21st CEUM

21st **C**entral **E**uropean NMR Symposium

&

Bruker **U**sers **M**eeting

BOOK OF ABSTRACTS

September 4-5, 2019

Belgrade, Serbia

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Preface

The 21st Central European NMR Symposium & Bruker users meeting (CEUM) is an important scientific and professional event for all NMR users in Central Europe.

It has already a long history and has travelled along many counties in Europe.

It has always been an important occasion to share experience and know-how inside the community and meet scientists working mainly in the close countries (but not only!) in order to built up a stronger NMR community and have also a vision on new applications of NMR and new developments in the Magnetic Resonance Technology.

We are sincerely grateful to the lectures and guests coming from Greece, Bulgaria, Romania, Moldovia, Croatia, Slovenia, Bosnia and Herzegovina, Hungary, the Netherlands, US and from different cities from Serbia to have jointed this meeting.

He sincerely hope that all our effort will be lead also this year in this very nice city to a interesting and successful conference.

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NMR SPECTROSCOPY OF NATURAL PRODUCTS: A USEFUL TOOL IN QUALITY CONTROL, FINGERPRINTING OF PLANT EXTRACTS AND TARGETED ISOLATION OF NATURAL PRODUCTS

Eleni Skaltsa

National & Kapodistrian University of Athens, School of Pharmacy, Department of Pharmacognosy & Chemistry of Natural Products, Panepistimiopolis, Zografou, 157 71, Athens., Greece

email: skaltsa@pharm.uoa.gr

The therapeutic use of plants and natural drugs has been systematically exploited for thousands years all over the world. Over the last decades, the demand of medicinal plants has vastly increased and various cultivations of medicinal plants have been developed in order to respond on this enormous market. NMR spectroscopy is a useful tool for the quality control of herbal products, as it can provide information on the fingerprint of herbal extracts and also on potential adulterations. Moreover, NMR spectroscopy is allowing in depth phytochemical investigations and the detection of even minor compounds in the extracts. A non-destructive structure elucidation can be obtained using NMR data. NMR strategy can also serve as guide for targeted isolation of natural products.

BEE HONEY - TELL THE REAL ONE BY NMR SPECTROSCOPY

Svetlana Simova*, Dessislava Gerginova

Bulgarian NMR Center, *Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str. Bl. 9, 1113 Sofia, Bulgaria*
* Corresponding author e-mail: sds@orgchm.bas.bg

Bee honey is well known as a sweetener and a medicine in folk medicine, pharmacy and cosmetics. High demand makes it among the most counterfeited food products in the world. Numerous studies over the past decade aim to develop faster and more reliable methods for determining its authenticity, botanical and/or geographic origin. The sugar profile, which characterizes the carbohydrate content of honey, has not been sufficiently studied and so far there is no public database of honey produced in different countries and harvested from different vegetation.

We applied NMR techniques to identify the components that distinguish honey from acacia, oak, lime, chestnut, sunflower, orange blossom and bouquet, produced in Austria, Argentina, Brazil, Bulgaria, Germany, Greece, Italy, Cyprus, Malta, Morocco, Northern Macedonia, Pitcairn Islands, Poland, Romania and the Czech Republic, collected by *Apis Mellifera*, as well as by stingless bees – *Meliponini* in Tanzania. Quantitative data for 35 substances, among which saccharides, amino acids and alcohols, were analysed by chemometric methods -ANOVA, PLS-DA, box charts. Statistical analysis identified 16 components to differentiate the botanical origin of honey, and 11 – the country of origin. Several samples from North Macedonia contain the monosaccharide quinovose that is widespread in plants, but has not been reported until now as a component of honey. Our results demonstrate the usefulness of the combination of NMR spectroscopy and chemometrics to successfully control the authenticity of honey from 16 countries and 7 botanical sources.

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^{1}H NMR-BASED METABOLOMICS AS A TOOL TO UNRAVEL DEFENSE MECHANISMS IN EUPHORBIA LATEX BEARING SPECIES

Luis Francisco Salomé-Abarca [1], Dejan Gođevac [2], Min Sun Kim [3], Geum-Sook Hwang [3], Young Pyo Jang [4], Cees, A. M. J. J. van den Hondel[5], Peter G. L. Klinkhamer [6], Young Hae Choi [1, 4,]

[1] Natural Products Laboratory, Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands
[2] Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Studentski trg 12-16, 11000 Belgrade, Serbia
[3] Integrated Metabolomics Research Group, Western Seoul Center, Korea Basic Science Institute, Seoul, Korea
[4] College of Pharmacy, Kyung Hee University, 02447 Seoul, Republic of Korea
[5] Department Molecular Microbiology and Biotechnology, Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands
[6] Plant Ecology and Phytochemistry, Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands

Latexes are considered to be one of the best models to study interactions between plants and various environmental factors. [1] Also some of their defensive mechanisms have been hypothesized (e.g. wound-sealing) and demonstrated (e.g. deterrence and cytotoxicity). [2] Considering the highly distinctive metabolic profiles of latexes, it is presumed that small molecules possess a special role in latexes. Therefore, unravelling latex metabolic variations in response to exogenous factors might provide a hypothesis of the evolution of plant defensive chemical selection. To prove it, a systematic research was conducted using three *Euphorbia* species collected across geographical locations of Serbia. The samples were analyzed by a ^{1}H NMR metabolomics-based approach. Environmental factors showed highly differentiated influence on roots, leaves and latexes. The chemical variation of leaves and roots were more affected by their location, while latex showed highly conserved chemical profiles. In every species, the level of triterpenes in latex were found to be extremely higher than in leaves and roots. However, during simulated plant-organism interactions, the metabolites of latexes did not displayed better activity than the metabolites from leaves and roots. Nonetheless, the distinctive defensive character of latex was well reflected on its mechanical defense, which is not provided by leaves nor roots. These effects were especially reflected on the restriction of the movement of bacteria and fungi. These results demonstrated that plant latexes are sophisticated defense systems using their chemical components separately or complementary to fight against general insect-herbivores and pathogens, which resulted in the constraint of their metabolic variation.

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NMR STUDIES OF PROTONS IN FAST EXCHANGE WITH WATER

Ivan Vučković, Prasanna K. Mishra, and Slobodan Macura*

Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota, 55905, USA
*Corresponding author e-mail: macura@mayo.edu

Normally, the solvent line is suppressed by selective irradiation or by suitable pulse sequences. However, when the solvent line is part of the study it should be kept unperturbed. In modern NMR spectrometers equipped with digital filters the dynamic range is less of a problem but at high fields and high sensitivity probes major problem is radiation damping (RD).

RD is a coupling between large (usually solvent) magnetization and B1 field this magnetization induces in the high sensitivity (high Q factor) RF coil. Additional B1 field is 90° shifted with respect to this magnetization driving the magnetization back into equilibrium much faster than original T1 processes will do. This shortens apparent T1 relaxation time and broadens respective NMR line (decreases effective T2). Because induced B1 field is relatively weak and on resonance of the strongest NMR line it acts selectively on the strongest signal, Figure 1A. Suppression of solvent line eliminates RD but also diminishes signals of interest, Figure 1C. Placing the sample in capillary tube and detuning the coil (both lowering the Q factor) eliminates RD but preserves the strongest line and its partners, Figure 1B.

Effects of RD and its elimination are demonstrated on NMR spectra of formalin fixative where numerous hydroxyl protons (from (poly) methylene glycol) are in fast exchange with water protons.

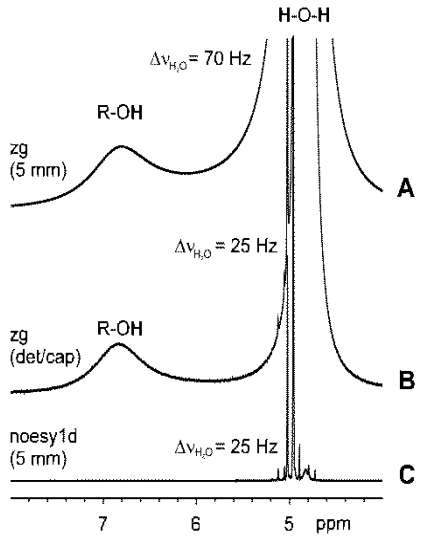

Figure 1. The 500 MHz spectrum of 16% formalin fixative in PBS at 25° C using cryo-probe. Spectra are scaled with respect to CH_2 resonance of methylene glycol (not shown, ~3.4 ppm):

A. Normal 1D in 5 mm tube (zg). The water line is broadened by chemical exchange and RD.

B. zg in detuned coil with the sample in the capillary inserted in 5mm tube with D_2O.

C. As A with solvent suppression using noesy1d sequence and solvent saturation.

In B and C the water line is broadened by chemical exchange only.

NMR INSIGHTS INTO METABOLIC DIFFERENCES OF BLOOD AND TISSUES CAUSED BY CANCER IN CHILDREN

Ljubica Tasic,[a,*] Melissa Quintero Escobar,[a,b] Danijela Stanisic,[a] Tássia B. B. C. Costa, [a] Mariana Maschietto,[c] Ana C. V. Krepischi[d]

[a] Institute of Chemistry, University of Campinas (UNICAMP), Campinas-Brazil.

[b] Faculty of Medical Sciences, University of Campinas (UNICAMP), Campinas-Brazil

[c] Boldrini Children's Hospital, Campinas-Brazil

[d] Institute of Biosciences, University of Sao Paulo (USP), Sao Paulo-Brazil

* ljubica@unicamp.br

Diseases alter human metabolism in many ways. Therefore, identifying differences among metabolites in tissues and/or fluids can lead to identification of biomarkers of the targeted disease with potential for its early diagnosis, which could also contribute to the understanding of the molecular pathways' alterations and lead to the tailor-made treatment with favorable outcome. Cancer affecting very young population, such as Wilms tumor, hepatoblastoma and osteosarcoma present a group of very peculiar diseases [1]. These three malignancies were studied by liquid and semisolid NMR. Hepatoblastoma metabolites from tissues taken from the cancer border and center were compared to the ones identified in healthy tissues from border and center from the same patients. Also, Wilms tumor tissue samples were studied in the same way. Osteosarcoma blood plasma metabolites were studied as to connect the observed alterations with clinical evaluation (metastasis and recurrence), and efficacy of the anticancer therapy. Among expected differences between cancer and healthy tissues and/or plasma [2], such as in glucose, lactate, and acetate, alterations in lipids (VLDL and LDL) and some amino acids (aromatic and histidine) were detected in cancer samples. Lipids play important roles in structure, signaling and organism energy balance, and metabolic alterations observed point to lipid biosynthesis (cholesterol and phospholipids) and lipogenesis, and aromatic amino acids and histidine roles in cancer. Thus, plasma and tissues biopsy by NMR may allow to understand the molecular changes occurring in the tumor in real-time, derived from intratumor heterogeneity and/or therapeutic pressure and may greatly add-in oncological research.

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NMR METABOLOMICS IN MEDECINE: MAYO CLINIC EXPERIENCE

Ivan Vučković,[a,b,*] Song Zhang,[b] Petras Dzeja,[a] Ian Lanza,[b,c] Slobodan Macura[a]

[a] Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota, 55905, USA
[b] Metabolomics Core Laboratory, Mayo Clinic, Rochester, Minnesota, 55905, USA
[c] Division of Endocrinology and Metabolism, Mayo Clinic, Rochester, Minnesota, 55905, USA
* Corresponding author e-mail: vuckovic.ivan@mayo.edu

NMR spectroscopy has been established as one of the principal analytical techniques in metabolomics research. Overshadowed by GC-MS and LC-MS in terms of sensitivity and resolution, NMR has few comparative advantages: ease of sample preparation, nondestructive nature, high-throughput and high level of reproducibility. In addition, NMR is intrinsically quantitative over a wide dynamic range, which made it preferred platform in long-term and large-scale clinical metabolomics studies [1].

At Mayo Clinic Metabolomics Core we leverage the power of both NMR and MS. Easy access to samples with well described diagnoses and patient histories, and close collaboration with clinicians makes us a unique place for research in the metabolomics field. Besides Bruker IVDr platform, we use various in-house developed protocols for sample preparation, data acquisition and metabolite profiling. We'll illustrate our methodology with three most commonly used types of biofluids: plasma, urine and tissue extract. First we'll show how we used Bruker IVDr for lipoprofiling to study the effect of omega-3 supplementation on human plasma, and how we combined NMR and MS to identify altered metabolites [2]. Then we will present our approach to NMR analysis of urine samples from patients with proteinuria. At the end, we will showcase the tissue analysis with the study of diet-induced thermogenesis in mice [3].

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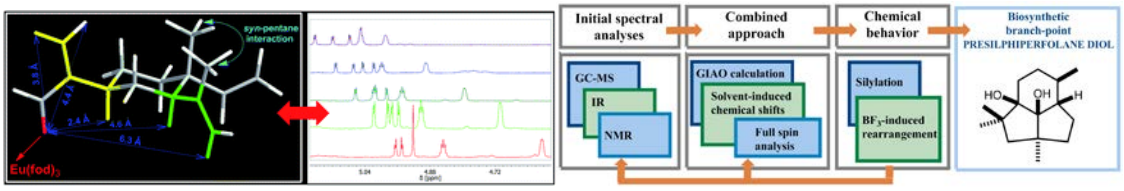
NMR IN THE EYES OF AN ORGANIC/NATURAL PRODUCT CHEMIST-EXAMPLES FROM THE PRACTICE

Niko S. Radulović [a,*]

[a] Department of Chemistry, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia

* Corresponding author email address: nikoradulovic@yahoo.com

Over the past 35 years there have been several thousand publications describing the use of 2D NMR to identify and characterize natural products, and many more dealing with NMR characterization of organic synthesis products. During this time period, the amount of sample needed for this purpose has decreased from the 20–50 mg range to under 1 mg but this approach still requires the availability of a sample of relatively high purity. Extensive analyses of mixtures of naturally occurring molecules of plant origin by this classical approach leave much "unidentified" and "unassigned" compounds. Metabolites displaying highly overlapped and higher order NMR signals are unattractive targets and require the application and development of innovative analytical methodologies. We investigated the potential usefulness of lanthanide-induced shift reagents for the resolution and assignment of overlapped ^{1}H NMR signals originating from different components of a complex natural mixture (i.e. for qualitative analysis). The incremental addition of Eu(fod)$_3$ leads to a simplification of NMR spectra in terms of signal overlap and removal of chemical shift degeneracy, allowing the mining of crucial data from the shifted NMR spectra. 2D-NMR spectra (^{1}H–^{1}H-COSY, NOESY, HSQC and HMBC) of the sample mixed with Eu(fod)$_3$ prove to be particularly valuable in this respect [1]. Additionally, we have demonstrated that combining solvent-induced removal of chemical shift degeneracy and theoretical (DFT-GIAO) prediction of NMR spectra with the analysis of ^{1}H NMR splitting patterns can facilitate structural elucidation of organic molecules with difficult-to-interpret NMR data [2]. Thus, herein examples of the application of a new chromatography-free methodology will be given that could be of value in structure elucidation of unknown compounds even if they are not available in pure state and present an approach that decreases the probability of an erroneous identification, and allows an unambiguous stereochemical elucidation and full NMR assignment.



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^{1}H-^{15}N 2D NMR SPECTROSCOPY – A USEFUL TOOL FOR STRUCTURE DETERMINATION OF AZAHETEROCYCLIC COMPOUNDS AND THEIR PRECURSORS

Valentin Badea*

Faculty of Industrial Chemistry and Environmental Engineering, Politehnica University Timişoara, C. Telbisz 6, 300001 Timişoara, Romania

*Corresponding author email address: valentin.badea@upt.ro

Azaheterocyclic compounds constitute a large and very important class of heterocyclic compounds and are becoming ever more important in all aspects of pure and applied chemistry. For azaheterocyclic compounds, ^{1}H-^{15}N correlation 2D NMR spectroscopy could be the key to prove the structure and regioselectivity. Like all 2D NMR methods (X,H), the crucial condition to have a signal in this type of correlation spectroscopy (^{15}N,H) is the presence of direct coupling with protons (HSQC) or over 2 and 3 bonds (HMBC). Due to the low natural abundance of the ^{15}N isotope (0.365%), which decreases the sensitivity in ^{15}N 1D NMR spectroscopy, the ^{1}H-^{15}N correlation NMR spectroscopy should be a good substitute for using costly ^{15}N-labeled compounds. Other problems to be overcome are the ^{15}N chemical shifts, which cover about 1100 ppm (from +620 to -420 ppm), and the J(^{15}N,H) coupling constants that sometimes have distinct values from compound to compound.

DIAGNOSING RARE METABOLIC DISEASES BY NMR METABOLOMICS. OR, IS RARE INDEED SO RARE?

Calin Deleanu [a,b]

[a] "C.D. Nenitescu" Centre of Organic Chemistry, Spl. Independentei 202-B, Bucharest, Romania.
[b] "Petru Poni" Institute of Macromolecular Chemistry, Aleea Grigore Ghica 41-A, Iasi, Romania.
*Corresponding author email address: calin.deleanu@yahoo.com

During its rather brief history, NMR spectroscopy (discovered in 1946 by Block & Purcell) was initially used by physicists. It was only in the 1960's when the chemical shift was discovered that the chemical community started to use it and the NMR field took off. Thus, NMR became an indispensable tool in structure elucidation of pure compounds and, until late 1980's, this remained the most important type of application. Once the high field NMR spectrometers entered the chemical community the method started to be used also for complex mixture analysis, penetrating new fields like medicine or food sciences.

In recent years there was an increasing scientific activity and public pressure for developing both diagnosis tools and therapies for rare diseases. As society evolves there is an increasing effort for tackling conditions affecting a small number of individuals even when this approach requires targeted research activities for each particular case.

As more statistics become available on various rare diseases, it turns out that the total number of humans affected by such diseases is impressively high. Thus, a significant number of new born babies are dying worldwide of treatable disorders simply because of the lack of diagnosis tools.

The paper deals with the NMR approaches in research of rare diseases and some related medical conditions requiring personalized medical diagnosis. Several cases and research directions from our laboratories are presented. Experimental factors, reproducibility, and data interpretation *via* either biomarker identification approach or blind statistical classifier approach are also discussed.

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INSIGHT INTO THE STRUCTURAL FEATURES OF G-QUADRUPLEX DNA

Peter Podbevšek[a,*]

[a] Slovenian NMR centre, National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia
* peter.podbevsek@ki.si

Guanine-rich regions are enriched in telomeric and promoter regions of the human genome and can adopt non-canonical secondary structures. Telomeric regions are found at ends of chromosomes and protect the chromosome termini from deterioration or from fusion with neighbouring chromosomes, while promoters are involved in regulating gene expression. Conditions in cells favour the formation of G-quadruplexes, four-stranded structures comprised of planar arrangements of four guanine nucleobases connected with Hoogsteen hydrogen bonds. G-quadruplexes exhibit polymorphism in terms of strand stoichiometry and polarity, orientation across glycosidic bonds, groove dimensions, sequence details, and structure of connecting loops. Among the four DNA nucleobases guanine has the lowest redox potential and is therefore the easiest to oxidize. We systematically probed guanine positions in the human telomeric oligonucleotide sequence (hTel) by substitutions with the major product of oxidation - 8-oxo-7,8-dihydroguanine (^{oxo}G) - and evaluated the G-quadruplex forming ability of such oligonucleotides with NMR. [1] Some positions in the hTel sequence were found to tolerate substitutions with ^{oxo}G. Accommodation of ^{oxo}G at sites originally in *syn* or *anti* in non-substituted hTel G-quadruplex requires a minor structural rearrangement or a major conformational shift, respectively. Similarly, we have shown that a G-quadruplex structure adopted by the vascular endothelial growth factor (VEGF) promoter sequence is disrupted with the introduction of an oxoG lesion. However, the G-quadruplex fold can be recovered by adding a short pyrene conjugated lesion-free G-rich oligonucleotide. [2]

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NMR INSIGHTS ON THE ACTIVATION MECHANISM OF A HEME ENZYME

Georgios A. Spyroulias,[a]* Aikaterini I. Argyriou,[a] Garyfallia I. Makrynitsa,[a] Styliani Chasapi,[a] Aikaterini Zompra,[a] Stavros Topouzis,[a] Andreas Papapetropoulos[a,b]

[a] *Department of Pharmacy, University of Patras, GR-26504, Patras, Greece*

[b] *School of Health Sciences, Faculty of Pharmacy, University of Athens, GR-15 771, Athens, Greece*

* G.A.Spyroulias@upatras.gr

Soluble Guanylate Cyclase (sGC) is the primary receptor for nitric oxide (NO) and a potential therapeutic target. sGC has a key role to essential and diverse physiological processes such as blood pressure regulation, memory formation, platelet aggregation and muscle relaxation. sGC is a heterodimeric hemoprotein which consists of an H-NOX domain, a Per/ARNT/Sim (PAS) domain, a coiled-coil (CC) domain and a catalytic domain. The heme domain of sGC belongs to a recently discovered family of heme-based gas sensor proteins called Heme-Nitric Oxide/Oxygen (H-NOX) binding domains that are conserved across eukaryotes and bacteria. Also, due to the implication of sGC in cardiovascular diseases, sGC stimulatory compounds are vigorously sought [1].

Overall, our research focuses on the Nuclear Magnetic Resonance (NMR) study of the heme-bound H-NOX domain from *Nostoc sp,* which shares 35% sequence identity with the H-NOX domain of the β1 subunit of human sGC [2]. Specifically, we examined the conformational dynamics of the HNOX domain during the interactions of this domain with activators and stimulators. For this purpose, heteronuclear NMR and UV-visible spectroscopy are used to investigate the structural integrity, the folding, the dynamics and the oxidation state of H-NOX. Hence, these results will provide new valuable insights for a better understanding of the activation mechanism, while they may provide a path forward for new drug discovery targeting sGC [3].

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NMR INSIGHTS ON THE INTERACTIONS OF MACROLIDES AND MACROZONES

Predrag Novak,[a]

[a] Department of Chemistry, Faculty of Science, University of Zagreb, Horvatovac 102 a, Zagreb, Croatia
* pnovak@chem.pmf.hr

Macrolide antibiotics, such as azithromycin have been widely used to treat infections of both gram-positive and gram-negative bacteria [1]. They exert their biological activity by binding to bacterial ribosome at or near the peptidyl transferase center thus inhibiting protein biosynthesis. Multi-drug resistant microbial pathogens present today a serious and challenging medical problems which demands novel antimicrobial agents to be obtained. In order to overcome this issue it is necessary to elucidate and understand the drug mode of action.

In order to discover and design more effective macrolide compounds, it is important to explore the interactions with ribosome and other biological targets and characterize bound conformations in solution [2–5]. We have prepared a series of bioactive azithromycin conjugates, the macrozones, by coupling thiosemicarbazones to azithromycin. Here we present results of interaction studies of macrolide antibiotics and some selected macrozones by employing a combination of NMR experiments such as transferred nuclear Overhauser effect spectroscopy (trNOESY), saturation transfer difference (STD), diffusion and solvent paramagnetic relaxation experiments and molecular modelling to characterize binding epitopes and asses bound conformations.

The data so obtained can serve as a basis for the design of novel compounds with an improved biological profile.

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EXPRESSION, PURIFICATION AND NMR STUDY OF THE 124-RESIDUES C-TERMINAL POLYPEPTIDE OF ARKADIA

Konstantinos D. Marousis[a], Maria Birkou[a], Aikaterini Zompra[a] and Georgios A. Spyroulias[a]*

[a] Department of Pharmacy, University of Patras, GR-26504 Patras, Greece
*e-mail: G.A.Spyroulias@upatras.gr

Arkadia is a RING domain ubiquitin ligase that positively regulates TGF-*β* signaling pathway by mediating degradation of the negative regulators Smad6 and Smad7 and the nuclear co-repressors Ski and Skil (SnoN) [1],[2]. The domains that are required for the substrate recognition and ubiquitin ligase activity are located in the highly conserved area of the C-terminal 100 amino acids. This region is composed by the NRG and TIER amino acid segments, as well as a RING domain which comprises two Zinc(II) ions in a cross-brace topology. NRG and TIER are required for substrate recognition while the RING domain is required for the ubiquitin ligase activity [3].

In the present study the polypeptide that comprises the 124-residues C-terminal polypeptide of Arkadia, including the NRG and TIER segments along with the RING domain was cloned, expressed in *Escherichia coli* cells and subsequently purified through affinity chromatography for NMR structure, dynamics and interaction studies. Moreover, the E3 ligase ubiquitin activity of the 124-residues C-terminal polypeptide was monitored *in vitro* through auto-ubiquitination experiments.

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COMPLETE ASSIGNMENT OF ^{1}H- AND ^{13}C-NMR SPECTRA OF 3-METHOXYCUMINYL ESTERS FROM THE ESSENTIAL OIL OF *PULICARIA DYSENTERICA* (L.) BERNH.

Marko Z. Mladenović,[a] Niko S. Radulović,[a,*] Dušan D. Vukićević[b]

[a] Department of Chemistry, Faculty of Sciences and Mathematics, Višegradska 33, 18000 Niš, Serbia
[b] Faculty of Medicine, Kragujevac, Svetozara Markovića 69, 34000 Kragujevac, Serbia
*Corresponding author: *nikoradulovic@yahoo.com*

Analyses by GC and GC/MS of two essential-oil samples of *Pulicaria dysenterica* (L.) Bernh. aerial parts disclosed the presence of 3-methoxycuminyl isobutyrate as the main constituent (25.5 – 31.1%). As the composition was somewhat different from the one in previously published reports [1-2], we were motivated to perform more detailed analyses which led to the tentative identification of two other 3-methoxycuminyl esters: 2-methylbutanoate (a new natural product) and 3-methylbutanoate (a rare natural product that was identified only as a constituent of *Inula viscosa* essential oil [3]). In order to confirm the tentative identifications, a small synthetic library of 5 esters (3-methoxycuminyl 2-methylpropanoate, butanoate, 2-methylbutanoate, 3-methylbutanoate, and pentanoate) was created. The obtained esters and intermediates in the synthesis of the starting alcohol (3-methoxycuminol) were subjected to a battery of 1D- (^{1}H and ^{13}C, including ^{1}H spectra with homonuclear and ^{13}C spectra without heteronuclear decoupling, DEPT90, and DEPT135) and 2D- (NOESY, gHSQC, gHMBC) NMR experiments in $CDCl_3$. A combination of data from these spectra allowed the assignation of all ^{1}H- and ^{13}C-NMR signals. The synthesized esters were also additionally characterized by MS, IR, UV–Vis, and chromatographic (RI) data.

ACKNOWLEDGMENTS

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ASSESSMENT OF PROTEIN AND MEMBRANE BIOPHISYCAL PROPERTIES BY EPR SPIN-LABELING METHODOLOGY

Aleksandra Pavićević,[a,*] Đura Nakarada,[a] Ana Vesković,[a] Milan Lakočević, [b] Ana Popović-Bijelić, [a] Miloš Mojović[a]

[a] EPR laboratory, University of Belgrade - Faculty of Physical Chemistry, Studentski trg 12-16, Belgrade, Serbia

[b] Clinic of Endocrinology, Diabetes and Metabolic Diseases, Clinical Centre of Serbia, Dr Subotića 13, 11000 Belgrade, Serbia

* aleks.pavicevic@ffh.bg.ac.rs

A wide variety of physicochemical techniques have been developed to study the biophysical properties of biomolecules and biomolecular structures. Among these techniques, electron paramagnetic resonance (EPR) spectroscopy is the method of choice for the study of protein, membrane and DNA structures. For this purpose, EPR-detectable paramagnetic compounds are first incorporated into the investigated biomolecular assemblies, and further detected by EPR spectroscopy. These compounds are known as spin-probes (SPs), if they are non-covalently bound to the examined structure, or spin-labels (SLs), if they are covalently attached to the biomacromolecule. Both SPs and SLs, are mostly aminoxyl radicals containing an >N-O$^{\cdot}$ group, with an unpaired electron located between the nitrogen and oxygen atoms. The proximity of the unpaired electron to either nitrogen or oxygen atom depends on certain environmental factors such as proticity, polarity and pH. The influence of these factors on the unpaired electron can be observed in the distance between the EPR spectral lines, a hyperfine splitting parameter, A_o (Fig. 1a). Furthermore, the degree of the immobilization of the SPs or SLs, upon their binding to the protein, or implementation in the lipid-based structures, also affects the overall shape of the EPR spectra. The sharp triplet arising from the freely rotating molecules undergoes the asymmetric broadening resulting in an altered spectrum characterized by spectrum width (outermost hyperfine splitting parameter) $2A_{max}$ (Fig. 1b).

Aminoxyl derivatives of certain lipids, mostly fatty acids and phospholipids, are typically used to study lipid-based structures, such as liposomes or membranes. These compounds usually contain a paramagnetic doxyl group attached to different carbon atoms of the methylene chain, thereby providing the opportunity to study the membrane/liposome fluidity at different depths in the lipid bilayer (e.g. 5- (5-DS) and 16-doxyl-stearic acid (16-DS)). In such an environment, SP molecules exhibit an axial motional symmetry, characterized by two EPR spectral parameters – parallel ($2A_{\parallel}$; this parameter coincides with $2A_{max}$) and perpendicular ($2A_{\perp}$) hyperfine splittings (Fig. 1). From these data it is possible to calculate the order parameter which is a quantitative measure of the degree of lipid bilayer fluidity [1]. It has been shown that this method is useful not only for the study of the

biophysical properties of the cell membranes, but it can also be used as a diagnostic test for certain metabolic diseases related to the altered lipid composition of the cell membranes (e.g. Gaucher disease, GD). Namely, using the Bruker ELEXSYS II E540 EPR spectrometer, it has been demonstrated that by spin probing of the blood cells (erythrocytes and peripheral blood mononuclear cells) membranes it was possible to distinguish between healthy controls and therapy-naive GD patients, as well as between two groups of GD patients – therapy-naive and those who are receiving the enzyme replacement therapy [2].

In case of the proteins, spin-probing and spin-labeling are able to provide information about conformational changes, ligand binding, distances within the certain parts of the protein molecule etc. It was also demonstrated that binding of the aforementioned 5-DS and 16-DS to main blood plasma protein responsible for fatty-acid transport, serum albumin, may provide insight into the interactions of albumin with drugs, which are very valuable information from the standpoint of the pharmacokinetics and pharmacodynamics [3,4]. Furthermore, it has been shown in literature that binding of 16-DS to blood plasma or serum can be used as a diagnostic test for various types of cancer, diabetes, liver failure, cirrhosis, sepsis etc. [5].

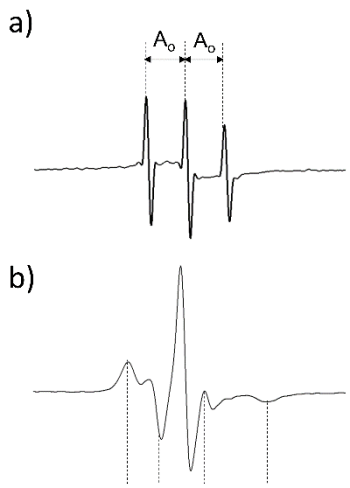

Figure 1. Typical EPR spectra of aminoxyl compounds: freely tumbling in aqueous solution (**a**) and of those incorporated into the lipid bilayer or protein (**b**).

In conclusion, EPR/spin-probing (labeling) methodology is a very useful tool for the investigation of protein and membrane structure, able to provide valuable biophysical information. Furthermore, it has a great potential for diagnostic applications as well.

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USING LOW TEMPERATURE X-BAND SPECTROSCOPY TO STUDY MITOCHONDRIAL DYSFUNCTION

A. Pavićević[a], Đ. Nakarada[a], A. Vesković[a], S. Kanazir[b], M. Mojović[a], A. Popović-Bijelić[a]*

[a] EPR laboratory, University of Belgrade - Faculty of physical chemistry, Studentski trg 12-16, Belgrade, Serbia
[b] University of Belgrade – Institute for Biological Research "Siniša Stanković", Bulevar despota Stefana 142, Belgrade, Serbia
*ana@ffh.bg.ac.rs

Metals may be studied by several physicochemical methods, but low temperature EPR spectroscopy gives slightly more information, which turns out to be quite useful in the research of biosystems. Although normal mode low T EPR can only "see" unpaired electrons, it is unprecedented in the study of metalloproteins, as it provides particular details about the metal. Namely, an EPR spectrum of a paramagnetic metal that is part of a biomolecule contains data about its oxidation state, as well as the nature of its ligands [1]. This is extremely important when examining the catalytic function of a metalloenzyme, its interaction with another biomolecule or drug. The downside of the technique is that the sample must be frozen, due to the fact that the spin-lattice relaxation times of metals are short, and therefore the signals are homogenously broadened beyond detection above 100 K [2]. Nonetheless, with an appropriate choice of microwave power, modulation amplitude, as well as temperature, the complicated signals are easily deconvoluted and interpreted. Furthermore, EPR can successfully be employed in the study of intact cells or tissues, therefore having an important role in biomedical studies. For example, mitochondrial dysfunction in an animal model of Alzheimer's disease (5xFAD mouse) may be studied using low T EPR.

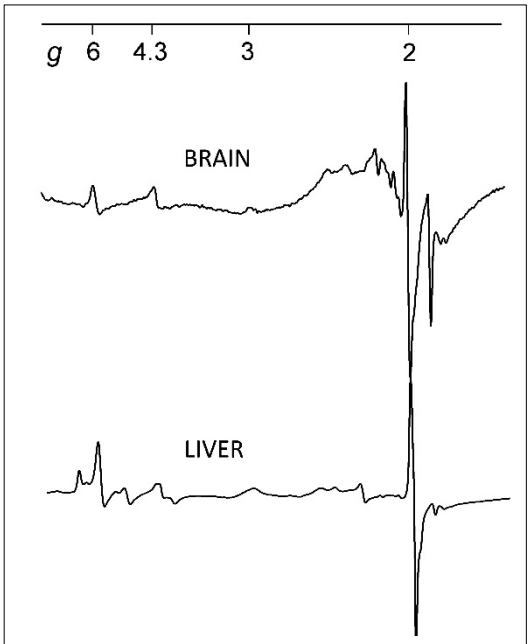

Figure 1. 20 K EPR spectra of 5xFAD mouse brain and liver.

In mammalian cells, the mitochondria are a major source of the superoxide radical ($O_2^{\bullet -}$) formation, as a result of electron leakage from complexes I and III [3]. It has been shown that $O_2^{\bullet -}$ can inactivate complex I, as well as aconitase, and succinate dehydrogenase, by oxidizing iron-sulfur (Fe-S) clusters. Certainly, ROS-related damage to

the mitochondrial Fe-S proteins has been observed in a number of neurodegenerative pathologies [4]. Our results from low T EPR spectroscopy of isolated 5xFAD mouse brains and livers (Figure 1) had showed that the ratio of oxidized-to-reduced Fe-S clusters was increased compared to healthy controls, which implied that these tissues were exposed *in vivo* to higher amounts of ROS. These types of findings may aid in the identification of both general and specific targets for therapeutic intervention strategies in a variety of diseases.

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EVALUATION OF *IN VIVO* OXIDATIVE STATUS BY L-BAND EPR SPECTROSCOPY

A. Vesković[a], A. Pavićević[a], Đ. Nakarada[a], B. Prokić[b], M. Perović[c], S. Kanazir[c], A. Popović-Bijelić[a]*, M. Mojović[a]

[a] EPR laboratory, University of Belgrade - Faculty of Physical Chemistry, Studentski trg 12-16, Belgrade, Serbia
[b] University of Belgrade – Faculty of Veterinary Medicine, Bulevar oslobođenja 18, Belgrade, Serbia
[c] University of Belgrade – Institute for Biological Research "Siniša Stanković", Bulevar despota Stefana 142, Belgrade, Serbia
*ana@ffh.bg.ac.rs

The blood brain barrier (BBB) breakdown in Alzheimer's disease has been reported to occur prior to neurodegeneration, dementia, and brain atrophy [1]. The mechanism of BBB disruption involves blood vessel damage and reduced brain perfusion that lead to amyloid β (Aβ) neuronal injury, followed by Aβ accumulation in the brain tissue [2]. Furthermore, there is evidence of Aβ toxicity to brain endothelial cells, which further induces the production of reactive oxygen species (ROS). Other sources of ROS that have been reported in Alzheimer's disease include red blood cell-derived hemoglobin and free iron, neutrophils adhered on activated endothelium, as well as activated microglia [1,3]. This indicates the importance of measuring the physiological levels of ROS associated with this pathology, as well as other neurodegenerative diseases.

EPR spin trapping is a technique in which short-lived ROS, and reactive nitrogen species (RNS), react with specially designed molecules (spin traps) to form relatively stable radical molecules which can be detected by EPR. However, this is limited to *in vitro* studies, as the spin traps, and the corresponding spin trap/RO(N)S adducts are oxidized *in vivo*. Therefore, it has been proposed that the oxidative status of a living system may be evaluated indirectly, using *in vivo* L-band EPR spectroscopy. This involves the use of spin probes, stable aminoxyl radicals, that when injected *in vivo*, are (non)enzymatically reduced in the cells and tissues. For this purpose, a number of aminoxyl radicals have been synthesized, which differ in their size, solubility, hydrophobicity and *in vivo* stability. Two of them, 3-carbamoyl proxyl (3CP) and 3-carboxy proxyl (3CxP) have been used extensively to study the redox changes that occur in several animal models of neurodegenerative diseases. It has been shown that *in vivo* reduction kinetics of 3CP may be used as a biomarker for the later stages of the ALS-like disease in the $SOD1^{G93A}$ rat model [4]. However, this probe has not proved to be useful for the study of the redox status in the 5xFAD mouse model of Alzheimer's disease (Figure 1), as the reduction kinetics were the same in the healthy control (wild-type) as in the 5xFAD model. It may be concluded that 3CP and 3CxP have different decay kinetics due to their different

cell-membrane permeability, and therefore the site of reduction. It is most probable that the reduction of 3CP occurs predominantly intracellularly, and of 3CxP extracellularly. There is an absolute demand for the development of a wide array of spin probes with different physicochemical properties that will allow for comprehensive *in vivo* assessment of oxidative stress in animal models for neurodegenerative and inflammatory diseases.

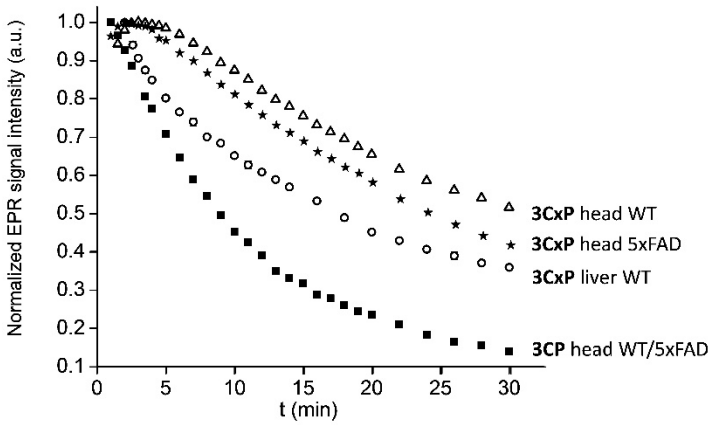

Figure 2. *In vivo* reduction kinetics of 3CP and 3CxP in the wild-type (WT) and the 5xFAD mouse model of Alzheimer's disease obtained by L-band Bruker E540 Elexsys II EPR spectrometer.

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CHEMICAL ANALYSIS OF SEIZED ANTIDEPRESSANTS BY ^{1}H NMR - A CRIMINAL COURT CASE

Boban Anđelković[1*], Ivana Sofrenić[1], Marina Todosijević[1], Ljubodrag Vujisić[1], Vele Tešević[1], Slobodan Milosavljević[1], Dejan Gođevac[2], Milka Jadranin[2], Mirjana Cvetković[2], Jovana Stanković[2], Vlatka Vajs[2]

[1]University of Belgrade - Faculty of Chemistry, Studentski trg 12-16, Belgrade
[2]University of Belgrade, ICTM-CH, Njegoševa 12, Belgrade
[*]Corresponding author e-mail: aboban@chem.bg.ac.rs

Detection of legal and/or illegal active substance with a pharmacological and physiological effect has been among the most fascinating objects for the practicing analytical chemist involved in forensic analysis. A development of fast identification and structural characterization is greatly facilitated by the advent of modern spectroscopic techniques, namely nuclear magnetic resonance spectroscopy (NMR) and modern mass spectrometry (MS) as well as separation techniques, such as gas and liquid chromatography (GC and LC). The NMR spectroscopy which is one of the most potent structural elucidation techniques allows us a quickly and rapidly compare a lot of different samples in short period of time, to simultaneously perform compounds identification and quantification.

In spring 2018 our laboratory received a demand from the Criminal court of Belgrade to analyze samples seized by police, containing antidepressants as the active ingredients. Our main task was to determine qualitatively and quantitatively the active substance and excipient type in all samples, comprising total of 475.5 tablets of different shapes, sizes and colors, as well as four powders.

The analytical samples were prepared using organic solvent extraction. NMR spectra of the samples were compared with literature data concerning the active compounds. The structures of the identified compounds were also confirmed with CG/MS or FTIR analysis. Quantitative measurements were evaluated by determining the recovery (91%), repeatability and precision of measurement. The quantification and measurement uncertainty were performed using the Quantitative Liquid-State ^{1}H NMR Spectroscopy (qNMR), Reference Procedures |2| Organic Analysis Bundesanstalt für Materialforschung und – Prüfung (BAM), Date: Feb. 2014/2016, reference procedure. The butylated hydroxytoluene (BHT) was used as internal standard in standard *zg30* pulls sequence with extended relaxation time (d1=10s) for quantification.

The Midazolam ((14.1 ± 0.6) mg/tablet), Clonazepam ((from 1.78 to 1.93 ± 0.6) mg/tablet), Alprazolam (0.91 to 1.00 ± 0.6) mg/tablet), Zolpidem ((6.2 ± 0.3) mg/tablet), Diazepam ((from 9.7 to 9.9 ± 0.4) mg/tablet) and Tramadol ((from 44 to 46 ± 2) mg/tablet) were found in the analyzed pills. Lactose and cellulose were recognised as excipients. One of the analyzed powder sample contained spectroscopically pure Zopiclone (102 g) and the remaining powders were identified as cellulose.

EVALUATION OF THE UNIVERSALITY OF NMR METABOLIC FINGERPRINTS OF SCHIZOPHRENIA

Katarina Simić,[a] Nina Todorović,[a] Zoran Miladinović,[b] Stefan Ivanović,[a] Snezana Trifunović,[c] Ljubodrag Vujisić,[c] Vele Tešević,[c] Vesna Jovanović,[c] Nataša Avramović,[d] Aleksanda Gavrilović,[e] Silvana Jovanović,[e] Tássia B. B. C. Costa,[f] Letícia Huan Liu,[f] Pedro Barros,[f] Danijela Stanišić,[f] Boris Mandić,[c,*] Ljubica Tasic[f]

[a] Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Belgrade, Serbia
[b] Institute of General and Physical Chemistry, Studentski trg 12-16, 11158 Belgrade, Serbia
[c] University of Belgrade - Faculty of Chemistry, Belgrade, Serbia
[d] Faculty of Medicine, University of Belgrade, Belgrade, Serbia
[e] Special hospital for psychiatric diseases "Kovin", Kovin, Serbia
[f] Institute of Chemistry, University of Campinas, Campinas, Sao Paulo, Brazil

* borism@chem.bg.ac.rs

Schizophrenia (SCZ) is a very disabling mental disorder whose molecular basis is a combination of many factors still not completely understood, with a diagnosis based on observed behavior, the person's reported experiences and reports of others that are familiar with the person, with no objective test. Also, up to date, there are no reliable markers for monitoring the SCZ. NMR-metabolomics [1] reported in 2017 bring some of the possible markers from blood serum of SCZ individuals linked strongly with known dopamine, glutamate and GABA dysfunction in SCZ. As to verify if these findings are universal, we have compared the SCZ patients from geographically different environments and cited interesting SCZ characteristics.

The first set of samples was collected in Belgrade, Serbia. 14 mental health patients (50% male) with 52.86 ± 7.27 years of age had a confirmed diagnosis of SCZ. The control group of 13 healthy individuals (69% male) had none of psychotic disorders, and individuals were 23.07 ± 2.79 years of age. Blood serum samples were collected and prepared for the analysis following the published methodology [1, 2]. NMR spectra were measured on a Bruker AVANCE III spectrometer (500.26 MHz for ^{1}H). The spectra were acquired at 298 K with 128 scans and 32 k. The serum samples were prepared and measured as triplicates.

On the other side, the group of individuals from Brazil that was matched in number, age, gender and history of mental illness with individuals from Serbia was previously described [1].

^{1}H NMR spectra were phase and baseline corrected using MestreNova and the lactate doublet was used as the chemical shift reference. The data were binned (0.005 ppm) in a spectral range 0.50 - 9.00 ppm, while the residual HDO peak (4.50-5.00 ppm) was excluded. Then, the data were normalized by the sum equal to 1000, the variables were mean centered and PCA and PLS-DA were performed using MATLAB.

It was shown that the mental health patients have clearly different blood serum metabolites when compared to the healthy ones independently from where the samples were obtained with almost identical marker set. Also, it was shown that the samples are different metabolically when Brazilian and Serbian samples were compared.

ACKNOWLEDGEMENTS

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PAULI MATRICES FOR TIME EVOLUTION OF THE DENSITY MATRIX

Dragana Drašković,[a] Nina Đapić[a,*]

[a] University of Novi Sad, Technical Faculty "M. Pupin", Đure Đakovića bb, Zrenjanin, Serbia
* Corresponding author email address: nidjapic@gmail.com

In this abstract focus is on calculating the time evolution of the density matrix for a $\frac{1}{2}$– spin particle in the initial state $\rho(0)$ when RF magnetic field is given by: $B_1 \cdot \vec{i}$. For the $\frac{1}{2}$– spin particle, the spin angular momentum component is $\tilde{S}_i = \frac{\hbar}{2}\sigma_i$, where σ_i are Pauli matrices, which are given as follows [1]:

$$\sigma_x = \begin{pmatrix} 0 & 1 \\ 1 & 0 \end{pmatrix}, \sigma_y = \begin{pmatrix} 0 & -i \\ i & 0 \end{pmatrix} \text{ and } \sigma_z = \begin{pmatrix} 1 & 0 \\ 0 & -1 \end{pmatrix}$$

In the case observed Hamiltonian is: $H = -\gamma \cdot B_1 \cdot \tilde{S}_x = -\omega_1 \cdot \frac{\hbar}{2}\sigma_x$, where γ is gyromagnetic ratio associated with the given particle [2]. Since Hamiltonian is not explicitly time dependent, easily can be obtained the propagator [2]: $U(t) = e^{i \cdot \frac{\omega_1 \cdot t}{2} \cdot \sigma_x}$. Matrix $i \cdot \frac{\omega_1 t}{2} \cdot \sigma_x$ is diagonalizable and that propagator can be calculated by:

$$U(t) = \begin{pmatrix} \cos\frac{\omega_1 t}{2} & i\sin\frac{\omega_1 t}{2} \\ i\sin\frac{\omega_1 t}{2} & \cos\frac{\omega_1 t}{2} \end{pmatrix}.$$

Given the propagator, expressed can be the time evolution of the density matrix by [3]:

$$\rho(t) = U(t) \cdot \rho(0) \cdot U^{\dagger}(t) = \begin{pmatrix} \cos\frac{\omega_1 t}{2} & i\sin\frac{\omega_1 t}{2} \\ i\sin\frac{\omega_1 t}{2} & \cos\frac{\omega_1 t}{2} \end{pmatrix} \cdot \rho(0) \cdot \begin{pmatrix} \cos\frac{\omega_1 t}{2} & -i\sin\frac{\omega_1 t}{2} \\ -i\sin\frac{\omega_1 t}{2} & \cos\frac{\omega_1 t}{2} \end{pmatrix}.$$

For example, when 90° RF pulse is applied on the $\frac{1}{2}$– spin particle in the initial state $\rho(0) = \tilde{S}_z$, the time evolution of the density matrix, in this example is:

$$\rho(t) = U(t) \cdot \rho(0) \cdot U^{\dagger}(t) = \begin{pmatrix} \cos\frac{\omega_1 t}{2} & i\sin\frac{\omega_1 t}{2} \\ i\sin\frac{\omega_1 t}{2} & \cos\frac{\omega_1 t}{2} \end{pmatrix} \cdot \tilde{S}_z \cdot \begin{pmatrix} \cos\frac{\omega_1 t}{2} & -i\sin\frac{\omega_1 t}{2} \\ -i\sin\frac{\omega_1 t}{2} & \cos\frac{\omega_1 t}{2} \end{pmatrix} = \frac{\hbar}{2}\begin{pmatrix} \cos\omega_1 t & -i\sin\omega_1 t \\ i\sin\omega_1 t & -\cos\omega_1 t \end{pmatrix}.$$

Applying 90° RF pulse obtained was:

$$\rho\left(\frac{\pi}{2\omega_1}\right) = \frac{\hbar}{2}\begin{pmatrix} \cos\frac{\pi}{2} & -i\sin\frac{\pi}{2} \\ i\sin\frac{\pi}{2} & -\cos\frac{\pi}{2} \end{pmatrix} = \tilde{S}_y.$$

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THE USE OF NMR ANALYZES IN DETERMINATION OF SOME UNUSUAL PRODUCTS OF REACTIONS

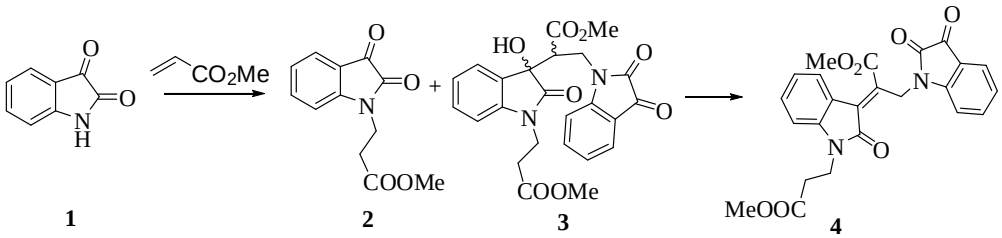
Natalia Sucman,[1,2] Calinicenco Serghei,[2] Fliur Macaev[1]

[1]Institute of Chemistry, Academy of Sciences of Moldova, Academiei str. 3, MD-2028, Chisinau, Republic of Moldova
2 Department of Agro-Technology, Comrat State University, 17,Galatsana str., Comrat, MD-3802, Republic of Moldova
E-mail: nicheli@yandex.ru

In most cases NMR spectroscopy serves to confirm the structure of estimated compounds, to determine some configurations of already known molecules. But it is quite difficult to establish the structure of an unknown compound with a complex construction. Nevertheless, in some cases this can be done, even if the construction of such molecules seems to be unreal.

Organic synthesis is fairly predictable. However, sometimes there are surprises when instead of the expected products other compounds with unusual structures are obtained. In this report, one of them will be presented.

The target product of reaction between indole-2,3-dione with methylacrylate (in absolute DMFA with equimolar presence of NaH) should to be compound **2.** But the main product of the reaction was another compound. Following the data of the NMR analysis, it was suggested that this is a mixture of two diastereomers **3**. To confirm the expected structure, dehydration was performed, which resulted in product **4**, the structure of which was confirmed by NMR spectra too. To determine the structure of described substances, various NMR analyzes were applied (one-dimensional (^{1}H, ^{13}C, DEPT-90^o and DEPT-135^o) and two-dimensional homo-(^{1}H/^{1}H COSY-45^o, ^{1}H/^{1}H NOESY) and heteronuclear (^{1}H/^{13}C HSQC and HMBC)).The structure of the **4** was later confirmed by the data of X-ray analysis.



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PERIODIC TABLE OF COMPOUNDS IN WINE USING ^{1}H NMR SPECTROSCOPY AND CHEMOMETRICS

Desislava Gerginova,[a,*] Yavor Mitrev,[a] Svetlana Simova[a]

[a] Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences; Acad. G. Bontchev str., Bl. 9, 1113 Sofia, Bulgaria
* Corresponding author: dpg@orgchm.bas.bg

Wine is a favourite alcoholic beverage and the oldest documented medicine. It contains more than 10 000 compounds, but the composition depends on many factors, including grape variety, climate, relief, soil, production recipe etc. High demand, value and variability of wine make it one of the frequently adulterated foods. Most often fraud is related to declaration of false botanical and/or geographical origin or vintage. NMR spectroscopy is robust and reproducible method, often used for authentication of botanical origin and quality control of wine in European countries [1], but not yet in the region of South East Europe. Bulgarian wine is famous for its aroma, taste, vinicultural traditions and high quality, with antioxidant properties proved to be among the highest in European wines [2].

60 samples of Bulgarian wine (Cabernet Sauvignon, Merlot, Syrah, Sauvignon Blanc and Chardonnay) were analysed using several NMR experiments. More than 40 compounds were identified, classified, quantified and presented in a so-called “Periodic Table”. Data has been analysed using chemometric methods. The quantity of 21 components in the studied wines allows unambiguous determination of their botanical origin. Experimental data for Bulgarian and wines produced in 13 additional countries are presented.

ACKNOWLEDGMENTS
Financial support of the Bulgarian National Science Fund (Projects B02-22/2014, UNA-17/2005 and DRNF-0213/2009) is gratefully acknowledged.

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THE UNREVEALED POTENTIAL OF LIPOSOMAL INTEGRATION METHOD AND EPR SPECTROSCOPY IN STUDIES OF ANTIRADICAL ACTIVITY OF COMPOUNDS POORLY SOLUBLE IN WATER

Đura Nakarada[a,*], Ana Vesković[a], Aleksandra Pavićević[a], Ana Popović-Bijelić[a], Boris Pejin[b], Miloš Mojović[a]

[a] EPR laboratory, University of Belgrade - Faculty of Physical Chemistry, Studentski trg 12-16, Belgrade, Serbia
[b] Department of Life Sciences, Institute for Multidiscipinary Research, IMSI, University of Belgrade, Kneza Višeslava 1, Belgrade, Serbia
[*] djura@ffh.bg.ac.rs

The main advantages of electron paramagnetic resonance (EPR) spectroscopy over other spectroscopic methods are its high selectivity, high sensitivity and the fact that its performance does not depend on the optical characteristics of the sample. These facts affirm EPR spectroscopy as the paramount method in terms of antioxidant and antiradical studies. This method proved to be faster compared to other spectroscopic and chromatographic methods in determination of overall antioxidant capacity of various natural products. The significant obstacle, potentially hampering EPR studies, is that many compounds are poorly soluble in water, and most of the biologically significant free radicals have to be generated in aqueous medium. This issue could be surpassed by incorporating water insoluble compounds into the liposome bilayer. The presented idea is not new, but it was applied only in a limited number of cases, to micelles, but not to biologically relevant free radical species.

In the present study, avarol was used as a model molecule. This secondary metabolite of marine origin has various beneficial biological activities, and the major problem preventing its clinical application is its poor solubility in water. After incorporating it into the membrane of DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine) liposomes, its antiradical activity was studied toward $^{\cdot}OH$, $O_2^{\cdot -}$ and $NO^{\cdot}$ radicals. Since the half-life of these radicals is very short, they could not be directly detected by EPR spectroscopy. Instead, the EPR spin-trapping technique was employed. This technique is based on the reaction of short-lived free radical species with compounds (spin-traps) which are transformed into more stable radicals, detectable by EPR spectroscopy. In the present study, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) was used as the spin-trap. The EPR measurements were performed using Bruker Elexsys II E540 EPR spectrometer.

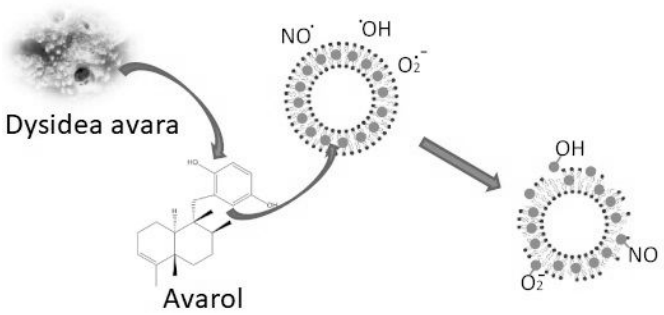

Figure 1. Antiradical activity of avarol liposomes.

The obtained results [1] indicate that avarol successfully eliminated ·OH (86.2 %), $O_2^{\cdot -}$ (50.9 %) and NO· (23.6 %) radicals. According to the available literature data, this is the first example of liposomal integration method and EPR spectroscopy combined in the studies of antiradical activity of compounds poorly soluble in water toward biologically relevant free radicals. The attained results are more than promising, and this method could be directly applied to other compounds that are not soluble in aqueous media, in order to assess their antiradical activity toward free radical species which must be generated in aqueous solutions.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant number III 41005).

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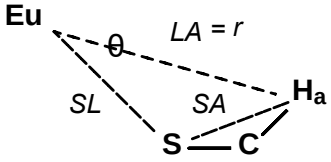
COMPUTER-BASED MODELING OF LSR COMPLEXES

Dragan Zlatković [a,*], Niko Radulović [a,*]

[a] Department of Chemistry, Faculty of Sciences and Mathematics, University of Niš, Serbia
* DZ: dragan.zlatkovic@gmail.com; NR: nikoradulovic@yahoo.com

Although the use of lanthanide shift reagents (LSRs) has somewhat declined in the last decades, complexes such as Eu(fod)$_3$ are still used for two main purposes: simplifying complicated spectra (qualitative) and determination of the geometry of LS (ligand-substrate) complexes (quantitative application) [1]. A shift in the resonance frequency, Δ, caused by the pseudocontact interaction, depends on the geometry of the complex and is given by the McConnell-Robertson equation, Eq. 1 (Fig. 1) [1]. The exact geometrical model of LS complex can be of utmost help in structure elucidation since it would allow differentiation of diastereoisomers [2,3]. So far, the process of modeling was basically trial and error [2,3] – initially, the position of the Eu center would be randomly selected, then the Δ values for several nuclei would be calculated and compared to the experimental values. The position of Eu in the model would be then slightly altered and new Δ values calculated. The process is repeated until a good correlation is achieved. This process is, however, extremely time-consuming and frustrating – in our experience it could take several weeks to obtain an accurate model [2,3]. Thus, we designed a simple and easy-to-use QBasic application which predicts the position of the Eu center relative to the substrate molecule. This is not the first computer application designed for this purpose – Gonçalves et al. [4] developed a FORTRAN program for the same purpose, however, their approach also included Powell's method (to optimize the lanthanid position) which made it much more complex. Our approach is significantly simpler since the LS model is optimized by the brute force approach. In praxis, the coordinates of the lanthanide atom are usually retrieved after several seconds when a modern processor is used. We believe that the application can be extremely useful to natural product chemists as it removes one of the greatest obstacles of LSR use – tedious manual work which is necessary to obtain a usable LS model.



$$LA = [(x_a - x)^2 + (y_a - y)^2 + (z_a - z)^2]^{1/2}$$

$$\cos\theta = \frac{LA^2 + SL^2 - SA^2}{2 \cdot LA \cdot SL}$$

$$\Delta = K \frac{3\cos^2\theta - 1}{r^3} \qquad \textbf{Eq. 1}$$

Fig. 1

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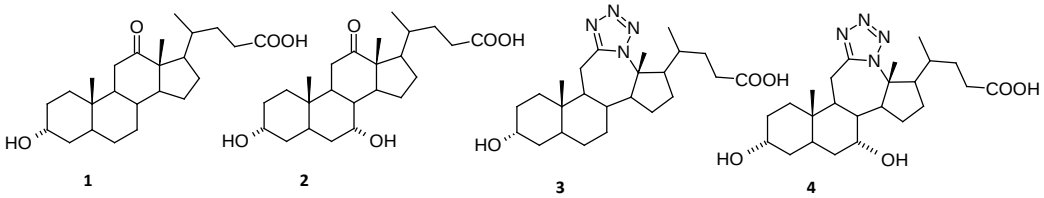
AGGREGATION STUDY OF SOME BILE ACID DERIVATIVES BY NMR

Dušan Škorić,[a] János Csanádi [a]

[a] Department of Chemistry Biochemistry and Environmental protection, University of Novi Sad Faculty of Sciences, 21000, Novi Sad, Serbia
* dusan.skoric@dh.uns.ac.rs

Since one of the main characteristics of bile acids and their derivatives is ability to form aggregates (micelles), techniques for studying aggregation behavior are very important for the bile acid research. NMR spectroscopy proved to be excellent technique to determine critical micelle concentration (CMC) and to study interactions in molecular aggregates of different bile acid derivatives [1].

In this work we present NMR spectroscopic study on aggregation of oxo bile acids **1** and **2** as well as study on two tetrazole analogues (**3** and **4**). Tetrazole derivatives were conveniently prepared from oxo bile acids *via* Schmidt tetrazole synthesis. D_2O solutions with different concentrations of the bile acids sodium salts were investigated by ^1H NMR techniques. Measurement of spin-lattice relaxation time as well as self diffusion measurement (DOSY-NMR) were used to examine micellization dependency on concentration of sodium salt. Results are showing that introduction of tetrazole ring instead of oxo group slightly elevates CMC for the bile acid derivatives.



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STRUCTURAL AND FUNCTIONAL INSIGHT INTO LA PROTEIN DOMAINS VIA NMR SPECTROSCOPY

Georgios Bouras,[a] Aikaterini Argyriou,[a] Maria Apostolidi,[b] Eleni Kaliatsi,[b] Detlef Bentrop,[c] Constantinos Stathopoulos,[b] Georgios A. Spyroulias[a,*]

[a] Department of Pharmacy, University of Patras, GR-26504 Patras, Greece
[b] Department of Biochemistry, School of Medicine, University of Patras, GR-26504 Patras, Greece
[c] Institute of Physiology II, University of Freiburg, D-79104 Freiburg, Germany
* G.A.Spyroulias@upatras.gr

Lupus antigen protein is a multi-domain RNA binding protein. It was first described as an auto-antigen in patients suffering from rheumatic systematic lupus erythematosus and Sjogren's syndrome[1]. La protein plays a key-role in the tRNA biogenesis by binding to UUU-3'-OH terminal motif of nascent RNA polymerase III transcripts. It guides accurate 5'end maturation of pre-tRNAs by RNase P, while protects their 3'ends from exonucleolytic digestion and prevents their misfolding through its chaperone activity[2]. Although La is located in the nucleus, it also facilitates translation of certain cellular and viral-encoded mRNAs, involved to subcellular trafficking and antiviral defense[3]. The protein consists of four distinct domains, namely La motif (LaM), two RNA recognition motifs (RRM1 and RRM2α) and a C-terminus region[4]. So far, the structural data of the La and RRM motifs from few eukaryotes (including human) that exist are bound to synthetic oligonucleotides providing inadequate information on the possible roles of the full-length La protein, in a more dynamic tRNA – dependent cellular network.

To elucidate the structural phenomena occurring during the interaction of the protein, from *Dictyostelium discoideum*, with RNA substrates, as well as the stability of the protein, we initiated an extensive structural and functional characterization of a "domain library" of La. Our research focuses on the NMR-driven structure determination of each domain, their pairwise combinations and the study of the whole protein in free state via 2D/3D NMR. Along with the structural investigation, the dynamical properties of the above constructs are studied through ^{15}N-relaxation measurements. The final goal is the functional analysis of those domains and the elucidation of the role of each one of them in RNA recognition and binding using natural and synthetic RNA substrates. Interaction studies are carried out through NMR-driven titration experiments. Results will be discussed and compared with the corresponding ones from human La.

ACKNOWLEDGMENTS
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"OPERATIONAL PROGRAMME EDUCATION AND LIFELONG LEARNING" and is co-funded by the European Social Fund (ESF) and National Resources (MIS 1225-D608 to C.S.).

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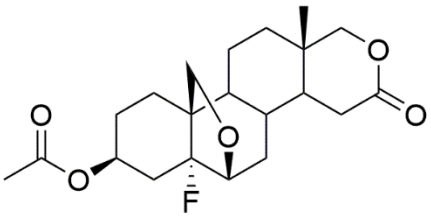
DETAILED ^{1}H AND ^{13}C NMR ASSIGNMENT OF NOVEL 5α-FLUORO-6β,19-EPOXY STEROID DERIVATIVE

Ivana Kuzminac,[a]* Marija Sakač[a]

[a] Department of Chemistry, Biochemistry and Environmental Protection, University of Novi Sad, Faculty of Sciences, Trg Dositeja Obradovića 3, 21000 Novi Sad, Serbia
* e-mail: ivana.kuzminac@dh.uns.ac.rs

C19-Modified steroid derivatives have shown significant inhibitor activity on enzyme cytochrome P450 aromatase. This fact made them good candidates for future breast cancer therapy, since aromatase is expressed in most postmenopausal breast cancers. Furthermore, steroidal halogen derivatives have shown potent antiproliferative activity toward different cancer cells [1]. With this in mind, we have synthesised 3β-acetoxy-5α-fluoro-6β,19-epoxy-17-oxa-17a-homoandrostan-16-one (**1**). NMR Analysis was carried out on the basis of 1D (^{1}H, ^{13}C and ^{19}F) and 2D (HSQC and HMBC) NMR spectra. The detailed assignment of ^{1}H and ^{13}C NMR chemical shifts was performed.



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ACKNOWLEDGMENTS

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"MIRACULOUS" DIETARY SUPPLEMENTS FOR WEIGHT LOSS IN BALKAN MARKET

Ivana Sofrenić[a]*, Boban Anđelković[a], Ljubodrag Vujisić[a], Dejan Gođevac[b], Vlatka Vajs[b], Vele Tešević[a], Slobodan Milosavljević[a]

[a]University of Belgrade – Faculty of Chemistry, Studentski trg 12-16, Belgrade
[b]University of Belgrade, ICTM-CH, Njegoševa 12, Belgrade
*Corresponding author: ivanasofrenic@chem.bg.ac.rs

People tend to self-treat overweight and obesity, and they are tempted to buy weight loss food supplements that are freely available in pharmacies, health food stores, groceries and on the Internet. These products are aggressively marketed with claims such as “all natural” and “easy weight loss” products. Often these claims are exaggerated as some unscrupulous manufacturers do not hesitate to add synthetic active pharmaceutical ingredients in order to improve the efficiency of their preparations.[1]

In summer of 2017 and winter of 2018 we analyzed "miraculous" dietary supplements for weight loss distributed by Slim Line Internacional DOO, Skoplje, North Macedonia. The dietary supplement Slim Line gelatin capsules and SLC ADVANCE capsules were provided to us directly from the pharmacy shelves. In both cases the distributor had the valid certificate of health safety. Since they were registered as dietary supplements, by law they only needed to be tested on presence of microorganisms, heavy metals and pesticides.

The method of choice was nuclear magnetic resonance (NMR) as nonselective, nondestructive and fast analytical method. The main advantage of this method is ability to detect all soluble compounds in single experiment and estimate their concentration ratios. Using different NMR experiments allows us to determine and confirm the structure of organic molecules in the solution. The simple solvent extraction procedure was performed with deuterated chloroform and heavy water for sample preparations, and butylated hydroxytoluene (BHT) was used as internal standard for quantification. The analysis was performed using 1D (^{1}H and ^{13}C) and 2D NMR (COSY and HSQC) experiments.

Comparison of the recorded spectra with literature data revealed the presence of antidepressant fluoxetine (Prozac) in Slim Line gelatin capsules, in concentration of 9 mg per dosage.

At the same time, Sibutramine, a prohibited sliming drug, was found in SLC ADVANCE capsules in concentration of 6.5 mg per dosage.

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Complete assignment of ^{1}H and ^{13}C NMR spectra of italidione I

Jelena Aksić,[a] Marija Genčić,[a,] * Niko Radulović,[a,] * Nicolas Baldovini[b]

[a] Department of Chemistry, Faculty of Sciences and Mathematics, University of Niš, Serbia
[b] Institut de Chimie de Nice, CNRS UMR 7272, Université Côte d'Azur, France
* denijum@yahoo.com; nikoradulovic@yahoo.com

Non-terpenic β-diketones I-V (italidiones) represent unique volatiles of *Helichrysum italicum* (Roth) G. Don fil., Asteraceae (**Fig. 1a**). Although these rare natural products have been described in the 1970s for the first time, their unequivocal NMR characterization was done almost 50 years later [1]. In solution, these β-diketones exist predominantly in diketo-forms, accompanied by a certain percentage of the corresponding enolic forms. Since all italidiones contain at least two chiral centers, all our synthetic efforts ended up in mixtures of diastereomers, that made the interpretation of NMR data difficult. Up to now, only two simpler italidiones, IV and V, and the corresponding enolic forms were completely assigned. Herein, we report the complete assignments of ^{1}H and ^{13}C NMR data of a synthetic standard of another (prenylated) italidione (I). This was achieved by extensive analyses of 1D and 2D NMR spectra in combination with Global Spectral Deconvolution and Spin Simulation algorithms incorporated in MestReNova. Under the studied conditions signals of both diastereomeric diketo-forms (KK1 : KK2 = 48 : 40) were detected, while two asymmetric β-keto-enol tautomers rapidly interconvert in solution resulting in only one set of NMR signals (EK + KE = 12%; **Fig. 1b**).

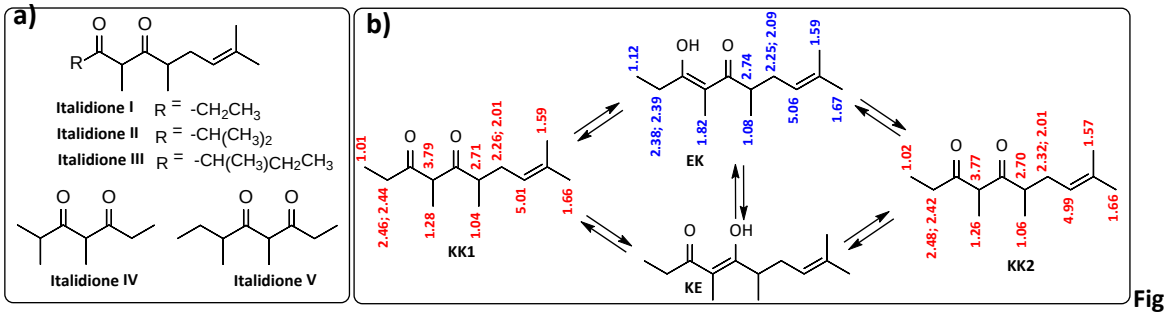

Fig 1. Structures of italidiones I-V (**a**) and ^{1}H NMR shifts (25°, $CDCl_3$, 400 MHz) of keto-enol tautomer and two diastereomeric diketo tautomers of italidione I (**b**)

ACKNOWLEDGMENTS

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NOVEL APPLICATION OF EPR SPECTROSCOPY FOR MONITORING OF PLGA PARTICLES BIODEGRADATION

A. Vesković, J. Kostić, Đ. Nakarada, A. Pavićević, M. Mojović, A. Popović-Bijelić*

EPR laboratory, University of Belgrade - Faculty of Physical Chemistry, Studentski trg 12-16, Belgrade, Serbia
*ana@ffh.bg.ac.rs

Poly(lactic-co-glycolic) acid (PLGA) is one of the most commonly used degradable polymers in a broad range of pharmaceutical and biomedical applications. Various PLGA micro- and nanoparticles have been fabricated for controlled drug release. The rate and mechanism of the release of therapeutic agents from polymer matrix are essential criteria for their particular application [1,2]. However, the phenomenon of PLGA biodegradation has not been conclusively described in literature.

This study investigates the *in vitro* degradation of PLGA microparticles modified with two aminoxyl radicals, 3-carbamoyl-, and 3-carboxy-proxyl (3CP and 3CxP) using X-band Bruker E540 Elexsys II EPR spectrometer. 3CP/3CxP-loaded PLGA microparticles were prepared by oil in water emulsion-solvent evaporation method with some modifications [3]. EPR spectra of 3CP- or 3CxP-modified PLGA microspheres acquired after synthesis confirmed the presence of both spin probes in the samples, suggesting an immobilization of 3CP, but not 3CxP. The results indicated that 3CP conjugates to the surface of PLGA particles, probably via interactions between the amino group of the spin probe and the carboxyl group of PLGA. On the other hand, the anionic spin probe 3CxP seemed to be entrapped inside the PLGA particle core, away from the terminal groups. The *in vitro* release of 3CP or 3CxP from the polymer particles was monitored and quantified by EPR spectroscopy during dialysis at 4 °C. 3CP showed a typical desorption profile, while 3CxP release kinetics were fitted with an appropriate pharmacological model (Figure 1).

PLGA microparticles modified with nitroxides have a potential to extend the *in vivo* application of spin probes in biostudies. 3CxP seems to be a more suitable spin probe for future experiments. Furthermore, EPR spectroscopy has proved to be an appropriate technique for PLGA particles biodegradation monitoring.

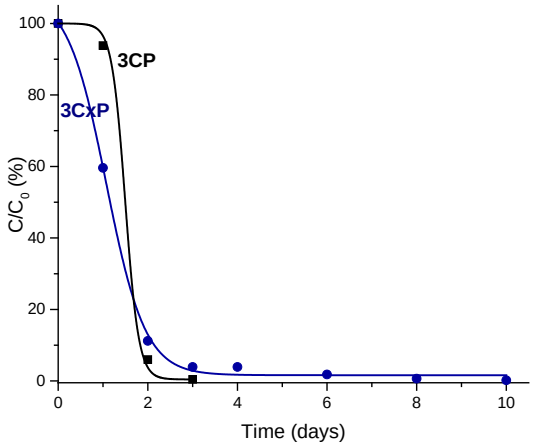

Figure 1. *In vitro* release profile of 3CP and 3CxP. C/C_0 - the amount of spin probe released from PLGA spheres during dialysis.

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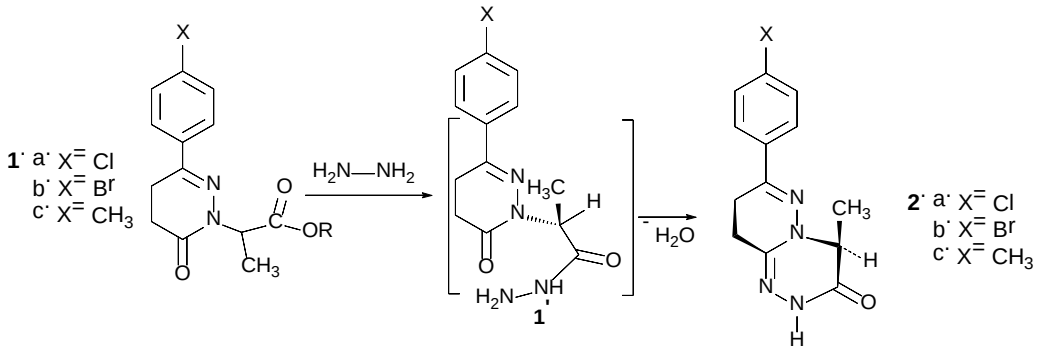
A NEW CLASS OF PYRIDAZINO-TRIAZINE: A NMR STRUCTURE ELUCIDATION

Violeta Mangalagiu,[b] Dorina Amariucai-Mantu,[b] Ionel Mangalagiu[a,b,*]

[a] Chemistry Department, Alexandru Ioan Cuza University of Iasi, Bd. Carol 11, 70506 IASI, ROMANIA.
[b] Institute of Interdisciplinary Research- CERNESIM Centre, Alexandru Ioan Cuza University of Iasi, Bd. Carol 11, 70506 IASI, ROMANIA.
* ionelm@uaic.ro; violeta.mangalagiu@uaic.ro

Azaheterocyclic derivatives with five and six membered ring, are invaluable motifs in medicinal chemistry, having a large variety of biological activities: anticancer, anti-HIV, antimicrobials (anti-TB including), anti-inflammatory, cardiovascular and antihypertensive, antithrombics, anticoagulants, antidepressant, anxiolytics, anticonvulsant, analgesic, diuretics, etc. [1-5]. In continuation of our work in the field of azaheterocyclic derivatives, we present herein the synthesis and structure determination of a new class of fused heterocycle with pyridazino [1,2,4]triazine skeleton. The synthesis is straight and efficient, the reaction of 4,5-dihydro-pyridazin-3(2H)-ones esters **1a-c** with hydrazine hydrate leading to the desired pyridazino [1,2,4]triazine derivatives **2a-c** [3-5].



The structure of compounds was proven by elemental (C, H, N) and spectral analysis. The NMR experiments (^{1}H-, ^{13}C- and two-dimensional experiments COSY, HMQC, HMBC) and X-ray measurements prove unambiguously the 8,9-dihydro-pyridazino[1,2,4]triazine structure of compounds **2**.

ACKNOWLEDGMENTS

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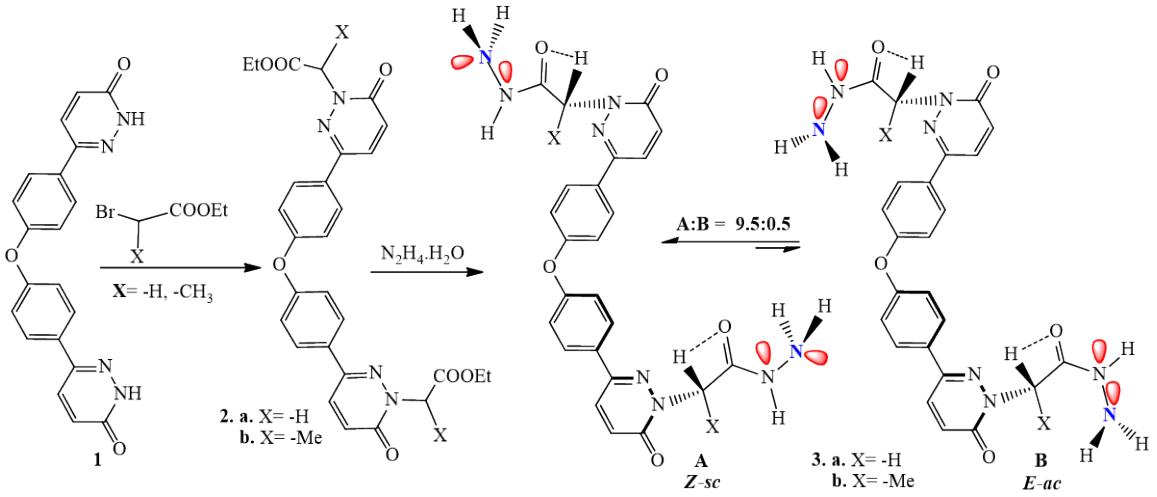
BIS-PYRIDAZINONE DERIVATIVES: A NMR STUDY OF STEREOCHEMISTRY

Ionel Mangalagiu,[a,b] Dorina Amariucai-Mantu,[b] Violeta Mangalagiu[b,*]

[a] Chemistry Department, Alexandru Ioan Cuza University of Iasi, Bd. Carol 11, 70506 IASI, ROMANIA.
[b] Institute of Interdisciplinary Research- CERNESIM Centre, Alexandru Ioan Cuza University of Iasi, Bd. Carol 11, 70506 IASI, ROMANIA.
* ionelm@uaic.ro; violeta.mangalagiu@uaic.ro

Pyridazinones derivatives, are widely discussed building blocks in medicinal chemistry, having extremely useful biological activities such as: cardiovascular and antihypertensive, anti-inflammatory, analgesic, antinociceptive, antimicrobial, etc [1-3]. As a result, some pyridazineones are already drugs in therapy, e.g. *Levosimendand* (heart failure), *Emorfazone* (analgesic, anti-inflammatory and antinociceptive). Taking into consideration these assertions, we synthetize a new class of bis-pyridazinones with acetylhydrazine skeleton. The synthesis involve an initially N-alkylation of bis-pyridazinones **1** with 2–bromoalkyl esters, when bis-pyridazinones eters **2a,b** are obtained, followed by a subsequent treatment with hydrazine, when the desired acetylhydrazines bis-pyridazinones **3a,b** are obtained [4].



The stereochemistry of the acetylhydrazines bis-pyridazinones **3a,b** was studied using the NMR experiments (^{1}H, ^{13}C, 2D HMBC) at room temperature, and reveal a conformational equilibrium of **3a,b**: *Z-sc* (around 90%) and *E-ac* (around10%) conformers. The NOE difference 1D experiments prove unambiguously the above considerations, only the major isomer *Z-sc* showing a strong NOE between the hydrazidic NH and the –CH-R group. A temperature dependence ^{1}H NMR study concerning conformational equilibrium has been performed, indicating the presence of a single stereoisomer at temperatures higher to 80 °C, the *Z-ac* conformer.

ACKNOWLEDGMENTS

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STRUCTURAL STUDIES OF MODEL SYSTEM FOR SENECIOBIPYRROLIDINE SKELETON SYNTHESIS

Milena Simić*, Nikola Micić, Miloš Petković, Predrag Jovanović, Gordana Tasić, Miloš Jovanović and Vladimir Savić

Department of Organic Chemistry, University of Belgrade, Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade
* milena@pharmacy.bg.ac.rs

Tetracyclic octahydro-dipyrroloquinoline architecture is not commonly found in natural products but some of them such as Incargranine B isolated from *Incarvillea mairei* var. *grandiflora* and seneciobipyrrolidine isolated from *Senecio scandens* are derivatives of this heterocycle [1-3], Figure 1. We envisaged potential synthetic pathway for this skeleton based around two key steps: (3+2) dipolar cycloaddition of azomethine ylides and unsaturated ketone and intramolecular Cu (I)-catalyzed amination to form the core structure (Scheme 1). Creation of several chiral atoms during this process prompted careful structural examination in order to establish correct stereochemistry and correlate it with the structure of natural products.

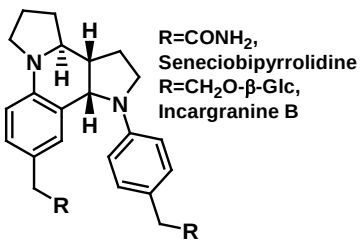
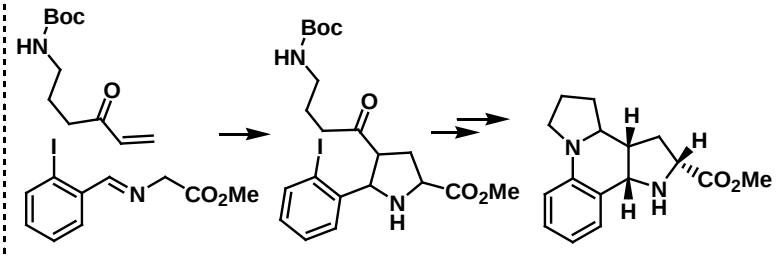


Figure 1



Scheme 1

ACKNOWLEDGMENTS

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ROTAMERS OF *N*-(4-METHOXYPHENETHYL)FORMAMIDES: AN NMR STUDY

Miljana R. Đorđević,* Niko S. Radulović*

Department of Chemistry, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, Niš, Serbia
* MĐ: miljanadjordjevic11@gmail.com; NR: nikoradulovic@yahoo.com

^{1}H and ^{13}C NMR characterization of a synthetic iodinated tyramide, *N*-(3-iodo-4-methoxyphenethyl)formamide (**1**) and its non-iodinated analog, *N*-(4-methoxyphenethyl)formamide (**2**) is reported herein [1]. All ^{1}H and ^{13}C NMR spectroscopic data provided evidence of the presence of two rotameric forms due to a slow rotation (on the NMR time scale) around the amide bond, N–CO (exchange peaks visible in their NOESY/ROESY spectra). These species could be completely characterized, while their ratio in $CDCl_3$ at 25 °C, based on the integration of well-separated signals in the ^{1}H NMR spectrum, was 1 : 5.56 (compound **1**) in favor of the *Z*-rotamer (*antiperiplanar* conformer). The major rotamer displayed a broad singlet formyl proton signal, whereas the minor (*synperiplanar* conformer) isomer gave a doublet with J = 9.4 Hz for the same proton in agreement with the stronger coupling expected for an antiperiplanar orientation of the HN–CH fragment. The non-iodinated formamide **2** showed similar conduct to that of **1**. The HSQC signal of C1-H1 (within the carbonyl) of the *Z*-rotamers of **1** and **2** appeared as "multiplets" (quartet-like) having the two farthest peaks corresponding to a $^{1}J_{CH}$ doublet (192.8 and 192.5 Hz, respectively; the precise values were determined from ^{1}H coupled ^{13}C NMR spectra), while the *E*-rotamers displayed broadened doublets having a splitting that is considerably lower than the measured $^{1}J_{CH}$ (189.3 and 188.7 Hz, respectively; values from ^{1}H coupled ^{13}C NMR spectra). These are probably artifacts due to a significant mismatch of d2, and/or d4 delays, optimized to $^{1}J_{CH}$ = 145 Hz, for the one-bond coupling of formamide ^{1}H/^{13}C nuclei in question. The value of the one-bond coupling constant is proportional to the bond order (electron density between the two atoms). One can expect different electron densities in the two rotamers and therefore different values of $^{1}J_{CH}$.

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TOPICAL DELIVERY OF LIPOSOME ENCAPSULATED ASCORBIC ACID - 2D EPR IMAGING STUDY

Dušan Mladenović [a], Aleksandra Pavićević [a], Đura Nakarada [a], Ana Vesković [a], Ana Popović-Bijelić [a], Miloš Mojović [a,*]

[a] Faculty of Physical Chemistry - University of Belgrade, Studentski trg 12-16, 11000 Belgrade
* milos@ffh.bg.ac.rs

Over the last two decades, liposomes have been intensively studied as potential drug-carriers for dermal and transdermal administration. When compared to conventional formulations, the effects of many drugs are increased several fold when applied to the skin encapsulated in liposomes. Although it is known that the enhancement of drug delivery into the skin is critically affected by the composition and physicochemical characteristics of liposomes, the exact mechanism of the enhancement of molecular penetration into the skin by liposomes is still not established. In order to spatially resolve the drug distribution and reduction data in different skin layers, EPR imaging measurements are performed [1]. Likewise, the *ex vivo* interactions of liposomes with pig ear skin, using electron paramagnetic resonance (EPR) spectroscopy and imaging methods, has shown to be a well-established experimental model for investigating topical drug delivery systems [2]. Since drug-containing liposomes generally do not have EPR signal, the spatial distribution and the reduction capability of liposomes is measured through their interaction with stable spin-probes which, depending on their chemical structure, follow the intra- or extra-cellular distribution. Once penetrated into the skin tissue, spin-probes could be reduced by antioxidants to their corresponding hydroxylamines, which are EPR inactive. As redox status indicators, a number of spin-probes have been extensively utilized in many applications including skin cells, skin tissue, and intact skin.

Herein, 2D EPR imaging technique has been used to measure the level of penetration and the antioxidative activity of liposomes containing ascorbic acid spread onto the *ex vivo* pig ear skin model. For this purpose, 3-carbamoyl proxyl (3CP) spin-probe has been selected since it is cell-membrane permeable, has a reasonably long half-life even in *in vivo* conditions, and is readily reduced by the ascorbic acid [3]. Liposomes were prepared using a thin-film method containing 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) and cholesterol in different ratios. The ascorbic acid was incorporated into liposomes upon rehydration. Uniform size of liposomes was obtained using extrusion and unencapsulated ascorbic acid was removed by dialysis. Samples were initially treated with liposomes containing ascorbic acid and subsequently with 3CP solution. All skin samples were put into the tissue-cell holder and recorded on Bruker Elexsys II E540 EPR spectrometer in L-band, using **ER 540R23** resonator in vertical orientation (Figure 1). From the 2D EPR image of

control and treated skin samples it could be observed that liposomes containing ascorbic acid have significantly reduced the EPR signal of spin-probe 3CP. Likewise, using this 2D EPR imaging method, it was possible to observe the level of penetration of different liposome formulations into the skin, their stability and antioxidative activity in *ex vivo* conditions. The same principle could also be used for *in vivo* investigations, using various experimental animal models.

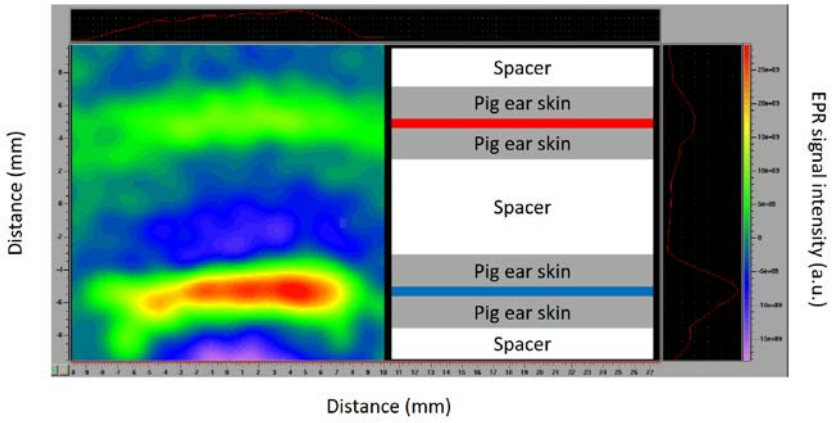

Figure 1: 2D EPR image of control and liposome-treated pig ear skin samples. The upper image represents the sample treated primarily with the liposomes (encapsulating ascorbic acid solution) and subsequently with spin-probe 3CP (red line on the schematic illustration). The lower image represents the control sample which was treated only by spin-probe 3CP (blue line on the schematic illustration).

2D EPR imaging was employed to perform *ex vivo* measurements of the penetration of liposome-encapsulated ascorbic acid evaluating its capability to reduce cell wall-permeable spin-probe 3CP introduced to the pig ear skin. 2D EPR image has shown that once penetrated into the skin tissue, the encapsulated antioxidant has successfully reduced the spin-probe reflecting its penetration profile and effective antioxidative activity.

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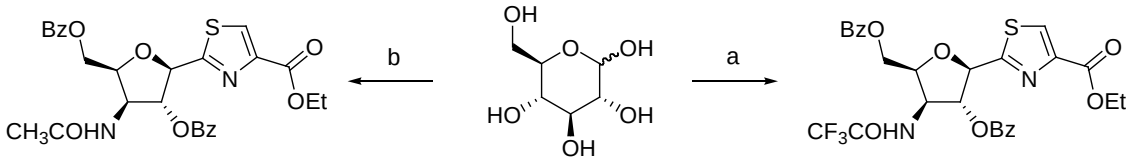
NMR INVESTIGATION OF INTRAMOLECULAR HYDROGEN BONDING INVOLVING AN ORGANIC FLUORINE

Mirjana Popsavin*, Ivana Kovačević, Sanja Đokić, and Velimir Popsavin

Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, Trg D. Obradovića 3, Novi Sad
* E-mail adress: mirjana.popsavin@dh.uns.ac.rs

Multistep stereospecific synthesis of protected thiazole *C*-nucleosides **1** and **2** has been achived starting from D-glucose.Two methylene hydrogens from the ethyl group in the compound **1** are in different chemical environments and therefore have different chemical shifts appearing as two quartets the ^{1}H NMR spectrum. If the temperature is raised, these signals are broaden and at $T > 42$ °C coalesce into a single quartet. Restricted bond rotation is due to N–H····N and F····H–C intramolecular hydrogen bonding. The free energy of activation for the process is determined by NMR. Unlike compound **1**, the ^{1}H NMR spectrum of **2**, shows only one quartet due to the resonance of two methylene protons from the ethyl group.



2 **D-glucose** **1**

Scheme 1. (a) 17 steps; (b) 16 steps.

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PHOTOINITIATED THIOL-ENE COUPLING REACTION USING LED NMR SPECTROSCOPY

Miroslav Dangalov[a, *], Nikolay G. Vassilev[a], Natalia Toncheva-Moncheva[b], Christo P. Novakov[b]

[a] Institute of Organic Chemistry with Center of Phytochemistry, 9, Acad. G. Bonchev Str., 1113 Sofia, Bulgaria
[b] Institute of Polymers, 103-A, Akad. G. Bonchev Str., 1113 Sofia, Bulgaria
* m.dangalov@orgchm.bas.bg

Versatile LED NMR spectroscopy [1] was successfully performed for *in situ* study of thiol-ene modification of (co)polymers without additional photoinitiator. For the purpose poly(allyl glycidyl ether) (PAGE) homopolymers was reacted with few mono- and dithiol oligo- or polyethylene glycols (PEG) via UV illumination (365 nm wavelengths). This methodology uses LEDs as light sources, thereby provides opportunities to conduct effective photoinitiated experiments in combination with whole variety of NMR methods for studies of photochemical reactions.

Thus the progress of the process was followed by ^{1}H NMR spectra and diffusion experiments (DOSY) allowing the study of the photochemical reactions as well as the process of association/aggregation of macromolecules in solution. Proton spectra and GPC traces of resulting products support the expected turnaround of “click” reaction and formation of pegylated (co)polymers. In some cases upon coupling with dithiol reagent, formation of a gel-fraction in a noticeable amount was observed, suggesting occurring of cross-linking reaction, which is subject of a further study.

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THE IDENTIFICATION OF THE STRUCTURE OF NEW 2-HYDROXY-JUGLONE DERIVATIVE

Natalia Sucman,[1,2] Timur Andrusenco,[1] Fliur Macaev[1].

[1]Institute of Chemistry, Academy of Sciences of Moldova, Academiei str. 3, MD-2028, Chisinau, Republic of Moldova

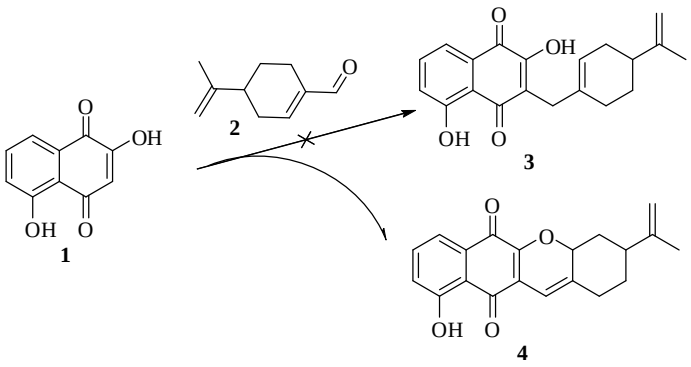
2 Department of Agro-Technology, Comrat State University, 17, Galatsana str., Comrat, MD-3802, Republic of Moldova

E-mail: nicheli@yandex.ru

Juglone is a naphthoquinone compound present in high amounts in the plants of the *family Juglandaceae*. Especially high amounts can be detected in the eastern black walnut (*Juglans nigra*) and common walnut (*Juglans regia*).

Juglone has a wide spectrum of biological activity, including antibacterial and antifungal properties [1].

The aim of our study was to obtain new compounds derivated from juglone, which could potentially have interesting biological properties. For this reason, a reaction was carried out between 2-hydroxy-juglone **1** with perillaldehyde **2**. We expected the formation of compound **3**, but the ^{1}H-, ^{13}C-NMR data indicated the formation of another one. The identity of it has been established by additional experiments including those two-dimensional (COSY, HSQC, HMBC, NOE). Moreover, the structure **4** was confirmed by X-ray analysis.



Scheme. The synthesis of juglone derivative **4**.

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IN SITU IRRADIATION NMR SPECTROSCOPY IN THE DESIGN OF NEW FUNCTIONAL MATERIALS

Nikolay Vassilev,[a,*] Miroslav Dangalov[a]

[a] Institute of Organic Chemistry with Centre of phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev str. Bl. 9, 1113 Sofia, Bulgaria

* Corresponding author niki@orgchm.bas.bg

In situ irradiation NMR spectroscopy includes illumination of the samples inside the NMR spectrometer [1]. The setup, which includes LEDs as light sources, allows application of whole variety of NMR methods to photochemical reactions. In addition to the standard NMR spectroscopic approaches feasible for reactions in the dark, a special hyperpolarization technique applicable exclusively to photoreactions, the Photo-CIDNP spectroscopy provides a molecular fingerprint of reactive intermediates at a nanosecond time scale.

The setup includes control unit connected directly to the NMR spectrometer through three BNC connections. The first connection can switch between CW and Pulse mode, the second one can setup the optical power of used LED source and third one can switch on and off the illumination. The setup has three LEDs (365-470 nm, 440-460 nm and 650-670 nm), which are coupled to optical fiber. The other end of the optical fiber is inserted in a coaxial insert and can illuminate the NMR sample.

The equipment was successfully tested on photoisomerization of azobenzene [2] by *in situ* UV irradiation using LED source at 365 nm. The Photo-CIDNP spectroscopy was demonstrated on photo-induces reaction between benzophenone triplet and hydroquinone [3]. The possibilities of novel methodology for thiol-ene modification of (co)polymers accomplished under LED UV-irradiation without any photoinitiator were tested [4].

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SOLID STATE NMR CHARACTERIZATION OF MODIFIED SBA-15 MESOPOROUS SILICAS AS DRUG DELIVERY SYSTEM OF VERAPAMIL HYDROCHLORIDE AND DICLOFENAC SODIUM

Pavletta Shestakova,[a] Margarita Popova[b], Charlotte Martineau-Corcos,[c] Agnes Szegedi,[d] Yavor Mitrev,[a] Ivalina Trendafilova,[b] Alessia Michelotti[c]

a Bulgarian NMR Centre, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. Georgi Bonchev St. Bl.9, 1113 Sofia, Bulgaria
b Laboratory Organic Reactions on Microporous Materials, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. Georgi Bonchev St. Bl.9, 1113 Sofia, Bulgaria
c Institut Lavoisier de Versailles, UMR 8180 CNRS, Université de Versailles Saint-Quentin-en-Yvelines, 78035 Versailles, France
d Research Centre for Natural Sciences, Institute of Materials and Environmental Chemistry, Hungarian Academy of Sciences, Magyar tudósok körútja 2, 1025 Budapest, Hungary
* Pavletta Shestakova: psd@orgchm.bas.bg

During the last decade solid state MAS NMR has been established as an efficient tool for structural characterization of drug delivery systems (DDS), including mesoporous silicas (MS) and zeolites.1,2 In the present study 1H and heteronuclear (27Al, 29Si, 13C) MAS NMR were applied to investigate Al and -SO3H modified SBA-15 MS loaded with Verapamil Hydrochloride (VH) and Diclofenac Sodium (DNa). The NMR data indicate that both drugs are successfully included within the pores of Al-SBA15 and SBA15-SO3H materials. The nature of drug-matrix interactions was elucidated by 1H-1H 2D RFDR and 1H→29Si 2D HETCOR experiments. The RFDR experiments show the presence of nonspecific interactions between VH and silanol groups of the MS, while in case of DNa the interaction involves specifically the CH2COO- function of drug molecules. The 1H and 23Na MAS NMR spectra evidence that the interaction between MS and drug molecules is associated with exchange of H+ (in case of VH) and Na+ ion (in case of DNa) from the drug molecules and protons from ether Al-OH+ species (in Al-SBA15) or SO3H groups (in SBA15-SO3H) of the modified MS materials. The 1D 23Na and 23Na MQMAS experiments were used to elucidate the hydrate form modifications of DNa upon its loading in the modified SBA15 materials. The results contribute to better understanding the factors governing the effective drug loading as well as to clarify how the drug-carrier interactions define the performance of the newly developed DDS.

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Financial support within Bulgarian-French Research Collaboration Program of the NSF, Bulgaria and MEAE-MESRI, France, Grant: № DNTS (ДНТС) 01/14-2017 is gratefully acknowledged.

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QUALITATIVE AND QUANTITATIVE NMR IN OPTIMIZATION OF METHYL B-D-FRUCTOFURANOSIDE SYNTHESIS CATALYZED BY INVERTASE GLYCOFORMS

Uroš Andjelković [a,b,*], Ivan Gudelj [c], Thomas Klarić [c], Hannes Hinneburg [d], Marijana Vinković [e], Karlo Wittine [b], Nebojša Dovezenski [f], Dražen Vikić-Topić [e,g], Gordan Lauc [c,h], Zoran Vujčić [i], Djuro Josić [b]

[a] Department of Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Belgrade, Serbia
[b] University of Rijeka, Department of Biotechnology, Rijeka, Croatia
[c] Genos Glycoscience Research Laboratory, Zagreb, Croatia
[d] Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, Potsdam, Germany
[e] NMR Centre, Ruđer Bošković Institute, Zagreb, Croatia
[f] Institute for Medical Research, University of Belgrade, Belgrade, Serbia
[g] Department of Natural and Health Sciences, Juraj Dobrila University of Pula, Pula, Croatia
[h] Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia
i Faculty of Chemistry, University of Belgrade, Belgrade, Serbia
* uros@chem.bg.ac.rs

Abstract

Glycoforms of invertase that exhibit high stability in hydrophilic organic solvent were purified from a crude mixture of a multitude of invertase N-glycoforms synthesized by *Saccharomyces cerevisiae*. Purification was achieved by specially designed subfractionation procedure on anion-exchange chromatographic monolithic supports. Among separated N-glycoforms (EINV1-EINV4), only EINV1 exhibited 2-5 times higher stability in 30% methanol. The difference of stability in the presence of high methanol concentration was used to improve the efficiency and yield of methyl β-D-fructofuranoside (MF) synthesis.
Formation of MF in the reaction mixtures was confirmed by COSY, HMBC and HMQC. Kinetic of MF formation was assessed by quantitative NMR using anisole as a standard. NMR analysis provided data that enabled identification of optimal reaction time and calculation of the reaction yield.
The efficiency and yield of MF synthesis was improved more than 50% when the most stabile N-glycoform was utilized. These data underline the importance of analysis of glycan structures attached to glycoproteins, demonstrates different impact of N-glycans on invertase stability in hydrophilic organic solvent reaction environment, and provide a platform for improvement of yield of biocatalytic processes by selection of N-glycoforms.

^{1}H Iterative Full Spin Analysis of Hotrienol from a Mixture of *Sambucus nigra* L. (Adoxaceae) Volatiles

Vidak Raičević,[a] Marija Sakač,[a] Milica Aćimović,[b] Marko Mladenović,[c] Niko Radulović[c,*]

[a] Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 3, Novi Sad, Serbia
[b] Institute of Field and Vegetable Crops, Maksima Gorkog 30, Novi Sad, Serbia
[c] Department of Chemistry, Faculty of Science and Mathematics, University of Niš, Višegradska 33, Niš, Serbia
* nikoradulovic@yahoo.com

Hotrienol (Figure 1) is an oxygenated monoterpene found in essential oils of many plant species and employed in the flavor and fragrance industry. As part of our ongoing study into the volatiles of *Sambucus nigra* L. (Adoxaceae), we isolated a hotrienol-rich fraction by chromatographing the diethyl ether extract from the distillation water of dry elderflowers. The collected NMR spectral data of this mixture were used to extract full NMR spectral data of hotrienol via ^{1}H iterative full spin analysis (HiFSA). While it is possible to execute automated HiFSA using computational tools like the PERCH software [1], a high purity sample is a necessity in that case [2]. Here, as performed previously on other molecules [3, 4], HiFSA of hotrienol was facilitated by manual iterations, using the MestReNova software spectrum simulation feature. The obtained data could prove useful for methods to quantify natural products in mixtures by means of NMR, such as the HiFSA–qHNMR approach [5].

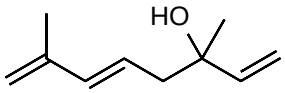

Figure 1. The structure of hotrienol

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Sugar profiling of honey – a quantitative HSQC approach

Yavor Mitrev,* Svetlana Simova, Dessislava Gerginova

[a]*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria*

* Corresponding author email address: yavor@orgchm.bas.bg

Honey is widely used in medicine, pharmacy and cosmetics, but also one of the most adulterated foods. Besides the simple validation for adulteration by ^{1}H NMR, the NMR analysis of honey samples is a challenging task, especially if a detailed sugar profile is required. For example, the anomeric region typically exhibits a multitude of overlapping signals in the ^{1}H spectrum, thus requiring ^{13}C detected 1D experiments for quantification of the individual saccharides. A viable alternative provide 2D HSQC experiments with ultra-high resolution in the indirect dimension [1], preferably acquired with non-uniform sampling (NUS) in order to reduce the overall experimental time.

Recently, it was demonstrated [2], that the combination of state-of-the-art techniques, like broadband homodecoupling, spectral aliasing and NUS, provides fast and sensitive method for quantitative HSQC analysis of metabolites. Using a similar approach, we present our initial results on the qHSQC technique for investigation of the saccharide composition of honey.

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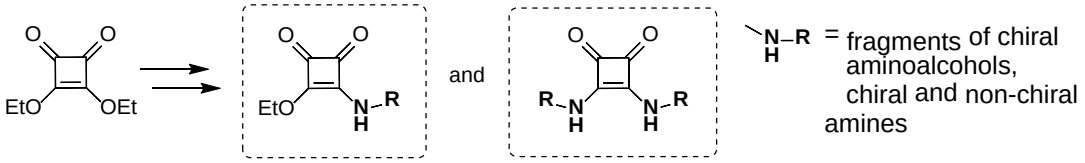
DOSY AND MULTINUCLEAR NMR EXPERIMENTS TO WITNESS A CATALYTIC SYSTEM BASED ON CHIRAL AMINOALCOHOLS AND THEIR SQUARAMIDES WITH $BH_3 \cdot SMe_2$

Yana Nikolova,[a] Pavletta Shestakova,[a]* Georgi Dobrikov,[a] Vladimir Dimitrov[a]

[a] Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria
* psd@orgchm.bas.bg

A series of squaric acid amides has been synthesized using a set of chiral aminoalcohols and amines. The catalytic activities of the squaramides and the corresponding free aminoalcohols have been comparatively studied in the model reaction of reduction of *α*-chloroacetophenone with $BH_3 \bullet SMe_2$ showing the better efficiency of the aminoalcohols as ligands.



The reactivity and stability of the *in situ*-formed borane complexes of chiral aminoalcohols and their squaric amides were investigated by 1D and 2D NMR. The ^{1}H and ^{11}B NMR spectra indicated that there was a coordination between the squaramide and $BH_3 \cdot SMe_2$, most probably through the N-atom from the ligand, however no definite structure was identified. Moreover the occurrence of destructive reactions resulting in disintegration of the ligand and the $BH_3 \cdot SMe_2$ was observed. The formation of complex between $BH_3 \cdot SMe_2$ and parent aminoalcohols was confirmed by ^{1}H diffusion ordered NMR spectroscopy (DOSY).

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List of participants

surname	name	email	institute	country
Aksić	Jelena	aksicjelena9@gmail.com	Faculty of Sciences and Mathematics, University of Niš	Serbia
Ana-Maria	Macsim	ana.iurascu@icmpp.ro	"Petru Poni" Institute of Macromolecular Chemistry	Romania
Anđelković	Boban	aboban@chem.bg.ac.rs	Faculty of Chemistry	Serbia
Andjelkovic	Uros	uros@chem.bg.ac.rs	University of Belgrade	Serbia
Asanin	Darko	dasanin@kg.ac.rs	University of Kragujevac, Faculty of Science	Serbia
Badea	Valentin	valentin.badea@upt.ro	Politehnica University Timisoara	Romania
Bečić	Ervina	ervina.becic@ffsa.unsa.ba	Faculty of Pharmacy	Bosnia and Herzegovina
Berinkeiné Donkó	Mária	berinkeinem@nebih.gov.hu	National Food Chain Safety Office, Hungary	Hungary
Bermel	Wolfgang	wolfgang.bermel@bruker.com	Bruker BioSpin GmbH	Germany
Bouras	Georgios	geo.mpouras@hotmail.com	University of Patras	Greece
Calinicenco	Serghei	casperleteha@gmail.com	Comrat State University	Moldavia
Cattaneo	Vittorio	vittorio.cattaneo@bruker.com	Bruker Italia srl	Italy
Csikorne Vasarhelyi	Helga	csikorneh@nebih.gov.hu	National Food Chain Safety Office	Hungary
Daković	Marko	marko@ffh.bg.ac.rs	Faculty of Physical Chemistry	Serbia
Dangalov	Miroslav	m.dangalov@orgchm.bas.bg	Institute of Organic Chemistry with Center for Phytochemistry-BAS	Bulgaria
Dedić	Mirza	mirza.dedic@ffsa.unsa.ba	Faculty of Pharmacy	Bosnia and Herzegovina
Deleanu	Calin	calin.deleanu@yahoo.com	Centre of Organic Chemistry	Romania
Denic	Aleksandar	denic.aleksandar@mayo.edu	Mayo Clinic	USA
Djapic	Nina	nidjapic@gmail.com	Technical Faculty "M. Pupin"	Serbia
Đorđević	Miljana	miljanadjordjevic11@gmail.com	Faculty of Sciences and Mathematics, University of Niš	Serbia
Draskovic	Dragana	dragana43809@gmail.com	Technical Faculty "M. Pupin"	Serbia
Đurović	Saša	sdjurovic@iofh.bg.ac.rs	Institute of General and Physical Chemistry	Serbia
Franich	Anđela	andjela.franich@pmf.kg.ac.rs	Prirodno-matematički fakultet	Serbia
Gáti	Tamás	tamas.gati@servier.com	Servier	Hungary
Gerginova	Desislava	dpg@orgchm.bas.bg	Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences	Bulgaria

Gođevac	Dejan	dgodjev@chem.bg.ac.rs	ICTM, National institute, University of Belgrade	Serbia
Gojgić Cvijović	Gordana	ggojgic@chem.bg.ac.rs	Institute of chemistry, technology and metallurgy	Serbia
Habinovec	Iva	ihabinovec@chem.pmf.hr	University of Zagreb, Faculty of Science, Department of Chemistry	Croatia
Imamović	Belma	belma.imamovic@ffsa.unsa.ba	Faculty of Pharmacy; University of Sarajevo	Bosnia and Herzegovina
Ivanović	Stefan	stefan.ivanovic@ihtm.bg.ac.rs	Institute of Chemistry, Technology and Metallurgy	Serbia
Jednačak	Tomislav	tjednacak@chem.pmf.hr	Universty of Zagreb, Faculty of Science	Croatia
Kessler	Pavel	pavel.kessler@bruker.com	Bruker BioSpin GmbH	Germany
Korać	Jelena	jskorac@gmail.com	Institute for Multidisciplinary Research	Serbia
Kostić	Jelena	jelena.kostic.995@gmail.com	Faculty of Physical Chemistry	Serbia
Krstic	Aleksandar	aleksandar.krstic@vin.bg.ac.rs	Vinca Institute of Nuclear science	Serbia
Kuzminac	Ivana	ivana.kuzminac@dh.uns.ac.rs	University of Novi Sad, Faculty of Sciences, Department of Chemistry, Biochemistry and Environmental Protection, Trg Dositeja Obradovića 3, 21000 Novi Sad, Serbia	Serbia
Macura	Slobodan	macura@mayo.edu	Mayo Clinic	USA
Mandić	Boris	borism@chem.bg.ac.rs	Faculty of Chemistry	Serbia
Mangalagiu	Ionel	ionelm@uaic.ro	Alexandru Ioan Cuza University of Iasi	Romania
Mangalagiu	Violeta	violeta.mangalagiu@uaic.ro	Alexandru Ioan Cuza University of Iasi	Romania
Margetic	Aleksandra	aleksandra@chem.bg.ac.rs	Institute of Chemistry, Technology and Metallurgy, University of Belgrade	Serbia
Marousis	Konstantinos	kmarouk@upatras.gr	University of Patras	Greece
Mathieu	Daniel	Daniel.Mathieu@bruker.com	Bruker BioSpin GmbH	Germany
Miladinovic	Zoran	zmiladinovic@iofh.bg.ac.rs	Institute of General and Physical Chemistry	Serbia
Milenkovic	Milan	milan_milenkovic@batut.org.rs	Institute of Public Health of Serbia "Dr Milan Jovanovic Batut"	Serbia
Miricioiu	Marius	marius.miricioiu@icsi.ro	National Institute of Research and Development for Cryogenic and Isotopic Technologies ICSI Rm. Valcea	Romania
Mitrev	Yavor	yavor@orgchm.bas.bg	Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Scinces	Bulgaria

Mladenović	Marko	markohem87@gmail.com	Faculty of Sciences and Mathematics	Serbia
Mojović	Miloš	milos@ffh.bg.ac.rs	Faculty of Physical Chemistry - University of Belgrade	Serbia
Nakarada	Đura	djura@ffh.bg.ac.rs	Faculty of Physical Chemistry	Serbia
Napoli	Claudia	claudia.napoli@bruker.com	Bruker Italia srl	Italy
Natalia	Sucman	nicheli@yandex.ru	Institute of Chemistry	Moldavia
Nešić	Milan	nesicmilanvl@gmail.com	University of Niš, Faculty of Sciences and Mathematics, Department of Chemistry	Serbia
Nicolescu	Alina	alina@icmpp.ro	"Petru Poni" Institute of Macromolecular Chemistry	Romania
Nikolic	Milica	milica.nikolic.1990@gmail.com	Faculty of Science and Mathematics, University of Nis	Serbia
Nikolova	Yana	ynikolova@orgchm.bas.bg	Institute of organic chemistry with centre of phytochemistry	Bulgaria
Novak	Predrag	pnovak@chem.pmf.hr	University of Zagreb, Faculty of Science, Department of Chemistry	Croatia
Parlov Vuković	Jelena	jelena.parlov-vukovic@ina.hr	INA d.d.	Croatia
Pavićević	Aleksandra	aleks.pavicevic@ffh.bg.ac.rs	Faculty of Physical Chemistry, University of Belgrade	Serbia
Pavlešić	Tomislav	tomislav.pavlesic@uniri.hr	University of Rijeka	Croatia
Petkov	Nikolay	nnp@orgchm.bas.bg	Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Science	Bulgaria
Petkovic	Milos	milosp@pharmacy.bg.ac.rs	Faculty of Pharmacy - University of Belgrade	Serbia
Podbevsek	Peter	peter.podbevsek@ki.si	National Institute of Chemistry	Slovenia
Popovic Bijelic	Ana	ana@ffh.bg.ac.rs	Faculty of Physical Chemistry - University of Belgrade	Serbia
Popsavin	Mirjana	mirjana.popsavin@dh.uns.ac.rs	University of Novi Sad, Faculty of Sciences	Serbia
Pupić - Bakrač	Mirko	mirko@aparatura.hr	Aparatura d.o.o.	Croatia
Radonjic	Leposava	leposava@donaulab.rs	Donau Lab Beograd	Serbia
Radulović	Niko	nikoradulovic@yahoo.com	Department of Chemistry, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia	Serbia
Raičević	Vidak	vidak.raicevic@dh.uns.ac.rs	University of Novi Sad	Serbia
Ripamonti	Angelo	angelo.ripamonti@bruker.com	Bruker Italia srl	Italy
Rohonczy	János	rohonczy@caesar.elte.hu	Eötvös Loránd University, Institute of Chemistry	Hungary

SALOME ABARCA	LUIS FRANCISCO	l.f.salome.abarca@biology.leidenuniv.nl	Leiden University, Institute of Biology	Netherland
Shestakova	Pavletta	psd@orgchm.bas.bg	Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences	Bulgaria
Simić	Milena	milena@pharmacy.bg.ac.rs	University of Belgrade, Faculty of Pharmacy, Department of Organic chemistry	Serbia
Simić	Katarina	katarina.simic@ihtm.bg	Institute of Chemistry, Technology and Metallurgy	Serbia
Simova	Svetlana	sds@orgchm.bas.bg	Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences (IOCCP-BAS)	Bulgaria
Škorić	Dušan	dusan.skoric@dh.uns.ac.rs	University of Novi Sad Faculty of Sciences	Serbia
Smit	Biljana	biljana.smit@pmf.kg.ac.rs	University of Kragujevac, Faculty of Science	Serbia
Sofrenić	Ivana	ivanasofrenic@chem.bg.ac.rs	Faculty of Chemistry	Serbia
Spyroulias	Georgios A.	G.A.Spyroulias@upatras.gr	University of Patras, Department of Pharmacy	Greece
Stanic	Petar	petar.stanic@pmf.kg.ac.rs	University of Kragujevac, Faculty of Science	Serbia
Steffen	Christian	christian.steffen@bruker.com	Bruker BioSpin GmbH	Germany
Steuernagel	Stefan	stefan.steuernagel@bruker.com	Bruker BioSpin GmbH	Germany
Stevanović	Milica	milica.stevanovic992@gmail.com	University of Niš, Faculty of Sciences and Mathematics, Department of Chemistry	Serbia
Stojanovic	Nikola	nikola.st90@yahoo.com	Faculty of Medicine, University of Nis	Serbia
Tasic	Ljubica	ljubica@unicamp.br	University of Campinas (UNICAMP)	Brasil
Tešević	Vele	vtesevic@chem.bg.ac.rs	Faculty of Chemistry	Serbia
Todorović	Nina	ninat@chem.bg.ac.rs	ICTM-Center for Chemistry	Serbia
Tóth	Gábor	drtothgabor@t-online.hu	TU Budapest	Hungary
Trifunović	Snežana	snezanat@chem.bg.ac.rs	Faculty of Chemistry, University of Belgrade	Serbia
Vassilev	Nikolay	niki@orgchm.bas.bg	IOCCP-BAS	Bulgaria
Vatic	Sascha	sascha.vatic@med.bg.ac.rs	University of Belgrade	Serbia
Vesković	Ana	ana.veskovic@ffh.bg.ac.rs	University of Belgrade - Faculty of physical chemistry	Serbia
Vuckovic	Ivan	vuckovic.ivan@mayo.edu	Mayo Clinic	USA
Vujisić	Ljubodrag	ljubaw@chem.bg.ac.rs	University of Belgrade - Faculty of Chemistry	Serbia
Wittine	Karlo	karlo.wittine@biotech.uniri.hr	University of Rijeka, Department of Biotechnology	Croatia

Zakrzewska	Joanna	jzakrzewska@iofh.bg.ac.rs	Institute of General and Physical Chemistry	Serbia
Zlatković	Dragan	dragan.zlatkovic@gmail.com	Faculty of Sciences and Mathematics, University of Niš	Serbia