FINAL REPORT

SOLUTION CONFORMATION AND PROTEIN-BINDING INTERACTIONS OF NOVEL BIOACTIVE CARBOHYDRATE- AND PEPTIDE DERIVATIVES STUDIED BY NMR AND OTHER BIOPHYSICAL METHODS

The project has been executed according to the major lines of the original aims, therefore we state that it conforms to the original research plan. The results are presented in the following two sections: **I. NMR methodological developments** and **II. Syntheses, NMR and molecular modelling studies of compounds with different biological or catalytic activities**. Since most of the results attained during the project of five-year duration have been published in peer-reviewed journals (***publications indicated with bold italics***) therefore the presentation of the present report is concise focusing only on the main achievements of the project.

I. NMR methodological developments

Nuclear magnetic resonance (NMR) spectroscopy is one of the most powerful techniques for investigation of structural, dynamical properties and interactions of molecules at atomic resolution. In order to gain reliable information from the measurements high quality NMR spectra are needed. Sensitivity and resolution are key parameters which primarily determine the quality of an NMR spectrum. In the last two decades the sensitivity of NMR measurements has increased by more than an order of magnitude due to electronic technological improvements, higher magnetic fields and the introduction of cryogenically cooled probes. In contrast, the spectral resolution has been enhanced by only a factor of two if we compare the highest magnetic field spectrometers available today with the ones operated 20 years ago. Therefore, there is a continuous demand for developing methods which can improve the resolution of NMR spectra. In the past five years our research group joined this dynamically growing NMR methodological research field with the goal of increasing the efficiency of NMR experiments by utilizing pulse sequence elements capable of broadband homonuclear decoupling.

Design of novel, state-of-the-art NMR methods utilizing broadband homonuclear proton-decoupling

a.) We improved the conventional 2D ^{1}H/X HSQC experiment by broadband homonuclear decoupling with the combined use of the BIRD^{d} pulse sequence element and a non-selective 180° proton pulse. The timing and phase program of the pulses applied in the proposed novel CLIP/CLAP-HSQC experiments were optimized by performing series of measurements on simple carbohydrate derivatives. The characteristic multiplet structure of correlation peaks was simplified, collapsed into singlets in the resulting spectra. An advantageous side effect of the BIRD^{d} pulse sequence element employed in the acquisition scheme is the efficient suppression of undesired long-range correlation peaks arising from strong coupling effects. The method developed was compared with previously published proton-decoupled HSQC experiment. Our method proved to be more robust, namely, it was less sensitive to the duration of INEPT/BIRD delays adjusted to one-bond heteronuclear couplings (***J. Magn. Reson. 2014***).

b.) We incorporated BIRD decoupling into a 'perfect echo' pulse sequence block. The resulting "perfectBIRD" block can remove splittings arising from geminal proton-proton couplings eliminating inherent limitation of the classical BIRD decoupling (***Chem. Comm. 2014***).

c.) A real-time (instant) broadband homonuclear decoupled variant of the sensitivity-enhanced ^{1}H-^{15}N HSQC method was developed for measuring biological samples in H_2O. Efficient water suppression could be achieved with field gradient pairs of appropriate strength and duration applied during acquisition flanking the BIRD^{d} block and the non-selective 180° proton pulse. We demonstrated that the proposed method was suitable for recording ^{1}H-^{15}N HSQC spectra of small proteins dissolved in H_2O even at natural ^{15}N isotopic abundance (***J. Biomol. NMR 2015***).

d.) We also developed the real-time pure shift variant of CLIP/CLAP-HSQC experiments for fast, robust and automated measurement of one-bond heteronuclear coupling constants which provide invaluable information for probing the structure and dynamics of bioactive molecules and their complexes via RDC analysis. To minimize the systematic errors in the apparent coupling constants measured, we devised a complex acquisition scheme, including cycling of radiofrequency pulse phases both from chunk to chunk during windowed acquisition, and from scan to scan during time averaging. The new real-time pure shift experiments designed speed up coupling constant determination, increase the sensitivity of measurement in favorable cases, and last but not least, simplify the extraction of accurate one-bond heteronuclear couplings from pure in- or anti-phase doublets using automated peak picking. The utility of the pulse sequences proposed has been demonstrated for coupling constant measurements under both isotropic and anisotropic conditions, on a small test molecule and also on a protein sample. The scope and limitations of the method have been critically reviewed (***RSC Adv. 2016***).

Multiple-bond heteronuclear coupling constants ($^{n}J_{XH}$) provide invaluable information for stereochemical and conformational analysis of molecules from synthetic and natural origin. Moreover, this information is often complementary to proton-proton coupling constants and NOE data. Determination of $^{n}J_{XH}$, however, is not a straightforward task owing to the low sensitivity of the relevant NMR experiments, and also to the fact that long-range heteronuclear ($^{n}J_{XH}$) and proton-proton ($^{2,3}J_{HH}$) coupling constants are typically the same order of magnitude. In the last two decades, much effort has been devoted to developing more efficient pulse sequences to measure $^{n}J_{XH}$ accurately. Our research group has also contributed to this field with the development of various broadband homonuclear decoupled HSQMBC methods. Recently, we have summarized and critically reviewed the scope and limitations of the different decoupling strategies applied and provided practical guidelines for the choice of the most appropriate method.

e.) We devised an interferogram-based, slice-selective broadband homonuclear decoupled CPMG-HSQMBC method for the accurate determination of multiple-bond heteronuclear coupling constants. The Zangger-Sterk (ZS)-type pulse sequence element was built in the CPMG-HSQMBC experiment to remove the undesired proton-proton splittings from the detected signals. As a result, the determination of long-range heteronuclear coupling constants was simplified to the measurement of frequency differences between peak maxima. We demonstrated on model compounds containing phosphorus that a broad range (~ 2-26 Hz) of multiple-bond heteronuclear coupling constants could be measured in a single experiment using the proposed method. The utility of ZS-decoupled CPMG-HSQMBC was also demonstrated for the extraction of $^{n}J(^{1}H,^{77}Se)$ and $^{n}J(^{1}H,^{13}C)$ values, respectively, in carbohydrates (***Chem. Eur. J. 2015a***).

f.) The ZS CPMG-HSQMBC experiment described above could be considerably speeded up with the application of real-time, windowed decoupling during acquisition. The limitations of the real-time acquisition strategy, namely, occurrence of inherent line broadening and minor

artefacts reducing the quality of spectra have been critically evaluated in an invited Mini review submitted for the 'Pure Shift' special issue of Magnetic Resonance in Chemistry. We demonstrated that the sensitivity of the experiment could be further improved by multiple slice selection using multiple-frequency modulated pulses. In this case, however, the careful choice of proton offsets for multiple excitation is critical to avoid accidental recoupling effects. Based on our experience the proposed real-time Zangger-Sterk CPMG-HSQMBC method could be the experiment of choice for determining multiple-bond heteronuclear coupling constants in those cases where the proton resonance frequency difference of coupling partners is large enough (> 90-100 Hz), allowing the use of short (max. ~10 ms) selective proton pulse for decoupling during acquisition (***Magn. Reson. Chem. 2017***, *accepted*).

g.) We also reported a broadband homonuclear decoupled PSYCHE CPMG-HSQMBC method for the precise and direct measurement of multiple-bond heteronuclear couplings. The PSYCHE-scheme built in the pulse sequence efficiently eliminates unwanted proton-proton splittings from the heteronuclear multiplets so that the desired heteronuclear couplings can be determined simply by measuring frequency differences between peak maxima of pure antiphase doublets. Moreover, PSYCHE CPMG-HSQMBC can provide significant improvement in sensitivity as compared to earlier Zangger-Sterk-based method. A remarkable feature of the PSYCHE CPMG-HSQMBC experiment is that the characteristics of the proton spin systems involved have practically no effect on the efficiency of homonuclear decoupling. As a result, the PSYCHE experiment can be performed routinely, without tedious fine-tuning of experimental parameters from sample to sample. In certain cases, the PSYCHE CPMG-HSQMBC can provide nearly an order of magnitude enhancement in sensitivity compared to the interferogram-based ZS-decoupled method. The performance of PSYCHE CPMG-HSQMBC method was illustrated by measuring $^{3}J_{CH}$ and $^{3}J_{SeH}$ values in various carbohydrates. These data can provide valuable information on the conformations around the glycosidic linkages. It is important to note that extraction of these couplings from the conventional, non-decoupled HSQMBC multiplets may require computer-aided fitting or in worse cases, may be impeded by partial signal cancellations in the mixed-phase signals (***Chem. Eur. J., 2015b***).

In conclusion, the broadband proton-decoupled heteronuclear correlation experiments developed during past five years can considerably help the assignment of NMR spectra of complex molecules and multicomponent systems (e.g. mixtures of diastereomers or reaction products, metabolites), supporting the structure elucidation or verification of these compounds. In addition, we have also developed broadband homonuclear decoupled NMR experiments for the precise and direct measurement of one- and multiple-bond heteronuclear coupling constants, which are invaluable and widely applied tools for structure elucidation and conformational analysis of organic compounds. The proposed novel NMR methods provide highly precise and accurate data to reliable disclosure of structure-activity relationships of bioactive compound, contributing to rational design and development of drug substances with improved biological profile.

Design of novel experiments for NMR-based binding studies

a.) Fragment-based drug design has been successfully applied to challenging targets where the detection of the weak protein–ligand interactions is a key element. NMR spectroscopy is well suited for fragment-based screening because it can reliably detect binding up to mM dissociation constant (K_d) values. ^{1}H saturation transfer difference (STD) NMR spectroscopy has emerged as one of the most popular and highly effective ligand-based NMR techniques but it requires pure homogeneous proteins as targets. In general, protein mixtures or impure protein samples cannot

be handled by current STD-NMR methods because the available target saturation schemes are not able to distinguish between the specific macromolecules present in solution. To resolve the problem of complex protein mixtures we have devised monoclonal antibody (mAb)-relayed ^{15}N-group selective STD experiment for target-specific NMR detection of protein–ligand interactions. During the preparation period of the experiment the ^{15}N-labelled target-specific mAb is selectively irradiated with a cascade of BIRD pulses and then the saturation is relayed through the target to the ligand by exploiting the selective molecular recognition of the target protein by a ^{15}N-labelled mAb. As a result, this method makes feasible the detection and identification of binding molecules directly from a protein mixture in a multicomponent system. As proof of principle, the experiment was tested on the anti-Gal-1 mAb/Gal-1/lactose system and also on a cell extract mixture (***Biochemistry 2014; J. Biomol. NMR, 2016***).

b.) A novel, sensitive approach was developed for selecting molecules that bind to carbohydrate-recognition proteins from a mixture of selenoglycosides using 2D ^{1}H-^{77}Se NMR correlation spectroscopy. The screening scheme developed relies upon the large multiple bond $^{n}J_{SeH}$ couplings for highly sensitive detection of ^{1}H-^{77}Se correlations, on the one hand, and on ^{1}H and ^{77}Se transverse relaxation enhanced by the binding process, on the other. Our method is highly efficient to pick up resonances of small molecule ligands that bind to the protein in the presence of non-binding partners. As a proof-of-concept, we selected four well-characterized lectin proteins (human Galectin-1, -3 and -7 and a plant lectin VAA) and monoseleno-digalactoside and diseleno-digalactoside (SeDG and DSeDG, respectively**)** as potential binders when applied in a complex mixture. ^{15}N-HSQC-titration experiments performed with the ^{15}N-labeled human lectins indicated significantly higher affinity for SeDG than DSeDG toward each lectin. In contrast, ^{1}H relaxation measurements indicated similar binding affinity to VAA for both ligands (*manuscript in preparation*).

II. Syntheses, NMR and molecular modelling studies of compounds with different biological or catalytic activities

a.) *Design and characterization of novel Kv1.3 selective blocker scorpion toxin analogs with reduced conformational flexibility*

The voltage-gated Kv1.3 K^+ channel plays a key role in the regulation of activation and proliferation of T lymphocytes in general and that of effector memory T cells (T_{EM}) in particular. Kv1.3 blockers selectively suppress the activation and proliferation of T_{EM} cells, which indicates the great potential of selective Kv1.3 inhibitors in the therapy of certain autoimmune diseases. Anuroctoxin (AnTx), a 35-amino-acid scorpion toxin is a high affinity blocker of Kv1.3, but also blocks Kv1.2 with similar potency. Our goal was to improve the selectivity of the toxin for Kv1.3 over Kv1.2 while keeping the high affinity for Kv1.3. Based on sequence comparisons with known Kv1.3 specific inhibitors we designed and produced three AnTx variants: ([F32T]-AnTx, [N17A]-AnTx, [N17A/ F32T]-AnTx). Patch-clamp technique was used to determine the blocking potency of the synthetic toxins on hKv1.3, mKv1.1, hKv1.2 and hKCa3.1 channels. Of the three variants the double substituted [N17A/F32T]-AnTx maintained the high affinity of the natural peptide for Kv1.3 but became more than 16000-fold selective over Kv1.2. NMR structure determinations showed that the residue-wise RMSD of atomic coordinates of the loop connecting the N-terminal end and the α-helix, between residues 6-9, is less well-defined in sAnTx than in [N17A/F32T]-AnTx. Multiple MD simulations with lengths of 100 ns, 1 μs and 10 μs for both sAnTx and [N17A/F32T]-AnTx were also performed to reveal the dynamics of the peptides. RMSDs of atomic coordinates of the backbone heavy atoms with respect to the starting and the average

structures along the trajectories show that the conformation of [N17A/F32T]-AnTx is more stable during the trajectory with consistently smaller deviations from the mean than that of sAnTx. Thus, the more rigid structure with restricted conformational space of the double substituted toxin compared to the flexible wild-type one is an important determinant of toxin selectivity.
The chemical synthesis of the Sec-analog of the double mutant, Sec-[N17A/F32T]-AnTx was also achieved, replacing all cystein (Cys) residues by selenocysteine (Sec) forming four diselenide bonds. We proposed a combined experimental and theoretical approach including NOE- and ^{77}Se-based NMR supplemented by MD simulations for conformational and dynamic characterization of the Sec-analog. This combined approach allowed us to attain unequivocal assignment of all four diselenide bonds and supplemental MD simulations allowed characterization of the conformational dynamics around each disulfide/diselenide bridge (***Scientific Reports 2015; RSC Chem. Sci. 2016***).

b.) *NMR characterization of adipokinetic (AKH) peptide hormone*

The spread of malaria by the female mosquito, Anopheles gambiae, depends, amongst other things, on its ability to fly. This in turn, is dependent on the adipokinetic hormone, Anoga-HrTH (pGlu-Leu-Thr-Phe-Thr-Pro-Ala-Trp-NH_2). We have used NMR restrained molecular dynamics to investigate the conformational space of this important neuropeptide in aqueous solution and when bound to a membrane surface. Our results gave information on the changes in conformation of insect AKH peptides during dynamics, and upon receptor binding and, hence, should be of interest to scientists working in diverse fields from genomics and insect neuopeptides to drug design (***Peptides 2013***).

c.) Design of novel glycosyl disulfide inhibitors against Trypanosoma cruzi

Trypanosoma cruzi is the etiologic agent of Chagas's disease, an endemic parasitosis in Latin America with 12–14 million people infected. Chronic *T. cruzi* infections have been associated with high rates of morbidity and mortality, and reports appeared about worldwide spreading due to international migration. Aromatic oligovalent glycosyl disulfides and some diglycosyl disulfides synthesized in our laboratory have been tested against three different *Trypanosoma cruzi* strains. Two of the tested molecules - di-(β-D-galactopyranosyl-dithiomethylene) benzene derivatives – proved to be active against all three strains of cell culture-derived trypomastigotes with IC_{50} values ranging from 4 to 11 μM at 37 ºC. Several molecules of the tested panel showed remarkable inhibitory effect against the intracellular development of *T. cruzi* amastigotes (***Bioorg. Med. Chem. Lett. 2013***).

d.) *NMR characterization of bioactive tyrocidine peptides*

The tyrocidine peptides are potential candidates as new therapeutic agents against a number of pathogens because of their broad spectrum of activity, their rapid membranolytic mechanism of action, and the possibility of alternative targets limiting resistance potential. The activity of antimicrobial peptides is determined by their three-dimensional structures and physicochemical properties. We have used NMR restrained molecular dynamics, circular dichroism, and mass spectrometry to investigate the structure of two major tyrocidines (TrcA, TrcC), antibiotic peptides from Bacillus aneurinolyticus, in aqueous environment. Our results show that both peptides readily form dimers by either associating sideways or stacking on top of each other. The ability of tyrocidines to form different types of dimers suggests that they might be able to form larger aggregates that are similar to those of the amyloid β-protein (Aβ). The resultant fibrils have been hypothesized to form channels that can destroy the membrane structure (***Biochemistry 2013***).

e.) *NMR characterization and folding studies of disulfide rich antifungal peptides*

The increasing interest in disulfide-containing antifungal peptides has led to an extensive search for suitable routes for their synthesis. Probing different approaches, we found that the oxidative folding of the synthetic linear PAF yielded a folded protein that has a structure and antifungal activity identical with that of the native antifungal protein from Penicillium chrysogenum. The disulfide pattern of the synthetic PAF was unambiguously confirmed using different methods, including bioinformatics, folding, mass spectrometry, and NMR structural studies. Our thermal unfolding studies showed that the population of hidden states could weigh up to 20–40% at 298 K. On the other hand, a low populated, 0.15%, state in slow exchange with the folded PAF was also identified by the sensitive ^{15}N-CEST NMR experiment. These findings denote the existence of a complex conformational landscape with multiple conformational states in dynamic equilibrium with diverse exchange rates (***Chem. Eur. J. 2013; Chem. Eur. J. 2015***).

We have compiled an invited review on 'NMR investigation of disulfide containing peptides and proteins' for the series of Specialist Periodical Reports - Amino Acids, Peptides And Proteins [(***ISBN:978-1-84973-585-8***)].

f.) *Design of novel selenosugar derivatives as promising leads with high inhibitory activities against infective African trypanosomes*

In collaboration with the group of M.Comini (Institut Pasteur, Montevideo) we have investigated the anti-trypanosomal activity and selectivity of a series of symmetric diglycosyl diselenides and disulfides. Of 18 compounds tested the fully acetylated forms of di-*β*-D-glucopyranosyl and di-*β*-D-galactopyranosyl diselenides displayed strong growth inhibition against the bloodstream stage of African trypanosomes. Both compounds induced redox unbalance in the pathogen. Nonacetylated versions of the same sugar diselenides proved to be, however, much less efficient or completely inactive to suppress trypanosome growth. *In vitro* NMR analysis indicated that diglycosyl diselenides react with glutathione, under physiological conditions, *via* formation of selenenylsulfide bonds. Our results suggest that non-specific cellular targets of the glucose and the redox metabolism of the parasite may be affected. These molecules are therefore promising leads for the development of novel multitarget antitrypanosomal agents (***ChemistrySelect 2016; Int. J. Parasitol.: Drugs & Drug Resist. 2017***).

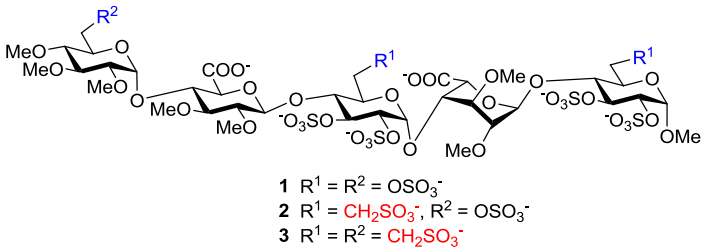
g.) *Novel sulfur- and selenium-containing carbohydrates as promising inhibitors of lectins*

Initiated by the group of Prof. H-J. Gabius (München) a collaboration was started to test the biological activities of some sulfur- and selenium-containing carbohydrates synthesized in our group. The inhibitory capacities of benzene-based mono- to trivalent dithiogalactosides increased with valency for the plant toxin VAA, potently blocking its binding to a lactose-presenting matrix and to human tumor cell lines. Interestingly, human galectins were much less sensitive to the disulfides than the toxin. Selenodigalactoside and diselenodigalactoside were also prepared and both compounds proved bioactive for the toxin. Remarkably, the Se-linked digalactoside proved active for human galectins as well (***Bioorg. Med. Chem. Lett. 2015***).

h.) *Syntheses, structure, dynamics and binding studies of heparin analog carbohydrates*

Heparin, isolated from natural sources, has been used as an antithrombotic agent in clinical practice from 1937. Since its administration is associated with rare serious side effects, several research groups – among them the group of Prof. Borbás at our University – are making efforts to synthesize heparin-analogue oligosaccharides displaying better pharmacological properties than heparin. Two novel synthetic pathways have been developed for the preparation of Idraparinux (**1**). Idraparinux is a fully O-sulfated, O-methylated, heparin-related pentasaccharide possessing selective factor Xa inhibitory activity and served as a reference for our NMR binding studies. During the project period we investigated the structure and dynamics of novel heparin-analog

pentasaccharides containing two or three sulfonatomethyl groups at specific positions and studied their interaction with antithrombin III (AT-III) using combined NMR experimental and MD computational approach.

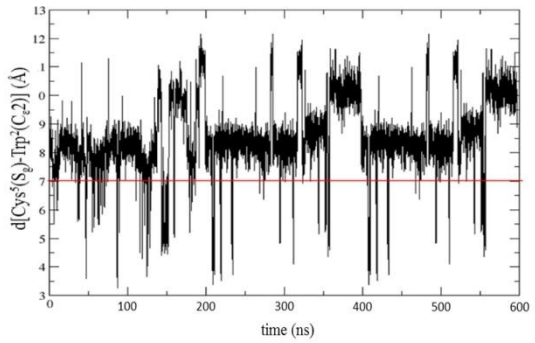
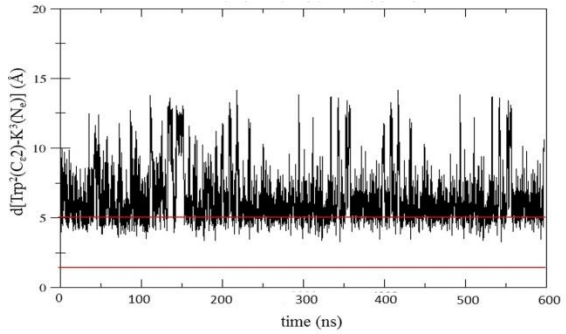
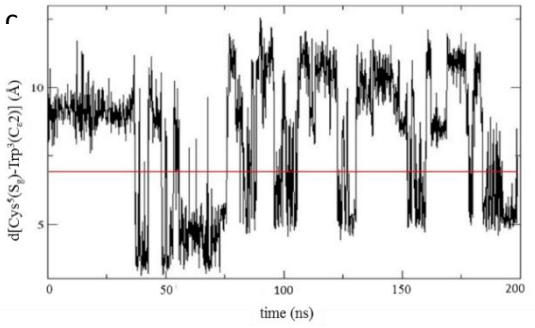
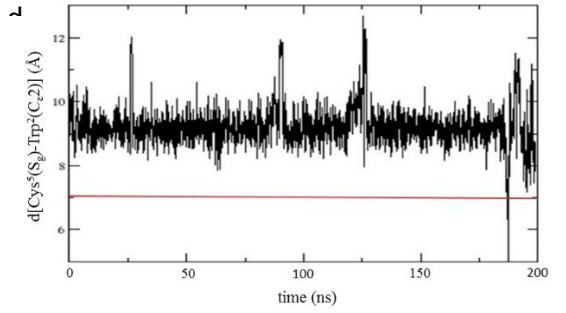


Examined heparin--analogue pentasaccharides

Although the chemical composition of these analogues is almost the same, the *in vitro* anticoagulation studies revealed significant differences in their biological activities. Specifically, the trisulfonate analogue exhibited markedly lower activity than the two other ones. In order to disclose the structural and/or dynamic factors behind the diverse biological profile, the conformation along with the conformational flexibility have been investigated in both the free and the antithrombin-bound forms of the three pentasaccharides. Pleasingly, the conformationally relevant proton-proton distances and torsion angles assessed by the NMR and MD approaches were in good agreement. The three heparin analogs in complex with AT-III displayed conformations alike, on the contrary, their free forms exhibited significant differences in both the conformation and conformational flexibility, which might explain the differences observed in their biological activities. We performed a series of ITC measurements to characterize the stoichiometry of the pentasaccharide-AT-III complexes and the thermodynamics of their interactions (***Tetrahedron 2013; Eur. J. Org. Chem. 2013; Tetrahedron 2014; Carb. Res. 2014; J. Biomol. Struct. Dyn. 2015***; EUROMAR 2016 *conf. abstract*; *manuscript in preparation*).

i.) *Photochemical and structural studies on cyclopeptide models*

In view of the impact of UV irradiation on the structure and function of proteins, it is of the utmost importance to further unravel the conditions of tryptophan amino acid residue mediated photolysis of disulfide bonds. In collaboration with the group of Zsuzsa Majer (Laboratory for Chiroptical Structure Analysis, Eötvös Lóránd University) we studied a series of cyclic model peptides containing aromatic amino acid residue and disulfide bridge in the sequence. To examine the relationships between the photoexcitation of a Trp and the associated reduction of disulfide bridges, we used NMR spectroscopy and MD simulations for determining the structure and predicting the distances between the aromatic amino acid – disulfide bridge and the possible cation-pi interactions. The evolved sulfhydryl group(s) upon illumination with near UV light was followed by its reaction with CPM and the formation of peptide-CPM derivatives detected by fluorescence spectroscopy. Detailed conformational analysis with the combined use of NMR and molecular modelling calculations was carried out on three Trp containing pentapeptide models - Ac-c(CWAGC)-NH_2, Ac-c(CWKAC)-NH_2, Ac-c(CAWAC)-NH_2.

a 	b 
Ac-c[CWKAC]-NH_2 Distance between the disulfide bridge and the tryptophan ring d[$Cys^5(S_\gamma)$-$Trp^2(C_\varepsilon 2)$] along three concatenated MD-trajectories	Ac-c[CWKAC]-NH_2 Distance between tryptophan ring and lysine sidechain d[$Trp^2(C_\varepsilon 2)$-$K^3(N_\varepsilon)$] along three concatenated MD-trajectories
c 	d 
Ac-c[CAWAC]-NH_2 Distance between the disulfide bridge and the tryptophan ring d[$Cys^5(S_\gamma)$-$Trp^3(C_\varepsilon 2)$] along the 200 ns long MD-trajectory	Ac-c[CWAGC]- NH_2 Distance between the disulfide bridge and the tryptophan ring d[$Cys^5(S_\gamma)$-$Trp^2(C_\varepsilon 2)$] along the 200 ns long MD-trajectory

Intramolecular atomic distances (in Å) relevant for photolytic processes. The corresponding distance criteria are represented by red lines.

Our results clearly confirmed that the structural characteristics of the three selected peptides are in good agreement with the desired and expected features proposed on the basis of simple molecular mechanics force field calculations. This also means that the peptides selected and examined in our work are suitably good models for studying the structural aspects of the photolytic mechanism of disulfide bond reductive cleavage and so, to prove (or disprove) the different hypotheses proposed so far in the literature (***Magyar Tudomány 2016***; *manuscript in preparation*).

j.) *Conformationally constrained nucleoside analogues with a new heterotricycle obtained from the D-ribofuranose unit*

Novel type of nucleoside analogues in which the sugar part is replaced by a new tricycle, 3,7,10-trioxa-11-azatricyclo[5.3.1.$0^{5,11}$]undecane have been produced by substrate-controlled asymmetric synthesis. Formation of one stereoisomer, out of the eight possible, was observed in all cases. In collaboration with the group of Anikó Borbás (Department of Pharmaceutical Chemistry, University of Debrecen) the absolute configuration of the new stereotriad-containing tricyclic systems was unequivocally established by conventional NMR experiments followed by chemical shift calculations using an X-ray crystal structure as reference that was in good agreement with H-H distances obtained from a new ROESY NMR method (***Org. Biomol. Chem., 2017***).

PhD and students' scientific activities within this research project

On this research project 3 PhD students worked (*István Timári, Mária Raics, Tamás Gyöngyösi*) under the supervision of the Principal Investigator (**Katalin E. Kövér**) and during the research period in 2016 *István Timári* obtained PhD degree 'summa cum laude'. *Tamás Gyöngyösi,* as former chemistry and pharmacy MSc student, presented the results of his research at the National Scientific Students' Associations Conference (OTDK) and won the First Prize in 2015. His diploma thesis was awarded by the Hungarian Chemical Society ('MKE nívódíj'). *Milán Tamás Nagy*, a chemistry MSc student, completed students' scientific research within the project and for his diploma work he received the award of the Hungarian Chemical Society ('MKE nívódíj'). In 2017 he received PhD scholarship from 'Richter Gedeon Talentum Alapítvány'.