STUDIES OF CARBOHYDRATES AND PEPTIDES BY NMR SPECTROSCOPY AND MOLECULAR MODELLING

Synopsis of PhD dissertation

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1. Introduction

Matter in the Universe is made up of the same 100 different types of atoms, still a vast variety of different materials are observed. The enormous diversity arises from the various ways the atoms are arranged. Therefore, knowing the 3D structure of molecules is of paramount importance today in chemistry, physics and biology, since it determines the large variety of chemical, physical and biological properties of materials.

In crystalline materials positions of the heavy atoms in the molecule can be determined by X-ray crystallography. Problems arise with X-ray crystallography when crystals cannot be grown or a molecule has a dynamic nature. An alternative method for structure determination is nuclear magnetic resonance (NMR) that is steadily gathered importance in the last three decades or so. Applications of high-resolution solution state NMR extend to numerous areas. As an analytical tool the result of chemical synthesis is checked in a fast and efficient way, as far as connectivity, stereochemistry and purity is concerned. In structural studies NMR spectroscopy has made the largest impact on the determination of protein structures in solution, and a pioneer in NMR was awarded the Nobel Prize in 2002 for his contributions to this area. The information provided by NMR structures is complementary to X-ray crystallography in many ways. It is performed on molecules in solution or most recently on powder crystals; therefore no single crystal is required. New structures can be obtained which are not available from X-ray studies or a meaningful comparison of the conformations in single crystals and in non-crystalline states can be obtained. It can provide additional information about dynamics on various time scales. The solution conditions for NMR studies (e.g., pH, temperature, ionic strength, buffers) can be varied over a wide range, which opens the possibility for comparative studies, or for investigations of intermolecular interactions with other solute molecules. The NMR studies are often combined or compared with the results of molecular modelling methods, since NMR parameters provide structural constraints at the atomic level that can be conveniently utilized in computations, while the analysis of calculations give further insight into the characteristics of the chemical system.

2. Objectives

This thesis describes conformational studies of two important classes of molecules, namely, lipophilic derivatives of cell-wall glycopeptides and novel disaccharide derivatives featuring disulfide interglycosidic linkages, by means of NMR spectroscopy and model calculations to reveal conformational features of possible biological relevance. Furthermore, modifications to known NMR methods are proposed to improve measurements of structurally relevant NMR parameters, in particular, measurements of residual one-bond ^{1}H-^{13}C dipolar couplings and assignment of oligosaccharides featuring strong overlap in their NMR spectra.

Conformational studies

1. Polymeric peptidoglycans of bacterial cell walls, and smaller glycopeptides derived from them, exhibit versatile biological activities including immunomodulating properties. Novel lipophilic derivatives of the peptidoglycan monomeric molecule displayed similar biological activities as the parent molecule isolated from the bacterial cell wall. In view of the similar in vivo behaviour of these three compounds it was of interest to determine whether these activities are related to similarities in the molecular conformations or, on the opposite, eventual conformational differences may not exert sizeable influence on the biological activity.

2. Carbohydrate derivatives containing disulfide interglycosidic linkages structures are of interest for several reasons: i) added flexibility within the resulting compounds with respect to the reference natural glycosides, ii) increased distance between components in terms of the number of connecting bonds (3 vs. 2), iii) extension of the available conformational space as a result of i) and ii), iv) altered electronic and steric properties of the linker atoms and v) inherent axial chirality of the disulfide bond. All these characteristics play a significant role in biological interactions involving carbohydrates such as in cell recognition or in carbohydrate metabolism amd also determines the chemical reactivity of the SS-bond. The objective of the present study was to gather information on the conformational features of interglycosidic linkage in these compounds.

Improving methods for determination of NMR parameters

3. Residual dipolar couplings (RDC) provide unique long-range constraints for structure determination of molecules and lately have therefore been extensively applied for structural studies of proteins, nucleic acids and carbohydrates in liquid state. Measurement of

the residual dipolar contributions presents a considerable experimental challenge for flexible or spherically shaped molecules whereas the RDCs are often small. Reliable measurement of RDC's by the conventional coupled HSQC experiment is compromised by the presence of long-range heteronuclear couplings in the indirect dimension, hence modification of the original sequence was neccessary.

4. The laboratory synthesis of a specific carbohydrate molecule is often a multistep procedure which require application of protecting groups. The result of the reaction is usually examined by NMR spectroscopy after each reaction step. Resonance assignment of NMR spectra of such protected oligosaccharides is, however, often difficult due to by the presence of intense signals arising from protecting groups overlapping with the signals containing structural information. Suppression of the disturbing signals by the modifying the sequence was needed in order to carrry out reliable resonance assignment.

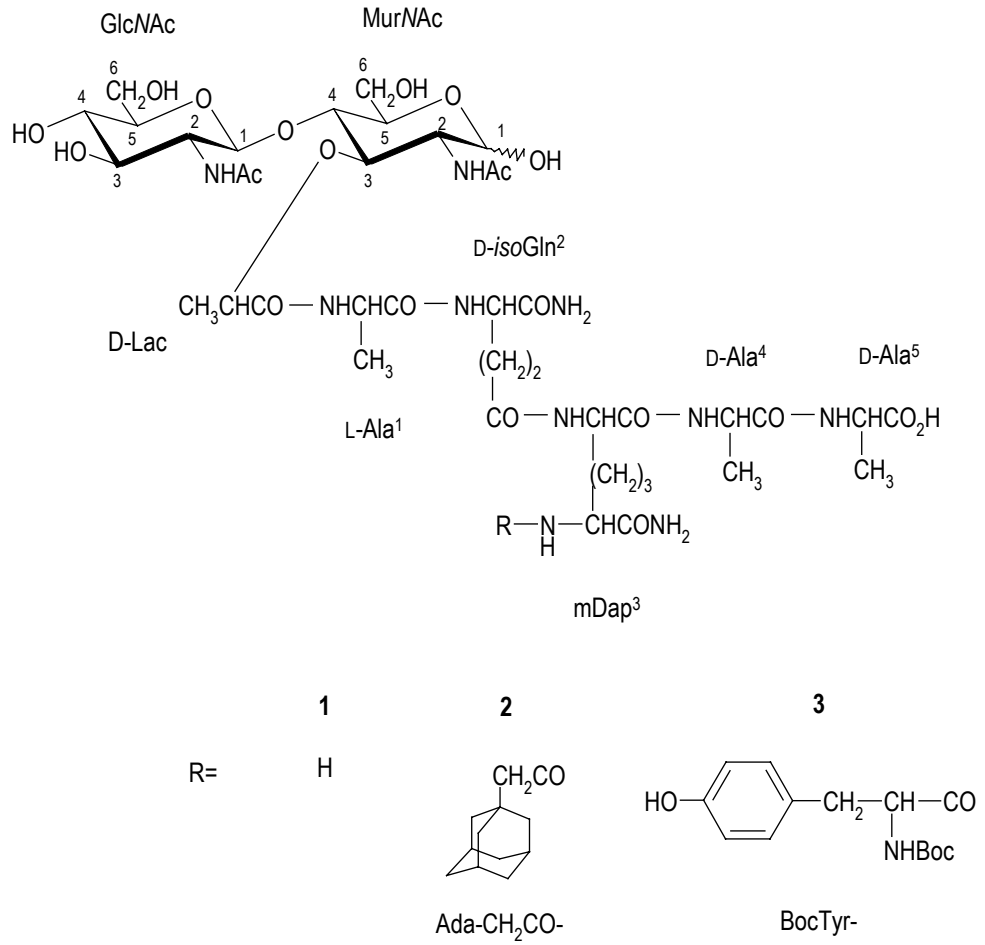
3. Applied methods

Conformational characteristics were studied by NMR spectroscopy and supplemented by molecular modelling calculations. Complete and unambiguous assignments of ^{1}H- and ^{13}C-spectra were achieved by the combined analysis of various 1D and 2D measurements such as COSY, 2D TOCSY, HSQC-TOCSY, selective TOCSY, edited HSQC and HMBC. NOESY/ROESY experiments have provided interproton distance information that were either used in distance geometry modelling calculations as distance restraints or compared to the results of the computer aided conformational search. Conformational search has been carried out including systematic as well as stochastic searches. The molecular mechanics calculations were carried out with the DISCOVER program implemented in the InsightII 2000. These data were supplemented with information available from chemical shifts, temperature dependence of amide proton shifts and proton-proton scalar couplings. ^{1}H chemical shifts and conformationally important homonuclear coupling constants were extracted from a resolution enhanced 1D spectrum or, in case of signal overlap, from selective TOCSY experiments. Solvent accessibilities were studied by temperature coefficients for the hydroxide and amide protons to identify possible H bonding sites. One-bond residual dipolar ^{1}H-^{13}C couplings were measured in weakly oriented liquid crystalline media. For the measurement of ^{1}H, ^{13}C residual dipolar couplings F1 coupled gradient selected, sensitivity enhanced HSQC spectra modified with a G-BIRD module were used.

4. Results

1. Conformational investigations of modified cell wall peptides

We have conducted a comparative study of the conformations of the peptidoglycan monomer isolated from *Brevibacterium divaricatum* and novel lipophilic derivatives of PGM bearing either (adamantyl-1-yl)-acetyl or Boc-Tyr substituents.



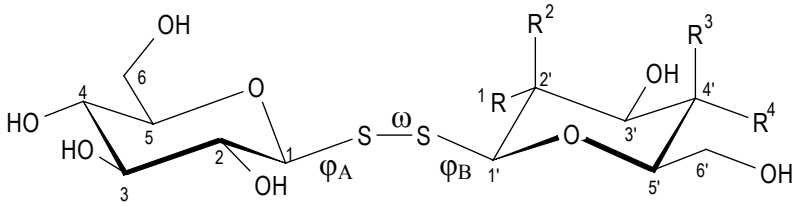
Structures of the peptidoglycan monomer and its derivatives

Analyses of the results suggest that the lipophilic substituents attached to the Dap^3-ε amino group of the parent PGM molecule introduce changes to the conformational preferences of the peptide moiety. In PGM electrostatic interactions between charged end groups apparently promote folded conformations with participation of the long Dap side chain. Derivatives wherein such interactions are suppressed by acylation of the Dap^3-ε amino group are characterized by more extended conformations of the peptide chain. The new

synthetic derivatives exhibit biological properties similar to those of the parent PGM. This may indicate that peripheral parts of the peptide chain such as the C-terminal and end groups of the long Dap side chain do not significantly contribute to the binding to receptors or enzymes under physiological conditions.

2. Conformational preferences of diglycosyl disulfides

Conformational preferences of a novel class of glycomimetics, non-symmetric oligosaccharides containing a three-bond disulfide linkage in place of the interglycosidic oxygen were investigated.



	R^1	R^2	R^3	R^4
Glc-S-S-Man	H	OH	H	OH
Glc-S-S-Gal	OH	H	OH	H
Glc-S-S-NAcGlc	NAc	H	H	OH

Temperature coefficients for the hydroxide and amide protons in DMSO solutions indicate that majority of the labile protons was exposed to the solvent and not involved in hydrogen bonding. The exchange with the solvent was found to be somewhat restricted in the case of the amide proton in Glc-S-S-NAcGlc.

NOESY/ROESY spectra recorded under similar conditions revealed a few conformationally relevant interannular NOEs contacts for all three derivatives.

One-bond residual dipolar ^{1}H-^{13}C couplings (RDC) were measured in aqueous weakly oriented media. Comparison of ^{13}C chemical shifts revealed no significant conformational changes between the structures in DMSO solution and in the aqueous media. Relative magnitudes of the RDCs were found to be consistent with the stereochemistry of carbon centres within the monosaccharide rings, but structure determination on the basis of RDCs was not attempted thus far.

Experimental NMR data were supplemented by force-field calculations. Systematic search around the M_A, M_B and T angles as well as simulated annealing calculations resulted in

18 minima in the conformational space for each of the three disaccharides investigated. The M angles indicate staggered conformations around the C1-S bond, that is $+60^o$ and -60^o for synclinal arrangements and 180^o for antiperiplanar arrangement. Conformers featuring exo-anomeric effect at their M angles dominate among the low energy conformers. The conformation around the disulfide bond, ω, was found to be close to $\pm 90^o$.

Conformational families were divided into subgroups of low energy of conformations. The larger subgroup includes the first 9 lowest energy conformations. Within these, a separate subgroup incorporating the first 4 lowest energy structures can be distinguished. 3 conformers within the latter group were found to be identical for all three derivatives with insignificant variation in the E_{pot}.

None of the minimum conformation can be used as 'single state model' to satisfactorily account for the experimental data observed. The intraresidual NOE's observed were found to be in agreement with conformations among low energy structures. A H bonding interaction was identified in the lowest energy conformer satisfying all the interresidual NOE's for derivative Glc-S-S-NAcGlc.

Experimental data suggested the presence of several different, time-averaged conformations, however the computational results were indicative of definite conformational preferences in these novel disulfide disaccharide mimics. Thus, the increase of the degrees of motional freedom, expected a priori upon extension of the interglycosidic linkage, gets largely offset by stereoelectronic effects, such as the exo-anomeric effect, the preference of the disulfide torsion angle towards $\pm 90^o$ and the existence of interresidual H bonding interactions in case of the Glc-S-S-NAcGlc.

3. Accurate determination of small one-bond heteronuclear residual dipolar couplings

A modification of the F1-coupled HSQC experiment was proposed for the accurate determination of one-bond heteronuclear residual dipolar couplings. The modification consists of inserting a G-BIRD$^{(r)}$ module to refocus the long-range coupling evolution of the heteronucleus during the t_1 frequency labelling period. As a result, the crosspeaks obtained are split only by the direct one-bond coupling that can be extracted by measuring simple frequency differences between singlett maxima. Additionally the decoupling of long-range multiple bond splittings leads to considerable sensitivity enhancement.

4. Bandselective suppression of unwanted signals in oligosaccharide spectra

A general scheme was proposed for eliminating disturbing signals of some protecting groups from the 2D NMR spectra of oligosaccharides by making use of a band-selective suppression – restoration sequence that serves as a preparation step to various 2D NMR experiments. This consists of a preparation step whereby band-selective suppression of unwanted signals is followed by a TOCSY transfer to recover useful resonances in the spectral region of interest. When the element is inserted in the place of the initial 90^{o} ^{1}H pulse of any sequence, it yields a transverse magnetisation arising from the useful signals that is further manipulated by the remaining parts of the specific sequence. Modified versions of 2D methods commonly used for assignment (such as COSY, TOCSY, HSQC, HMBC) yielded spectra displaying signals of useful anomeric and sugar ring protons without disturbing signals arising from protecting groups, thus, unambiguous assignments of the substances become possible.

5. Possible utilization of the results

The peptidoglycan monomer and its derivatives have been shown to possess versatile biological activity, investigations of their conformation can, therefore, help to reveal the structural basis of the biological functions. The result of such studies could be of value in design of further analogues of medical importance.

Understanding the conformational features of disaccharides featuring disulfide interglycosydic linkage might play a role in devising new potential inhibitors of glycoside hydrolyse enzymes.

In recent years residual dipolar couplings has become increasingly important in structure determination of molecules by NMR spectroscopy. The proposed modification of the measurement F1 coupled HSQC technique offer an accurate and simple way to determine such NMR parameters, and therefore is expected to provide more reliable data.

The modification of common NMR techniques suggested for suppressing disturbing signals might find application in aiding resonance assignments of oligosaccharides with NMR spectra of overlapping signals such as intermediate products bearing protecting groups of multistep carbohydrate synthesis.

6. List of publications

1. Kövér KE, ***Fehér K***, Szilágyi L, Borbás A, Herczegh P, Lipták A (2000). "2D NMR spectra of oligosaccharides enhanced by band-selective suppression of unwanted signals." ***Tetrahedron Letters*** 41(3): 393-396.

2. ***Fehér K***, Berger S, Kövér KE (2003). "Accurate determination of small one-bond heteronuclear residual dipolar couplings by *F1* coupled HSQC modified with a G-BIRD$^{(r)}$ module", ***Journal of Magnetic Resonance,*** *in press*

3. ***Fehér K***, Pristovšek P, Szilágyi L, Ljevaković Đ, Tomašić J (2003). Modified Glycopeptides Related to Cell Wall Peptidoglycan: Conformational Studies by NMR and Molecular Modelling, ***Bioorganic & Medicinal Chemistry,*** 11: 3133-3140.

Other publications

1. Szilágyi L, and ***Fehér K*** (1998). "Oligomycins B and C: complete ab initio assignments of their H-1 and C-13 NMR spectra and a study of their conformations in solution." ***Journal of Molecular Structure*** 471(1-3): 195-207.

2. Borbás A, Szabovik G, Antal Z, ***Fehér K***, Csávás M, Szilágyi L, Herczegh P, Lipták A (2000). "Sulfonic acid analogues of the sialyl Lewis X tetrasaccharide." ***Tetrahedron-Asymmetry*** 11(2): 549-566.

Oral presentations in conferences

1. "Structural investigations of macrocyclic antibiotics and carbohydrates by NMR spectroscopy" 1998, *National Scientific Student Conference, Debrecen, Hungary*

2. "Assignments and Conformational Studies of Oligomycin Antibiotics by ^{1}H and ^{13}C NMR" 1999, *14th NMR Valtice, Meeting of the Central European NMR Discussion Groups, Valtice, Chech Republic*

3. "Band-selective suppression of signals in the 2D NMR spectra of oligosaccharides" 2000, *15th NMR Valtice, Meeting of the Central European NMR Discussion Groups, Valtice, Chech Republic*

4. "Using residual dipolar couplings for structural studies of molecules" 2002, *Doktorandenseminar des Instituts für Analytische Chemie, Wilhelm-Ostwald-Tagungsstätte, Großbothen, Germany*

Poster presentations in conferences

1. "Enhancement of 2D NMR spectra by band-selective suppression of unwanted signals" *K. E. Kövér, K. Fehér and L. Szilágyi,* ***1999, Central European NMR Symposium and Bruker User Meeting, Szeged, Hungary***

2. "NMR investigations of sialyl Lewis X analogs" *K. Fehér, L. Szilágyi, A Borbás, A Lipták* ***2000, Conference of the Hungarian Chemical Society, Debrecen, Hungary***

3. "Modified Cell Wall Glycopeptides: Conformational Studies by NMR and Molecular Modelling" *K. Fehér, L. Szilágyi, M. Kvederč, B. Koji-Prodič and J. Tomašič* ***2000, 22th Discussion Meeting of the Magnetic Resonance Spectroscopy Division of the German Chemical Society, Regensburg, Germany***

4. "Conformational preferences in diglycosyl disulfides", *L. Szilágyi, K. Fehér, A. Bényei, T-Z. Illyés*, ***2001, 11th European Carbohydrate Symposium, Lisbon, Portugal***

5. "Solute-solvent interactions in carbohydrate-water-TFE system studied by intermolecular NOE", *K. Fehér, S. Berger,* ***2002, 16th European Experimental NMR Conference, Prague, Chech Republic***

6. "Measurement of small one-bond residual dipolar couplings by a modified HSQC sequence", K. Fehér, K. E. Kövér, L. Szilágyi, S. Berger, ***2002, 24th Discussion Meeting of the Magnetic Resonance Spectroscopy Division of the German Chemical Society, Bremen, Germany***