristics of the test material to be used as a fluorescent marker. However, despite the inherent advantages of the fluorophores, more research is yet to be done done in order to improve specific properties or to circumvent certain limitations (M. Shigeta, 2012). Although organic fluorescence markers that include compounds with emissions in the electromagnetic ultraviolet and near infrared spectrum are known, there are still limitations for the probes of higher wavelength, which are of particular importance for many biological applications (Ute Resch-Genger, 2008). Thus, there is a great need for new fluorophores or derivatives of fluorophores which are known to have good solubility in water and high fluorescence quantum yields. The biggest challenge is undoubtedly the design and synthesis of fluorophores that possess these characteristics. There are, of course, the additional requirement that such compounds will include a functional group capable of effective covalent attachment to various biomolecules.

The synthesis of fluorescent molecules *was performed using enzymatic catalysis, microwaves, ultrasounds and non-toxic solvents, ionic liquids. The results of this phase consisted of the successful cyclization reactions using as* ***starting materials*** *several nitrogen heterocycles, various* ***halogenated reactives***, ***quaternary ammonium salts*** *and* ***compounds with triple bond*** *activated by an electron-withdrawing group. The byocatalized reactions were multiple because we varied both biocatalysts and starting compounds to obtain different functionalized compounds which can have different properties –* ***bioactive***, ***fluorescent*** *– due to the substituents present in the molecule.*

Regarding the influence of solvents on the absorption and emission spectra of cycloadducts, from the recorded absorption spectra it can be seen that in all the studied solvents, the investigated compounds show a continuous spectrum absorption, unstructured, suggesting a planar conformation of molecules in both the ground state and the excited state (Figure 4) in which an absorption band corresponding to the transition S0-S1 cycloadduct 4 (i) is presented.

From the presented spectra it can be seen batocrom effect of aprotic solvents (eg. Chloroform) on this band.

The emission spectra recorded in different solvents shows that the fluorescence maxima of the curves are in the blue-green area, as can be seen from Table 2 for compound 4a (i).

The shape of the emission curve is approximately the same, continuous and unstructured, as can be seen in Figure 5. Concerning the fluorescence spectrum, the solvents haven't a great influence on the wavelength of the emission maximum, but exerts a greater influence on the fluorescence intensity maximum. Thus, in protic solvents, the fluorescence is higher than in the aprotic non-polar solvents or slightly polar solvents, such as chloroform. At the same time, in aprotic solvents (chloroform, ethyl acetate, acetone), there may be a hypsochromic shift of the maximum of fluorescence.

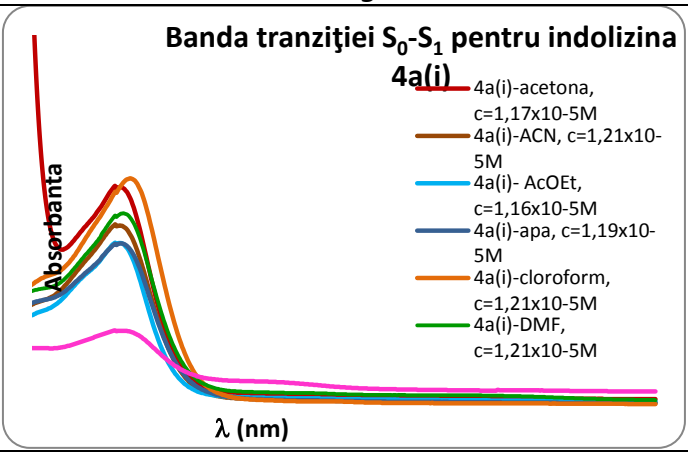
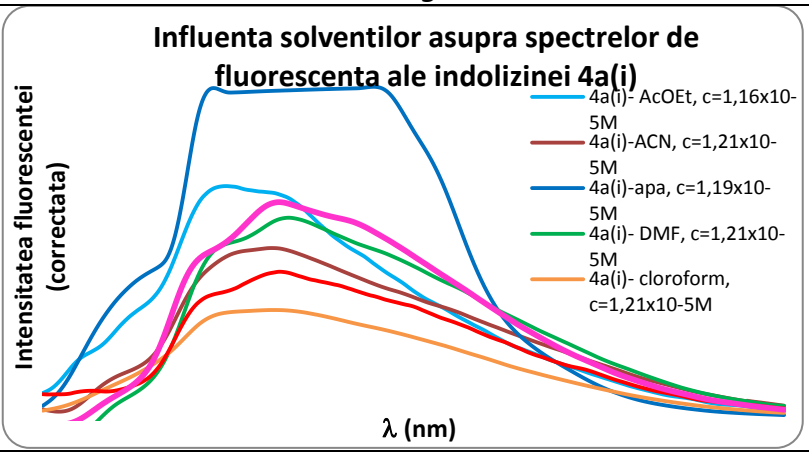
Figure 4	*Figure 5*
	

Table 2. The spectral parameters of compound 4

Compusul 4a(i)	*Solvent*						
	H_2O	*AcOEt*	*EtOH 95%*	*CH_3CN*	*DMF*	*$(CH_3)_2CO$*	*$CHCl_3$*
$\lambda_{max,\ exc.}$ (nm)	*366*	*366*	*366*	*366*	*369*	*366*	*372*
$s_{max,\ em}$ (nm)	*534*	*474*	*496*	*493*	*499*	*490*	*488*
$I_{F,\ max}$	*866*	*607*	*565*	*445*	*524*	*383*	*283*

Regarding the studies realised in order to determine the influence of internal factors (substituents, structure) of mono- and bis-indolizinelor fluorescence, it can be seen that the absorption spectra have the same continuous aspect with bands S1 and S2 completely separate, whatever the nature of the substituents and the mode of binding of the indolizine nucleus (Figure 6). From the emission spectra of some synthesized cycloadducts, recorded in the ethyl acetate solutions, there is observed strong fluorescent properties of all the studied compound swith the maximum of the emission band in the blue-violet region (Table 3 and Figures 7 and 8); the grater fluorescence belonging to the bis-indolizine cycloadduct 6a derived from 4,4'-bipyridyl, probably due to the direct conjugation between the indolizine ring.

The recorded fluorescence spectra were showing that the donor groups (eg. methoxy) linked to phenyl ring produce a bathochromic movement and an increase in fluorescence intensity, while the electronwithdrawing groups (nitro) although causes a bathochromic shift of the emission maximum they have the effect of "quenching" of fluorescence.

*Also, the presence of the ester group as substituents ($COOC_2H_5$ and $COOCH_3$) in the 1 and / or 3 position of the indolizine nucleus, lead to the high fluorescence yields and the maximum emission band results in slightly hypsochromic shifts (**unpublished results**).*

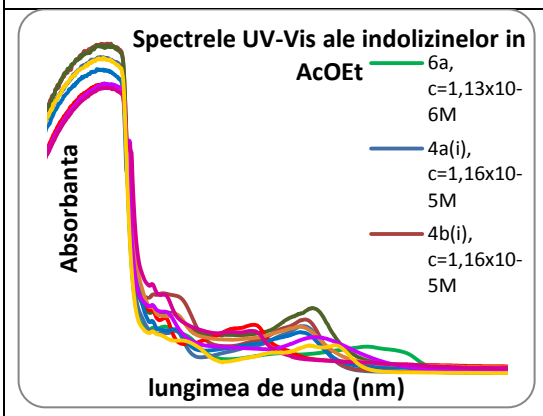
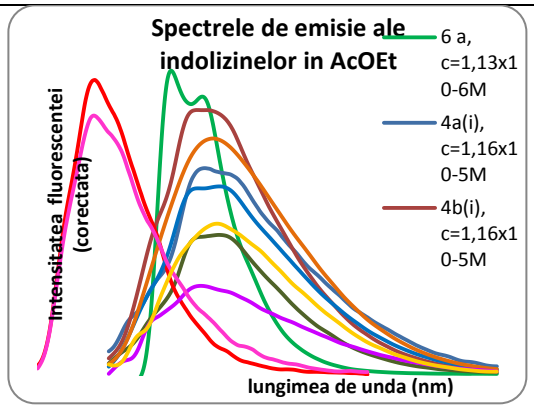
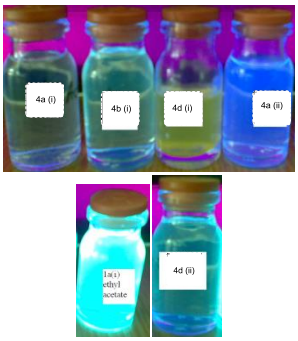
Figure 6	Figure 7	Figure 8
		 analyzed fluorophores

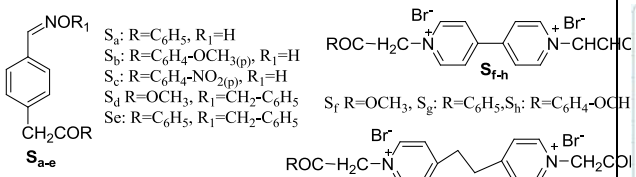
Table 3. Spectral parameters of the analyzed compounds

AcOEt	*Compounds*									
	4a(i)	***4b(i)***	***4c(i)***	***4d(i)***	***4a(ii)***	***4b(ii)***	***4c(ii)***	***4d(ii)***	***5a***	***6a***
$\lambda_{max,\ exc.}$ ***(nm)***	*366*	*369*	*374*	*335*	*366*	*366*	*376*	*335*	*388*	*410*
$\sigma\lambda_{max,\ em}$ ***(nm)***	*474*	*477*	*483*	*389*	*467*	*478*	*481*	*388*	*468*	*448, 472*
$I_{F,\ max}$	*607*	*778*	*410*	*866*	*553*	*694*	*259*	*762*	*443*	*893 817*

O.III.2. Biological characterization of synthetised compounds

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 (schema 9). 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(figure. 9). Two other salts, Sg and Sh, have demonstrated a broad spectrumof action, but moderate inhibition (Figures 10,11).*



Schema 9. Quaternary ammonium salts analyzed in terms of antimicrobial activity

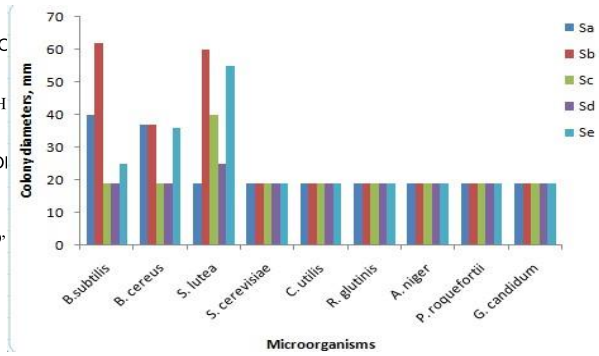


Figure 9. Bacterial inhibition activity

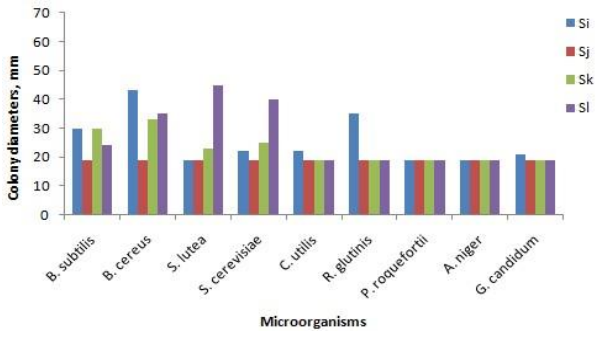


Figure 10. Antimicrobial activity of salts Si-Sl

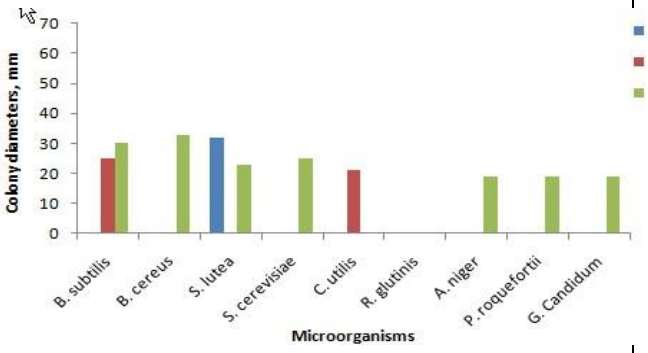
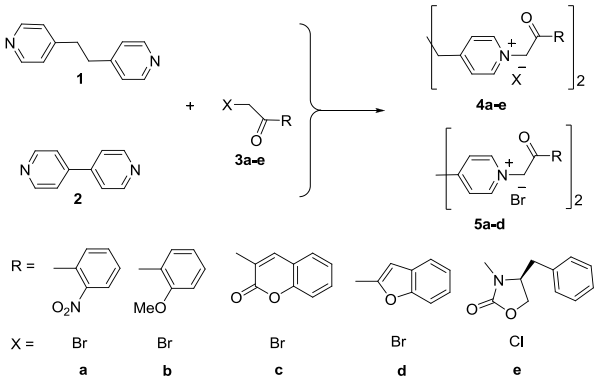


Figure 11. Fungal inhibition activity

Another series of quaternary bis-pyridinium salts (bis-PyQAs) were evaluated for their antimicrobial activity on non-pathogenic microorganisms. The compounds were tested on bacteria, molds and yeasts; the activities were expressed through the minimum inhibitory concentrations (MIC) (Table 3). It was subsequently analyzed the relationship between structural descriptors (LogP, polarizability, polar surface area (2D) and Van der Waals surface area (3D)) and the biological activity of the bis-PyQAs compounds in the diagram 10 below. The literature data shows that these descriptors can help predict the cytotoxicity of chemical compounds (J. Ranke et all, 2007).



Schema 10. **Analized compounds**

The compounds 4a-d, 5a and 5d show efficient inhibitory properties at least against one bacterial strain. However, one of the most active bis-PyQAs was compound 5b, with broad spectrum activity (table 4). We found that antibacterial properties appear to correlate well with structural descriptors LogP and the values for the polar surface area and van der Waals (Table 4).

Table 4. The diameter of the inhibition zones (Diz), expressed in mm, for compounds bis-PyQAs -4a- d and 5a-d

Compounds tested / *MO*	*DIZ (mm)*								
	4a	*4b*	*4c*	*4d*	*5a*	*5b*	*5c*	*5d*	*H2O*
B. subtilis	*35.00±0.57*	*30.33±0.33*	*19.16±0.16*	*40.83±0.44*	*41.33±0.33*	*45.00±0.57*	*19.83±0.44*	*25.33±0.33*	*0*
B cereus	*41.66±0.33*	*43.00±0.57*	*19.33±0.33*	*37.33±0.33*	*35.50±0.28*	*50.50±0.28*	*19.50±0.28*	*21.16±0.16*	*0*
S. lutea	*22.33±0.33*	*19.50±0.28*	*41.00±0.57*	*19.50±0.28*	*46.00±0.57*	*45.33±0.33*	*21.50±0.28*	*41.50±0.28*	*0*
S. cerevisiae	*22.00±0.57*	*22.66±0.66*	*19.66±0.66*	*19.16±0.16*	*19.50±0.28*	*23.16±0.16*	*19.33±0.16*	*19.33±0.33*	*0*
C. utilis	*22.66±0.66*	*22.50±0.28*	*19.33±0.33*	*19.33±0.33*	*19.16±0.16*	*22.66±0.66*	*19.33±0.33*	*19.16±0.16*	*0*
R. glutinis	*40.33±0.33*	*35.33±0.33*	*19.50±0.28*	*19.83±0.44*	*19.66±0.33*	*53.50±0.28*	*19.50±0.28*	*19.50±0.50*	*0*
A. niger	*19.66±0.66*	*19.33±0.33*	*19.83±0.44*	*19.33±0.33*	*19.33±0.33*	*32.33±0.33*	*19.33±0.33*	*19.16±0.16*	*0*
P. roquefortii	*19.50±0.28*	*19.16±0.16*	*19.16±0.16*	*19.16±0.16*	*19.16±0.16*	*19.16±0.16*	*19.16±0.16*	*19.50±0.28*	*0*
G. candidum	*20.00±0.57*	*21.66±0.66*	*19.33±0.33*	*19.33±0.16*	*19.33±0.33*	*33.16±0.16*	*19.16±0.16*	*19.33±0.33*	*0*

The values are presented as average ± SEM. The diameter of the disc of paper - 19 mm

All the compounds assayed showed significant in vitro antibacterial activity against bacterial strains, compounds 4b and 5b having superior antimicrobial activity against all microorganisms under study. Our preliminary studies indicate that some of these salts are also capable of destroying the formation of biofilm formed by microorganisms in the food industry.

Table 5. Physico-chemical properties of the synthesized salts

Comp.	*M.p. (°C)*	*LogP*	*Polar surface area (2D)*	*The surface area of Van der Waals (3D)*	*Polarizability*	*Hydrophobicity (RM)*
4a	*>250*	*-3.4*	*133.5*	*711.4*	*52.5*	*2.091±0.027*
4b	*241-243*	*-3.6*	*60.3*	*730.7*	*53.6*	*2.325±0.014*
4c	*>300*	*-3.0*	*94.5*	*732.4*	*59.5*	*2.122±0.047*
4d	*>300*	*-3.2*	*68.2*	*707.4*	*57.6*	*1.938±0.023*
4e	*nd*	*-3.0*	*100.9*	*888.2*	*65.9*	
5a	*258-259*	*-4.3*	*133.5*	*652.1*	*50.3*	*1.739±0.035*
5b	*252-253*	*-4.5*	*60.3*	*667.1*	*51.6*	*2.142±0.024*
5c	*>300*	*-3.9*	*94.5*	*671.0*	*57.3*	*1.917±0.049*
5d	*301-302*	*-4.1*	*68.2*	*646.0*	*55.4*	*2.092±0.023*

LogP, polar surface and van der Waals areas and polarizability were calculated using MarvingSketch 6.0.2 (http://www.chemaxon.com). The RM Values are presented as average ± SEM

A part of the results obtained in this study were published in the journal Molecules 2014, 19.

The study of the antimicrobial activity of the heterocyclic compounds obtained through biocatalysis reactions was performed by determining the biological activity on pathogenic microorganisms through the diffusion method and the cell viability assay, MTT.

For the study of the antimicrobial activity on pathogenic microorganisms, we conveyed the results obtained for the quaternary ammonium salts which are one of the most used classes of disinfectants, with wide application in hospital environments, water treatment, textiles, paint and food industry due to their relatively low toxicity for humans and animals.

*The compounds analyzed proved intense antimicrobial activity toward Escherichia coli, Streptococcus spp., Staphylococcus sp.; the antimicrobial effect is enhanced by the presence of nitrofurans groups and the presence of halogens in the molecule; there were obtained rather low values of the MIC (31.25 mg/ mL). The most potent compound studied from the viewpoint of antimicrobial action is compound **2**, followed by **5** and **1**. The activity was strongest against Staphylococcus sp., Streptococcus sp., and E. coli, and the lowest was against Candida albicans. Compounds **1** and **2,** whose Sstaphylococcus and Streptococcus MIC are very small (≤ 250 mg/ mL), may be of therapeutic interest.*

*Data on cell viability have long been obtained from in vitro cytotoxicity tests (Manish Raj Pandey et all, 2014). Today the emphasis is on markers for cell death, **the cell survival assay with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)** being widely used to measure the cytotoxic potential of a chemical compound; this way an assessment of the oxidative metabolism and the response of a cell population to external factors can be made. MTT measures the mitochondrial function and is often used to detect the loss of cell viability/ survival of cell due to a drug or toxin. Sensitivity, speed, and low cost of this method recommend it to be one of the most used tests for this purpose. The toxicity of the compounds obtained in this project was assessed by studying the cell viability on a pathogenic bacterium, Escherichia coli, using MTT. After treating cells with chemical compounds several different time periods (0, 2, 4, 24 hours), the culture medium was removed and cells were treated with solution 1 mg/ mL of MTT; the tetrazolium ring is reduced by the mitochondrial dehydrogenase succinate to a purple precipitate which was determined spectrophotometrically in the 490-600 nm range with a Tecan 900 Pro microplate reader. The MTT method showed that, following treatment with the compounds tested, the mitochondrial activity of the Escherichia coli cells was inhibited*

especially in the compounds containing methoxy and nitro functional groups(results published in J. Biotechnology, 2015, IF 3,108).

*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 activity of some known reference compounds for the biological activity analyzed (rutin, BHA and vitamin C for the antioxidant activity) (**unpublished results**).*

*The **determination of antioxidant activity using the DPPH free radical scavenging** methodrequired 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.

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 .

The biological studies conducted proved remarkable biological activityof 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. 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.

O.III.2. Biological characterization of synthetised compounds

Studies of toxicity and cytotoxicity

*Current research demonstrates that **quaternary pyridinium salts** and **bisquaternary salts** have many broad-spectrum antibacterial properties, inclusive against strains of methicillin-resistant Staphylococcus aureus or vancomycin-resistant Enterococcus faecalis (Pernak& et all., 2001, Chanawanno& et all., 2010). Oxime functions grafted onto these salts generate important*

properties in treating organic phosphate poisoning and also antibiotic (Lorke, 2008). A number of bispyridinium-oxime has a very good reactivation activity of AChE inhibited by sarin and paraoxon (Acharya& et all., 2011, Bharate& et all., 2009). Another interesting application of this type of compounds is generated by their ability to hydrolyze DNA, acting as artificial nucleases, thus being possible to become useful tools in biotechnology, in gene therapy or chemotherapy (Fernandes& et all., 2008).The class of ***indolizine*** *compounds is particularly interesting because of their fluorescent properties and biological activity. Thus, some indolizines have anti-inflammatory, antiviral and antioxidant activity, being targeted to treat cancer, cardiovascular diseases and HIV infection (Shen& et al., 2010). Studies have also shown that indolizine derivatives exhibit cytotoxicity against cancer cell lines resistant to antibiotics (David A. James & et all, 2008).* ***Triazole*** *derivatives obtained through* ***"click chemistry" reactions*** *are more than passive linkers; they can easily associate biological samples by hydrogen bonds and dipolar interactions (B. S. Sekhon, et all., 2012).*

Given the current research, in this stage we continued the study of the biological activity of the intermediate and final compounds of the biocatalysed reactions (achieved in previous steps), quaternary pyridinium salts, indolizines and triazoles, studies that were conducted in the following directions:

i. *the intermediate compounds (used in biocatalysis) toxicity study on several microorganisms;*
ii. *cytotoxicity studies carried out on the Saccharomyces cerevisiae;*
iii. *the toxicity study of the compounds on plant germination.*

The toxicity tests performed by us consisted in the use of methods taken from literature, sensitive, inexpensive and accurate means with which one can evaluate the toxicity and cytotoxicity and/ or cytostatic effects of various chemical compounds.

i. The intermediate compounds (used in biocatalysis) ***toxicity study*** *was based on the assessment of the toxicity degree of the reactants of a model reaction (pyridine derivative, halogen derivative reactive and ethyl propiolate) against certain strains of* ***bacteria***, ***yeasts*** *and* ***molds*** *(C. robusta MIUG 3.2, C. robusta MIUG 3.3, C. tropicalis MIUG 3.4, C. utilis MIUG 3.5, Sacharomyces cerevisae MIUG 3.6, Yarrowia lipolytica RD 14 MIUG, Yarrowia lipolytica RD 15 MIUG, Yarrowia lipolytica RD 16 MIUG, Saccharomycopsis fibuligera), belonging to the collection of microorganisms of University "Dunarea de Jos" of Galati's "Bioaliment" Platform, with MIUG indicative. In the selection process from the MIUG collection of microorganisms samples were analyzed resulting from incubation of 21 microorganisms for 5 days in the presence of four different concentrations of chemicals (0.5 mM, 1 mM, 1.5 mM, 2 mM). Among the tested strains were found three strains of yeast, one of mold and a bacterium. The concentration of 1.5 mM is optimal for biocatalysis reactions. The Yarrowia lipolytica RD13 MIUG, Yarrowia lipolytica RD14 MIUG, Yarrowia lipolytica RD15 MIUG, Geothricum candidum MIUG 27 and Pseudomonas fluorescens MIUG MP11 strains were selected as resistant to the tested compounds and as biocatalysts in "click-chemistry" reactions of forming nitrogen-containing heterocyclic compounds, in an aqueous neutral medium(published results in J. Biotech, 2015, IF 3,108 şi* Scientific Study & Research, Chemistry & Chemical Engineering, Biotechnology, 2016*).*

ii.The cytotoxicity studies were carried out on Saccharomyces cerevisiae, a microorganism which has several genetic and biochemical characteristics similar to human cells.

The biological models have long been used to determine the cytotoxicity and the cytostatic activity of many chemical compounds natural and/ or synthetic. Current analysis techniques usually require the use of chemical reagents or expensive technological equipment, or they lack appropriate test sensitivity.

The purpose of this study was to evaluate the cytotoxicity induced by the compounds synthesized through survival tests on Saccharomyces cerevisiae, an microorganism which has several genetic and biochemical characteristics similar to human (T. S. Gonçalves et all, 2014, N. P. Poletto et all, 2008). It has been widely used as a model organism for the study of diseases in mammals and the evaluation of toxic compounds such as genotoxic and cytotoxic agents (Armour CD et all, 2005).

There were evaluated three groups of compounds: quaternary pyridinium salts, indolizine and triazole derivatives obtained through biocatalysis cyclization reactions.

In this study we used a pure culture of Saccharomyces cerevisiae MIUG D 27. This culture is pricked out on malt agar gravy culture medium (MMA); the culture medium is slant and incubated at 25° C 48 hours before the inoculum preparation. The inoculum is prepared in sterile saline and is dimensioned through counting with the Thoma chamber.

After 24 and 48 hours of cultivation samples are taken to assess the degree of budding and autolysis by direct counting with the Thoma chamber.

The increase in colony of yeast cells was measured in culture medium containing increasing concentrations of chemical agents. The results showed that the test was able to clearly differentiate the cytotoxic effect of the compounds, the cytotoxic effect following an exponential curve with increasing concentrations.

The degree of autolysis is determined by reporting the number of cells autolysed to the total number of cells in a microscopic field, performing the counting in 10 fields. In the case of bispiridiletan derived indolizine (BPE), the results are remarkable. At a maximum of concentration the degree of autolysis was comparable to that determined in the absence of the compounds and at the lowest concentration tested, 10^{-6} M, approximately 60% fewer autolysates cells have been identified than in the control samples.

By adding one micromol of indolizine derivative of the bipyridyl/ L culture medium, a degree of autolysis of cells of 13.88% can be obtained, lower than that determined in normal conditions (23.09%) after 96 hours of submerged culture using YPD culture medium, specific for yeast, without the addition of heterocyclic compounds.

In the case of the quaternary salt derived from pyridine (fox), the addition of 0.3 mg of compound/ L culture medium leads to a decrease in the degree of autolysis compared to the blind, to a value of about 12%.

The budding index is determined by reporting the number of budding cells to the total number of cells in a microscopic field, performing the counting in 10 fields.

After the first 24 hours of cultivation, the extent of sprouting in the controls is comparable to the values of the samples supplemented with indolizine derived from bispiridiletan. After 48 hours of cultivation, the extent of sprouting in the presence of 10^{-5} M concentrations is greater than 24%.

In the case of the indolizine derived from bipyridyl (bpy), at the lowest concentration used in this experiment there is a degree of cell budding lower than in the controls, with 20% less after 48 hours of submerged cultivation.

At the maximum concentration tested, the presence of the compound fox (pyridine derivative) has produced the budding of more than 20% of the yeast cells, while a concentration of 10^{-6} M in the culture medium led to values comparable to those of the control samples, after 48 hours of submerged cultivation.

i. *The toxicity study of the compounds on plant germination*

The impact of different types of compounds on superior plants has been studied extensively (Hong și Otaki, 2006; Lin și colab., 2009; Seeger și colab., 2009). When growing, plants absorb relatively large amounts of essential and non-essential elements, which can be toxic to certain concentrations. Wheat is an important crop for human consumption worldwide and is considered a model for the monocotyledonous species for research in molecular biology.

To test the effect of compounds synthesized by biocatalysis (quaternary pyridinium salts, indolizines and triazoles) on the germination of wheat seeds, the experiment was carried out under laboratory conditions using wheat seeds purchased from a local market in Galati. The healthy seeds were selected and the surface was sterilized with 10% solution of sodium hypochlorite for 10 min, then rinsed heavily with distilled water sterilized before the transfer to petri dishes with a double layer of filter paper. After 4 days the grains that did not germinate, the sprouted grain's plumules are counted and the plumules' height is measured in order to calculate the average height .

The germination of wheat grains in the presence of indolizine derivatives of bpy and BPE compounds led to a height of the plumules at least equal to that of the controls, even if in higher concentration. In the case of the compound derived from bpy, 54% of the grains had plumules after 4 days in culture, compared with 40% in control samples.

As a result of this experiment, it was found that two of the tested compounds decrease the water sensitivity of the grains of wheat. It is noted that the addition of higher concentrations of the compound has a beneficial effect. A concentration of 10^{-5} M of a quaternary pyridinium salt (fox) leads to a decrease in the percentage of seeds which do not germinate by 75% compared to the controls. In order to advance cytotoxicity studies and carry out tests on mammals (lab mice), there were developed advanced studies of crystallization of proteins (lysozyme), subsequently used as an encapsulating material for the compounds obtained in the project with the purpose of carrying out pharmacological studies . Some of the results obtained in this study are unpublished and others were published in the Romanian Journal of Chemistry, 2014, 65, No. 8.

The interaction of the compounds with DNA

DNA plays an important role in biological processes since it carries the hereditary information codes required for the synthesis of all proteins and enzymes. DNA directly or indirectly controls the structure and function of the cell. Ever since the discovery of the structure of DNA, it has been a prime target for various therapeutically important small molecules that belong to different classes of drugs, ranging from anticancer drugs to antibiotics. Apart from interacting with DNA associated proteins or interacting through DNA–RNA hybrids, small molecules may directly bind to the DNA helix. Such interactions result in diverse downstream processes like interfering with the activity of various important enzymes and proteins involved in maintaining the structure and functions of the cell. The interaction of small molecules with DNA has been

studied extensively. These studies provide insights into the development of effective therapeutic drugs that could control gene expression. Newer and more effective DNA-targeted drugs against several diseases can be easily developed. Understanding the mechanism of action of various anti-cancer drugs became possible by studying the drug–DNA interactions(Sayeed Ur Rehman et all, 2015).

Spectroscopic studies (absorption and fluorescence) can provide important information on how the nucleic acid binds fluorescent molecules (fluorophores) or drugs (Modukuru N. K. et al., 2006, Muhammad Sirajuddin et al.,2013; Sayeed Ur Rehman et al, 2015).

The photophysical behavior of indolizines has been studied and efforts have been made to develop derivatives with improved and exceptional fluorescent properties. Due to their characteristic fluorescence behavior, indolizine derivatives have been used as photochemical sensors, hydrogen bonding sensors and as fluorescent sensitive probes to VOCs (Matthieu Becuwe et al., 2011, Bankim Chandra Ghosh et al., 2011, Mustafa K. Bayazit et al., 2014).

They have been used for studies of DNA interactions and as photochemical dyes (Dinică et al., 1999, Furdui et al., 2007).

The researches on the interaction of nucleic acids with different biologically active molecules synthesized in the earlier stages of the project contribute to the understanding of the mechanism of interaction. The interaction of nucleic acid with organic compounds, differently functionalized (products of cyclization reactions - fluorescent indolizines, figure 12), obtained in the previous stages through biocatalysis, was done by spectral methods (UV-VIS and IR).

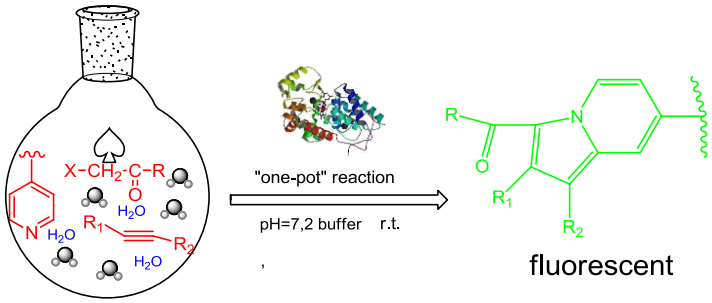
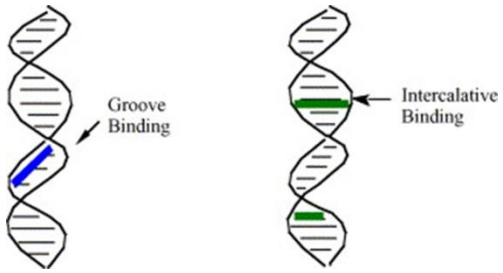


Figure 12 Indolizine synthesis

UV-vis absorption measurement is a simple, but effective method in detecting DNA-compound complex formation. Generally, when a small molecule interacts with DNA to form a new complex, the changes should appear in absorbance and in the absorption maximum position. If the binding type is intercalating, the π∗ orbital of the intercalative molecule can be coupled with the π orbital of DNA base pairs, thus leading to a decrease in energy tranzition π∗ → π resulting in a bathochromic effect. On the other hand, the coupling of the π orbital partially occupied by electrons lead to a reduction of the probability of transition and at the same time resulting in a hypochromic effect. Generally, hiperchromic and hypochromic effects are spectral characteristics of the double helix DNA structure; hiperchromic means breaking the secondary structure of DNA and hypochromic means that the binding molecule to DNA is through electrostatic or interlacing effect that can stabilize duplex DNA, while the redshift indicates the destabilization of the DNA duplex.



The mode of binding of a compound is affected by the geometrical, steric and electrostatic characteristics. Thus, binding by intercalation is common for aromatic and heterocyclic plane molecules, while electrostatic interaction may occur in the presence of cationic functions and of the side chain.

Samples and DNA preparation

To study the interaction of the fluorophore with the nucleic acids, in a first stage of laboratory investigations the absorption spectra of indolizines were determined. Indolizine stock solutions were prepared by dissolving an appropriate amount of compound in buffer Tris-HCl, pH 7,6. The UV-VIS spectra of the dyes were recorded between 200 and 800 nm, in aqueous buffer solution. Indolizines possess two absorbtion bands in the UV-Vis region .

Steady state fluorescence measurements were conducted with a Infinite 200 Pro NanoQuant Microplate Multimode Reade (300 µL / well) and the measurements were performed at 24°C. The dye concentration ranged from 2 to 20 µM depending on the experiment.

Figura 13. Indolizine solutions

The recorded fluorescence spectra showed that the donor groups (eg. methoxy) linked to phenyl ring produce a bathochromic movement and an increase in fluorescence intensity, while the electronwithdrawing groups (nitro) although causes a bathochromic shift of the emission maximum they have the effect of "quenching" the fluorescence.

The concentration of calf thymus DNA

Deoxyribonucleic acid sodium salt from calf thymus (Sigma Chem. Co., USA) was used without further purification, and its stock solution was prepared by dissolving an appropriate amount of DNA in doubly distilled water and stored at 4 ∘C. The concentration of DNA in stock solution was determined by UV absorption at 260 nm using a molar absorption coefficient ε_{260} = 5000 L mol^{-1} cm^{-1}. Purity of the DNA was checked by monitoring the ratio of the absorbance at 260 nm to that at 280 nm. The solution gave a ratio of >1.8 at A_{260}/A_{280}, which indicates that the DNA was sufficiently protein-free. For measurements, the DNA was dissolved in 10 mM Tris-HCl, pH 7 buffer and was used for all the spectroscopic binding analysis.
The concentration of nucleic acids was determined with the same microplate reader which has a special quartz plate suitable for 16 samples which guarantees high performance and a high rate of reproducibility for the quantification of nucleic acids. The plate is compatible with an eight-channel pipette to easily distribute samples for analysis. 2 µl of each DNA dilution were transferred on the special NanoQuant plate which subsequently was read. All measurements were performed in triplicate.

Effect of DNA on UV-VIS spectra

The spectra were recorded by progressive addition of DNA solution to indolizines. Absorption titrations were carried out by keeping the concentration of the solution sample constant, while adding concentrated solution of DNA in progressively increasing amounts in physiological conditions, until the saturation in hypochromism was observed.

The absorption spectra of DNA-indolizine solutions are shown in the figure below. DNA binding to indolizines leads to the red shift of the absorption band of the dye, visible at high DNA concentration.

The red shift (or blue shift), hyperchromic (or hypochromic) effects, and the isochromatic point are spectral properties of DNA-drug interaction, which are closely related with the double helix structure. The hypochromicity at the maximum absorption of DNA (260 nm) indicates the compaction of DNA due to the electrostatic interaction. Intercalation induces the hyperchromicity at this wavelength (Reza Hajian et al., 2013).

With the increasing of indolizine concentration, the spectrum shows the decrease of the absorbance intensity. Since the indolizine nucleus contains aromatic rings which may facilitate the intercalation, a conventional intercalative interaction is possible.

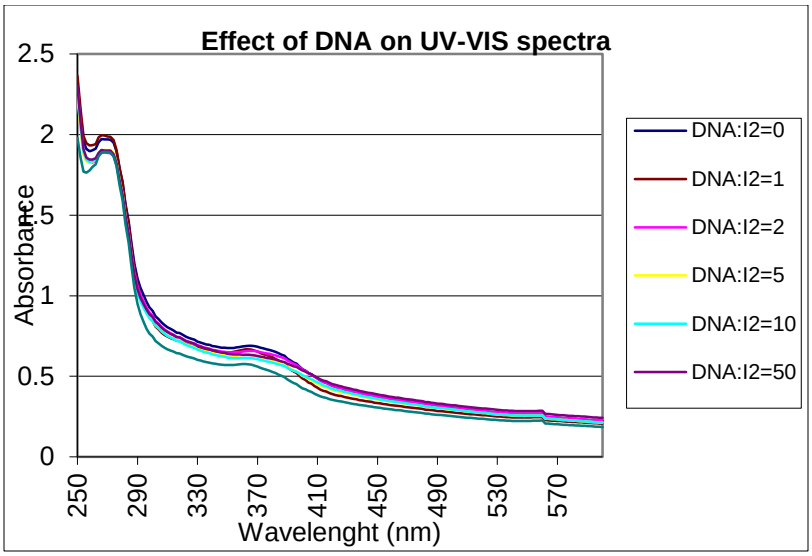


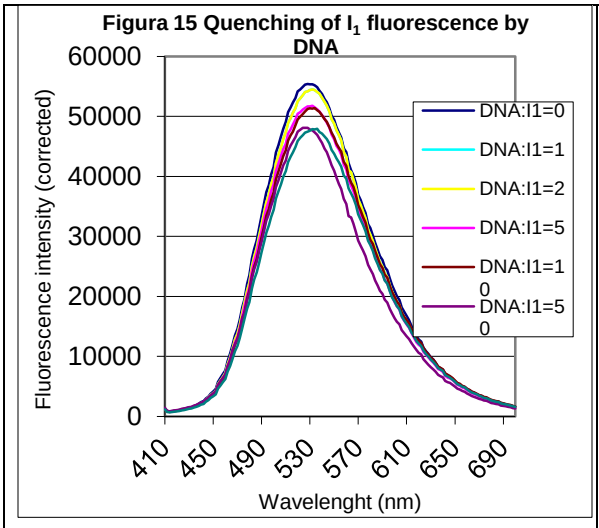
Figure 14 Absorption spectra of indolizine I2 in the absence and presence of increasing quantities of DNA

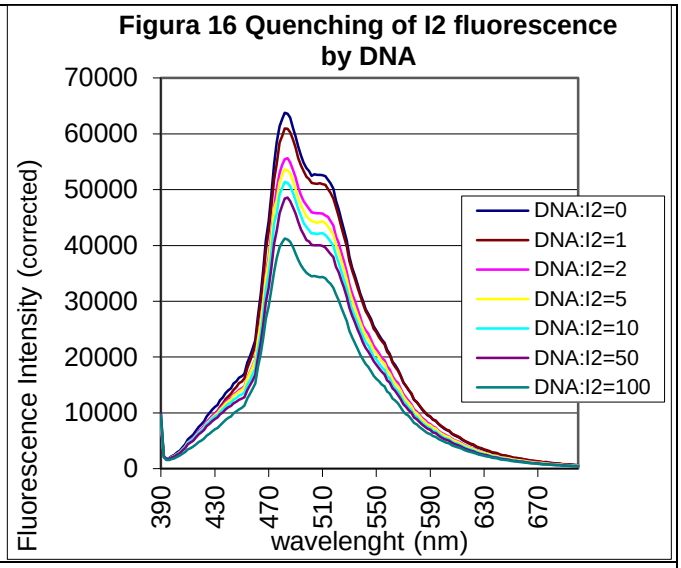
Fluorimetric titration. Quenching of fluorescence by DNA

Fluorescent quenching techniques involve a variety of molecular interactions such as excited state reactions, formation of ground-state complex, molecular rearrangements, energy transfer, and collision. In this context, fluorescence quenching experiments were undertaken to investigate the interaction of synthesized compounds, indolizines, with DNA. The binding of compounds with DNA, by maintaining the concentration of compounds constant and varying the concentration of DNA was studied by fluorescence spectroscopy
The fluorescence spectra was recorded at different molar ratios DNA:indolizine, with excitation of the samples at the wavelength of the lowest energy maximum absorption band.

The absorption and emission spectra have stressed the strong interactions between DNA and studied fluorophores, which is manifested by fluorescence quenching with increasing molar ratio DNA: fluorophore. Titration curves (fluorescence intensity to molar ratio of DNA: indolizine) show exponential decrease in fluorescence intensity with increasing concentration of the indolizine.

The data from the photometric titrations were used to determine the particular ratio of bound ligand molecules to DNA, plotting the relative fluorescence intensity against the molar ratio of DNA : indolizine, in order to establish the molar ratio of maximum interaction. Upon addition of DNA, the fluorescence intensity of the indolizine decreased.





- *strong DNA-fluorophores interaction*
- *fluorescence quenching with increase of DNA concentration*

- *maximum fluorescence extinction at ~10 equiv. p.b. DNA*
- *I2 compound shows the strongest DNA affinity*

The effect of ionic strength

Study of ionic strength on the interaction of the DNA molecule is an important method to analyze the binding type between small molecules and DNA. Strong electrolytes, such as NaCl, are used for this purpose. Adding NaCl to the free ligand in the absence of DNA should have a low or no effect on the fluorescence yield. However, in the presence of DNA, Na^+ partially neutralizes the negative phosphate groups of the DNA strand resulting in reduction in the electrostatic repulsion between them.

Electrostatic attraction between a small molecule and DNA surface is weakened by the addition of Na^+ ions. When the molecule binds on the DNA surface, an electrostatic interaction occurs out of the groove and the fluorescence intensity is quenched upon interaction with DNA. A small molecule located in the groove of the DNA helix is more exposed to the ionic strength in the surrounding solvent than a intercalated molecule. The addition of NaCl weakens the interaction and leads to the release of the compound from DNA surface resulting in increased of fluorescence intensity. (S.U. Rehman et al., 2015).

The effect of salt concentration was studied by recording fluorescence spectra in the presence of different NaCl concentrations in order to establish the nature (intercalative or electrostatic) of dye-DNA interaction.

KI quenching experiments Studies of potassium iodide fluorescence quenching are typically used to determine how DNA binds the drugs that are fluorescent. KI is a small compound which posseses fluorescence quenching properties and can be used to determine the binding type of drugs to DNA.

Quenching experiments indicate where molecules bind, either outside or inside the DNA helix. Iodine ions are negatively charged and can quench small molecule fluorescence efficiently in a saturated solution, in an aqueous medium.
The negative iodide ions are repelled by the negatively charged phosphate groups of the DNA strand. Any small molecule intercalated in the DNA helix is well protected from being quenched by the saturated anionic solution the fluorophore being restricted. However, electrostatic molecules are exposed to the solvent medium and are not well protected by the saturated anions, even in the presence of DNA.
The studies performed have lead to the conclusion that the salt concentration doesn't influence significantly the fluorescence intensity in the presence of DNA, so the presence of electrostatic interaction can be excluded(fig. 17,18) ***(Results to be published).***

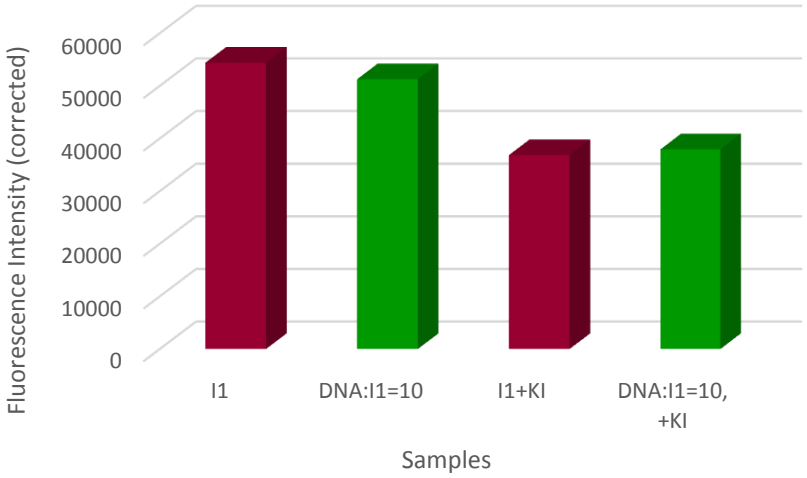


Figure 17. Fluorescence quenching with the indolizine I1 and indolizine- DNA

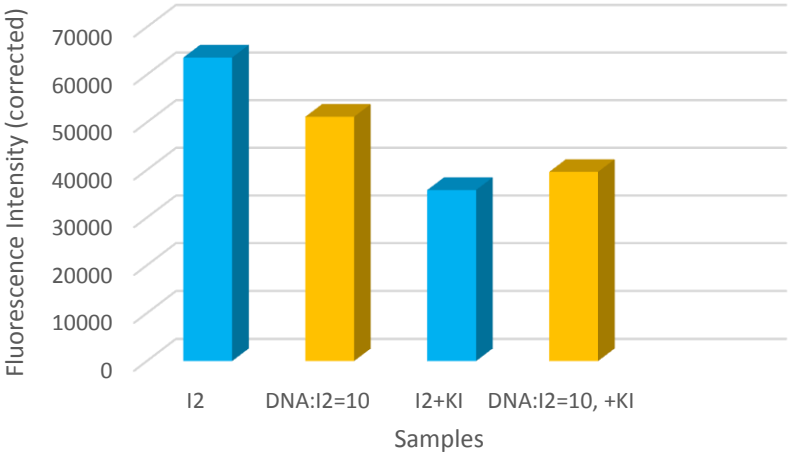


Figure 18. Fluorescence quenching with the indolizine I1 and indolizine- DNA

The interaction of molecules with nucleic acids by FTIR spectroscopy studies

Fourier transform infrared spectroscopy (FT-IR) has numerous applications in biological sciences. This provides an easy way to identify functional groups present in the molecule, and the identity of the pure compound can be determined. Data results are shown as a range that acts as a molecular fingerprint of the compound or molecule. Drug-DNA interaction study highlights the changes in intensity and spectral shifts of the bands. Pure DNA molecular fingerprint is in the spectral region of 1800-700 cm^{-1} due to plane vibration of nitrogenous bases, stretching vibrations (asymmetric and symmetric) of phosphate groups and stretching vibration of deoxyribose. The transition conformation of the DNA double helix to form B to form A or to form B to form Z can be detected readily by binding of drugs to DNA. (T. Zhao, 2014, S.T. Saito, 2012, D.K. Jangir, 2011, H. Arakawa, 2000).

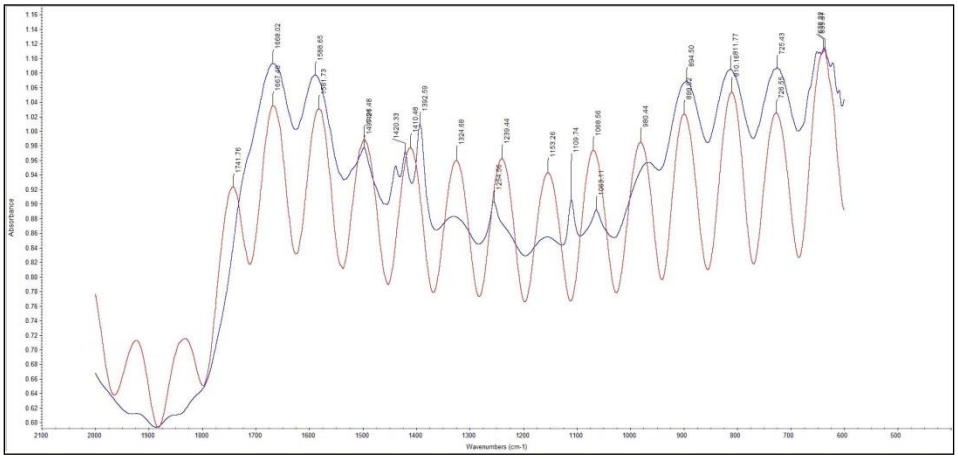


Figure 19. FTIR spectra: DNA and DNA-indolizine 1

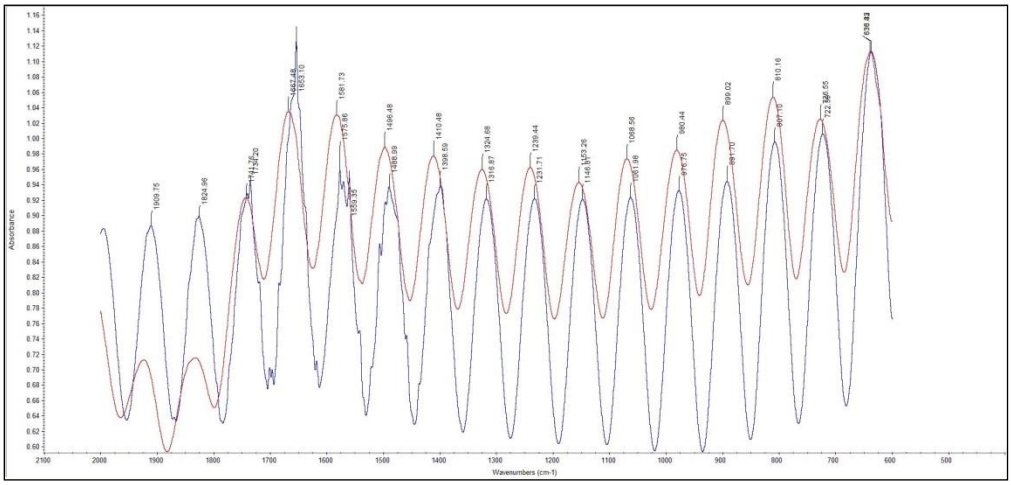


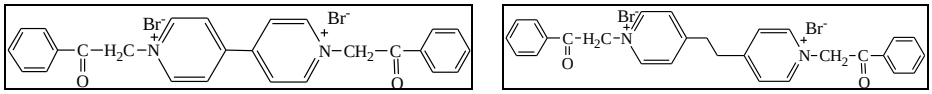
Figure 20. FTIR spectra: DNA and DNA indolizine 1

Figures 19 and 20 show the FTIR spectra of calf thymus DNA in the absence and in the presence of indolizines I1 and I2.
*Linking the analyzed molecules to DNA bases are demonstrated by evaluating spectral bands at 1741 cm-1 which is mainly attributed to stretching plan vibration of guanine (G), the spectral bands at 1668 cm^{-1} caused by basic vibrations of thymine (T), and the spectral bands at 1581 and 1496 cm cm^{-1} due to the stretching vibration of adenine (A) and cytosine (C) (**Results to be published).***

O.IV.2 Studies of electrochemical properties

Studies of cyclic voltammetry

Viologen compounds present a significant interest due to their changing properties and colour in connection with the reaction medium. In this work, the electrochemical behaviour of two pyridinium heterocyclic compounds has been recorded. The compounds were studied for the first time by cyclic voltammetry in aq. media. New heterocyclic pyridinium compounds as N,N' di-(p-bromophenacyl)-4,4'-bipyridinium dibromide (Lr) and N,N' di-(p-bromophenacyl)-1,2-bis(4-pyridinium)-ethane dibromide (Lm), synthesized in the previous stages of the project, were investigated applying cyclic voltammetry to evaluate their electrochemical behaviour. The stability of the new heterocyclic pyridinium compounds in aq. media depends on pH and the dependence was correlated with spectrophotochemical data. Ethylenic group from Lm induces changes on the stability and on the electrochemical performances of the ligand. The quasireversible process on electron transfer between functional groups depends on pH and also on the scan rates of the potential applied. The alkaline pH of aq. media is more favorable than acidic pH for the ligands stability and the electron transfer process on platinum electrode. The study of the redox potential (cyclic voltametry) of the two ligands indicatse the role as mediator candidate in reduction mechanism. We believe that the present work will stimulate the investigations of the chemical features of ligands and their role in biological and medicinal chemistry.



Schema 11. Structure of bipyridinium salts Lr and Lm

The elucidation of the electro-changes of the synthesized compounds is useful for the electrochemical investigation [R. Palin et al.„ 2002]. Compounds can be used as biological redox indicators, electrochemical sensors, electronic transporters and precursors for compounds such as indolizines, biologically active compounds which can serve as potential markers and fluorescent ligands for estrogen receptors [M. Eda et al., 2008, D. H. Evans et al., 2001]. This research also helps us to understand the mechanism of cycloadditon of these ligands with dipolarophils for obtaining indolizine compounds [Dinica et al., 2013]. The behaviour of the compounds bearing aryl substituents on the quaternarized nitrogen atoms has been examined showing that the spectroscopic and electrochemical properties are fine tuned by the nature of the nitrogen ring. These quaternisation reactions will be done in aq. media. The redox potentials

of the compounds are interesting for determining the donor - acceptor properties of the radicals [F. Tepl et al.„ 2012]. Another aspect that we will study in the future would be understanding reactions of cycloaddition and substitutions, realized in aq. Media.

Stability of ligands

The synthesized compounds were N,N' di-(p-bromophenacyl)-4,4'-bipyridinium dibromide (named rigid Ligand-Lr) and N,N' di-(p-bromophenacyl)-1,2-bis(4-pyridinium)-ethane dibromide (named mobile Ligand-Lm) [3]. The stability of ligands derived from 4,4'-bipyridil (Lr) and 1,2-bis(4-pyridil)-ethane (Lm) respectively was performed by spectrophotometric analysis and open circuit potential (OCP) measurements. The aq. compounds (0.1 mM) were prepared and the analysis was conducted by adjusting the pH in range 3-11. Solutions shown variable colours depending on pH, beginning from light yellow to violet (N,N' di-(p-bromofenacyl)-4,4'-bipyridinium dibromide (Lr) and orange (N,N' di-(p-bromofenacyl)-1,2-bis(4-pyridinium)-ethane dibromide (Lm). A discoloration of the solution was observed when the ligand concentration was lower than 10-5 M.

The UV/VIS spectra of aq. solutions were analyzed at 264 nm (λmax) [Furdui et al., 2012] with the purpose to study the initial stability and after 24 hours (Figure 21). The highest absorbance are registered for Lr on pH 3 (weak yellow colour) and pH around 7 (weak violet colour) when protonated species are formed. Fresh aq. Lr solution presents most stable form in lower alkaline medium (pH around 8). On the other hand, Lm is more stable in lower acidic medium (pH around 6) and the dication form is unstable in alkaline media. This behaviour suggests the presence of the anionic species AH- at the addition of base excess and transition from AH2 to AH-.

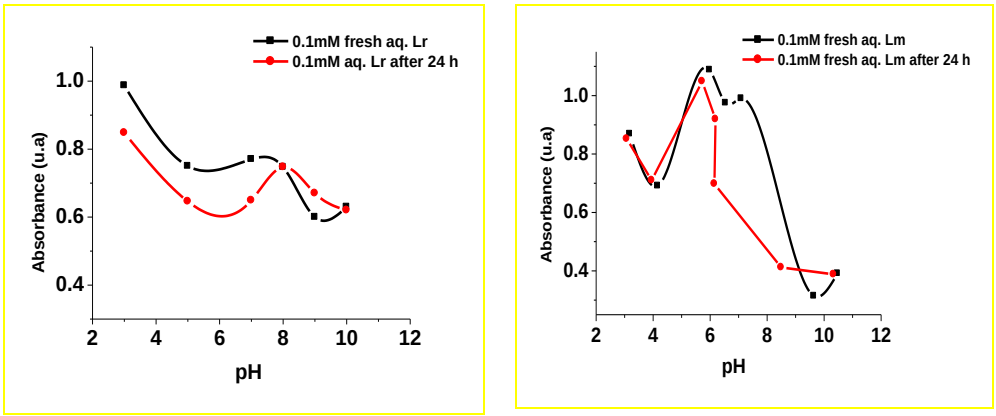


Figure 21. Overview of absorbance vs pH of aq. Lr and Lm

The ligands solutions kept at room temperature shown after 24 hours a slightly absorbance change; for Lr it decreases with about 2 units. The electro-oxidation process is more active due to favourable dynamic acid-basic equilibrium of the electrons transfer reaction from the dication to produce radical cation (AH.+), and then neutral species. At the same time Lm indicates stability in acidic medium (until pH 6), but an evident decrease of absorbance values in

neutral and alkaline medium. This behaviour could be explained by the ethylenic group from Lm, that is more favourable for some cycloarrangement additions in alkaline than in acidic media.

The absorbance from UV/VIS spectra are well correlated with OCP measurements. Bispyridinium compounds are organic oxidants and electrochemical techniques are useful to allow the characterization of the electro-oxidation behaviour of electroactive species, to establish the redox potential and to predict the reaction mechanism.

After 24 hours, a difference of about of 30 mV between the acid aq. ligands was determined for the OCP values. The fresh Lm solutions show larger variations of OCP values for the entire pH range because it is more unstable due to the position of ethylenic group in base excess. Meanwhile Lr which does not have the ethylene group in its structure is more stable in the same conditions.

Thus, OCP values sustain the ligands structure shown in schema 11 and explain their stability as confirmed by the spectrophotochemical measurements. The data results are helpful to provide an explanation for the proposed electro-oxidation mechanism of the ligands but are insufficient data for the reaction kinetics in aq. solutions.

Cyclic voltammograms measurements

The electrochemical measurements give information on the electrochemical state of the organic compounds on the working electrode in an active or passive state. CV provides information about the redox couples and this remains the most attractive gain of this technique. CVs data are useful for the presence of the protonated/deprotonated structures and supply information about the kinetics of reactions. A series of CVs measurements were obtained in aq. media of the ligands at variable pH, from acidic to alkaline, and different scan rates of the potential applied.

Effect of the pH. The pH has an important role for organic compounds as a result of the proton concentration from aq. media. Changes in CVs are the result of the different ligand structures in aq. solutions with variable pH (Figure 22 and 23). The shapes can explain the influence of functional groups from the ligand molecule in aq. media correlated with their stability in time, depending on pH.

A similar behaviour appears in the redox process on platinum electrode in both compounds mainly for the anodic current peak. The peak corresponding to the initiation of the anodic oxidation indicates that at least a prototropic couple (AH2-/AH-) of the organic compounds is present. The anodic current peak (ia) around of + 300 mV vs. SCE is more evident for Lr and it appears diminished for Lm, with a difference of 0.5 μA and shifted to a less positive potential.

The voltamogramms sustain the electrons transfer between functional groups of ligands and the prototropic couple, depending on the pH of the solution. If the pH solutions are changed, the typical voltammograms will be observed. Figure 14b shows the voltammograms of aq. ligands at a pH of 9.0. The electron transfer is indicated by a characteristic cathodic peak on Pt electrode which is similar in both salts under the same potential conditions and scan rates applied (E = ± 1 V; 50 mV s-1). Lm indicates a slight modification to more negative potential as a result of a favourable rearrangement of ethylenic group present in the structure. An anodic peak

can be observed related to the oxidation of Lr to Lr product (radical anion), as an irreversible process due to its higher reactivity.

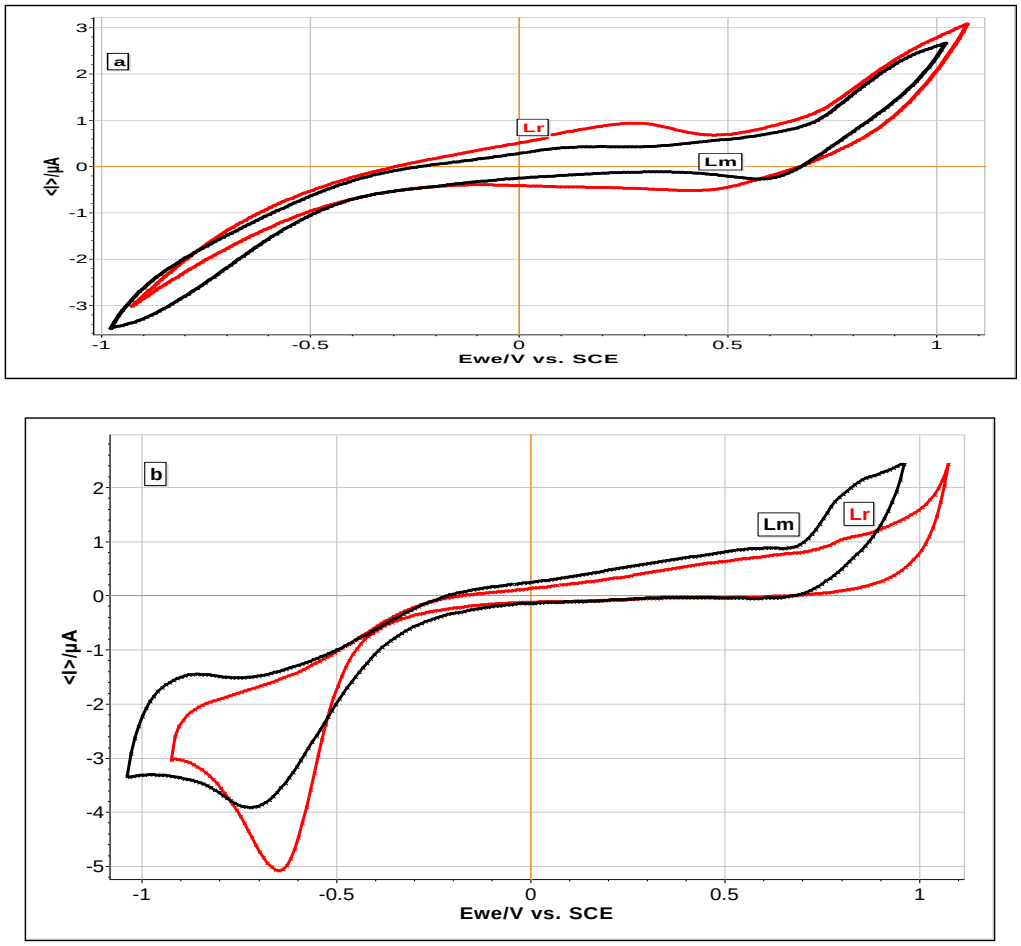


Figure 22. Cyclic voltammograms in aq. Lr and Lm from fresh solutions with initial pH (a) and at pH 9.0 (b); E = ± 1.0 V vs. SCE, from a negative direction, scan rate 100 mV s-1(a), scan rate 50 mV s-1(b).

The compounds described by CVs measurements could be more complex and show more than a single redox transaction. Water as proton acceptor suggests that in this case, the proton charge is delocalized over the primary shell of water molecules firmly formed around the pyridinium ligand. Lr indicates an evident reduction process in the alkaline domain (around pH 9), having quite a different behaviour, compared to the acidic and neutral domains (Figure 23a). The scan rate also remains an important condition in these electron transfers (Figure 23b). In alkaline pH, the current peak is shifted to more positive potential around +800 mV while the cathodic reduction is an intensive active process. At the same time Lm does not indicate an obvious electro-oxidation transformation (Figure 24).

It is evident that in the cathodic process on Pt electrode and at alkaline pH the deprototropic conjugate couple (from AH- to AH) changes slower in comparison with acidic and

neutral pH. A blockage of the electron transfer by adsorption of the reduced pyridinium ligands on the Pt substrate can suggest this behaviour.

Effect of the potential scan rate *CVs on platinum electrode have been registered for both aq. ligands (0.1 mM) at different scan rates (100 – 20 mV s-1) for E = ± 1 V (Figures 3b and 4a). No significant shifts were observed in the redox potential when the scan rate was increased from 20 to 100 mV·s-1in CVs of fresh aq. Lr at 6.00 pH (Figure 23b). Moreover, an increase of the anodic current was obtained when the scan rate was higher as an effect of faster prototropic conjugate in the form of AH2/AH-·or AH.-/AH2 from ylides stage that immediately formed when a potential is applied [Furdui, 2012].*

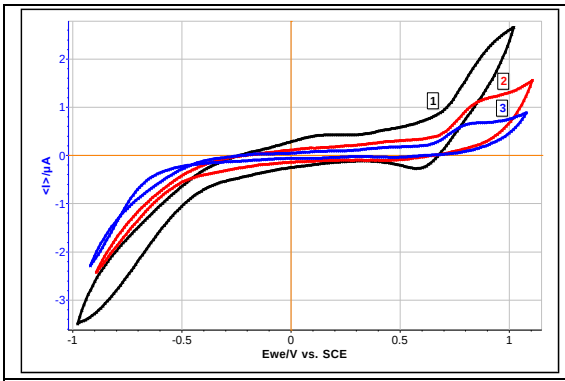


Figure 23a. Cyclic voltammograms in aq. fresh Lm (pH 5.50), at different scan rate: 100 mV s-1(1); 50 mV s-1(2) and 20 mV s-1(3), E = ± 1.0 V

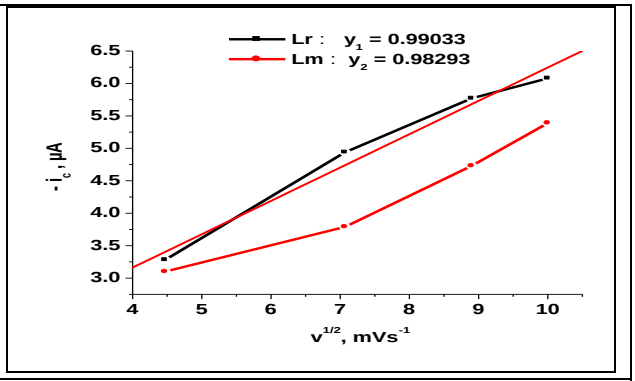


Figure 23b. Plot of cathodic current vs. square root of scan rates

The cathodic peak position obtained at 100 mV s-1 corresponds to the redox changes from potential (Epc) of + 350 mV for Lr to the potential of + 600 mV for Lm (Figure 23a). A comparative study of aq. alkaline pH shows the different behaviour between the pyridinium heterocyclic ligands. The electrochemical parameters are presented in Table 6.

Table 6. Electrochemical data vs. scan rate of the potential applied for aq. pyridinium ligands at pH 9.00

scan rate (mV·s-1)	*- Epc (mV vs SCE)*		*- ic (µA)*		*Epa (mV vs SCE)*		*ia (µA)*	
	Lr	*Lm*	*Lr*	*Lm*	*Lr*	*Lm*	*Lr*	*Lm*
100	*670*	*901*	*6.08*	*5.39*	*910*	*876*	*2.85*	*2.35*
80	*652*	*886*	*5.65*	*4.73*	*880*	*780*	*2.54*	*2.27*
50	*647*	*874*	*4.94*	*3.79*	*790*	*760*	*1.66*	*2.15*
20	*613*	*818*	*3.28*	*3.10*	*690*	*660*	*1.45*	*1.87*

The reduction peak of ligands was significantly shifted towards a more negative potential, whereas a small shift in the oxidation peak was obtained at aq. Lr. Lm presents a different potential between the anodic peak and the cathodic peak that is considered an effect

of quasireversible redox process of electronic transfer, due to the presence of the ethylenic group. Both ligands show characteristic peaks as a result of electron transfer between the functional groups, more evident on cathodic range in alkaline solutions. The cathodic peak at very lower potentials cannot be clearly assigned to reduction.

Assuming that the electron transfer rate is faster, the current Ia is measured while the potential decreases, directly related to the diffusion rate of oxidized species on the Pt surface, by the flux governed by Fick law (A. M. O. Brett et all., 2003). Thus, the oxido-reduction process involves a transfer of hydrogen proton and electron transfer of pyrididium ligands functioning as mediators in the aq. media. The peak is characterized by Ep, Ia and there is a peak shift while the pH increases.
The pyridinium ligands generate some current waves in cyclic voltammogram which are described as two typical one-electron transfer steps. The first step is reduction of AH2 (AH2/AH-) and the second step is the role of electron carrier of pyridinium ligand (AH-/AH.-). To explain this behaviour, a mechanism can be proposed when a catalytic reduction of the H+ ion takes place via a neutral (alkaline) intermediate of 1H adsorbed on the Pt electrode in aq. media. The addition of proton donor produces changes, not only to the electroactive species, but also to the overall mechanism of reaction, making it concerted. Aq. alkaline ligands indicate an irreversible electron transfer, confirmed also by the cathodic peak on electrode, which is more evident on Lr than on Lm; ligands exhibit different mechanisms depending on aq. pH.
Spectrophotochemical absorbance registered in different solutions, fresh and kept at constant temperature indicates that pH 6-7 is better for stability of aq. ligands. The open circuit potential is a useful tool to characterize the ligands stability in aqueous media on Pt electrode. CV has been applied to characterize the electron transfer of aq. pyridinium ligands and the possibility to become a useful mediator. Electrochemical measurements offer the possibility to study the redox potential and the influence of pH and scan rates applied.
A good correlation was found between the electro-oxidation potential and the stability in aq. media of the ligands at acidic and neutral pH. Moreover, at alkaline pH, the pyridinium ligands indicate a difference taking into account the acidic-basic and electrochemical properties which might result from the most favourable arrangement. The presence of the ethylenic group diminishes this effect as a result of more cyclofavourable addition in alkaline pH. We believe that the present work will stimulate further investigations; CV can be used to characterize the reduction ability and electrochemical behaviour of the compounds as pyridinium ligands and their role in biological chemistry. This study helps us better understand the ligands mechanism in aq. media and our purpose is to use them in cycloaddition to obtain indolizines in catalytic systems and for the synthesis (results published in Studia Chemia, 2015).

Study of lipase interaction and compounds involved in the reactions "click" by CV

*We investigated also through cyclic voltammetry the **lipase enzyme interaction** with new pyridinium ligands derivated from 4,4'-bipyridine. The biocatalytic properties of the lipase interaction were also evaluated by analyzing the behaviour of a precursor of the pyridinium ligands, phenacyl bromide, and a dipolarophyle, ethyl proprionate, used in cycloaddition*

reactions in the next step to obtaining the indolizine ring (results published in J. Electrochem. Sci., 2016).

Solutions were prepared by dissolving 0.1 mM from each pyridinium ligand in 0.1 M KNO3 using deionized sterile water. Two freshly-made ligands solutions were analyzed with and without lipase, one with dissolved oxygen and the other in absence of oxygen by introducing for 5 minutes into inert atmosphere of nitrogen. The precursor, phenacyl bromide, and the dipolarophyle ethyl propiolate involved in the biocatalytic proceess with lipase were also investigated. Different concentrations of lipase were added to pyridinium ligands and precursors solutions. Data was collected at room temperature (20±1°C) from fresh-made solution and during a certain period of time (1-14 days). The pH and conductivity was monitored with a Consort C862 multiparameter and the spectrophotometric data was collected with UV-VIS T90+ equipment.

The physicochemical properties of the lipase enzyme interaction with pyridinium ligand solutions were characterized by cyclic voltammetry (CV) technique, using Bio-logic SP50 equipment. The measurements were performed using a carbon electrode immersed in the prepared solutions at various scan rates between 0.5 V·s-1 – 0.02 V·s-1 for a potential range from a negative direction of E = -1.0 V to +1.0 V vs Ag/AgCl. All measurements were registered at 20±1°C and also at 40°C. The free redox potential (open circuit potential – OCP) and cyclic voltammetry (CV) measurements were repeated five times to mark the significant changes that might appear in solutions.

The morphology of the obtained lipase after electrochemical investigation was characterized by scanning electron microscopy (SEM) and energy X-ray spectroscopy (EDX) using Quanta 200. After being filtered, the lipase was dried in air at room temperature and it was placed on carbon-coated-copper grid.

The stability of pyridinium ligands in the absence and presence of lipase

The enzyme's influence on the rigid ligand (Lr) and mobile ligand (Lm) was investigated through physico-chemical properties .

Lr has shown a pH of 6.5 at fresh-made aq. solution and remains stable after 2 days. By adding a smaller amount of lipase (0.05 mg/mL), no essential modification in the pH on Lr electrolyte was observed after 2 days from the initial contact with enzyme. With the increase of the lipase amount, the pH decreases slowly. After 7 days the system with 0.5 mg/mL lipase had shown a decrease in pH with one unit of pH compared to Lr without enzyme (Figure 24).

Lm was characterized initially by a weak acid pH and varied over 14 days from pH 6.4 to pH 6.8. The presence of lipase induced significant changes in the pH of the Lm solution, mainly at a higher amount of enzyme (0.5 mg/mL), a neutral to weak alkaline pH being recorded. As time passed and an optimum operating pH of enzyme (neutral pH) was achieved, an increase of OH- ions is noticed and the enzyme becomes more active (Figure 24).

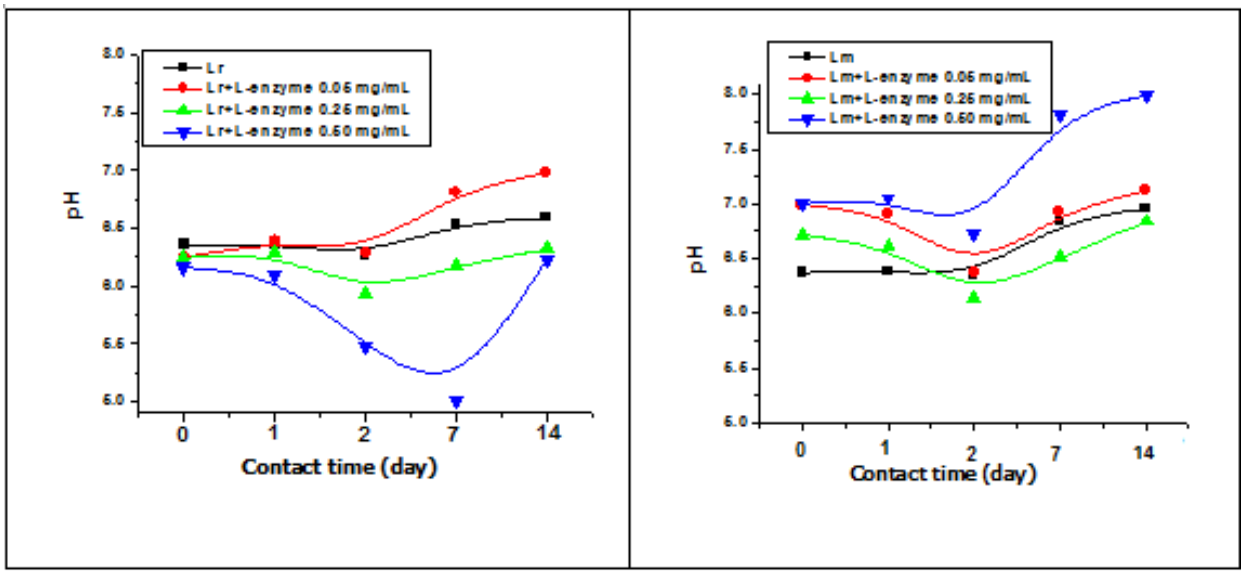


Figure 24. Time evolution of pH of the rigid and mobile ligand (Lr and Lm) with and without lipase

Aq. ligands (0.1 mM) electrolyte without lipase present a conductivity of 12-13 mS/cm In all systems, a constant decrease of conductivity in time was recorded with 2 mS/cm after first and second day and after that remains almost constant (Figure 25). Anyway, a slight variation of conductivity is obtained more for Lm in the presence of lipase of 0.05 mg/mL and 0.25 mg/mL after 7 days.

There were recorded UV-Vis spectra for all aq. solutions with and without lipase. The maximum wavelength (λmax) of Lr and Lm is 264 nm (UV). The absorbance indicates a shift for both of the ligand aq. solutions when they are in contact with lipase. The highest value of the absorbance when adding lipase was obtained for Lr, compared with Lm. The addition of lipase on the ligands' structure has induced the diminution of the absorbance more obvious for Lm than Lr. The lowest values of absorbance were recorded for a lipase concentration of 0.5 mg/mL, as a consequence of the inhibition of the enzyme major component in the ligands electrolyte. The same downward trend of the absorbance was maintained in the presence of the lipase for both pyridinium ligands in time over 14 days (Figure 26).

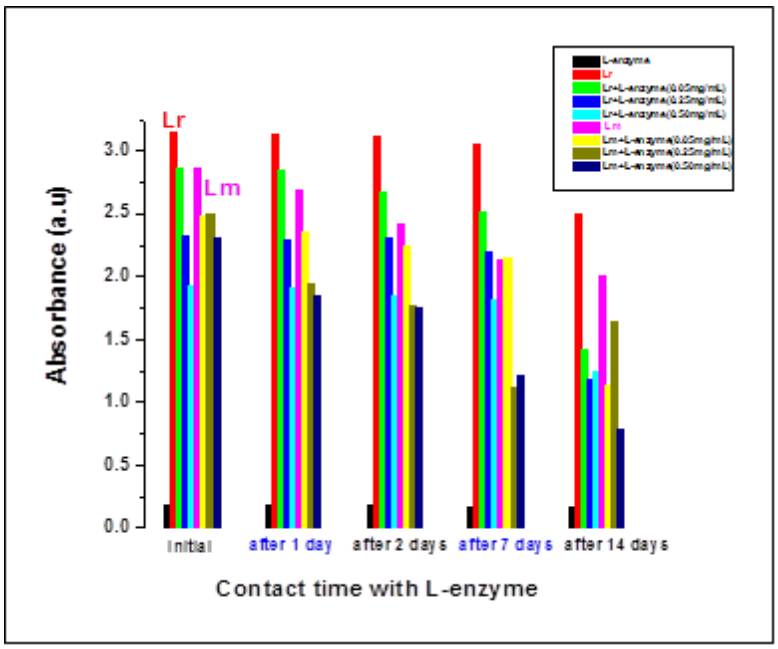


Figure 26. Time evolution of absorbance for pyridinium ligands with and without lipase

Temperature effect on pyridinium ligands in the absence and presence of lipase

An enzymatic reaction is affected by temperature and many studies showed that the optimum activity of many enzymes occurs at a temperature between 35 - 40°C In case of lipase, the optimal temperature was reported at 37-40°C. The ligand electrolyte solutions were prepared as previously described, adding lipase at 40°C (keeping the temperature constant) without stirring and emulsifying agent. With the increment of the temperature, for both Lr and Lm, changes of pH were recorded, showing a growth of the assessed values (Figure 27). Lr showed a decrease of pH in the presence of enzyme. On the other hand, the pH of Lm increased to a more alkaline one at the addition of lipase, varying between 6.8 (at lower enzyme concentration) and 8.2 (at higher enzyme concentration).

The conductivity was drastically reduced at 40°C, being situated in this case in the range of µS/cm. The ionic dissociation of the ligand electrolyte solutions without enzyme indicates a difference of cca 200 µS/cm more for Lm comparative with Lr. In the presence of lipase a different behaviour of the dissociation process was observed. Lr from fresh-made solution without lipase indicates a conductivity reduced by half (114 µS/cm) in contact with 0.05 mg/mL lipase and a slight increase of values up to 135 µS/cm for 0.50 mg/mL lipase (Figure 27). At the same time, Lm showed in the presence of 0.05 mg/mL enzyme a decrease of conductivity with 145 µS/cm from the values obtained with the fresh electrolyte without lipase (343 µS/cm) and a slight increase up to 317 µS/cm for more lipase added. Therefore, an inhibition of ionic dissociations occurs by raising the temperature of both ligands in the presence and absence of lipase depending of the enzyme amount.

The lipase interaction was evaluated at 40°C using the absorbance from the UV-Vis spectra. The absorbance showed an intensive lipase interaction with the molecules of ligands. In both

systems a decrease of absorbance was obtained, as an effect of the enzyme activity and increased temperature.

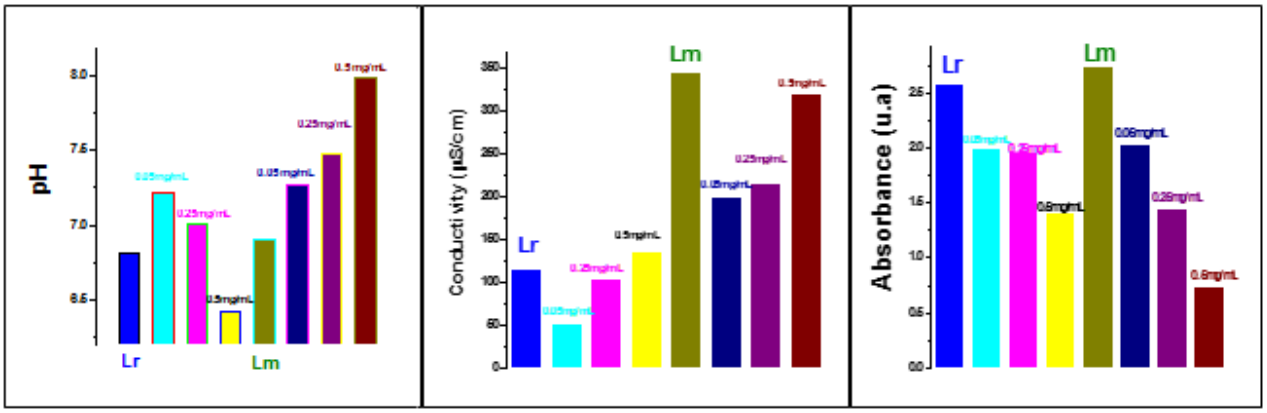


Figure 27. The effect of temperature at 40°C in the evolution of pH, conductivity and absorbance (λmax 264 nm) of the pyridinium ligands in presence of L-enzyme

Cyclic voltammetry studies

The ligand electrolyte solutions, initial and after 1, 2, 7 and respectively 14 days kept at constant temperature (20°C) were analyzed by electrochemical measurements. Cyclic voltammetry measurements were performed as a useful electroanalytical method to characterize the reduction ability and electrochemical behaviour of new pyridinium ligands which are involved in biocatalyzed cycloaddition by lipase enzymes. Oxidation processes (anodic reactions) manifest themselves in positive current peaks, and reduction processes (cathodic reactions) in negative peaks useful in understanding the mechanism of the reaction .

OCP measurements of the Lr electrolyte without enzyme showed a potential ranging from 0.034 V to 0.045 V vs Ag/AgCl till 2000 s and for Lm between 0.052 - 0.056 V vs Ag/AgCl, as effect of the zwitterions ligands' structure. Ligands without lipase showed for Lr an anodic current of 0.24 μA compared to Lm's almost constant anodic current which was no more than 0.054 μA. There were recorded CVs for potential applied between E=±1V vs Ag/AgCl on both ligands 0.1mM in electrolyte KNO3 0.1 M with and without lipase. The lipase content in the ligand electrolyte solutions has substantial effects on their physicochemical properties as well as voltammetric response, more on Lr compared with Lm, and this effect was dependent on their structure and also on the lipase concentration and scan rate of the potential applied.

Effect of the lipase concentration

CVs generated as an effect of the lipase concentration in both ligands electrolyte are presented in Figure 5. When 0.05 mg/mL lipase was added for Lr the anodic current increased with 0.25 μA and two current peaks were observed compared with the ligand without enzyme. When more enzyme was added to Lr electrolyte a relatively distinct anodic peak current (peak a) at a potential of 0.5 V vs Ag/AgCl and increase with 1.50 μA at 0.5 mg/mL lipase was obtained. The Lr molecule readily undergoes electrooxidation. Primarily, it is the reduction of AH2 (AH2/AH-) and the second step is the role of electron carrier of pyridinium ligand (AH-/AH.-). The radical intermediate subsequently undergoes AH- to the formation of a new radical on the ligand AH.-. When the concentration of enzyme was gradually increased from 0.05 mg/mL to 0.50 mg/mL

there was a gradual increase in the peak current response and this response was finally saturated at 0.25 mg/mL (Ia of 1.76 µA) of the lipase where the anodic current values observed were almost constant (Figure 28). Lm in interaction with lipase had shown an anodic current between 0.08 µA to 0.1 µA without any distinct peak.

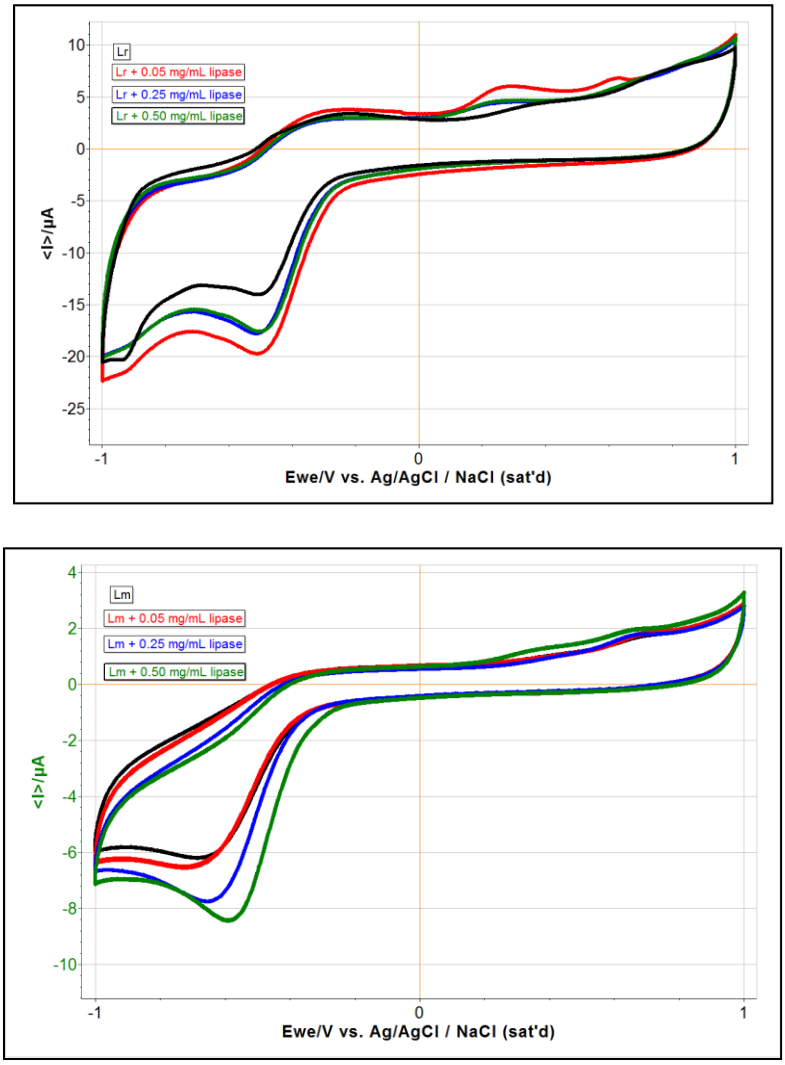


Figure 28. CVs generated of Lr and Lm electrolyte 0.1 M KNO3 in the presence of different concentration of L-enzyme, E = ±1 V vs Ag/AgCl, 0.5 V·s-1 for Lr and 0.1 V·s-1 for Lm

For further studies the oxygen was removed by solutions and CV was generated (Figure 6). In the ligands electrolyte in the absence of oxygen (by introducing for 5 minute into inert atmosphere of nitrogen), with and without lipase CVs shown a reducing of the anodic current in the absence of oxygen in electrolyte with lipase and the effect is also depending of the scan rate of the potential applied (Figure 29).

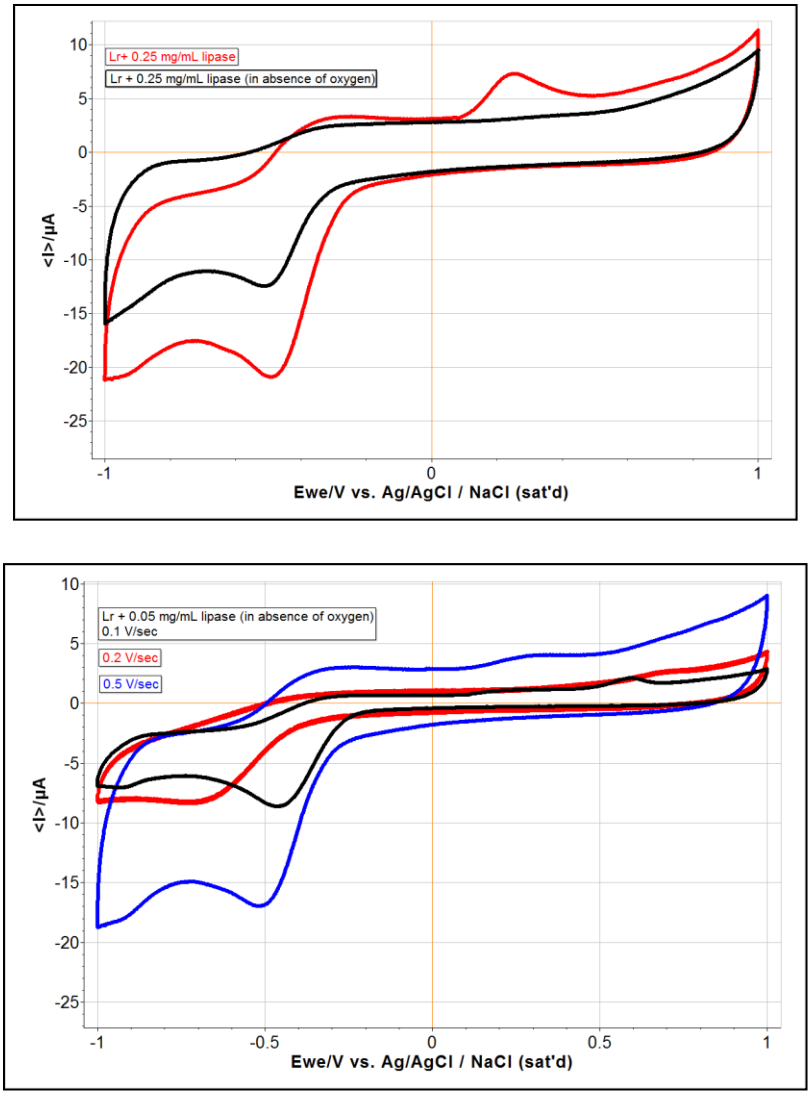


Figure 29. CVs generated of Lr in electrolyte 0.1 M KNO3 in the presence of lipase with and without oxygen, E = ±1 V vs Ag/AgCl, 0.5 V·s-1

Effect of the scan rate

The lipase interaction at room temperature with pyridinium ligands is intensively affected by the scan rate of the potential applied. CVs shown change of waves in both ligands when scan rate of the potential applied was changed from 0.02 V·s-1 to 0.5 V·s-1. Figure 30 shows CVs of the pyridinium ligands in the absence and in the presence of 0.25 mg/mL lipase (pH 7.0) at the different scan rate (E = ±1 V vs Ag/AgCl). An increase of Ia is obtained for Lr from 2.77 µA (at 0.2 V s-1) to 6.3 µA (at 0.5 V s-1) and a shift of potential from E1 of 0.22 V to E2 of 0.28 V vs Ag/AgCl. Lm does not indicate an evident anodic peak but an increase of Ia with the scan rate of the potential applied was observed.

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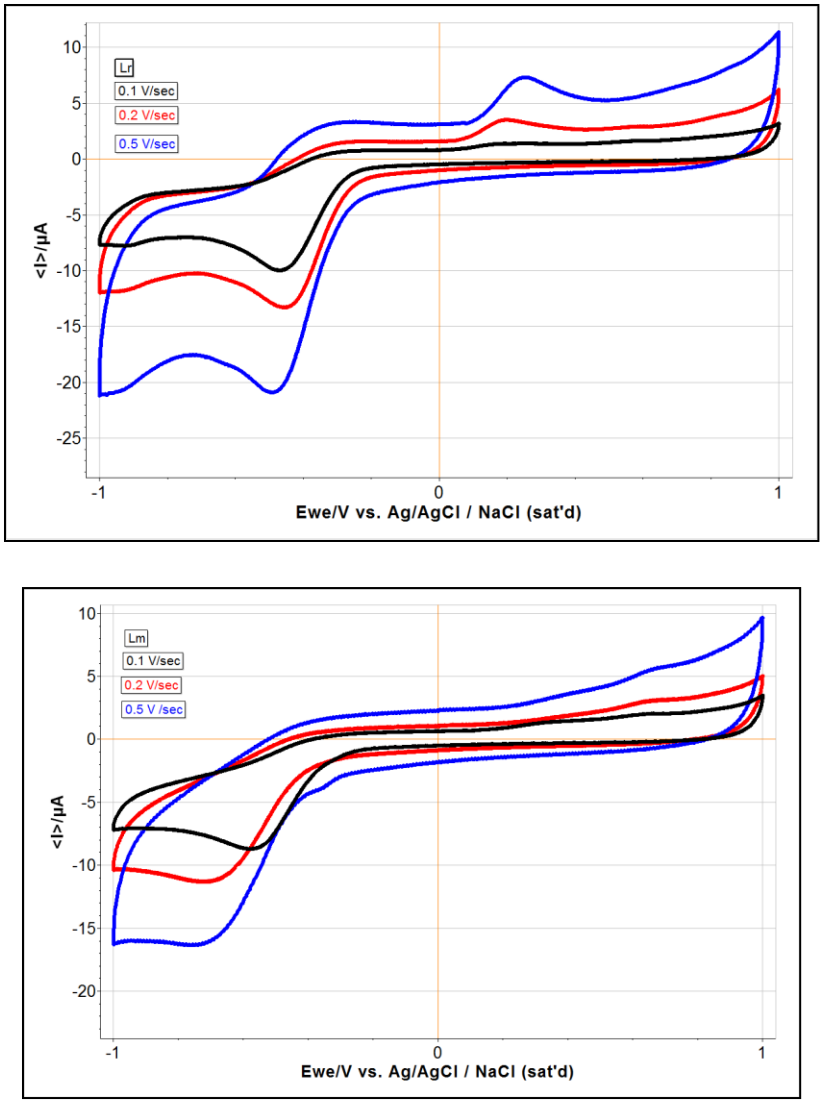


Figure 30. CVs generated of the Lr and Lm electrolyte in the presence of 0.25 mg/mL of L-enzyme at different scan rate, E = ±1 V vs Ag/AgCl

Figure 30 shows CVs of both ligands in the presence of 0.50 mg/mL lipase enzyme (pH 7.0) at the scan rate of 0.5 V s-1. A distinct anodic peak is obtained as effect of an intensive oxido-redox process, more evident at Lr than Lm. The behaviour of ligands is different, lipase shown a positively effect on Lr compared with Lm. This catalytic lipase activity difference is mainly due to there being more functional groups active on Lr structure than Lm. The presence of the mobile ethylene group marks changes in electrochemical performances of the Lm. The enzymatic activity of L-enzyme is dependent on structure. The Ia value for Lr is higher than Ia of Lm (ΔIa= 45 µA), which could explain a more rapid electron transfer process for Lr, as a result of the favorable its structural arrangement, comparative with Lm.

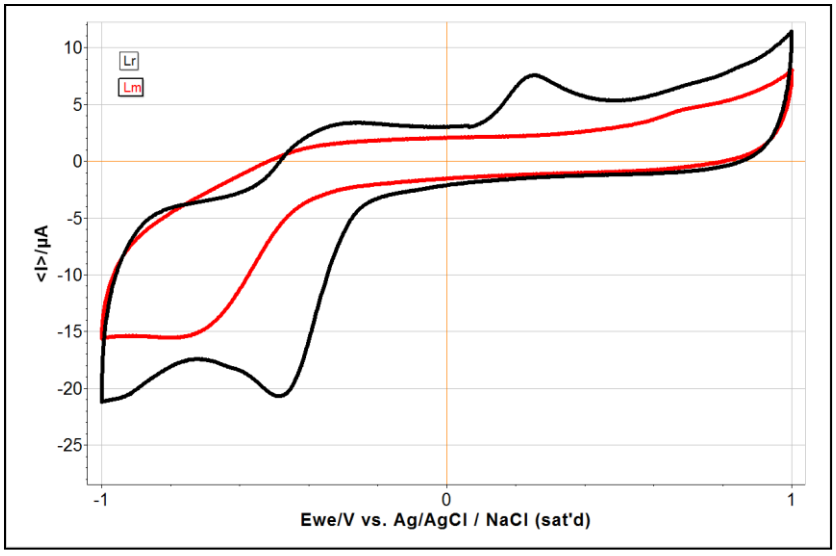


Figure 31. CVs generated of the Lr and Lm electrolyte in the presence of 0.50 mg of L- enzyme, E = ±1 V vs Ag/AgCl, 0.5 V mV·s-1

No reduction wave was observed in the presence of the precursor of pyridinium ligands, phenacyl bromide and respectively on the ethylpropiolate (synthon) in presence or absence of lipase. The lipase interaction on precursor is not observed (Figure 32). CVs registered only the effect of the diffusion process on the carbon electrode. These results demonstrated that pyridinium ligands with a different structure than precursor shown an electro-oxidation behaviour and lipase has an effect on them (Figures 29-31).

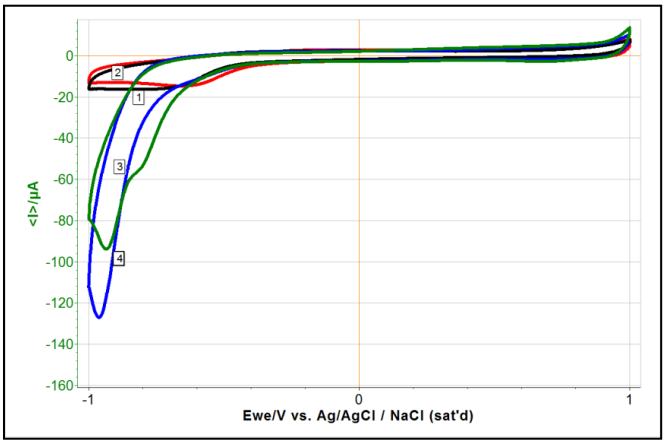


Figure 32. CVs generated of phenacyl bromide (1) in the presence of L-enzyme 0.25 mg/mL (2); ethylpropiolate (synthon-3) in the presence of L-enzyme 0.25 mg/mL (4), E = ±1 V vs Ag/AgCl, 0.5 V·s-1

Structural characterization

The lipase was analyzed before and after interaction with ligands electrolyte. The enzyme that was in contact with ligands from CV measurements was recovered by filtration and dried in air

at room temperature. The SEM images and elemental analysis (EDX) was performed when lipase was placed on carbon-coated-copper, to observe the morphology and structural changes. SEM images showed a modification in the structure of lipase before of experiment and after interaction with ligands, in the presence or absence of oxygen (Figure 33).

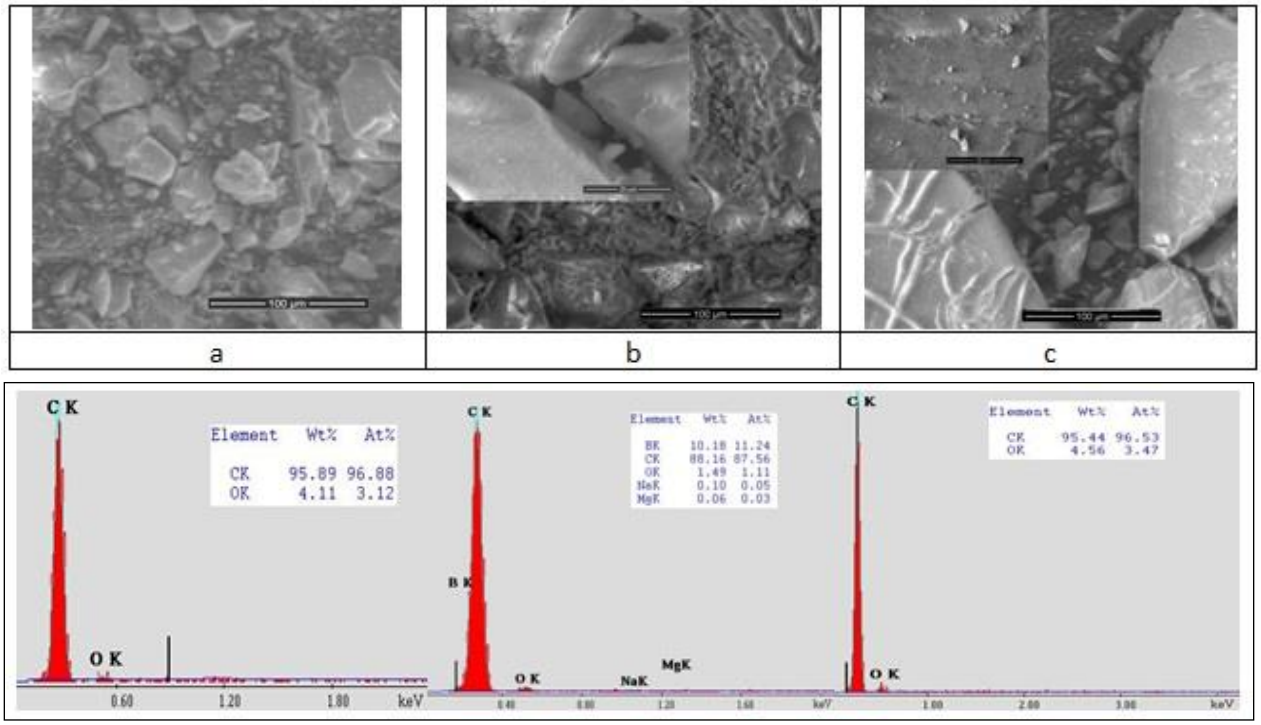


Figure 33. SEM images and EDX analysis of L - enzyme (a); lipase interaction with Lr in the presence of oxygen (b) and without oxygen (c)

The grain size of lipase (Figure 33a) was modified significantly in the presence of Lr electrolyte (Figure 10c), even the chemical analysis indicated almost same concentration, carbon 95.89 wt % versus 95.44 wt % and respectively oxygen 4.11 wt % versus 4.56 wt %. The reduction of carbon at 88.2 wt % and oxygen at 1.5 wt %, boron (10.18 wt %), also Na and Mg in small content with a strengthening role in the cell is evident a result of a contact of lipase with Lr (Figure 33b).

In conclusion, the stability of new ligands in KNO3 10-1 M as support electrolyte is influenced by the quantity of lipase added and a good correlation was observed during a certain period of time between the pH, conductivity and UV/Vis spectra measurements.

The electro-oxidation processes of the electron transfer depend on the ligands' structure. The lipase content in the ligand solutions has substantial effects on their physicochemical properties as well as voltammetric response. The recorded voltammograms showed an intensive electronic transfer due to the Lr interaction with lipase compared with Lm because of the absence of a mobile ethylene groups from the Lr chemical structure. In the presence of oxygen, the pyridinium ligands act differently, taking into account all physicochemical properties and the

redox potential. This might result from the most favorable arrangement of their molecular structure, depending of the lipase concentration and the scan rate of potential applied also.
The study is useful to understand the mechanism of cycloaddition reactions in which compounds could participate as synthon. Quaternary ligands were designed as precursors for fluorescent indolizine synthesis.
These were the first spectroscopic and electrochemical studies performed on the compounds synthesized in the previous stages. As a result of these studies, remerkable biological and electrochemical properties of the compounds obtained through biocatalysis were identified.

O IV.3.Senzors obtaining essay using different techniques

In recent years, numbers of applications have been shown requiring the deposition of organic molecules, biomolecules or polymers as a substrate. Many studies with a wide variety of targeting biological applications have made it a fast growing research topic for specific medical technologies. Polymer films for applications in organic electronic devices and sensor have also been investigated.
The primary targets for MAPLE (matrix assisted pulsed laser evaporation) has been for biological molecules and polymers. MAPLE is derivate from pulsed laser deposition for delicated as polymers, complex biological molecules etc., and materials in undamaged form. Using MAPLE, proteins and enzymes have been also deposited. The enzyme lipase found in almost biological system (with role of catalyst) is used in biosensors, but an important issue is immobilization/ deposition as a substrate. The molecules deposited by MAPLE preserve the conformational characteristic of the molecules.
This technique is derivate from pulsed lasers deposition (PLD) with the difference in the composition of the target material. A frozen sample (e.g. polymer 1-5% mass concentration)/ solvent solution represent the target. The molecular species is ionized and the target is solid at room temperature. The most of important conditions are the laser wavelength and matrix material (A. Piqué, 2011). A pulsed laser, usually ultraviolet or infrared, having a spot from 1 to 25 mm2 is directed into a vacuum chamber through a laser window. The laser fluence is chosen to minimize degradation of the solvent/solute system, ranging from 0.01-5 J cm-2. A few mL of target solution is frozen in a metallic cup (Al) and inside the vacuum chamber, a rotating target holder is cryogenically cooled to keep the frozen during experiment. Initial a low vacuum (10-2 to 10-7 torr). The substrate holder is positioned parallel to the target holder at a distance from 3 cm to 10 cm. The monitor of the thickness during deposition is also provided in some equipment. The characteristic in our study was: a pulsed KrF excimer laser source (λ = 248 nm, τ = 25 ns, v = 10 Hz). Many variables exist for this technique: laser wavelength, fluence, pulse duration, solvent, concentration, background pressure, gas species, substrate temperature etc(Datcu, A. et all., 2015)..*

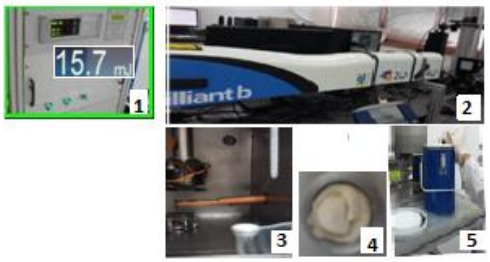


Fig. Schematic overview of MAPLE techniques: 1- Nd-Yag laser source; 2-laser beam energy, 3 vaccum chamber vacuum under Ar atmosphere, 4 – target sample in metallic cup; 5 –frozen sample

Mechanism deposition in the MAPLE process. The initial photophysical interaction between laser and target material is responsible for material ejection and film formation.

In our preliminary study we showed that is possible using MAPLE to deposit novelty compounds based on lanthanide with heterocyclic ligands on a substrate of silicium and respectively quart.

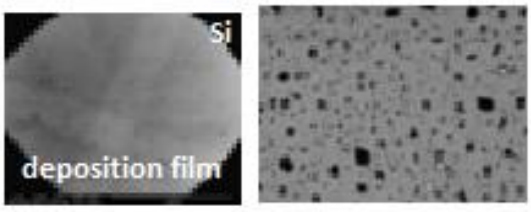
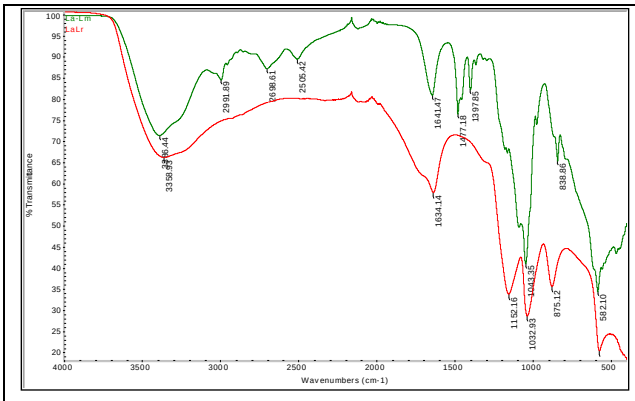
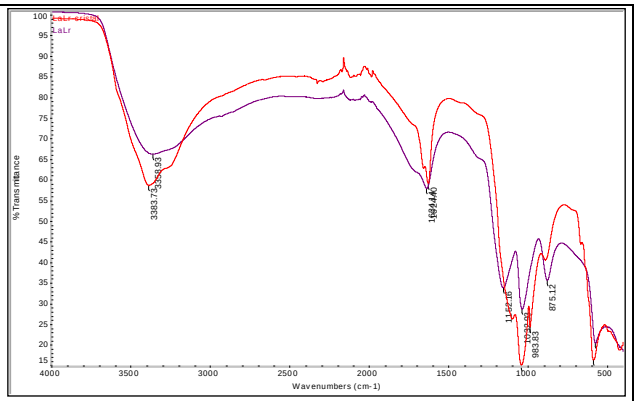


Fig. The deposition film and SEM images

SEM observation preserving it's conform structure as shown by FTIR analysis (Fig. x). In our study we try to show that is possible using MAPLE technique to deposit organic molecules, on a substrate preserving its conformational structure, as shown by FTIR analysis.



FTIR analysis of compounds of La-Lr si La-Lm in solution



FTIR analysis of compound of La-Lr in solution and respectively as deposited film by MAPLE

The FTIR analysis confirm that a deposited of compounds on film using MAPLE technique.

Several articles had shown the enormously process of the MAPLE technology in the obtaining of biosensors(Touloupaki E et all, 2012).. We do not present or discuss a particular biosensor; we just demonstrated its feasibility presenting the results of preliminary test. Immobilized in a polymer composite for biosensor applications and demonstrated that the structure and activity of the biomolecules .
Anyway, the coordination of simulations and experiments is necessary to design films with desired properties using such technique. MAPLE is a gentler laser process approach for depositing films as a versatile deposition technique that enables additional control over the films growth process, as compared with other methods.

Conclusions

- ✓ *The objectives of the PCCE project have been fully performed.*
- ✓ *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, and the catalytic properties of the biocatalyst. In the case of microorganisms, bioconversion evolves 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.*
- ✓ *For the first time were obtained new compounds, with fluorescent properties, by "one-pot" reactions (which involves three components) using enzymatic catalysis.*
- ✓ *For the first time in cycloaddition reactions we used biocatalysts commercial enzymes, microorganisms from the UDJ collection (MIUG) or enzymes from plant sources (Amoracia rusticana). The reactions took place in an aqueous medium or ionic liquids at temperatures of 25-50^{o}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 reactions are made under mild conditions, with the enzymes that can be obtained easily and are renewable materials.*
- ✓ *The advantages of using biocatalysts are compared with the processes performed by classical reaction.*
- ✓ *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.*
- ✓ *For the first time were performed toxicological and cytological studies on model eukaryote microorganisms such as yeast Saccharomyces cerevisiae. Following studies, remarkable*

biological activity was proved for some compounds synthesized by the biocatalytic reactions.

- ✓ *For the first time spectroscopical and electrochemical studies were performed on the synthetised compounds obtained in previous phases. Following studies, remarkable biological and electrochemical properties were proved for some compounds synthesized by biocatalytic reactions.*
- ✓ *Fluorescence studies demonstrated strong fluorescent properties of all analyzed compounds with maximum emission band in blue-violet region.*
- ✓ *The interaction with DNA was made by UV-Vis, IR and fluorescence spectroscopy to study the nature of DNA-fluorophore binding. We found that there is a strong DNA-indolizine interaction, indolizine fluorescence is quenched at the increase of the molar ratio DNA:fluorophore. The possible interaction type of indolizines with DNA was determined to be groove binding.*
- ✓ *The electrochemical properties ware evaluated by cyclic voltametry. We believe that the cyclic voltametry studies will stimulate further investigations; CV can be used to characterize the reduction ability and electrochemical behaviour of the compounds as pyridinium ligands and their role in biological chemistry. This study helps us better understand the ligands mechanism in aq. media and our purpose is to use them in cycloaddition to obtain indolizines in catalytic systems and for the synthesis of new Ln complexes.*
- ✓ *For the first time they were realized electrochemical studies for sensor obtaining and testing of the compounds synthesized by MAPLE teqhnique. Following the studies the remarkable electrochemical activity of the synthesized compounds was proved.*
- ✓ *The biocatalytic processes used by us for the first time in obtaining said heterocyclic compounds require lower power consumptiondue to the mild reaction conditions (cheap biocatalysts, low T), fewer stepsand 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 for a process to be considered as conducted in the concept of sustainable chemistry.*
- ✓ *The research results contribute to the elucidation of mechanism of biocatalysts action in cycloaddition reactions.*
- ✓ *The collaboration between the involved partners in the project had as a result the development of papers published or submitted for publication, papers containing results of the project interdisciplinary research. Indicators provided were made; some of the results were published in peer reviewed papers.*
- ✓ *The results obtained during the project period were valued as follows: 9 papers published in ISI journals, 4 papers in ISI proceedings; 1 article BDI, 34 papers presented at international conferences, three manuscripts submitted for publication.*
- ✓ *It was published a monograph on electrochemical methods applied in the last phase of the project.*

- ✓ *A book chapter, Perspective-Breakthroughs in indole and indolizine chemistry - new synthetic pathways, new applications, was published in Scope of Novel Heterocycles from Organic and Pharmaceutical, edited by InTech-open acces.*
- ✓ *Two doctoral thesis completed by two members of the project (Ioana otilia Ghinea and Simon Bonte).*
- ✓ *The project has supported 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 3 projects that have resulted in a project proposal in the competition Parteneriate 2013, PNCDI III (colaborari bilaterale şi Proiect experimental demonstrativ, 2016).*
- ✓ *The results obtained concerning the biological activity contributed to participation and winning a COST project (Active and intelligent fiber-based packaging - innovation and market introduction- ActInPak COST FP1405, oc-2014-1-18987).*
- ✓ *The results of the research led to the habilitation title obtaining of the project manager and the development of new research directions with the debut of a new doctoral thesis (Veronica Andreea Dediu).*
- ✓ *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.*
- ✓ *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.*

Project manager,
Prof.dr. habil.Dinică Rodica Mihaela