LECTURES

IN-CELL NMR: A POWERFUL APPROACH FOR DRUG DISCOVERY

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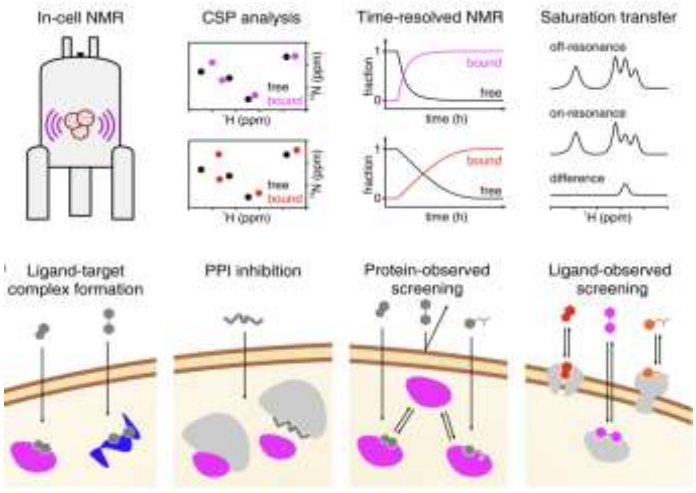
In-cell NMR, i.e. high resolution NMR spectra of biomolecules in intact, living cells, represents one of the highest impact applications of magnetic resonance, as it allows to obtain information on the conformational and functional properties of biomolecules and their interactions at atomic resolution in conditions as close as possible to the physiological ones.

A striking application is drug screening in real time at cellular level, in human living cells.



Methodological aspects need to be developed and implemented to address the various critical aspects of these systems from the NMR point of view: from the low sensitivity to the short life time of the sample, to the broadening of the signals, often beyond detection. Furthermore the selective detection of the biomolecules of interest among the myriads of those present in the cell also needs to be addressed. The innovative approaches towards these challenges will be discussed and a few examples of the striking power of this method for early and effective drug screening will be presented. Particular focus will be on the cellular uptake and target binding in the cellular milieu of drugs and leads and on the meaningful differences observed between drug-target binding in living cells versus in vitro. Furthermore the power of 19F in cell NMR both as protein-detected and as ligand-detected will be presented and discussed.

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HETEROCOVARIANCE, A POWERFUL TOOL FOR THE DISCOVERY OF BIOACTIVE NATURAL PRODUCT

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Natural products possess significant and well-documented biological properties representing the bioactive chemodiversity hot-spot in the chemical space, however the great majority of existing compounds remains still unexplored. Traditional isolation and scale-up procedures are inefficient and often become the bottleneck in natural products dereplication in order not to re-identify known compounds that are responsible for the activity.

Spectral data reflecting concentration differences of the components of an extract can correlate statistically with measurable dose-dependent properties such as enzyme inhibition, on the basis of a Heterocovariance approach, identifying the bioactive components prior to purification. Medicinal plants can be extracted and fractionated creating series of different concentrations of each component in consecutive fractions. The NMR and MS spectroscopic data of the fractions can be correlated with bioactivity assays using statistical spectroscopy approaches and the structure of the active components can be deduced.

In conclusion plant metabolomics provides a very powerful tool that can revolutionize natural products discovery. Inherent or carefully planned variance in concentration of plants' secondary metabolites as conveyed by NMR and MS spectra can be correlated with any dose-response property and unmask active constituents in the complex extract or fraction mixtures prior to any purification step. This highly innovative activity-based-metabolite-profiling can dramatically accelerate the discovery of active natural products challenging global biodiversity and chemodiversity.

VARIABILITY IN STRUCTURE AND FUNCTION OF DECORIN-BINDING PROTEINS AMONG EUROPEAN *BORRELIA*

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Borrelia spirochetes, the causative agents of Lyme disease, utilize a diverse set of adhesin proteins to invade host tissues, primarily by interacting with extracellular matrix components such as the proteoglycan decorin (mediated by decorin-binding proteins, DbpA/DbpB) [1]. These Dbp proteins exhibit an affinity for glycosaminoglycan (GAG) moieties, with variations in these interactions among different species driving tissue tropism [2]. Despite limited sequence similarity, Dbps maintain a conserved three-dimensional structure, with the "linker" region proved to be the key GAG-binding site in North American species [3].

This study investigates how subtle conformational variations in DbpA and DbpB from European *Borrelia* strains (*B. afzelii*, *B. bavariensis*, and *B. garinii*) influence their binding affinities for GAGs. The Dbp proteins were over-expressed in *E. coli* as isotopically labeled recombinant proteins and analyzed using NMR-based secondary structure prediction and backbone dynamics characterization. Binding affinities to GAGs were assessed through NMR titration and hydrogen-deuterium exchange mass spectrometry [4].

Results indicate that while Dbp proteins from European strains bind to all GAG ligands, their specific affinities and interacting residues exhibit clear species-specific differences. Notably, the binding affinities of European Dbps significantly differ from those of North American strains, despite similar overall structures [3,5]. These findings support a model of highly selective interactions between Dbps and GAGs, providing insight into the distinct tissue tropisms observed among Borrelia species.

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STRUCTURAL DYNAMICS OF MEMBRANE PROTEINS, FAST MAGIC-ANGLE SPINNING, HIGH MAGNETIC FIELDS

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The study of membrane proteins has always posed significant challenges due to their complex nature and the difficulty of mimicking their native environments. Recently, the development of magic-angle spinning (MAS) NMR has provided groundbreaking tools to overcome these obstacles. Notably, fast MAS rates, exceeding 100 kHz, have enabled the direct detection of proton resonances with unprecedented sensitivity and resolution. This, combined with the use of higher magnetic fields, has transformed our approach to atomic-level protein investigations. These technological advancements offer several key advantages: they allow the examination of larger molecular systems with precise site specificity, reduce the necessity for extensive isotopic labeling (especially deuteration), and streamline the processes of resonance assignment, structure calculation and dynamical parameter acquisition.

In this presentation, we will explore the methodologies that underpin these advancements and demonstrate their application in the detailed characterization of various transmembrane channels and transporters within lipid bilayers. Furthermore, we will highlight the impact of new instrumentation capable of achieving even faster MAS spinning rates (up to 160 kHz) and operating at higher magnetic fields (up to 1.2 GHz). We will also discuss advances in sample production, including the use of bacterial outer membrane vesicles for the in-situ characterization of bacterial envelope proteins and the expression in Pichia pastoris for labeling human proteins. These advancements open new avenues for the study of complex biological systems, providing unprecedented insights into their structural and dynamic properties.

EX-SITU AND IN-SITU SOLID STATE NMR STUDIES OF MECHANOCHEMICAL TRANSFORMATIONS OF ACTIVE PHARMACEUTICAL INGREDIENTS (APIS) AND RELATED PRODUCTS

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Mechanochemistry is an innovative, versatile platform for the production of a variety of compounds that in many cases are difficult to obtain using classic "wet" methods. Mechanosynthesis eliminates the need for solvents, which reduces waste and minimizes environmental impact and typically operates at room temperature leading to energy savings. Recently, it has been shown that modern analytical tools facilitate the study of solid-state processes under real operating conditions (operando). Various analytical probes such as X-rays or laser beams, vibrational spectroscopy (Fourier transform infrared spectroscopy, Raman spectroscopy) were used to control the reaction mechanism and the formation of intermediates. Unfortunately, high-resolution solid-state NMR spectroscopy has not been used very often until now, and its potential is not fully apparent. Recently, we have proven that NMR spectroscopy, in particular the 1D and 2D Magic Angle Spinning (SS MAS NMR) techniques, can be treated as a complementary tool to other analytical methods.[1,2] SS MAS NMR is particularly suitable for *in situ* monitoring processes when substances during transformation form low-melting eutectic mixtures (e.g. cocrystal formation). If the final products obtained by the ball mill method and the thermal process are exactly the same, Variable Temperature (VT) SS NMR is the bridge between the two methods and provides unique information about the mechanism of transformations. In this sense, MAS NMR can be thought of as a "prepanostic" device (preparation and diagnostics). The application of ^{19}F MAS NMR as probe for investigation of mechanochemical loading of APIs into the mesoporous carriers[3] and ^{133}Cs MAS NMR as support in mechanosynthesis of peptides [4] will be discussed.

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DNA STRUCTURAL MODULATIONS DRIVEN BY ADDITION OF CATIONS

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Instabilities of genomic segments with reiterating 1-6 base-pairs, i.e. short tandem repeats, are coupled to normal physiology mechanisms, while on the other hand, can also lead to neurodegenerative or other dreadful diseases. It remains unclear how expansions and contractions of repeats drive (aberrant) events, and furthermore, why repetitive or repeat-derived sequences comprise over half of the human genome.

The ongoing studies continuously underline that biological roles of short tandem repeats are fundamentally linked to formation and details of non-canonical DNA. These are perceived as structures in which hydrogen-bonded base pairs and nucleobase stacking geometries are different compared to the most common double-strand B-DNA helix. In particular, G-quadruplexes and i-motifs that can form within guanine- and cytosine-rich DNA, respectively, have been extensively studied to demonstrate their occurrences in regulatory genomic regions and furthermore their involvement in DNA transcription, gene expression etc.[1] Formation of G-quadruplexes depends on the presence of K+ and Na+ ions, while i-motifs are stabilized at acidic and sub-neutral conditions, altogether pointing out that both structural families act as peculiar biosensor systems.

NMR-based studies of oligonucleotides (prone to) fold into G-quadruplexes, i-motifs and other non-canonical structures remain a cornerstone of deciphering fundamental cellular processes related to DNA folding tuned by microenvironmental variations. Herein we will focus on solution-state NMR analysis directed to investigating polymorphisms of different DNA oligonucleotides comprising short tandem repeats.[2-5] In order to stimulate discussions on peculiar DNA structures, the presentation will include insights into dimeric DNA structures formed in the presence of divalent cations, which have been elucidated in detail with the use of NMR, while they might be hard to perceive by using other biophysical methods.

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IDPS: NOVEL EXPERIMENTAL APPROACHES AND ASSESSMENT OF STRUCTURAL PROPENSITIES

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Intrinsically disordered proteins and protein regions (IDPs/IDRs) are proved to play crucial roles in many biological processes. They possess no defined secondary, tertiary structure, instead they can be described as molecular ensembles. Regarding the amino acid composition, they lack residues with aromatic sidechains but are abundant in charged residues and prolines. Proline has a special status in IDPs, as it is possible to detect both *cis* and *trans* isomers.

Compared to globular proteins, IDPs possess distinct hydrodynamic properties, that are reflected in the translational diffusion coefficients.[1] Atomic level characterization of such systems can be given exclusively by NMR spectroscopy. Measurements under physiological conditions rule out the use of classical $^{1}H_{N}$ detected experiments, and to overcome this, we developed tools based on $^{1}H\alpha$ detection. We introduced the selective Hα,Cα-HSQC (SHACA-HSQC), with extensive hetero- and homonuclear decoupling that provides an uncompromised resolution and sensitivity for IDPs.[2] Focusing on the proline residues both major and minor prolines can be detected. We developed proline selective 3D experiments, that help in the determination of the *cis/trans* isomer.[3]

Further on, based on statistics on our collected dataset we tried to figure out how the nature of the neighboring residue will affect the *cis* amount. The established regularities for the varying amounts of *cis* isomer were tested on short peptides.[4]

Assessment of structural propensities in IDPs is challenging, as the difference between the measured value and the random coil value for a given environment is usually small and the random coil values depend on the predictor applied. Using statistical tools and comparing the different random coil chemical shift predictors we offer solutions to better assess a secondary structural propensity.[5]

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DECOUPLING IN APPLICATION AND THEORY

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The suppression of apparent coupling evolution can be achieved in various ways. The most common way of decoupling involves the inversion of one of the coupling partners by a single 180° pulse. Other decoupling schemes involve multi-pulse sequences, like classical heteronuclear decoupling or the suppression of evolution via planar or isotropic mixing conditions.

Modern examples for the different types of decoupling schemes will be shown, starting from a fast DOSY experiment with mixing, going to $^{1}H_{N}$ and $^{1}H\alpha$ detected experiments [1,2] with pulse inversion and chunked homonuclear decoupling. After this, the second part of the talk will deal with classical heteronuclear decoupling and a detailed theoretical description of properties of a selection of commonly used decoupling sequences. With the simulation program PulseDecoupler [3] their properties are studied, like the dependence of decoupling with respect to coupling constants and linewidths. Signal intensities and the appearance of sidebands will be discussed and our attempt to optimize an extremely low-power decoupling sequence called BROCODE.

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[3] will soon be released on github.

DOSY NMR SPECTROSCOPY FOR COMPLEX MIXTURE ANALYSIS

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Diffusion-ordered (DOSY) NMR spectroscopy is a powerful tool for the analysis of components in complex mixtures according to their diffusion properties. In a pseudo-two-dimensional DOSY NMR experiment, one dimension is represented by chemical shifts and the other corresponds to translational diffusion coefficients which depend on the shape and size of a molecule or an aggregate in the sample. As can be observed in Figure 1, different mixture components can be separated in the diffusion dimension and the diffusion coefficients can be calculated.

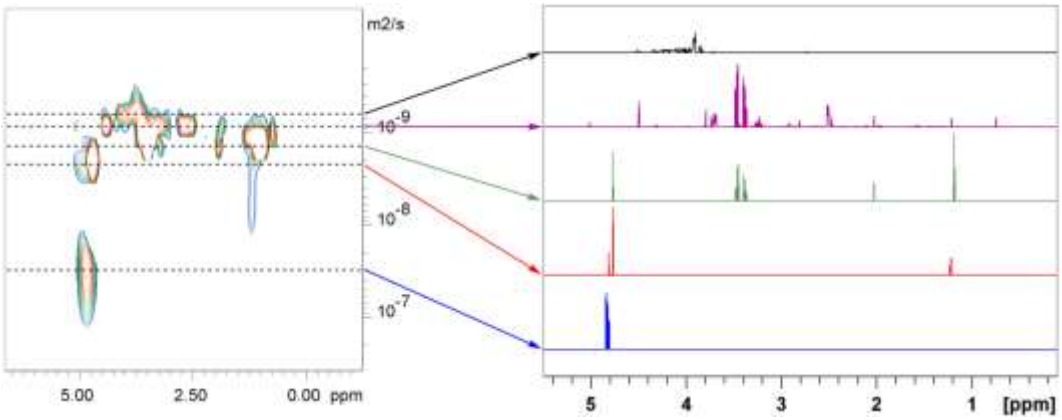

Figure 1. Representative ^{1}H DOSY NMR spectrum of a wine sample with 1D projections of selected spectral regions.

DOSY NMR spectroscopy has shown great potential in petroleum industry for crude oil and asphaltene analysis and in food technology to study milk, juices, honey and wine.[1–3] When combined with multivariate statistical methods, useful information can be obtained on various sample attributes, such as origin, authenticity, content, preparation procedure and physico-chemical properties. Recent applications of this approach in the analysis of complex mixtures have been shown on representative examples from petroleum engineering and food science.

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SYNTHESIS AND CHARACTERIZATION OF AZO, AZOXY AND AZODIOXY-LINKED POROUS ORGANIC POLYMERS FOR CO_2 CAPTURE

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Porous organic polymers (POPs) are crystalline or amorphous materials constructed via strong covalent bonds between organic molecular building units. One of the most important potential applications of POPs is the capture of CO_2, which is the main greenhouse gas whose elevated emissions into the atmosphere are closely related to global warming. POPs with nitrogen functionalities have recently emerged as particularly compelling candidates for efficient and highly selective CO_2 capture and separation. Incorporation of azo (-N=N-) groups into the POPs proved to be especially effective in increasing CO_2 uptake and CO_2/N_2 selectivity.[1] However, the drawback of such POPs is that due to the irreversible formation of azo bonds, they are usually amorphous. In contrast, the formation of similar azodioxy (-ON=NO-) linkages occurs reversibly by polymerization of aromatic polynitroso compounds and provides a way to produce crystalline porous organic networks.

In our recent studies, we focused on the synthesis and characterization of new POPs with different nitrogen-nitrogen linkages (azo, azoxy and azodioxy) and various central moieties (*e.g.*, benzene, pyridine, triazine, amine and porphyrin).[2–5] Due to the insolubility of the synthesized polymers in common organic solvents, ^{13}C CP/MAS NMR spectroscopy has proven to be particularly important for the identification of incorporated linkages and functional groups, for evaluating the degree of polymerization and for providing complementary structural information to IR spectroscopy. The structural and functional properties of the obtained POPs were additionally characterized by powder X-ray diffraction, elemental analysis, thermogravimetric analysis, nitrogen adsorption–desorption measurements and computational chemistry methods. Several new promising candidates for CO_2 capture have been identified.

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HRMAS NMR: APPLICATION IN UNDERSTANDING THE MOLECULAR ORIGIN OF HIGH TOUGHNESS AND REMARKABLE MECHANICAL PROPERTIES OF DOUBLE NETWORK HYDROGELS

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Polymeric hydrogels are materials with high swelling capacity in water, and with promising potentials to advanced pharmaceutical applications (gastric retention, controlled drug delivery) as well as in the field of tissue engineering and regenerative medicine (as scaffolds, tissue culture media). The main drawback of hydrogels is their poor to medium mechanical performance limiting their even wider practical applications. Exceptions are the Double Network (DN) hydrogels displaying extremely high toughness and remarkable mechanical properties.

HRMAS NMR spectroscopy enables in situ analysis of solid materials swollen in a suitable solvent. In our study HRMAS NMR was applied to gain insight at molecular level into the structure of DN hydrogels prepared from PAMPS (poly(2-acrylamido-2-methyl-1-propanesulfonic acid)) and PAAm (poly(acrylamide)) with different ratios of the two polymers, using N,N'-methylene *bis*(acrylamide) (MBAA) as a cross-linker. Diffusion filtered ^{1}H spectra, ^{2}H, ^{13}C, HSQC and ROESY spectra were used to investigate the influence of chemical composition on structure and properties of the DNs. The analysis of the HRMAS NMR data of PAMPS/PAAm hydrogels prepared with increasing AAm concentration shows the formation of covalent bonds between the two polymer networks through the non-reacted double bonds of the cross-linker MBAA. This result allowed elucidating the morphological characteristics of the studied DNs confirming the formation of the so-called "connected double-network gels" (c-DNs) rather than the "truly independent double-network gels" (t-DNs).

^{1}H and ^{2}H HRMAS spectra revealed the existence of a pool of amide protons or deuterons displaying respectively 1:1:1 triplet like ^{1}H and ^{2}H resonance patterns characterized as the rarely observed but nevertheless well-known $^{1}J(^{1}H\text{-}^{14}N)$ and $^{1}J(^{2}H\text{-}^{14}N)$ scalar couplings. In combination with the characteristic ROESY cross-peaks these data evidenced the formation of hydrogen bond network between the side chain fragments based on N-H group of the PAMPS as hydrogen bond donor and C=O group of the PAAm as hydrogen acceptor. These findings contribute to clarify the molecular origin and interactions responsible for the exceptional mechanical properties of the DN hydrogels.

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LIPIDOMICS IN CARDIOVASCULAR DIAGNOSIS AND ENVIRONMENTAL RESEARCH USING IVDr NMR

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In terms of number of NMR applications, after chemistry-related topics, metabolomics became a very successful and highly popular NMR topic in the last 30 years. However, although it took a longer time to prove its potential, nowadays NMR lipidomics seems to have an even higher impact in medical research than NMR metabolomics. This fast evolution of the NMR lipidomics was made possible by introduction of carefully developed SOPs, and industry standard solutions for spectra processing, as well as publication of trustful papers demonstrating impressive reproducibility of data, including transferability from various instruments, laboratories and operators into the same statistical model.[1-4] We are applying NMR spectroscopy in metabolomics [5-7] and lipidomics [8-10] for studying complex organisms such as plants,[6,8] animals,[7] and humans[5,9,10] with the purpose of advancing scientific knowledge in food sciences,[6,8] environment,[10] and medicine.[5,9] We describe here some NMR lipidomics applications in medical diagnosis and environmental sciences. In order to achieve reliable NMR lipidomics results we are extensively employing Bruker IVDr hardware, software and SOPs.

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SYNERGISTIC INSIGHTS: LEVERAGING ORGANIC CHEMISTRY AND NMR SPECTROSCOPY FOR ENHANCED SPECTRAL INTERPRETATION – A PERSONAL PERSPECTIVE

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As an organic chemist, my journey with NMR has been one of discovery and revelation, where the principles of organic chemistry have provided crucial insights into spectral data, and conversely, NMR has unveiled the subtleties of molecular structures that textbooks often simplify. This personal perspective delves into the synergies that arise when these two disciplines converge, offering not only a deeper understanding of molecular interactions but also practical strategies for tackling challenging spectral interpretations.

One of the most striking examples of this synergy is the "pure substance problem", where the assumption of purity in a compound can lead to misleading conclusions in spectral analysis. In my experience with halogenated carvacrol derivatives, the existence of rare sp^2-sp^3 atropisomers presented a significant challenge. [1] The intricate dance between these molecular forms demanded a nuanced approach, blending organic chemistry principles with NMR data to unravel the underlying structure.

Similarly, the elucidation of a new functional group in organic chemistry—α-iminoamidines—highlighted the complexity of NMR interpretation. The dynamic nature of these compounds, particularly their isotopic exchange with the solvent, added a layer of difficulty to their analysis. Here, the interplay between organic chemistry and NMR was crucial in deciphering the structure, as conventional approaches were insufficient to capture the full picture.

Moreover, the freedom afforded to an organic chemist, as opposed to an inorganic chemist, is exemplified in the context of "burying the possible hydrogen bond to gold (Au^{III}···H-C)" deep into the published article. [2] This unique flexibility allows for a broader exploration of chemical space, where NMR spectroscopy becomes a key tool in navigating the intricate relationships within organic molecules.

Through these examples, I will illustrate how the convergence of organic chemistry and NMR spectroscopy has refined my analytical approach, ultimately leading to more accurate and insightful interpretations of NMR spectra. This synergistic interplay not only enhances our understanding of molecular structures but also might pave the way for future discoveries in both fields.

Acknowledgements. This work has been supported by the Ministry of Science, Technological Development and Innovations of the Republic of Serbia, contact No. 451-03-65/2024-03/200124.

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POSTERS

MOLECULAR MAGNETIC RESONANCE IMAGING OF PROSTATE CANCER USING 9.4T MRI AND PARAMAGNETIC NANOPARTICLES

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Prostate cancer (PC) is the most common and the second leading cause of cancer death (1). Currently, the gold standard for PC diagnosis is prostate-specific antigen (PSA) testing and digital rectal examination (2). Computed Tomography and Positron Emission Tomography (3) are used for PC diagnosis and staging, yet they are of limited value. Molecular MRI using targeted contrast agents may rectify limitations of these methods.

To improve the tumor contrast we have developed new core/shell $NaDyF_4/NaGdF_4$ nanoparticles changing both T_1 and T_2 relaxation times of surrounding water molecules and conjugated them with tumor specific antibodies.. We also investigated toxicity, biodistribution and clearance of the new contrast agent. The relaxation times (T_1 and T_2) of the nanoparticles with various core/shell sizes and concentrations were measured at 9.4T MRI and 3T MRI. We performed in vivo imaging using mouse animal model and 9.4T MRI system. We imaged nude mouse with the tumor before and after the injection of targeted and non-targeted contrast agents.

Our results show that the new contrast agents may allow earlier detection of cancer than standard T1- or T2-only contrast.

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COMPLEXATION OF NABUMETONE BY CUCURBIT[7]URIL IN AQUEOUS SOLUTION

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Nabumetone (NAB) is a non-steroidal anti-inflammatory prodrug with extremely low water solubility, which is the reason for its limited bioavailability. It is used to treat pain and inflammation associated with rheumatoid arthritis and osteoarthritis. [1] One of the methods used to improve the bioavailability and bioactivity of drugs is the encapsulation of drug molecules in macrocyclic molecular containers. Cucurbit[n]urils (CB [n], with n = 5, 6, 7, 8, 10 or 14) are a relatively young family of macrocycles consisting of glycoluril units bridged by methylene groups, forming a macrocycle with a hydrophobic cavity accessible through two identical portals. They have been shown to form host-guest complexes with a variety of organic and inorganic small molecule drugs, with encapsulation facilitated by hydrophobic effects within the cucurbituril cavity and further stabilized by hydrogen bonding or ion-dipole interactions with the cucurbituril portal. [2] CB[7] has received most of the attention of the CB[n] family due to its good water solubility.

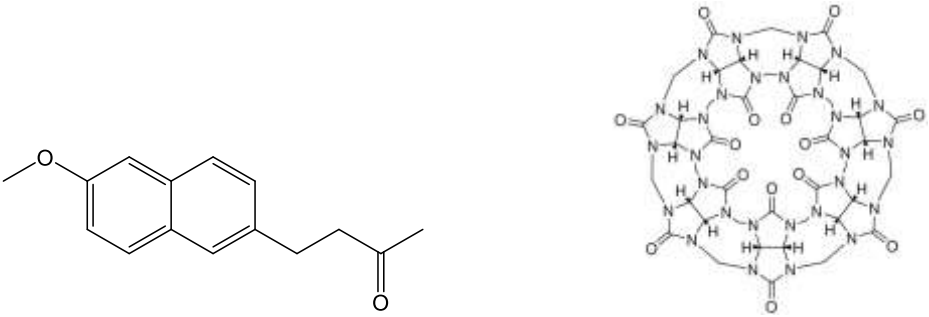

Figure 1. Chemical structures of NAB and cucurbit[7]uril

The complexation of NAB with cucurbit[7]uril was investigated by NMR spectroscopy and isothermal titration calorimetry (ITC) in water. One- (1H and 13C) and two (COSY, HSQC, HMBC, NOSY and ROESY) dimensional NMR spectra were recorded and analyzed. NMR studies confirmed that the complex is formed by the inclusion mechanism where NAB enters the CB[7]. The thermodynamic parameters were determined by ITC (log$K$, $\Delta_r H$, $\Delta_r S$, $\Delta_r G$). The evaluated thermodynamic profile indicates a strong and spontaneous interaction.

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NMR - BASED METABOLOMICS INTO WINE MATURATION AND GRAPE ALES DISTINCTION

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Wine is a popular alcoholic beverage and the earliest recorded medicine. Its composition varies depending on several factors, such as grape variety, climate, terrain, soil, and production methods. In Bulgaria, grape seeds dating back to 6000 BC have been found at Neolithic sites, and the Thracians, known for their "sweet wine of Thrace", lived in the region around 1000 BC. Bulgarian wine is renowned for its aroma, flavour, winemaking traditions, and high quality, with some of the highest antioxidant levels of any European wine. As wine ages, complex chemical reactions between sugars, acids, alcohols and phenolic compounds alter its flavour, often enhancing it.

Grape ales are hybrid drinks that combine beer brewing techniques with elements of winemaking. They typically involve the addition of grape juice, must or whole grapes during the beer fermentation process. This produces a beer with distinct wine-like flavours and aromas, introducing fruity, tannic, or acidic qualities not usually found in traditional beers.

The chemical profile of young and aged wine has been determined by means of ^{1}H-NMR spectroscopic analysis, with the aim of studying the ageing process that influences the taste and composition of this beverage. Additionally, the method was applied to grape ale – a relatively new style of beer, introduced in Italy in 2006. Quantification of the identified compounds was carried out, with further application of statistical methods in the differentiation of the grape products.

Acknowledgements. This work was supported by the Bulgarian National Science Fund, Project KP-06-M79/2. Research equipment purchased by Project No BG05M2OP001-1.002-0012 and part of the Distributed Research Infrastructure INFRAMAT, supported by the Ministry of Education and Science, were used in this investigation.

REMOTE NMR (R-NMR) - MOVING NMR INFRASTRUCTURES TO REMOTE ACCESS CAPABILITIES

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As part of the EU Remote-NMR (R-NMR) project, we want to create a common base that enables all users to perform NMR measurements remotely with an experience comparable to on-site access. This requires the development of standardised procedures for remote spectrometer access. R-NMR is a comprehensive network of European NMR infrastructures with the aim of establishing protocols for remote NMR use, including the dissemination of research protocols, sample shipping and CO_2 monitoring.

Hereby we will present the results of the R-NMR project.

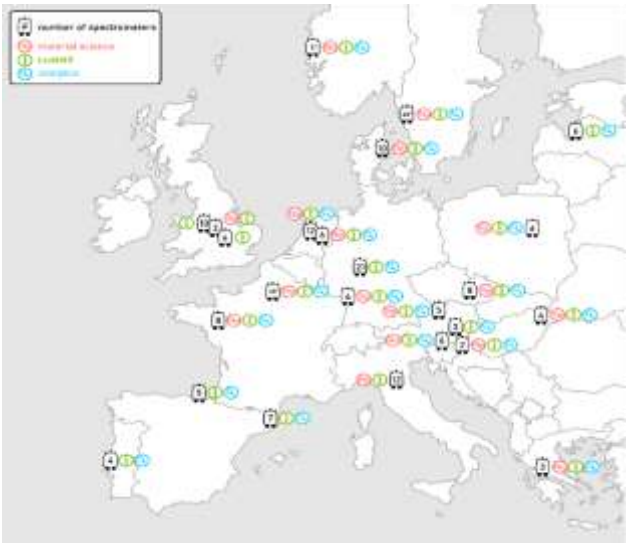

Figure 1. Members of the R-NMR consortium.

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AMINO ACID SUPPLEMENTATION PARTIALLY RESCUES THE GROWTH DEFECT OF TAZ1 DEFICIENT YEAST CELLS

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Barth syndrome is a rare recessive genetic disorder. It mostly affects mitochondria-rich organs like the heart and skeletal muscles.[1] However, there is a wide range of symptoms like cardiomyopathy, skeletal myopathy, hypertrophy, fibrosis, increased urinary excretion of 3 methylglutaconic acid, learning disabilities, attention deficit and so on. [2] The disorder is caused by a mutation in the tafazzin (TAZ) gene on the X chromosome. TAZ gene codes for the tafazzin enzyme that participates in a mechanism of cardiolipin (CL) maturation. [2] CL is a phospholipid located in the inner mitochondrial membrane. It provides membrane homeostasis and cell integrity. CL mutations make mitochondrial energy production less effective and shorter cell life. Since the physiology behind CL synthesis is not completely understood patients with Barth syndrome are treated symptomatically. However, with a better understanding of biochemistry and molecular mechanism connected to CL, new treatment methods could be considered. One of them is a high-protein diet. Proteins are macromolecules comprised of long chains of amino acids. Amino acids are building blocks for a variety of compounds. In some research amino acid supplementation improves cell growth. That is why we are interested in what effects supplementation of amino acids has on TAZ-deficient *S. cerevisiae* cells.

Acknowledgements. This work has been supported by Faculty of Biotechnology and Drug Development and University of Rijeka

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EXPLORING THE THERMOLYSIS OF 2,5-DIAZIDO-3D,6-DI-T-BUTYL-1,4-QUINONE: KINETIC AND MECHANISTINC INVSTIGATIONS BY MEANS OF NMR SPECTROSCOPY

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The thermal cleavage of 2,5-diazido-3,6-di-tert-butyl-1,4-benzoquinone (DADtBuBQ) leading to tert-butylcyanoketene (TBCK), published in 1970 by Moore and Weyler, opened a fertile research domain: the chemistry of cyanoketenes.[1,2] DADtBuBQ can be converted to 2-cyano-4-azido-2,5-di-tert-butyl-1,3-cyclopentadienone through photolysis with 3600-Å light. Cyclopentadienone, on thermal decomposition in refluxing benzene quantitatively yields TBCK. According to the authors two pathways would be possible: either a concerted ring contraction to the ketene dimer which then dissociates to a ketene or an electrocyclic ring opening to a zwitterionic intermediate cleaving to the cumulene.[3]

The aim of our study was to investigate the kinetics of TBCK formation *via* azide cleavage starting from DADtBuBQ but this time observing the changes in the molecule's structure revealed by solution and solid-state NMR spectrometry. Kinetic experiments have been done at 50, 55, 60 and 65 °C in C_6D_6 in the NMR tube, recording spectra every 15 minutes at the first 2 temperatures and every 5 minutes for the last two at the start of the experiments. According to our results (Figure 1), the formation of TBCK from the DADtBuBQ starting material undergoes through a TBCK dimer.

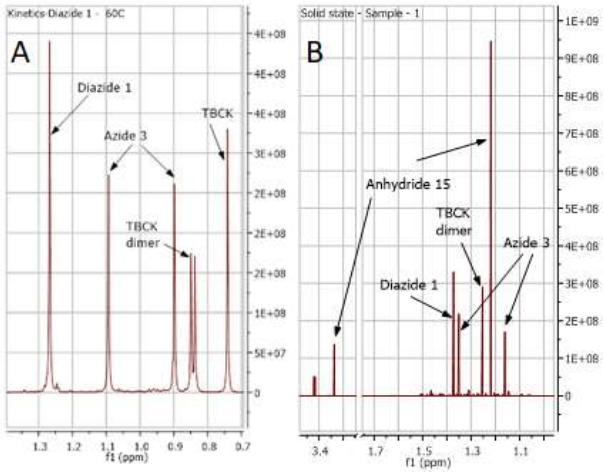

Figure 1. (A) Solution and (B) solid-state TBCK thermolysis at 60 °C after 45 minutes.

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SYNTHESIS AND CHARACTERIZATION OF A 9a-MACROZONE DERIVED FROM 4-AMINOBENZOIC ACID AND SALICYLALDEHYDE DERIVATIVES

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The overuse of antibiotics has led to bacterial resistance to antibiotics and the development of new antibiotics is urgently needed. One of the most commonly used antibiotics is azithromycin, an antibiotic that belongs to the macrolide class. Macrozones are compounds formed by the reaction of macrolides, i.e. azithromycin, and thiosemicarbazones.[1,2] Thiosemicarbazones can have antibacterial and anti-inflammatory effects, and their complexes with transition metals such as copper or nickel further enhance biological activity.[3] The aim of this work is to prepare and characterize the macrozones shown in Figure 1. Thiosemicarbazones derived from 4-aminobenzoic acid and salicylaldehyde derivatives were prepared. Subsequently, the corresponding macrozones were prepared by the reaction of the 9a-aminopropyl derivative of azithromycin and previously prepared thiosemicarbazones. Finally, macrozone complexes with nickel(II) and copper(II) were prepared. The compounds synthesized in each step were characterized by NMR spectroscopy and their biological activities were evaluated.

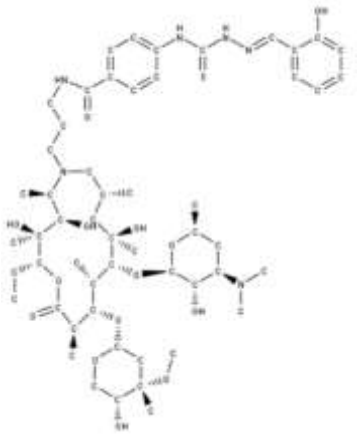

Figure 1. Structural formula of the 9a-macrozone derived from 4-aminobenzoic acid and salicylaldehyde.

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THE DETERMINATION OF *CIS* AND *TRANS* CONFORMER RATIO OF α-ACYLAMINO BENZAMIDE IN DIFFERENT ORGANIC SOLVENTS

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Central nervous system diseases, such as Alzheimer's disease (AD) and Parkinson's disease, are among the most significant healthcare challenges of the 21st century. Treating AD primarily involves increasing the levels of the neurotransmitter acetylcholine (ACh) by inhibiting the enzymes acetylcholinesterase and butyrylcholinesterase, which hydrolyze ACh.[1] Through the Ugi multicomponent reaction,[2] α-acylamino amides have been developed as a new class of cholinesterase inhibitors. The ^{1}H and ^{13}C NMR spectra of various α-acylamino amides show double signals, indicating the presence of *cis* and *trans* conformers.[3] It was found that the ratio of *cis* and *trans* conformers is different in polar and non-polar solvents.[4]

In this work, the synthesis of the *N*-isobutyl-*N*-[(cyclohexylaminocarbonyl)methyl]benzamide was performed through the Ugi reaction. The product was characterized by standard analytical methods (FTIR, 1D and 2D NMR, HRMS). In the ^{1}H and ^{13}C NMR spectra double signals were observed, which indicated the presence of *cis* and *trans* conformers. The ratio of *cis* and *trans* conformers was calculated[5] and found to be similar when determined by ^{1}H NMR in polar aprotic solvents (acetone 44 % : 56 %, acetonitrile 46 % : 54 %, dimethyl sulfoxide 48 % : 52 %) as compared to the ratio in a polar protic solvent like methanol (48 % : 52 %). However, in chloroform as non-polar solvent, the ratio was significantly different (19 % : 81 %).

Acknowledgements. This work was financially supported by the Croatian Science Foundation as part of the project Target-guided synthesis of cholinesterase inhibitors supported by machine learning (IP-2022-10-9525).

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QUANTITATION IN NMR METABOLOMICS

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Metabolomics is nowadays an important topic in a wide range of scientific areas, including medicine, pharmacology, nutrition and metabolism, food sciences and environmental research. Quantitation and reproducibility are key issues in metabolomics due to the fact that large numbers of data have to be included in the same statistical model. Ideally, data recorded with different instruments, by different operators and at different time intervals (covering several years) should produce highly reproducible data compatible with a single statistic model.

Recent studies shown impressive interlaboratory reproducibility of NMR spectra, but such studies involved trained personnel.[1-3] The purpose of the present study was to expand our preliminary study,[4] in order to evaluate the NMR reproducibility for metabolomics, when combining both industry-standard NMR solutions and multipurpose NMR equipment, and to compare the reliability of NMR metabolomics data when involving both dedicated NMR operators (including researchers and technicians) and chemistry users from outside the NMR group (including researchers and students). Thus, we report on an expanded dataset of samples and operators and we are evaluating results from experiments performed over intervals longer than two years. Both old and new NMR datasets have been reprocessed with the recently introduced Bruker IVDr Biobanking QC Package (B.I.BioBank Tool 1.0), in addition to our previously introduced reproducibility tests.[4] The results shown a 4% variability for most of the operators, including users which are newcomers to metabolomics/analytical chemistry. This variability matches the specifications of the IVDr NMR equipment producer and is lower than the nature-induced variability for most of the metabolomics studies.

Acknowledgements. This study was supported by a grant of the Ministry of Research, Innovation and Digitization, CNCS-UEFISCDI, contract no. 30ROMD/2024, project PN-IV-P8-8.3-ROMD-2023-0249 (DiMoMeD).

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A MAGNESIUM SUPPLEMENT AND ITS INTEREFERENCE WITH DIAGNOSIS OF SOME METABOLIC DISORDERS

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Magnesium is important in maintaining the healthy state of the organism as it contributes to keeping the heart rhythm and blood pressure in normal ranges as well as maintaining the bones strong. Recommended magnesium doses for adults are ranging between 300-450 mg/day.[1] There are currently several magnesium products available as food supplements or over-the-counter pharmaceuticals. These products include either magnesium alone as metallic ion, or in combination with other ions like calcium and zinc. For human use, magnesium is conditioned either as inorganic salts (e.g. oxide, chloride, hydroxide, carbonate) or organic salts (e.g. citrate, orotate, lactate, aspartate) the organic ligands presumably enhancing the body absorption. We describe a study on several healthy volunteers subjected to various doses of a commercial magnesium orotate supplement. We have monitored by urine NMR metabolomics the body clearance of the orotate ligand. The study is significant for possible false positive diagnosis of several inborn errors of metabolism associated with hyperammonemia.[2] Thus, we have previously diagnosed by NMR a severe argininosuccinic aciduria case with high orotate excretion in urine as one of the significant markers,[3] and we have also described several amonemia episodes in methylmalonic aciduria patients.[4] In conclusion, with an increased number of organic ligands used in metallic ions supplements, unexpected interferences with diagnosis of some metabolic diseases may occur.

Acknowledgements. This study was supported by a grant of the Ministry of Research, Innovation and Digitization, CNCS-UEFISCDI, contract no. 30ROMD/2024, project PN-IV-P8-8.3-ROMD-2023-0249 (DiMoMeD).

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NMR STRUCTURAL ELUCIDATION OF DESULFOGLUCOSINOLATES

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Glucosinolates (GSLs) are specialized metabolites that are biosynthesized from amino acids. [1] Only ninetyone GSLs have been conclusively demonstrated to occur in nature out of 140 known distinct GSLs produced by Brassicales species. [1] This means that large number of GSLs still require extensive spectroscopic characterization, including NMR, MS and satisfactory interpretation. The final confirmation of a GSL structure is usually based on the NMR of GSLs or desufloGSLs. While the isolation of GSLs can be more demanding than the isolation of desulfoGSLs, their NMR analysis is equivalent. [2] Insight into the qualitative and quantitative analysis of plants containing GSLs allows the selection of the species as the sources of GSLs for their isolation in the form of desulfoGSLs and confirmation by spectroscopic techniques. In this study, a collection of 5 desulfated GSLs with different side chains, has been studied by ^{1}H, ^{13}C, COSY and HSQC NMR with D_2O used as the solvent. 3-(Methylsulfinyl)propyl desulfoGSL was isolated from *Anastatica hierochuntica* (Brassicaceae), 4-hydroxybenzyl desulfoGSL from *Sinapis alba* (Brassicaceae), methyl desufloGSL from *Capparis spinosa* subsp. *rupestris* (Capparaceae), 2-hydroxy-2-methylpropyl desulfoGSL (Figure 1) from *Reseda alba* (Resedaceae) and 4-(α-L-ramnopyranosyloxy)benzyl desulfoGSL from *Moringa olefiera* (Moringaceae). The accurate interpretation of the NMR spectra enabled the determination of the nature of side chains and sulfation states. Additionally, it was noticed that the order of ^{1}H and ^{13}C NMR shift values in the D-glucopyranosyl unit was subjected to change, depending on the nature of the side chain.

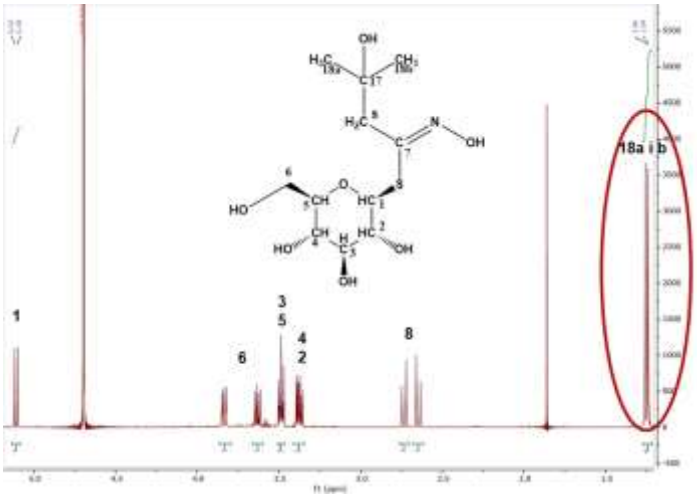

Figure 1. ^{1}H NMR of 2-hydroxy-2-methylpropyl desulfoglucosinolate.

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KRAS CATALYTIC INTERMEDIATES VIA A NEW NMR-DRIVEN PROTOCOL

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The catalytically significant states of the oncogenic G12C variant of KRAS, those of Mg^{2+}-free and Mg^{2+}-bound GDP-loaded forms, have been determined using CS-Rosetta software and NMR-data-driven molecular dynamics simulations. Due to the high flexibility of the Switch-I and Switch-II regions of KRAS, which also happen to be the catalytically most significant segments, only chemical shift information could be collected for the most important regions of both systems. CS-Rosetta was used to derive an "NMR ensemble" based on the measured chemical shifts, which, however, did not contain the nonprotein components of the complex. We developed a torsional restraint set for backbone torsions based on the CS-Rosetta ensembles for MD simulations, overriding the force-field-based parametrization in the presence of the reinserted cofactors. This protocol resulted in complete models for both systems that also retained the structural features and heterogeneity defined by the measured chemical shifts.

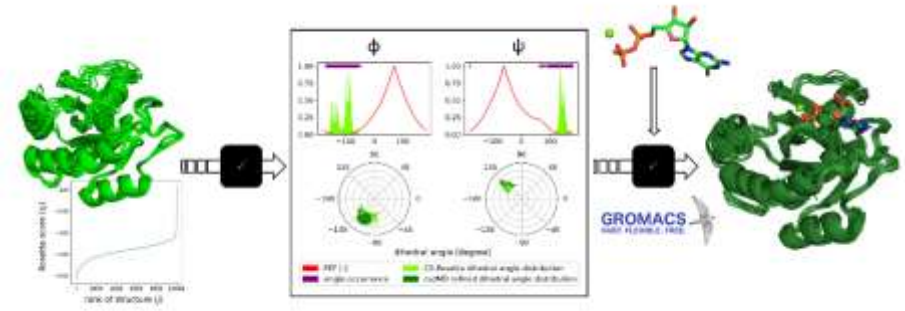

Figure 1. The workflow of our NMR-data-driven MD simulations, which can be used as a structure refinement method. We developed a torsional restraint set for backbone torsions based on the CS-Rosetta ensembles for MD simulations, overriding the force-field-based parametrization in the presence of the reinserted cofactors.

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NMR CHARACTERISTICS, CRYSTAL STRUCTURE, ANTIMICROBIAL PROPERTIES AND DUPLEX DNA STUDIES FOR THE MACROCYCLIC Co(III) COMPLEX OBTAINED BY TEMPLATE SYNTHESIS

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Polyazamacrocyclic compounds are known for important bio-applications. We report here on solution ^{1}H and ^{13}C NMR characterization, solid-state structure and study of some biological properties of a novel Co(III) coordination compound $[Co(HL)Cl_2]\cdot CH_3OH$ **1**. It was obtained *via* condensation between 2,6-pyridinedicarbohydrazide and 2,6-diacetylpyridine, using the Co^{2+} cation as a template. SCXRD study of **1** shows that the hexacoordinated Co(III) atom is located at the center of the planar macrocyclic ligand, forming coordination bonds with four of its nitrogen atoms and two trans-located Cl^- anions. Compound **1** has demonstrated selective bactericidal activity against Gram-positive bacteria, with highest activity against *Bacillus cereus* ATCC 11778 (MIC: 62,5 µg/mL; MBC: 125,0 µg/mL). Binding activity of **1** to duplex DNA (Drew-Dickerson Dodecamer- (DDD)) was studied by Circular Dichroism (CD) and ^{1}H NMR titrations. CD data have proved that compound perturbs the secondary structure of DDD, while having a minimal impact on base pair stacking interactions. Accordingly, broadening of the signals for imino- protons of DDD homodimer at constant resonance frequency in ^{1}H NMR spectra argue on weak nonspecific interactions between the major DNA conformer and **1.**

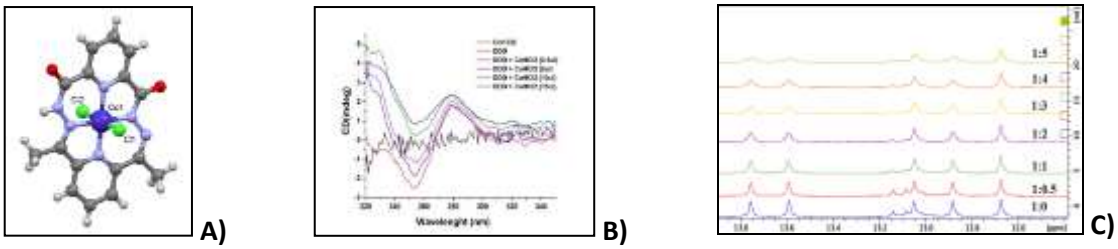

Figure 1. Bioactive macrocyclic Co(III) complex: A) view of the molecular structure of **1**; B) CD titration spectra of DDD with varying amounts of **1** in 100 mM KPi (pH 7.4) and KCl; C) Imino-region of the ^{1}H NMR titration spectra of DDD in 10 mM KPi (pH 7.0), 10 % D_2O (25 °C, 600 MHz). Ratio DDD-**1** is shown at the right side of the spectra.

Acknowledgements. E.G. thanks the CERIC for the access to facilities of Slovenian NMR Centre for NMR studies on DNA; V.L., I.B., V.K., P.B. thank the subprograms 010602 and 011202.

1D AND 2D NMR ANALYSIS OF NOVEL ASYMMETRIC *N*,*N*-DIETHYLAMINOPHENYL PORPHYRINS

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Porphyrins are the best-known members of the tetrapyrrolic family, with favorable properties for a variety of applications from medicine to materials chemistry.[1] Their most studied role is that of a photosensitizer (PS) for photodynamic therapy, due to their high stability, good optical properties and, above all, their structural versatility, which makes it possible to modify the porphyrin structure to obtain better physicochemical properties.[2]

In addition, asymmetric porphyrins have been shown to be more effective than symmetric porphyrins, as they pass through membranes better and cellular uptake.[3] However, 4-diethylaminophenylporphyrin and its asymmetric analogs (**Figure 1.**) in combination with 4-acetamidophenyl substituents have not yet been investigated as PSs for PDT.

Herein, a design and synthesis of novel asymmetric porphyrins following the mixed Alder-Longo condensation reaction with 4-diethylaminobenzaldehyde and 4-acetamidobenzaldehyde as aldehydes with pyrrole in refluxing propionic acid is presented. Four successfully isolated porphyrins were analyzed using 1D NMR techniques such as ^{1}H and ^{13}C NMR as well as COSY and HSQC for 2D NMR analysis.

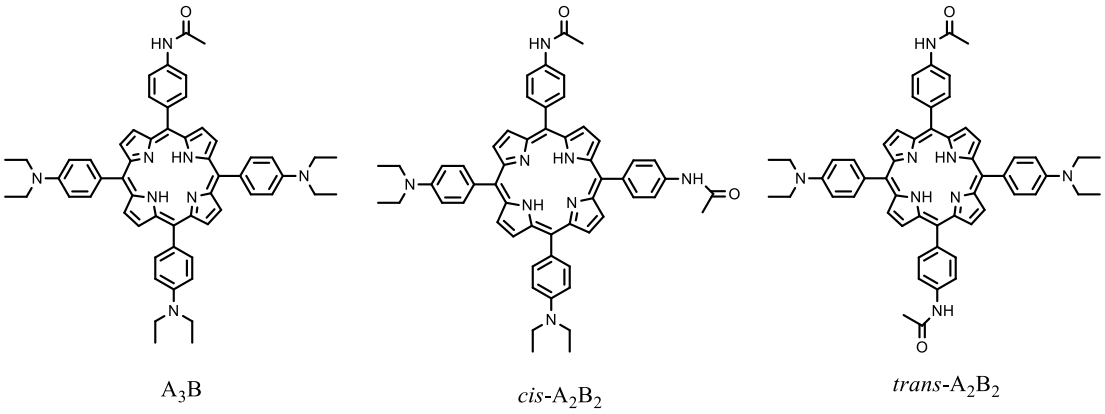

Figure 1. The structures of the analyzed novel asymmetric *N*,*N*-diethylaminophenylporphyrins.

Acknowledgements. This work has been supported by the University of Rijeka's grant assigned to Dr. Nela Malatesti (uniri-prirod-18–173 and uniri-iskusni-23-2).

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HESPERIDIN IN CROATIAN CITRUS FRUITS BY 1D AND 2D NMR

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Hesperidin, a secondary metabolite from citrus fruits, is known for its antiviral, anti-inflammatory and antioxidative properties, preventing cancer and cardiovascular diseases [1]. Most importantly in contemporary context it is also a potential inhibitor against SARS-CoV-2 infection [2].

In this work we assess hesperidin content in citrus fruit juices from orange (Citrus sinensis) and tangerine (Citrus reticulata), commercially acquired and homemade juices and peel extract with 1D 1H and 2D NMR. We also outline a machine learning approach to unravel complex spectra.

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A NMR STUDY OF STEREOCHEMISTRY OF ACETYLHYDRAZINE PYRIDAZINE DERIVATIVES

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Pyridazine derivatives, are widely discussed scaffolds 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 the above considerations, we decide to synthetize a new class of bis-pyridazine derivatives with acetylhydrazine skeleton. The synthesis involve an initially N-alkylation of nitrogen atom from pyridazine derivatives with 2–bromoalkyl esters, followed by a subsequent N-acylation to hydrazine moiety, when the desired acetylhydrazines pyridazinones are obtained [4]. The stereochemistry of the acetylhydrazines pyridazinones was studied using the NMR exeperiments (1H, 13C, 2D HMBC) at room temperature, and reveal a conformational equilibrium: *Z-sc* (around 90%) and *E-ac* (around10%) conformers. The NoeDiff 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 1H 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.

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PYRIDINE-IMIDAZOLIUM SALTS AND YLIDES: A NMR STUDY OF ZN-ACETATE COMPLEXATION

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Pyridine, imidazole derivatives and imidazolium salts in particular, are described as compounds with a largy variety of biological properties such us antibacterial, antifungal, antiplasmodial and antimalarial, anthelmintic, antitubercular, antiviral, anticancer, anti-inflammatory, antihypertensive, diuretics, antithrombics, anti-coagulants, antidepressant, anxiolytics, anti-Alzheimer's, anticonvulsant, analgesic, etc. [1-3]. On the other hand, chemosensors with imidazolium skeleton and anthracene fluorophores have a great interest in host–guest chemistry, due to their biological and environmental significance [4,5]. Having in view these considerations we synthesized two different classes of compounds having imidazol units: bis-imidazolium pyridine (A) and anthracene-imidazolium (B) salts. The syntheses were done in two steps only: N-alkylation (step I) and quaternization reactions (step II). The structures of new salts were proved by NMR experiments (1H-, 13C- NMR, 2D: COSY, HMQC, HMBC). The studies of complexation with zinc acetate were done using NMR measurements.

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NMR INSIGHTS INTO THE STRUCTURAL FEATURES OF NEW AZO-BRIDGED POROUS ORGANIC POLYMERS

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Azo-bridged porous organic polymers (azo-POPs) are very promising candidates for the design of new functional materials for efficient CO_2 adsorption. Recent studies revealed that the incorporation of azo bonds into the POP structure can increase the polymer's affinity for CO_2 and selectivity for CO_2 over N_2, even at high temperatures.[1,2] Therefore, it is necessary to investigate how different synthesis methods and starting building units influence the final porosity parameters of azo-POPs and their capacity to bind CO_2.

Herein, a series of new azo-POPs with various central units were synthesized by three different synthetic methods: reduction of aromatic nitro monomers, oxidation of aromatic amino monomers and condensation reactions between aromatic nitro and amino monomers under basic conditions.[3,4] Powder X-ray diffraction showed that the obtained polymers are amorphous solids, while thermogravimetric analysis revealed their good thermal stability. The formation of azo bonds in the synthesized azo-POPs, which are insoluble in common organic solvents, was confirmed by ^{13}C CP/MAS NMR spectroscopy and additionally supported by IR spectroscopy. Porosity parameters and CO_2 binding capacity of the azo-POPs were determined from recorded N_2 adsorption-desorption isotherms at 77 K and thermogravimetric CO_2 sorption analysis, respectively. The results indicated that several azo-POPs have high Brunauer-Emmett-Teller (BET) surface areas (up to 608 $m^2\ g^{-1}$) and good CO_2 binding capacity (up to 42 mg g^{-1} at 306 K), which makes them good candidates for the efficient CO_2 adsorption. Periodic DFT calculations and GCMC simulations provided additional insight into the effect of different central units on the geometrical and adsorption properties of the selected model azo-POPs.

Acknowledgements. This work has been fully supported by Croatian Science Foundation under the project IP-2020-02-4467.

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SYNTHESIS AND 1H NMR-NOESY ANALYSIS OF DIASTEREOMERIC SIGMA 1 RECEPTOR ANTAGONISTS

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Sigma (σ) receptors are transmembrane proteins involved in a large number of cellular functions.[1] They are divided into σ1 and σ2 receptor subtypes. σ1 Receptors (σ1R) work as molecular chaperones in the mitochondria-associated endoplasmic reticulum membrane and their role is related to cellular stress response and cellular homeostasis.[2] They are involved in different addiction pathologies (to alcohol, psychostimulants), as well as other disorders (Alzheimer's disease).[2] This makes σ1Rs promising targets for the investigation of neurological disorders. In this research, we will focus on the synthesis of novel ligands with high σ1R affinity, the purification and the isolation of diastereomers synthesised, and the stereochemical analysis of such compounds using NOESY studies. A spipethiane analogue **1** (containing 1,3-benzodioxane nucleus) was used as a starting molecule for the synthesis of diastereomers **2** and **3**, due to its high σ1/σ2 selectivity of 2515. Diastereomers **4** and **5** were produced as side-products in the synthesis of **2** and **3** (Figure 1), and were separated by flash column chromatography. The stereochemical relationship between the N-benzylpiper-idine moiety and the phenyl substituent of *cis* and *trans* diastereomers of **4** and **5** was determined by 1H NMR analysis (NOESY studies). In case of **4b**, nuclear Overhauser effect (NOE) for mentioned substituents in positions 2 and 4 was observed at 4.48 and 4.65 ppm, suggesting that both are equatorially-placed and that **4b** adopted *cis* orientation. Similarly, for **5b**, a NOE was observed between the axial protons in positions 2 and 4 at 4.36 and 3.78 ppm, suggesting the equatorial positioning of the N-benzylpiperidine moiety. Hence, **5b** is in *cis* orientation as well.

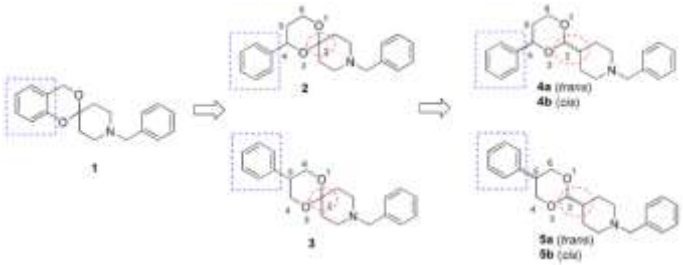

Figure 1. Synthesised ligands **2**-**5**, starting from spipethiane analogue 1.

Acknowledgements. This work has been realised under the supervision of professors Fabio Del Bello and Wilma Quaglia, from the University of Camerino, Camerino, Italy; from January 2020 to September 2023.

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DFT CALCULATION AND NMR SPECTROSCOPY OF AN OXAZOLINE AMINO ACID BIOCONJUGATE: SUPRAMOLECULAR DIMERS VS. MONOMERS

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In this presentation, an organic molecule consisting of two L-alanine amino-acids and one oxazoline bonded to the central aromatic ring was studied, Figure 1. In particular, two sets of calculated structural models were tested against experimental ^{1}H and ^{13}C liquid-NMR data using a modified protocol of Willoughby et al.[1,2] The protocol contains four main steps: (1) generation of the two sets of thermally populated structural models in solution (dimers and monomers), (2) DFT-optimization of structural models in each set, (3) determination of NMR parameters for each conformer in each set by GIAO computation and (4) appropriate Boltzmann averaging of data and correlation between computed and experimental parameters. The original procedure was modified by the usage of programs developed by Grimme and coworkers;[3] namely, a CREST program[4] is used for step (1), a CENSO protocol[5] for steps (2) and (3), including ORCA-5.0.2 as the basic DFT-program. The parameter MAE (Mean Absolute Error)[2] is calculated for each set of structural models, i.e. for dimers and for monomers, Figure 1. It was found that the MAE value for an ensemble of 41 dimers better fits to the ^{1}H and ^{13}C experimental NMR data in comparison to the MAE value for an ensemble of 47 monomers; the accuracy is better at lower temperatures.

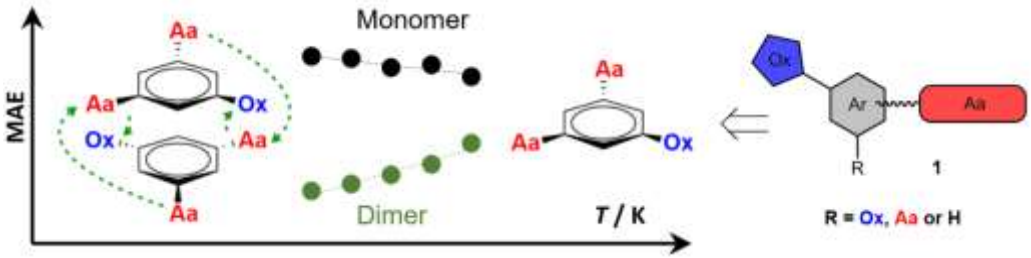

Figure 1. Comparison of MAE for dimers (green) vs. monomers (black) at different temperatures.

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INVESTIGATION OF THE -ASNGLY- ISOMERIZATION REACTION IN TETRAPEPTIDES BY NMR SPECTROSCOPY AND *AB INITIO* CALCULATIONS

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During isomerization (deamidation and hydrolysis) of peptides and proteins containing the -AsnGly- sequence, the side chain of the asparagine is converted to either α-aspartic acid or β-aspartic acid derivative.[1] The aim of our research is to better understand the spontaneous isomerization reaction of -AsnGly- containing peptides (Ac-NGXA-NH_2 (X=$E^{(-)}$: glutamate - sidechain negatively charged, $R^{(+)}$: arginine and $K^{(+)}$: lysine - sidechain positively charged, A: alanine)).

The isomerization reaction was monitored by time-dependent 1D ^{1}H-NMR experiments at different temperatures and at different pH values in sodium phosphate buffer. From the NMR experiments, the reaction rate coefficients (k_1) and half-lifes ($t_{1/2}$) of the isomerization were determined for the rate-determining step (deamidation). Furthermore, a kinetic model for the isomerisation was established.

The rate-determining step was also investigated by IRC path and NBO calculations at B3LYP/6-31++G(d,p) level of theory in vacuum. Frequency calculation was completed and Δ*G* values were determined at each point of the IRC paths. Endpoints of the IRC paths and transition states were further optimized at DFT B3LYP/6-31++G(d,p) level of theory in vacuum and using IEFPCM water model.

Acknowledgements. This work has been supported by the Ministry for Innovation and Technology from the Hungarian NRDI Fund (2020 1.1.6 JÖVŐ 2021 00010). The authors thank to Gábor Mező for the oligopeptide synthesis. This work was completed in the ELTE Thematic Excellence Programme (Szint+) supported by the Hungarian Ministry for Innovation and Technology. ELTE Institutional Excellence Program (1783-3/2018/FEKUTSTRAT) supported by the Hungarian Ministry of Human Capacities and grants from the European Union and the State of Hungary, co-financed by the European Regional Development Fund (VEKOP-2.3.3-15-2017-00020, VEKOP-2.3.2-16-2017-00014). Project no. 2018-1.2.1-NKP-2018-00005 has been implemented with the support provided from the National Research, Development and Innovation Fund of Hungary, financed under the 2018-1.2.1-NKP funding scheme. Project number RRF-2.3.1-21-2022-00015 is implemented with the support of the European Union's Recovery and Resilience Instrument.

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CUCURBITURILS VS. CYCLODEXTRINS; THE HYDRATION AND TEMPERATURE INFLUENCE ON THE INCLUSION THERMODYNAMICS

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The supramolecular recognition of non-polar guest by cyclodextrins and cucurbiturils has received much attention over the years.[1,2] Contrary to the endothermic micellization (classical hydrophobic effect), the complexation with both receptor classes is often exothermic, even completely enthalpy driven (non-classical hydrophobic effect). [1,2] This thermodynamic blueprint was, in the case of classically hydrated guests ($\Delta_{hyd}H° < 0$), rationalized by the dehydration of receptor cavity containing less associated water compared to the solvent bulk.[1,2] However, most complexation reactions were investigated at 25 °C. Since the hydration enthalpies of non-polar compounds exhibit a particularly strong temperature dependence one would expect notable temperature dependence of their complexation enthalpies and entropies with mentioned macrocycles. Apart from that, the comparative calorimetric studies of cyclodextrin and cucurbituril binding affinities are still rare, and the influence of guest dehydration on the complexation thermodynamics for larger non-polars remains largely unknown.

With this in mind, we examined the complexation of linear and (poly)cyclic aliphatic guest with size-compatible cyclodextrins and cucurbiturils by means of ITC and NMR over a wide temperature range (278-338 K). A pronounced $\Delta_rH°(T)$ and $\Delta_rS°(T)$ dependence due to temperature-induced disordering of the guest-hydrating water was observed for all studied pairs.[3,4] This gradual "melting" of hydration spheres significantly affected the thermodynamics governing the cyclodextrin complexation reactions, however the binding with cucurbiturils remained predominantly enthalpy driven irrespectively of temperature.[3,4] The comparative calorimetric studies also revealed that the dehydration of cucurbituril cavities is accompanied with lower entropy changes than the analogous process involving cyclodextrins.[3,4] In addition, the endothermic binding of larger diamondoid guest with γ-cyclodextrin indicated their classical hydration at lower temperatures.[4]

Acknowledgements. This work has been supported by Croatian Science Foundation (IP-2019-04-9560 and UIP-2017-05-9653)

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THE ROLE OF GLUCOSE MOIETIES IN ALKALI METAL CATION COMPLEXATION BY LOWER-RIM TERTIARY-AMIDE CALIX[4]ARENES

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Calixarenes are probably the most widely used macrocyclic scaffolds for a range of ion receptors and binders of neutral species. These macrocyclic ligands can be relatively easily functionalized at both upper and lower rim, which allows the preparation of ligands selective for most diverse classes of guests in a range of media, but very rarely in water. To overcome this obstacle we have recently introduced a new class of water-soluble glycocalixarenes designed for efficient hosting of first-group cations. (Figure 1.)[1] In this work the role of triazole and glucose functionalities in the coordination reactions is explored by studying the binding of alkali metal cations with tertiary-amide lower-rim calix[4]arenes (Figure 1.) in methanol, *N,N*-dimethylformamide, and acetonitrile.[2]

The obtained results reveal that all studied reactions are enthalpically controlled and the peak affinity of receptors for sodium cation is observed. The complex stabilities are the highest in acetonitrile, followed by methanol and *N,N*-dimethylformamide. The solvation of the reactants and products of the complexation reaction plays a a very important role in the binding process, especially the inclusion of solvent molecules in the calixarene hydrophobic cone. Importantly, the ligand solubilities are greatly affected by the presence of sugar subunits which hardly influence the calixarene receptor properties.

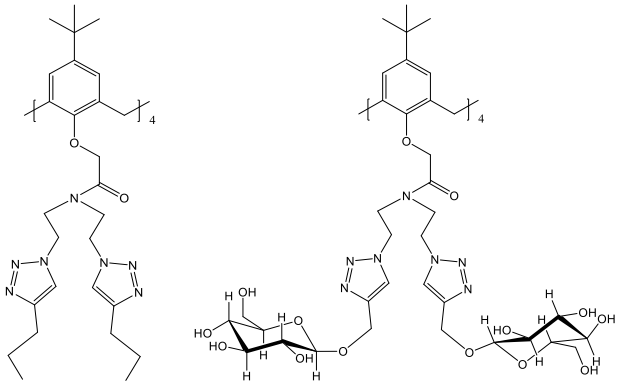

Figure 1. Structure of investigated calixarenes.

Acknowledgements. This work has been supported by Croatian Science Foundation (IP-2019-04-9560)

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SYNTHESIS AND NMR CHARACTERIZATION OF MORPHOLINE-BASED UGI PRODUCTS

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Multicomponent reactions like Ugi four-component reaction offer significant advantages over traditional multi-step reactions, primarily due to their ability to synthesize complex organic molecules at lower costs and with faster reaction times. Ugi four-component reaction is a single-pot synthetic method that involves the condensation of an amine, an aldehyde or ketone, an isocyanide and a carboxylic acid to produce a wide range of peptidomimetic compounds.[1] Due to their structural diversity and potential biological activity, these products serve as valuable intermediates in pharmaceutical research and drug discovery. [2, 3]

In this work, Ugi four-component reaction was used for the synthesis of the selected compounds where the carboxyl component was varied whilst paraformaldehyde, benzylamine and 4-(2-isocyanoethyl) morpholine were selected as other necessary components for Ugi's reaction. The products were characterized by FTIR, 1D and 2D NMR, HRMS. The prepared compounds can exist as a mixture of conformers because they contain amide bonds around which rotation is possible that is slow enough so that this effect is observed in the NMR spectra. ^{1}H NMR spectra of prepared compounds show duplicated signals, as well as ^{13}C NMR spectra, which indicated the presence of *cis* and *trans* conformers. The ratio of *cis* to *trans* conformers was determined[4] using ^{1}H NMR in different solvents like a polar aprotic solvent (dimethyl sulfoxide), polar protic solvent (methanol) or non-polar solvent (chloroform).

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COPPER(II) COMPLEXES OF SCHIFF BASES DERIVED FROM SALICYLALDEHYDE AND 8-AMINOQUINOLINE: SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL ACTIVITY

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The reaction between copper(II) acetate and reduced Schiff base ligands derived from salicylaldehyde and its 5-chloro- or 5-bromo-substituted derivatives and 8-aminoquinoline was investigated. The reaction resulted in oxidative dehydrogenation of secondary amines and coordination of the corresponding Schiff base ligands to copper(II) ions, leading to the formation of three new copper(II) complexes. All complexes were characterized by CHN analysis, IR, EPR and NMR spectroscopy, conductometry and single crystal X-ray diffraction. A detailed characterization of the secondary amines and the corresponding Schiff bases was performed using several different 1D and 2D NMR experiments. Based on the experimental geometries, quantum mechanical calculations of the complexes were performed at different DFT levels of theory. The complexes and ligands showed promising antibacterial and antifungal activity. [1,2]

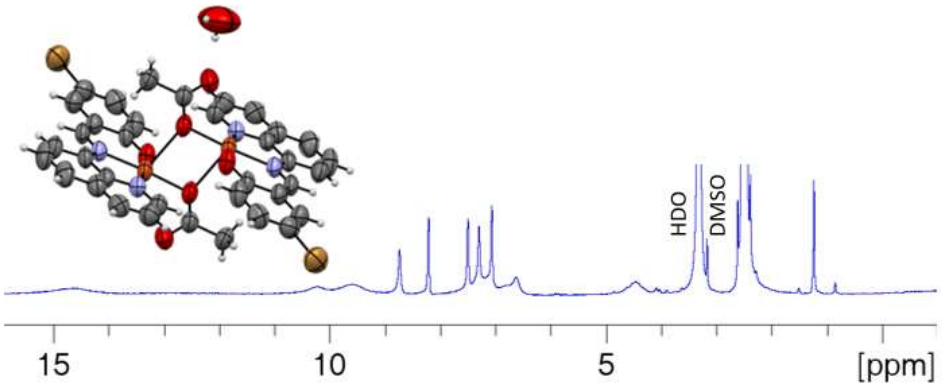

Figure 1. Crystal structure and ^{1}H NMR spectrum (600 MHz) of $[CuL(OCOCH_3)]_2$ x H_2O.

Acknowledgements. This work has been supported by the Ministry of Science, Higher Education and Youth of the Canton of Sarajevo through GLUKOVAN project no. 1432 (Adnan Zahirović).

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SOME NMR AT >225 KHZ MAS

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Faster spinning offers several new perspectives for NMR. While evolution of H-MAS applications in structural biology is well established, domain of other, in particular quadrupole nuclei, is much less explored. Nuclei with spins of 5/2 (like 17O) and 9/2 are of particular interest due to smaller powder spread of the second order quadrupolar interaction on satellite transitions as compared to the central transition. We show how increased spinning rates fight the first order quadrupolar interaction and allow to concentrate more intensity in the spinning centerband, also how fine control of magic angle will affect the spectra.

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ISOLATION AND CHARACTERIZATION OF 2-HYDROXYPHENYL THIOSEMICARBAZONE 4"- MACROZONE DERIVATIVES USING HPLC-SPE-NMR

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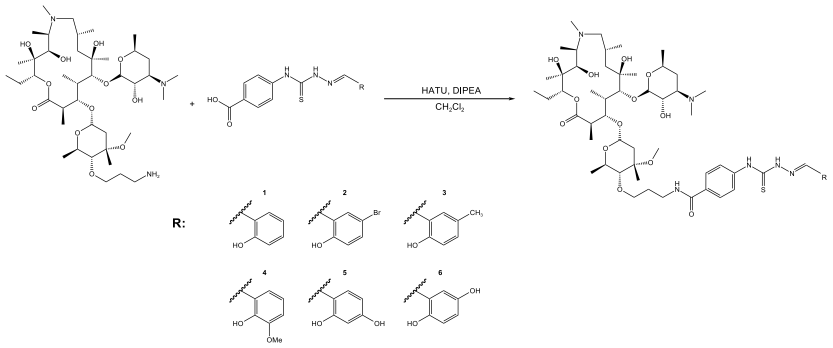
Macrozones are novel compounds prepared by conjugating azithromycin and thiosemicarbazones. Azithromycin, a 15-membered macrolide and a top-selling antibiotic, is favored for its excellent pharmacokinetics, including high cellular accumulation and stability. However, widespread misuse of antibiotics globally has led to the development of bacterial resistance to existing antibiotics, including macrolides, which now poses a significant threat to human health. Consequently, it is imperative to discover new macrolide scaffolds to restore and enhance their antibacterial efficacy. In this study, we successfully prepared 2-hydroxyphenyl thiosemicarbazone 4"- macrozone derivatives and analyzed the reaction mixtures components. A hyphenated HPLC-SPE/NMR system has become an indispensable tool for complex mixture characterization and was used here to analyze macrozone reactions outcomes. The 2-hydroxyphenyl thiosemicarbazone 4"- macrozone derivatives were prepared by coupling 4"-(*γ*-aminopropyl) azithromycin with six thiosemicarbazones (Scheme 1). HPLC was employed for separation of the reaction mixtures components, while post-column trapping of the selected compounds on SPE cartridges enhanced sensitivity for detecting analytes at low concentration. The results of NMR studies provide detailed insights into the molecular structure of the newly synthesized 4"- macrozone derivatives.



Scheme 1. The final step in the synthesis of 4"- macrozone derivatives.

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LIGAND BASED NMR SPECTROSCOPY FOR MONITORING INTERACTIONS OF SMALL MOLECULES AND PROTEINS

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Revealing interactions between small molecules and proteins are one of the key points of drug discovery in pharmaceutical research. [1] Nuclear magnetic resonance spectroscopy (NMR) represents one of the most powerful techniques for studying intermolecular interactions, providing many advantages over other methods, such as the ability to directly observe chemical compounds and target biomolecules. In particular, ligand-based NMR approaches, including NOE-based NMR techniques, diffusion experiments and relaxation methods, are excellent tools widely used nowadays in academia and industry. [2]

NMR provides an opportunity to determine ligand epitope mapping and ligand binding affinity with the target protein, which can reveal details about the mechanism of binding to particular proteins, metabolism, and toxicity in the body. NMR is also a suitable technique for the atomic level detection and identification of various substances, making it a very favorable technique for kinetic studies of enzyme activity. [3]

In this study, the ligand observed reverse ^{1}H NOE pumping experiment was used to identify ligand group epitope mapping for highly toxic compounds (organophosphorus and carbamate compounds) with bovine serum albumin (BSA). Beside this, *in situ* ^{1}H NMR experiment revealed binding kinetics of selected carbamate compounds and butyrylcholinesterase (BuChE).

The developed ^{1}H NOE pumping experiment provided the possibility to observe interactions using a single experiment without using reference spectra. It has been shown the advantage of the *in situ* ^{1}H NMR experiment as a confident method for monitoring the inhibition and reactivation of enzymes due to the possibility of following the reaction at the molecular level and having insight into all the steps that take place.

Acknowledgements. This work has been supported by Organisation for the Prohibition of Chemical Weapons (OPCW) Internship program, Project Nos. L/ICA/ICB-46/21 and L/ICA/ICB-307/23.

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APPLYING NMR TO MONITOR CHEMICAL SYSTEMS – A KINETIC AND MECHANISTIC APPROACH

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The unique ability of NMR to track individual atomic nuclei in different chemical environments makes it a very powerful tool for kinetic and mechanistic analysis. If the reaction is slow enough, various useful information for the characterization of reaction pathways and intermediers can be obtained. By tracking the changes in intensity of appropriate signals of reactants and products, important data (such as changes in concentration) can be acquired, that are then used for the determination of kinetic parameters of the reaction. This is ideal for determining mechanisms of organic reactions.[1] Figure 1 shows ^{1}H NMR spectra of the reaction of alanine methyl ester and allyl isothiocyanate, in which a 2-thiohydantoin derivative is formed. Using the obtained NMR data, a novel reaction mechanism was proposed, intermediates were characterized and kinetic parameters were determined, such as rate constants and reaction orders. This approach is not limited to organic reactions, as metal complexation reactions can be monitored as well, as long as there are changes in the chemical environments of the spin active nuclei in the ligand molecules, as well as the starting and obtained complexes.[2]

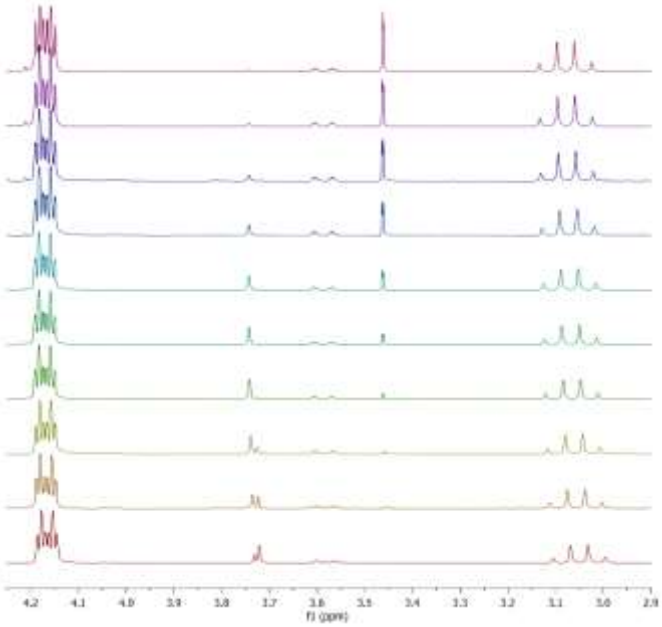

Figure 1. ^{1}H NMR monitoring of the reaction of alanin methyl ester and allyl isothiocyanate.

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ANALYSIS OF THE ADDITION OF 2-HYDROXYJUGLONE AND HYDROXYCITRONELLAL BY NMR

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Juglone is a biologically active naphthoquinone derivative with notable anticancer properties[1,2]. In this study, we reacted 2-hydroxyjuglone with hydroxycitronellal in the presence of Hantzsch ester, yielding both the expected product and an additional compound, ^{1}H NMR analysis of which revealed that two molecules of hydroxycitronellal attached to one molecule of 2-hydroxyjuglone. The attachment of one hydroxycitronellal molecule at the third position of 2-hydroxyjuglone is supported by the fact that the hydrogens of the first methylene group of hydroxycitronellal (2.54 ppm) interact with carbons at 191.2 ppm, 153.7 ppm, and 124.3 ppm, corresponding to the carbons at the fourth, second, and third positions of the 2-hydroxyjuglone molecule, respectively. Additionally, the carbon atom of the second methylene group, with a signal at 35.1 ppm, interacts only with the hydrogens of the remaining aliphatic moiety and does not interact with the aromatic hydrogens of the 2-hydroxyjuglone ring. Moreover, according to the HMBC data, the hydrogens of the second methylene group, with signals in the multiplet region at 1.57-1.27 ppm, show interactions with the carbon of the first methylene group at 20.3 ppm. These hydrogens also interact with the carbons of the remaining aliphatic moiety and only with the quaternary carbon atom (signal at 124.3 ppm) of the 2-hydroxyjuglone ring where the substitution occurred. Collectively, these data confirm that the attachment of one hydroxycitronellal molecule took place at the third carbon atom. Similarly, it was found that in the HMBC spectrum, the protons of the first methylene group of the second hydroxycitronellal moiety interact with the aromatic carbon atoms at 159.8 ppm, 142.0 ppm, and 134.2 ppm, corresponding to the carbons at the fifth, sixth, and seventh positions of the 2-hydroxyjuglone ring, respectively. There is no observed interaction with the quinonoid carbon atoms at the first and second positions, indicating that the substituent is sufficiently distant from these positions. This is possible only if the substitution occurs at the 7th or 6th position of the 2-hydroxyjuglone core. However, in the ^{1}H NMR spectrum, two doublets of protons with a coupling constant of 7.0 Hz were observed. This value is characteristic of neighbouring hydrogen atoms also confirmed by the two-dimensional COSY experiment. In the HMBC spectrum, there is also a signal indicating interaction of the carbon atom of the first methylene group (27.7 ppm) with the aromatic hydrogen at C7, further confirming the proposed structure of compound 2,5-dihydroxy-3,6-bis((S)-7-hydroxy-3,7-dimethyloctyl)naphthalene-1,4-dione.

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JUGLONE AND BUTYLAMINE REACTION PRODUCTS: ISOLATION AND STRUCTURAL CHARACTERIZATION

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Naphthoquinones, considered privileged structures in medicinal chemistry, act as anticancer agents through a diversity of mechanisms involved in all types of cancer. Some of the most important mechanisms broadly reported in the literature are: -DNA damage through reactive oxygen species (ROS) generation;[1] -inhibition of Topoisomerase II;[2] -NQO1 (NADPH: Quinone Oxidoreductase 1) inhibition;[3] regulation of tumour suppressor factors p53 and p73; [4] - induction of apoptosis via Endoplasmic Reticulum Stress. [5] Juglone is naturally occurring naphthoquinone isolated from plants with diverse biological properties along with being used as models for structural modifications.

As a result of the reaction of juglone with butylamine in the presence of copper(I) bromide and paraformaldehyde, a compound with a simple but unexpected structure was obtained with a good yield of 29%. NMR analysis confirmed that the structure of the obtained compound corresponded to 2-butylaminojuglone **1** (Fig.1). Two other substances were also isolated in this reaction. One of them, with a yield of 1.3%, was identified as 3-butylaminojuglone **2**. The second compound **3**, with a yield of 3.5%, was an adduct of juglone, two molecules of butylamine, and two molecules of formaldehyde.

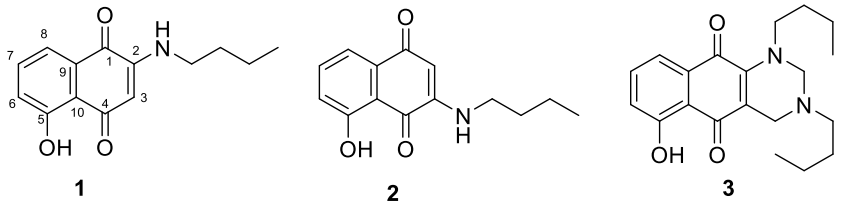

Figure 1. The Products of the reaction of juglone with butylamine and paraformaldehyde.

Determining the structure of the obtained compounds allowed us to propose a reaction mechanism, which was further confirmed in reactions with other amines.

Acknowledgements. This work has been supported by a research project 23.00208.5007.04/PD funded by ANCD and a research project MetNatVal (010601) supported by MEC.

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ANALYSIS OF CROATIAN AND SLOVENIAN SPARKLING WINES BY NMR SPECTROSCOPY

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Wines have very complex chemical composition with hundreds of different compounds in varying concentrations. Determining the chemical composition of wine is important for establishing the quality and value of wine, as well as detecting counterfeits and frauds such as unauthorized chaptalization or dilution with water.[1]

Among various spectroscopic methods used in wine analysis, NMR spectroscopy has gained more popularity due to its simpler and non-destructive sample preparation as well as application in both targeted and non-targeted analysis. DOSY NMR spectroscopy is an NMR technique with significant potential, since it separates components according to their translational properties and diffusion coefficient without physical separation. The DOSY NMR spectrum is therefore a pseudo-two-dimensional spectrum where proton chemical shifts represent one dimension, while the other dimension displays translational diffusion coefficients. It can be used for analyzing complex chemical mixtures[2] and determining wine origin, authenticity, cultivar, as well as grape variety.[3]

In this work NMR spectroscopy was used to characterize Croatian and Slovenian sparkling wines. The wine samples originated from different vintages and grape varieties. ^{1}H and DOSY NMR spectra provided information on different compounds present in the sparkling wines.[2] Furthermore, quantitative inverse-gated ^{13}C NMR spectra were also recorded to determine percentages of particular chemical components present in the complex wine samples such as aliphatics, aromatics, alcohols, carboxylic acids etc. Data so obtained will further be used for classification of wines by applying deep learning analysis.[3]

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SPONTANEOUS ISOMERIZATION OF -AsnGly- AMINO ACID PAIRS IN DEPENDENCE OF SEQUENCE AND STRUCTURE

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Peptides and protein sequences with -AsnGly- (-NG-) amino acid pairs show spontaneous isomerization under physiological conditions. The backbone atoms of the -NG- dipeptide will rearrange themselves first through succinimide ring closure, leading then to both β-Asp and α-Asp formation with hydrolysis. However, the kinetics of this process shows striking variability, as it may take place within just a few hours, or can last up to several days or even weeks. Previously, we demonstrated on a row of short model peptides the pH and temperature dependence of this process.[1] However, these data still do not fully explain the wide range of conversion rates known so far. In our present work, we aim to shed light on the influencing effect of buffers, neighboring amino acids, flanking sequences, as well as their inherent structural rigidity and/or flexibility with NMR spectroscopy.

Therefore, we observed 15N-HSQC spectra of naturally occurring proteins with sequences containing the -NG- dipeptide, such as protein PEP-19 or Calmodulin, in a time dependent manner. We compared the kinetic analysis of each protein to the analysis of peptides of the same sequence, but different lengths, analyzed in different buffers.

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CHEMICAL FATE OF ANTICANCER DRUGS. ^{1}H, ^{13}C, AND ^{31}P NMR STUDY OF CHLORINATION OF CYCLOPHOSPHAMIDE AND IFOSFAMIDE

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Among the many factors that determine the chemical fate of a drug in the environment, water chlorination and the sunlight irradiation are of special importance.[1,2] ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy was used to study the chlorination of two isomeric anticancer drugs (Figure), cyclophosphamide (**CP**) and ifosfamide (**IFO**). In addition, the hydrolytic stabilities of the two pharmaceuticals were tested in acidic and basic aqueous medium. To monitor reactions without a daylight exposure, a two-chamber borosilicate glass NMR tube, which allows for in-tube mixing, was used. In the reaction between HOCl and **CP** (**IFO**), the only product detected, and isolated, is the *N*-chlorinated compound **CP-Cl** (**IFO-Cl**). The product is stable for days in dark (or in the magnet), but is dechlorinated back to the parent structure when exposed to a daylight.

Both hydrolysis and chlorination reactions were sufficiently slow to fit the ^{1}H and ^{31}P NMR time window. However, the latter channel was superior in terms of kinetic analysis.

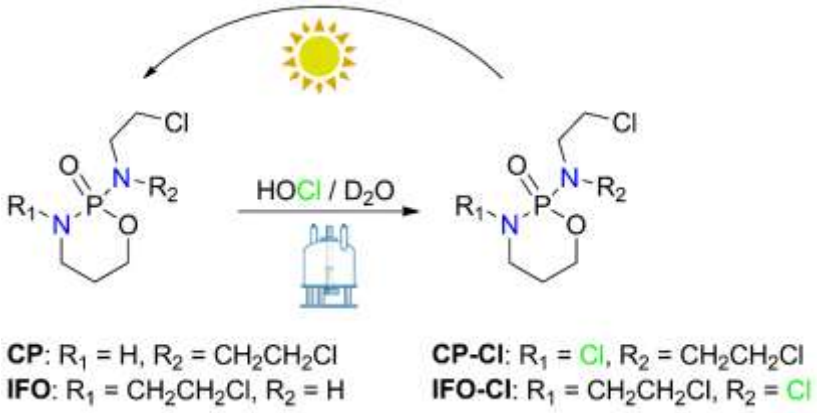

Figure. Chlorination of cyclophosphamide (**CP**) and ifosfamide (**IFO**) in deuterated water.

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CHARACTERIZATION OF CROATIAN OLIVE OILS BY NMR SPECTROSCOPY

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Vegetable oils play an essential role in the human body. They are an indispensable part of the human diet providing essential fatty acids. They can also be very rich sources of micronutrients and bioactive substances such as carotenoids, polyphenols and alkaloids. For more than 50 years, the "Mediterranean diet" has been considered one of the healthiest diets in the world as it traditionally uses olive oil as the main source of fat. Olive oils improve health owing to their nutritional value and antioxidant properties. They are a complex mixture of different molecules that are generally divided into a saponifiable fraction, which includes tri-, mono- and diglycerides, and an unsaponifiable fraction, which includes hydrocarbons, aliphatic alcohols, sterols, tocopherols, pigments, phenolic and volatile compounds.[1] The great interest in the quality of oils is also linked to the rapidly growing awareness of consumers who want to use safe and nutritious products with positive effects on health.

An understanding of vegetable oils is of particular interest for the research and development of production processes, production control and the determination of product quality. Edible oils, especially olive oils, are also susceptible to various types of adulteration.[2] Croatia is a growing market for extra virgin olive oil and there is a great need for the establishment of an olive oil bank. Over the last decades, nuclear magnetic resonance spectroscopy (NMR) has proven to be a powerful tool for studying the composition and properties of vegetable oils.[3,4] We report here NMR study of three different olive oil samples from the Adriatic region: Istria 1, Istria 2 and Dalmatia 1. We characterized these oils by using different one- and two-dimensional NMR techniques and determined the fatty acid distribution and iodine number.

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