Scientific Report

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

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

Between January - December 2014

Phase IV

The objectives for Phase IV of the project were fully achieved. The collaboration between the partners involved in the project resulted in published papers or papers written for publication, in which the various project activities are presented.

This phase aimed to biologically characterize the compounds obtained in the previous stages and the study of fluorescence spectra of the synthesized compounds. The results of the activities included in stage IV are further summarized.

Biological characterization of the compounds synthesized

4.1 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 all, Eur. J. Med. Chem., 2001 Chanawanno & all, Eur. J. Med. Chem., 2010). Oxime functions grafted onto these salts generate important properties in treating organic phosphate poisoning and also antibiotic (Lorke, Curr. Med. Chem 2008). A number of bispyridinium-oxime has a very good reactivation activity of AChE inhibited by sarin and paraoxon (Acharya & all, Eur. J. Med. Chem., 2011, Bharat & all, Curr. Med. Chem 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 & all, Bioorg. Med. Chem. Lett. 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 & all, Eur. J. Med. Chem. 2010). Studies have also shown that indolizine derivatives exhibit cytotoxicity against cancer cell lines resistant to antibiotics (A. David James & all, Bioorganic & Medicinal Chemistry Letters, 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 (BS Sekhon, J Pharm Educ Res Vol. 3, 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. the toxicity study of heterocyclic compounds on pathogenic and non-pathogenic microorganisms;
iii. cytotoxicity studies carried out on the Saccharomyces cerevisiae;
iv. 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*

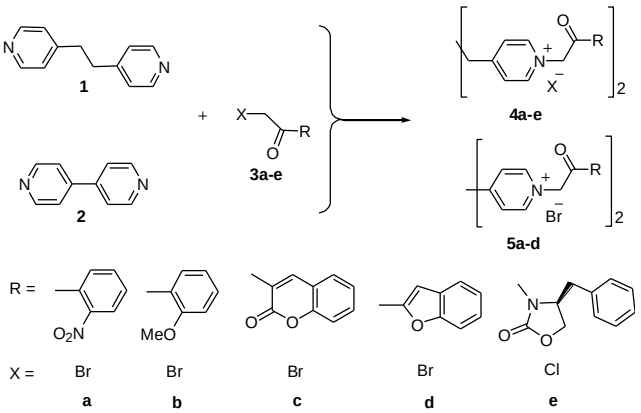
*ethyl propiolate) against certain strains of **bacteria**, **yeasts** and **molds** (C. robusta MIUG 3.2, C. robusta MIUG 3.3, C. tropicalis MIUG 3.4, C. utilis MIUG 3.5, Sacharomyces cerevisae MIUG 3.6, Yarrowia lipolytica RD 14 MIUG, Yarrowia lipolytica RD 15 MIUG, Yarrowia lipolytica RD 16 MIUG, 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.*

Some of the results obtained in this study were submitted to Letters in Applied Microbiology.

*ii. **The toxicity study** of heterocyclic compounds obtained through biocatalysis reactions was performed by determining **the biological activity on non-pathogenic and 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 (diagram1), 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 staphylococcus 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, Toxicology in Vitro 28, 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 (diagram 3), 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.*
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 below. The literature data shows that these descriptors can help predict the cytotoxicity of chemical compounds (J. Ranke et all, Ecotoxicology and Environmental Safety 67, 2007).

Diagram 3



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. 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 3. 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 4. 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.

iii. 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 cells (TS Gonçalves et all, BioMed Research International, 2014 NP Poletto et all, Basic & Clinical Pharmacology & Toxicology, 104, 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 (CD Armour et all, Curr Opin Chem Biol 2005 9).

There were evaluated three groups of compounds: quaternary pyridinium salts, indolizine and triazole derivatives obtained through biocatalysis cyclization reactions (diagrams 4-6).

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 in order to inoculate 100 ml of culture medium with 1 mL of cell suspension containing 10^8 cfu/ mL.

In the sample vials was added a solution of the compound whose effects are analyzed. Aqueous stock solutions were prepared with concentrations of 10-3 M, adding 1 ml to obtain a concentration of 10^{-5} M and 0.1 ml to obtain a concentration of 10^{-6} M. In the control tubes is contained only the inoculated culture medium, without the addition of the solution. The cultivation was carried out in Erlenmeyer flasks in 100 mL of YPD culture medium (g/ L: yeast extract 10, peptone 10, glucose 20) under aeration and agitation at 150 rpm in orbital shakers, thermostated at 25° C. 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. In order to determine the budding index wet microscopic preparations are carried out, using 10 ml of homogenized fermentation medium. In order to determine the degree of autolysis 0.5 mL fermentation medium is taken and to that is added 0.5 mL of a blue methylene-citrate (1: 1 v/ v); after 5 minutes, 10 ml of this mixture was taken and wet microscopic preparations were made – the autolysed cells are colored in blue. After 48

hours under stirring and aeration submerged cultivation, the bottles are kept in a steady state for 48 hours, after this time assessing the degree of autolysis. The fermentation media are transferred to Falcon tubes and centrifuged at 6000 rpm, 4° C for 15 min, performing two washes with sterile saline. Then the total amount of biomass and the percentage of moisture content of each sample can be determined.

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. Relevant results of this study are shown in Figures 8-10.

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.

iv. The toxicity study of the compounds on plant germination

The impact of different types of compounds on superior plants has been studied extensively (Hong and Otaki, 2006; Lin et al., 2009; Seeger et al., 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. Ethanolic stock solutions 10^{-3} M concentration are prepared from each compound to be analyzed. One petri dish and two filter papesr cut to the dimensions of each sample are also prepared. One of the two discs of filter paper is lodged in the Petri dish and it is imbibed with solution: 1 mL to obtain a concentration of 10^{-6} M and 10 mL to obtain s concentration of 10^{-5} M. For witnesses just swell with ethanol. The Petri dishes' lids are placed and the discs thus prepared are dried in drying stove at 85 ° C for 6 hours. 100 grains of wheat are counted for each petri dish, selecting only the grains with intact caryopsis. The grains are deposited on the dry disc of filter paper. Distilled water is added, 4 mL for the sample in which the germination energy is determined and 8 mL for the sample in which the sensitivity to water is determined; everything is covered with the second sheet of filter paper and the Petri plate's lid is placed. 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 (Figure 11).

The germination energy is determined by calculating the percentage of grains that did not germinate. Due to the fact that in this experiment this percentage was negligible and similar for the samples and the control samples, we used the average height of the plumules as indicator. 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 (Figure 12).

The water sensitivity of a plant is determined by adding a large amount of water and assessing the percentage of seeds which do not germinate. There was a higher percentage of grains which did not germinate than in the experiment for determining the germination energy, but a larger number of plumules with greater height. 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 (Figure 13). 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 were published in the Romanian Journal of Chemistry, 2014, 65, No. 8.

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 (Jefferson Chan, C. Sheel Dodani, Christopher J. Chang, Nature Chemistry, 4, 2012, 973-984) with the purpose of analyzing the intracellular composition of different microorganisms (Rakesh Nair, Sheetal Raina, Tajalli Keshavarz, JP Mark Kerrigan, Fungal Biology 115, 2011, 326-334) as well as investigating the course of action of drugs in vivo or in vitro (Bivash Mandal, Pavan Balabathula, Nivesh Mittal, George C. Wood, Himanshu Bhattacharjee, J Fluoresc., 22, 2012, 1425-1429). 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 (Shigeta, M., Morita and Gen-ichi Mifumi Konishi, Molecules, 17, 2012, 4452-4459). 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-Volume Editor, Standardization and Quality Assurance in Fluorescence Measurements./ Techniques, Springer-Verlag Berlin Heidelberg, 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 (compounds which have been synthesized in the first stage of the project – diagrams 4-6). 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.*

In order to study the properties of the synthesized cycloadducts photoluminescent absorption and emission spectra were recorded in solvents with various polarities: water, ethyl acetate, 95% ethanol, dimethyl formamide, acetonitrile, acetone, chloroform. The recording of all emission and absorption spectra was performed with approximately constant concentration solutions (1.06-1.39 x 10-5 M for compounds 4a-c) (i, ii) and 5a, 1.10-1.18x10-6M for compounds 6a and 4d (i, ii)) working at room temperature (T=25°C). The recording of absorption spectra was performed with UV-Vis spectrophotometer Labomed Inc., working at wavelengths between 200 and 600 nm. The recording of emission spectra was performed on a Perkin-Elmer spectrophotometer with excitation at wavelength λmax located furthest in the absorption spectrum (S0-S1 transition band), working in the window 420-700 nm for 5a, 400-700 nm 4a-c (i, ii), and 6 and 345-600 nm, respectively for the compounds 4d, e (i, ii).
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 (for example, Figure 14, 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 5 for compound 4a (i).

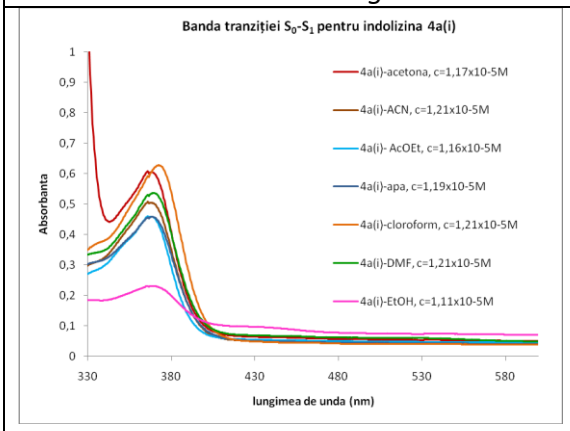
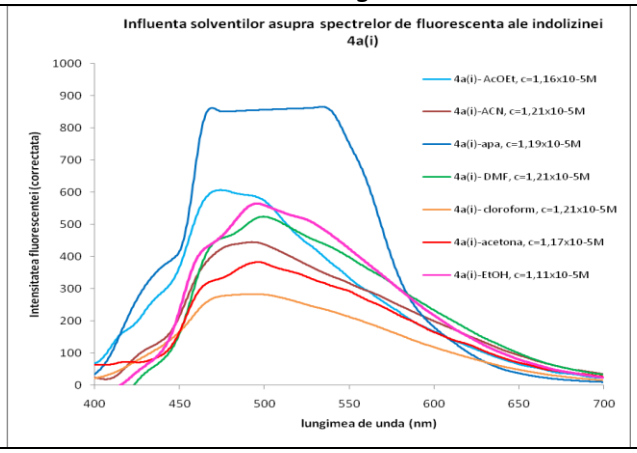
Figura 14	Figura 15
	

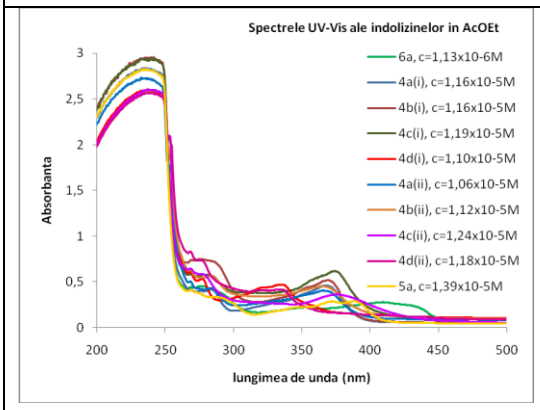
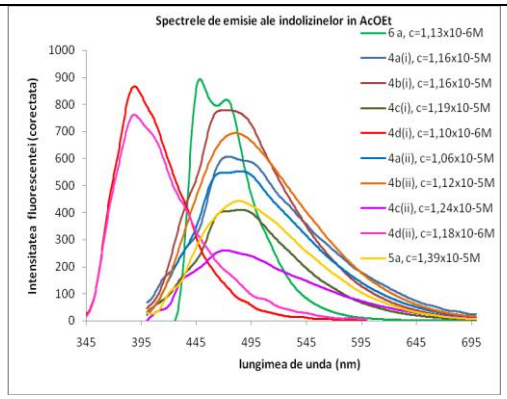
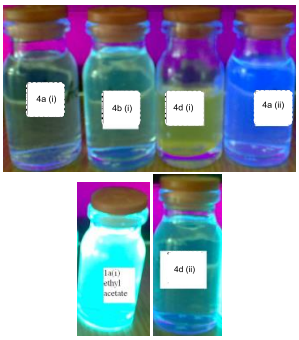
Table 5

Compound 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***

The shape of the emission curve is approximately the same, continuous and unstructured, as can be seen in Figure 15. 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.

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 16). 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 6 and Figures 17 and 18); 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.

Figure 16	Figure 17	Figure18
		

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.

Table 5

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

Conclusions *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. Fluorescence studies demonstrated strong fluorescent properties of all analyzed compounds with maximum emission band in blue-violet region.*

The objectives of this phase have been fully performed. The collaboration between the partners involved 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 as Molecules and Romanial Journal of Chemistry or communicated to international conferences (CISA, in 2014 and the French-Romanian Collocviul Medicinal Chemistry, Iasi, October 2014).

Also, it was published a monograph on electrochemical methods of analysis to be applied in the last phase of the project, in order to obtain and study the sensors containing compounds synthesized in the previous steps. Other results are submitted for publication or are in progress. The project supports two bilateral cooperation projects, one with French and one with China, projects involving several members of this project. Also, the results obtained in step on 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) several members of the project being also members of this COST action.

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