NMR Studies of Glycopeptides

Γ-HMBC: An NMR Experiment for the Conformational Analysis of the *o*-Glycosidic Linkage in Glycopeptides**

*Sergey Ilin, Carlos Bosques, Christopher Turner, and Harald Schwalbe**

Carbohydrates play a major role in many processes, such as the immunological response, and in cellular recognition events in general.[1] The structural characterization of carbohydrates by NMR spectroscopy has been a challenge because of the lack of experimental parameters and the intrinsic high mobility and resulting conformational averaging of oligosaccharides in solution. For example, the conformation around

[*] Prof. Dr. H. Schwalbe
Institut für Organische Chemie und Chemische Biologie
Zentrum für Biomolekulare Magnetische Resonanz
Johann Wolfgang Goethe-Universität Frankfurt am Main
Marie-Curie-Str. 11, 60439 Frankfurt am Main (Germany)
Fax: (+49) 69-7982-9515
E-mail: schwalbe@nmr.uni-frankfurt.de

S. Ilin, Dr. C. Bosques, Dr. C. Turner
Department of Chemistry and MIT/Harvard Center for Magnetic Resonance at the Francis Bitter Magnet Laboratory
Massachusetts Institute of Technology, Department of Chemistry
170 Albany Street, Bldg. NW14, Cambridge, MA 02139 (USA)

[**] We thank M.I.T., the Winnacker Foundation, and the Sloan Foundation for financial support. Spectra were recorded at the Bitter Laboratory (supported by NIH grant RR-00995) and the Spectroscopy Facilities at M.I.T. We thank Dr. J. Klein-Seetharaman for stimulating discussions.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  0044-8249/03/11512-1432 $ 20.00+.50/0

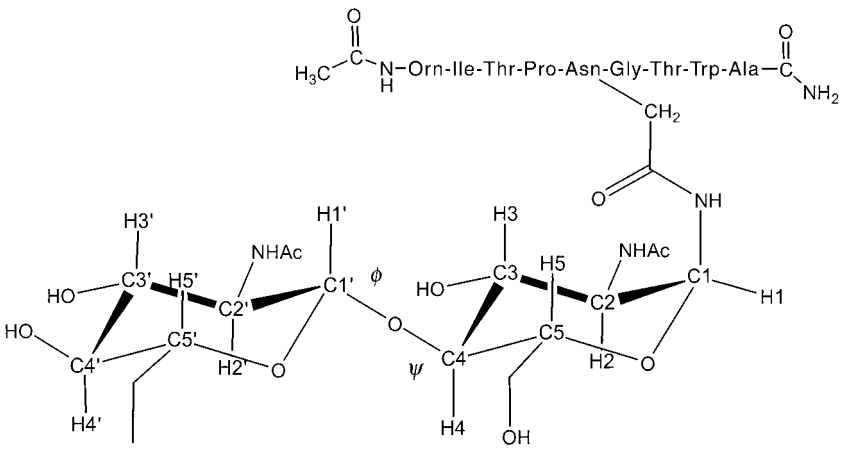

Figure 1. {[GlcNAc-β(1-4)-GlcNAc-α-]Asn}$^{286}_{281-290}$-hemagglutinin peptide **1** used as a model substance for a glycopeptide. The nomenclature follows IUPAC recommendations for carbohydrates, where φ and ψ are the O5-C1-OX-CX and C3-C4-OX-CX torsion angles, respectively; three-letter codes are used for amino acids.

the exocyclic glycosidic bonds cannot unambiguously be determined from measurement of inter-residue ^{1}H–^{1}H NOE measurements.[2] For oligosaccharides in solution, residual dipolar couplings that are partially oriented in bicelles [3] are not very sensitive to conformational averaging, as shown by NOE experiments. Only two ^3J(C,H) coupling constants can be observed as a result of varying the exocyclic torsion angles, and current methods for their measurement are insensitive for larger molecules.

We have therefore developed a new experiment, the *Γ*-HMBC (HMBC = heteronuclear multiple-bond correlation), in which four NMR parameters, ^3J(C,H) couplings, and cross-correlated relaxation rates $\Gamma^c_{C_iH_i,C_iH_j}$ are measured that can describe the conformation of the glycosidic torsion angles ϕ and ψ in oligosaccharides.[4] It relies on a correlation developed by Vincent et al.[5] in which coherence transfer between hydrogen and carbon atoms across the glycosidic linkage is achieved by C–H-dipolar cross-correlated relaxation. In the experiment, an initial antiphase operator $2H1'_zC1'_x$ (representing the annotated atoms in Figure 1) evolves into the operator $2H4_zC1'_x$ during a constant time delay CT (Figure S1 in Supporting Information). Correlation peaks in the experiment are generated through cross-correlated relaxation[6] between two dipoles centered on the same carbon atom. The cross-correlated relaxation rate is given by Equation (1):

$$\Gamma^c_{C_iH_i,C_iH_j} = \frac{2}{5}\frac{\gamma_H^2\gamma_C^2}{r^3_{C_iH_i}r^3_{C_iH_j}}\left(\frac{\mu_0}{4\pi}\right)^2\hbar^2\left(S^c_{ij}\right)^2\left(\frac{3\cos^2\theta_{ij}-1}{2}\right)\tau_c \qquad (1)$$

In this equation, γ_C and γ_H are the gyromagnetic ratios, μ_0 is the susceptibility of the vacuum, $r_{C_iH_i}$ and $r_{C_iH_j}$ are the carbon–proton separations, $\hbar$ is the Planck constant, S^c_{ij} is the order parameter, and τ_c is the overall correlation time. This variable depends on the projection angle (θ_{ij}) between the two dipole tensors which, in turn, depends on the torsion angles ϕ and ψ.

The operator $2H4_yC1'_z$ generated prior to t_2 can be detected by NMR and is modulated by the ^3J(C1′,H4) coupling. Therefore, ^3J(C,H) coupling constants can be determined from the spectra shown in Figure 2a–d by fitting spectral peaks with simulated multiplets with Lorentzian line shapes. This approach is related to the Keeler–Neuhaus–Titman approach for the determination of ^3J(C,H) coupling constants from ω_2-coupled HMBC-spectra.[7] However, in the original HMBC experiment, cross-peaks are phase-modulated by evolution of homonuclear ^3J(H,H) couplings during a fixed delay, which causes a complex line shape. The phase distortion in the HMBC requires the acquisition of two separate experiments to simulate the line shape of the target multiplet.

In contrast, in the experiments described here, the signal detected in t_2 is not phase distorted and split only by a single J(H,C) coupling in antiphase.[8] Experimental and simulated multiplets for H1′C1′ and H1′C4 cross-peaks in the glycopeptide **1** (Figure 1) are shown in Figure 2. The cross-peak H1′C4 (Figure 2b) is modulated by the same ^3J(H,H) couplings and transverse relaxation rate as either the upfield or downfield part of the related, direct cross-peak (H1′C1′) (Figure 2a); it differs only by its magnitude and by convolution with an antiphase long-range ^3J(C,H) coupling. Homonuclear ^3J(H,H) coupling constants and proton transverse relaxation rates were determined by fitting to both the upfield and downfield components of the cross-peak H1′C1′ that is observed with a high signal-to-noise ratio. These parameters were kept fixed in the simulation for the long-range ^3J(C,H)

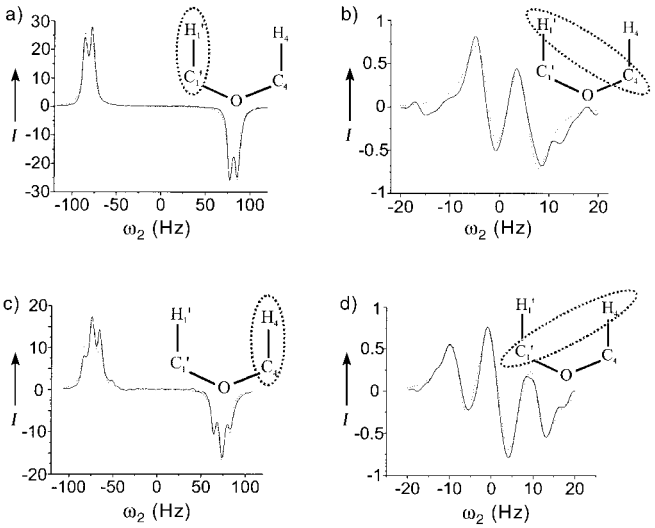

Figure 2. Experimental (-----) and simulated (•••••) multiplets. a) H1′C1′; b) H1′C4, ^3J(C,H) = 3.3 ± 0.2 Hz; c) H4C4; d) H4C1′, ^3J(C,H) = 1.2 ± 0.2 Hz. The measurement time was 48 h, and the sample concentration was 4 mM. Experimental parameters are given in Figure S1 of the Supporting Information.

  0044-8249/03/11512-1433 $ 20.00+.50/0

Table 1: Experimental transverse relaxation rates R_2, ^3J(H,C) and ^3J(H,H) coupling constants, and regression coefficient R^2 fit to the multiplets shown in Figure 2 a–d.

	H4C1′	H1′C4	H1C5
R_2 [s^{-1}]	6.9	6.5	6.7
^3J(H,H) [Hz]	8.3, 10.4	8.2	5.1
^3J(H,C) [Hz][a]	1.2 ± 0.2	3.3 ± 0.2	6.5 ± 0.2
R^2	0.97	0.93	0.97

[a] Active heteronuclear ^3J(C,H) coupling constant leading to the indicated cross-peak.

couplings in the H1′C4 cross-peak, as shown in Figure 2 b and d for the fits with the lowest χ^2 value. The complete statistics on the calculations of cross-peak parameters are given in Table 1. The torsion angles ϕ and ψ were calculated using the Karplus equation for ^3J(C,H) in oligosaccharides, polysaccharides, and glycoconjugates;[9] the results are listed in Table 2. The angles determined by this method are ambiguous because each coupling (J) can arise from up to four different torsion angles, and the conformation around the glycosidic bond is described by the two torsion angles ϕ and ψ. This ambiguity can be resolved by calculating the cross-correlated relaxation rates from the experimental intensities of the multiplets (Figure 2) according to Equation (2).

$$\frac{\sinh(\Gamma^{c}_{C_1'H_1',C_1'H_4}CT)}{\sinh(\Gamma^{c}_{C_5H_5,C_5H_1}CT)} = \frac{I_{C_1'H_1',C_1'H_4}}{I_{C_5H_5,C_5H_1}} \qquad (2)$$

The cross-correlated relaxation rate measured for cross-peaks across the glycosidic bond was internally referenced to the known cross-correlated relaxation rate $\Gamma^{c}_{C_5H_5,C_5H_1}$ observed within the sugar ring.[10] Variation of the relaxation rates, as a function of the torsion angles ϕ and ψ, is shown in Figure 3 using standard parameters (bond lengths: C–H 1.09 Å, C–O 1.42 Å; bond angles: H-C-O 109.7°, glycosidic C-O-C 115.5°, internal C-O-C 112.0°). The H1C5 cross-peak was used as a reference (torsion angle of −175°).

Out of sixteen possible combinations of ϕ and ψ angles based on coupling data, the cross-correlated relaxation data support two pairs of angles, where $\phi = 10°$ or 110°, and $\psi = 12°$ (Figure 3). Molecular dynamics (MD) simulations and an X-ray crystal structure are available for an analogue (GlcNAc-β(1-4)-GlcNAc) of the sugar moiety in the glycopeptide **1** investigated here. The MD simulation shows considerable flexibility (with $0° \le \phi \le 70°$ and $-75° \le \psi \le 15°$) and average torsion angles of $\phi = 46 \pm 13°$ and $\psi = -10 \pm 16°$.[11] In the crystal structure, ϕ and ψ assume values

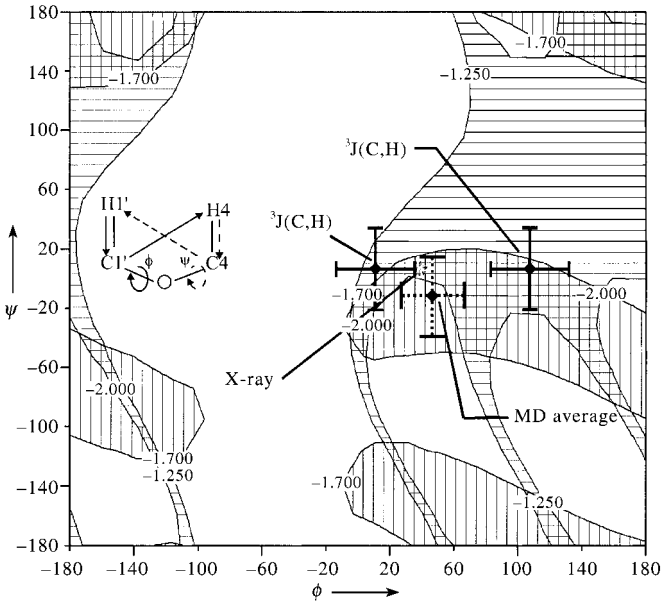

Figure 3. Overlay of simulated cross-correlated relaxation rates $\Gamma^{DD,DD}_{C_4H_1',C_4H_4}$ (horizontal line) and $\Gamma^{DD,DD}_{C_1'H_4,C_1'H_1'}$ (vertical line) as a function of φ and ψ. Crossover indicates experimentally allowed combinations. Single points with error bars indicate torsion angles derived from ^3J(C,H) coupling constants; "X-ray" and and "MD average" indicate angles derived from the crystal structure and MD simulations, respectively.

Table 2: A summary of coupling constants, torsion angles, and relaxation times.

	J [Hz][a]	φ,ψ [°][b]	$\Gamma^{DD,DD}$ [s^{-1}][c]
^3J(H1′,C4)	3.3 ± 0.2	ϕ:10 ± 20,110 ± 20,79 ± 20,-162 ± 20	$\Gamma_{C_4H_1',C_4H_4} = -1.4$
^3J(H4,C1′)	1.2 ± 0.2	ψ:12 ± 20,55 ± 20,-132 ± 20,-175 ± 20	$\Gamma_{C_1'H_4,C_1'H_1'} = -4.4$
^3J(H1,C5)	6.5 ± 0.2	±175 ± 5	$\Gamma_{C_5H_1,C_5H_5} = -1.5$

[a] ^3J(H,C) coupling constants. [b] Torsion angles from ^3J(H,C) coupling constants based on Karplus equation: $^3J(H,C) = 5.7\cos^2\alpha - 0.6\cos\alpha + 0.5$. Estimated torsion angle uncertainties are given. [c] Cross-correlated relaxation rates.

of 36.3° and 11.7°, respectively.[12]. NMR data of the species in solution supports a similar region of preferred conformation ($\phi = 10 \pm 20°$ and $\psi = 12 \pm 20°$). While identical values are found for ψ in the crystal and in solution, the torsion angle ϕ is shifted both in the MD and in the NMR data. Values of angle ϕ of approximately 40° are in agreement with predictions based on the exo-anomeric effect.

In conclusion, a novel method is introduced to determine the conformation around glycosidic bonds based on ^3J(C,H) couplings and C–H-dipolar cross-correlated relaxation by solution NMR spectroscopy. Using conventional NMR probes, the data can be obtained for glycopeptides without ^{13}C labeling at a concentration of 5 mM. The experiment is shorter than normal HMBC experiments as long periods with transverse proton magnetization are avoided. Extension of the method to the study of large polysaccharides is expected to add to a structural understanding of the role of carbohydrates in cell recognition[13] and may aid in the design of novel carbohydrate-based drugs.[14]

Experimental Section

Sample preparation: Glycopeptide **1** {[GlcNAc-β(1-4)-GlcNAc-α-]Asn}$^{286}_{281-290}$-hemagglutinin was synthesized using standard 9-fluorenyl methoxycarbonyl (Fmoc) chemistry and solid-phase peptide synthesis techniques. The α-linked chitobiose moiety was introduced as the Fmoc-Asn (TBDMS-chitobiose)-OH building block. The predicted mass for glycopeptide **1** (1421.5) was confirmed by ESI-MS. Although GlcNAc-β(1-4)-GlcNAc is the natural core in N-linked glycopro-

  0044-8249/03/11512-1434 $ 20.00+.50/0

teins, the anomeric center on the first sugar moiety in glycopeptide **1** investigated here was in the α conformation. All data were acquired on Bruker DRX 600 and Homebuilt 600 spectrometers with a 5 mm Bruker TXI ^{1}H {$^{15}N/^{13}C$} z-grad probe and Nalorac triple resonance probes, respectively. Information about the pulse sequence shown is given in the Supporting Information. The concentration of glycopeptide **1** was 4.5 mM (6 mg mL^{-1}) in a volume of 300 μL using a Shigemi tube. The sample temperature was 278 K.

Received: November 15, 2002 [Z50564]

Keywords: conformational analysis · glycosides · NMR spectroscopy · oligosaccharides · structural biology

[1] *Polysaccharides: Structural Diversity and Functional Versatility* (Ed.: S. E. Dumitriu), Marcel Dekker, New York, **1998**; P. M. Rudd, T. Elliot, P. Cresswell, I. A. Wilson, R. A. Dwek, *Science* **2001**, *291*, 2370–2375; S. E. O'Connor, B. Imperiali, *J. Am. Chem. Soc.* **1997**, *119*, 2295–2296; *NMR Spectroscopy of Glycoconjugates* (Ed.: J. Jiménez-Barbero, T. Peters), Wiley-VCH, Weinheim, **2002**.

[2] S. W. Homans, *Prog. Nucl. Magn. Reson. Spectrosc.* **1990**, *22*, 55–81; C. A. Bush, M. M. Pastor, *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 269–293.

[3] F. Tian, H. M. Al-Hashimi, J. L. Craighead, J. H. Prestegard, *J. Am. Chem. Soc.* **2001**, *123*, 485–492; H. Neubauer, J. Meiler, W. Peti, C. Griesinger, *Helv. Chim. Acta* **2001**, *84*, 243–258.

[4] O. Jardetzky, *Biochim. Biophys. Acta* **1980**, *621*, 227–232; A. Parfondry, N. Cyr, A. S. Perlin, *Carbohydr. Res.* **1977**, *59*, 299–309; M. L. Hayes, A. S. Serianni, R. Barker, *Carbohydr. Res.* **1982**, *100*, 87–101; J. M. Duker, A. S. Serianni, *Carbohydr. Res.* **1993**, *249*, 281–303.

[5] S. J. F. Vincent, C. Zwahlen, *J. Am. Chem. Soc.* **2000**, *122*, 8307–8308.

[6] B. Reif, M. Hennig, C. Griesinger, *Science* **1997**, *276*, 1230–1233; H. Schwalbe, T. Carlomagno, M. Hennig, J. Junker, B. Reif, C. Richter, C. Griesinger, *Methods Enzymol.* **2001**, *338*, 35–81.

[7] J. J. Titman, D. Neuhaus, J. Keeler, *J. Magn. Reson.* **1989**, *85*, 111–131; J. M. Richardson, J. J. Titman, J. Keeler, *J. Magn. Reson.* **1991**, *93*, 533–553.

[8] H. Schwalbe, W. Samstag, J. W. Engels, W. Bermel, C. Griesinger, *J. Biomol. NMR* **1993**, *3(4)*, 479–486.

[9] I. Tvaroska, M. Hricovinin, E. Petrakova, *Carbohydr. Res.* **1989**, *189*, 359–362; S. A. Wacowich-Sgarbi, C. C. Ling, A. Otter, D. R. Bundle, *J. Am. Chem. Soc.* **2001**, *123*, 4362–4363.

[10] Uniform ^{13}C-T_1 relaxation times of 0.32 ± 0.02 s were measured for **1**. s^c_{ij} values were therefore assumed to be similar for the corresponding peaks generated through cross-correlated relaxation.

[11] R. J. Woods, A. Pathiaseril, M. R. Wormald, C. J. Edge, R. A. Dwek, *Eur. J. Biochem.* **1998**, *258*, 372–386.

[12] F. Mo, L. H. Jensen, *Acta Crystallogr. Sect. B* **1978**, *34*, 1562–1569.

[13] A. Varki, *Glycobiology* **1993**, *3*, 97–130.

[14] J. McAuliffe, O. Hindsgaul, *Chem. Ind.* **1997**, *5*, 14–26.

  0044-8249/03/11512-1435 $ 20.00+.50/0