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Breaking Pseudo-Symmetry in Multiantennary Complex N-Glycans Using Lanthanide-Binding Tags and NMR Pseudo-Contact Shifts**

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Molecular recognition is of vital significance for life. Understanding the chemical basis of these interactions between cellular receptors and their specific ligands not only gives a functional meaning to structures and changes occurring in diseases but also helps devise innovative therapeutic approaches. In terms of biological coding and translating signals into cellular effects, glycans have gained a particular status, owing to their unsurpassed coding capacity and widespread presence of receptors (lectins) to read the encoded information.[1] The glycan sequence and shape and the intimate interplay between a glycan determinant and its cognate lectin ensure the flow of information of sugar coding.[2] Thus, in addition to their peptide scaffold, glycoproteins carry a second source of bioinformation in their glycan chains, realized, for example, by recognition of N-glycans by lectins.[3]

In this context, we herein present a novel NMR approach to individually monitor the behavior of each arm, A and B, of N-glycans (Scheme 1) and thereby provide a global perspective of their conformational and interaction features in solution. Structurally, N-glycans have a common pentasaccharide core composed of an *N*,*N*′-diacetyl chitobiose (*N*-glycosidically linked to the Asn at the protein chain) substituted at position O-4 of its nonreducing moiety by a trimannoside, as shown in Scheme 1. Further extensions of the external Man A and Man B residues generate the different N-glycan classes, and internal substituents at the core further increase the panel size of N-glycans.[4,5]

The conformational features of the different classes of N-glycans (from complex- to high-mannose type) have been investigated using different techniques, especially NMR spectroscopy,[6] since the flexibility of the glycosidic linkages has usually hampered their study by X-ray crystallography. However, typical NMR parameters (*J* couplings and NOEs) provide only short-range structural information (within a few Ångström).[7] Therefore, it is not easy to define the global shape of these nonglobular and relatively flexible molecules. Of course, NOEs and *J* couplings may define possible accessible conformations around each glycosidic linkage, but they cannot provide a global conformational model in solution. Especially, in our target molecule **1** (Scheme 1), the existence of eight glycosidic linkages amplifies the level of uncertainty in the relative positions of the two terminal Gal (A and B) moieties with respect to the reducing-end GlcNAc unit. In general, the limited number of available interresidual restraints in symmetrical complex-type N-glycans and the recurrent signal overlapping, precludes obtaining nonambiguous NMR parameters with conformational information.

In fact, for **1**, all the ^{1}H and ^{13}C chemical shifts of the terminal Gal and GlcNAc moieties as well as those of the C3, C4, and C6 atom pairs of Man residues A and B are isochronous. Therefore, the individual behavior of the two inner glycosidic linkages of the A and B arm cannot be estimated by typical NMR analysis. Differing only in their attachment points, compound **1** displays a pseudo-symmetry for the two arms, which renders them indistinguishable by conventional NMR techniques. However, biological recognition of N-glycans tends to be branch-specific in many cases. Despite the lack of molecular rationales for these findings, sialylation, galactosylation, and lectin recognition of N-glycans were found to show branch specificity.[8]

Our method is based on exploiting pseudo-contact shifts (PCS) induced by paramagnetic lanthanide ions.[9,10] Recent studies have demonstrated their applicability in carbohydrate conformational analysis of small oligosaccharides.[11–15] It is well-known that paramagnetic metal ions (such as most lanthanides) cause PCS, enhanced nuclear relaxation (PRE), and molecular alignment in the presence of magnetic fields.[16] Lanthanides offer a wide range of paramagnetic properties, and thus allow access to detailed structural information,

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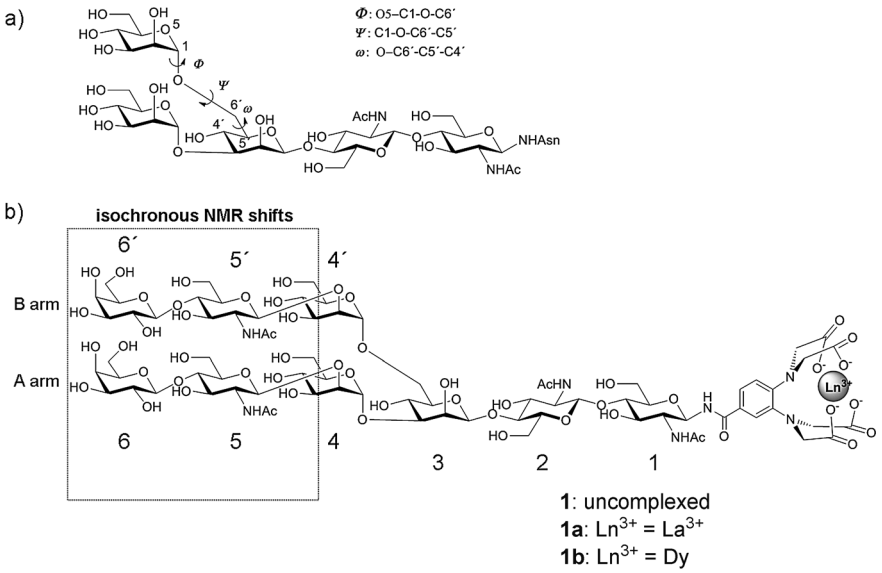

Scheme 1. a) Common pentasaccharide core of N-glycans. The torsional angles that define the conformation around the Manα1-6Man linkage are defined. b) Nonasaccharide derivatives studied in this work. The 1–3 and 1–6 arms attached to the β-mannose unit are labeled as A and B arms, respectively.

because of the spatial anisotropy of the induced paramagnetic effects.[17]

In the context of oligosaccharides, lanthanide-binding tags have been designed to decrease the effect of the paramagnetic relaxation enhancement (PRE), by separating the metal from the biomolecule by a rigid structure.[11–13] The synthesis of **1** was based on the corresponding anomeric azide[18] and planned following our previously tested strategy (see the Supporting Information).

As first step, ^{1}H, ^{1}H–^{13}C HSQC and HSQC-TOCSY NMR spectra were acquired for **1** in the presence of one molar equivalent of either La^{3+} (**1a**) as diamagnetic reference or Dy^{3+} (**1b**) as paramagnetic ion (Figure 2). The ^{1}H/^{13}C signals of the terminal Gal, GlcNAc, and Man (3, 4, and 6 positions) have isochronous chemical shifts in the HSQC spectra of both **1** and **1a**. In the case of **1b**, the aromatic signals as well as those of the GlcNAc-1 residue (and most of GlcNAc-2) disappeared since they are very close to the metal, while the other sugar signals were clearly visible in the HSQC spectra. Significant shifts were observed because of the presence of the paramagnetic ion and, strikingly, signal separation was seen in the Dy^{3+}-containing sample **1b** for the identical residues of the A and B arms (see Figure 2). Therefore, in the presence of the paramagnetic metal, the ^{1}H and ^{13}C resonances for each α-Man, GlcNAc, and Gal residues at either A or B arms can be easily distinguished. In fact, 34 ^{1}H NMR PCS (see Table S1 in the Supporting Information) could be measured in a nonambiguous manner, ranging from 0.03 (H4 Gal A) to 0.94 ppm (H3 GlcNAc-2). The estimated distance between H4 Gal A and the metal is larger than 30 Å.

Recently, a detailed theoretical MD analysis of the biantennary N-glycan part of **1** has been presented.[19] This work describes the existence of five conformers (Figure 1) in equilibrium, especially differing in the ψ and ω angles of the Manα1→6Man linkage. According to the MD data, all the Φ glycosidic torsions were defined by the *exo*-anomeric effect, while the rest of the ψ angles displayed moderate flexibility around the global minimum. The different conformers of the Manα1→6Man linkage were populated between 11% ("extended gt conformer", with ψ 180°, ω 180°) and 41% ("back-folded" conformer, with ψ 90°, ω 60°). Additional contributions of the "extended gg" (15%, with ψ 180°, ω 60°), "half back-folded" (15%, with ψ 60°, ω 60°), and "tight back-fold" (17%, ψ 60°, ω 180°) were also predicted by the simulations.[19]

Thus, the observed PCS were interpreted considering the presence of the postulated low-energy conformers[19] for the oligosaccharide of **1** (Figure 1). Inspection of the geometries of the five different conformers indicated that the

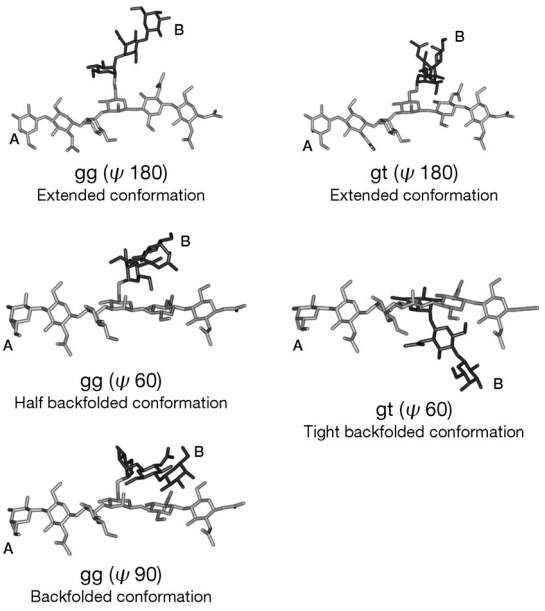

Figure 1. Minimum-energy conformations of free **1** according to MD calculations.[19] Structures were built and energy minimized using MacroModel/Batchmin package (version 9.6) and the MM3* force field.[20] The B arm trisaccharide is shown in black.

nuclei of the B arm are always closer to the paramagnetic metal than their analogs at the A arm, independently of the chosen conformation. Therefore, as first approximation, the peaks arising from the larger PCS were assigned to the nuclei of the B-arm. This initial assumption was backed by comparison of the back-calculated PCS values with the experimental ones.

The individual fittings for the five independent conformers of **1**[19] using MSpin software[21] are given in Table S1.

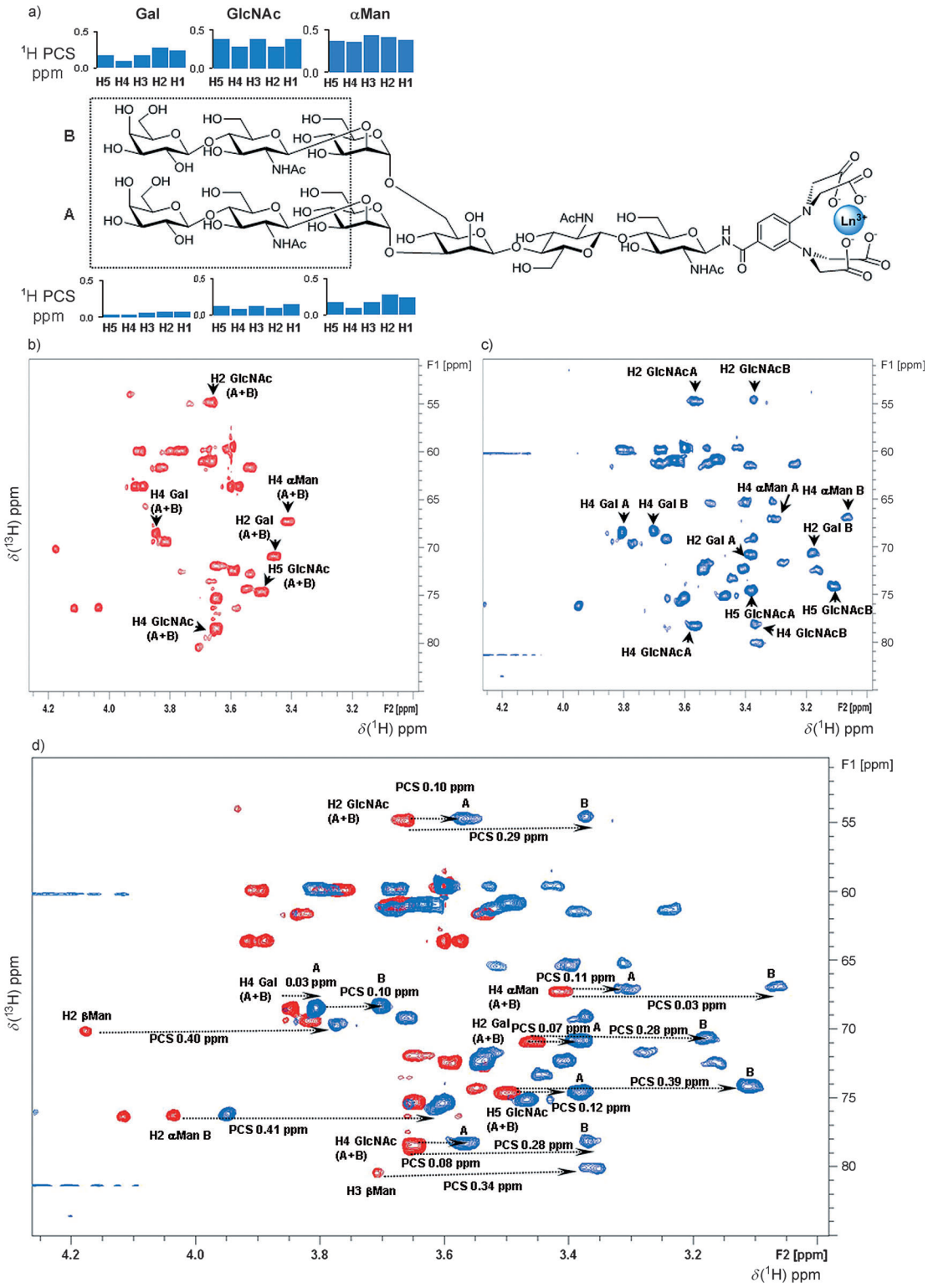

Figure 2. Pseudo-contact shifts arise from dipolar interactions between the unpaired electrons of a metal ion and the nuclei in the vicinity. PCS give rise to changes in chemical shifts that decrease with the distance between the metal ion and the nuclear spin with a $1/r^3$ dependence. PCS are measured by comparison of the spectrum of a complex with a diamagnetic ion with that of a paramagnetic ion. a) PCS values measured for the two arms of **1** are shown as bar graphs. b) ^{1}H-^{13}C HSQC spectrum of **1a**, (diamagnetic reference, $Ln^{3+} = La^{3+}$). c) ^{1}H–^{13}C HSQC spectrum of **1b**, (paramagnetic sample, $Ln^{3+} = Dy^{3+}$). The signals of Gal, GlcNAc and αMan of arms A and B (inside the box in 2a) display identical chemical shifts in the reference spectrum. Fittingly, they can be individually observed in the presence of Dy^{3+}. d) Superimposition of the ^{1}H–^{13}C HSQC spectra (600 MHz) acquired for **1a** and **1b**. The PCS for some of the signals are assigned.

When individually taken, none of them provided a satisfactory fit to the experimental PCS, especially for the B arm. The comparison of the experimental versus calculated PCS values for the extended and half backfolded conformations (see Figure 1) are shown in Figure 3. Indeed, the global-minimum geometry for all glycosidic torsions constituting the A-arm gave a very good fit between expected and observed PCS (Figure 3a), independently of the conformation at the B arm. Regarding the B arm, the best Q factor (0.145) and SVD condition (28.75) was obtained for the "extended gg" conformer (green dots in Figure 3b). The predicted global minimum by MD [9] gave a poorer fitting (Q factor of 0.451). Overall, a good correlation between experimental and back-calculated PCSs could not be obtained considering just one conformer.

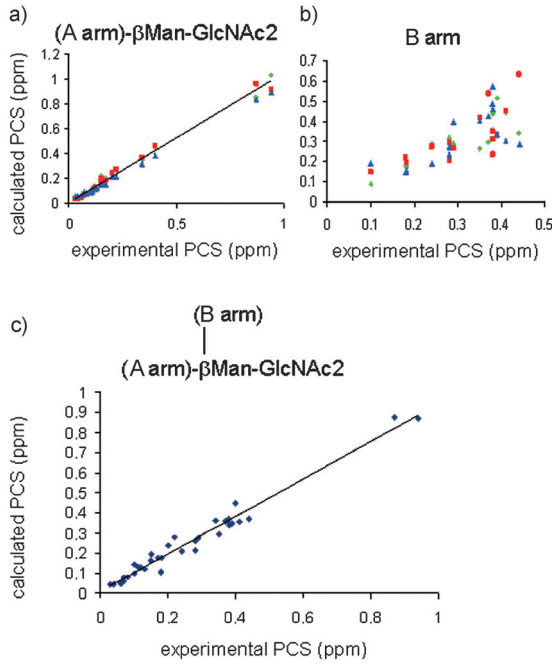

Figure 3. a,b) Correlation between experimental and computed PCSs for the protons at the "A" and "B" arms. PCSs calculated from the individual fitting of the structures of the extended conformations (green, gg (ψ 180); red, gt (ψ 180); blue, gg (ψ 60), half backfolded conformation). c) Correlation between experimental and computed PCSs for **1** calculated considering the presence of four conformations (gg (ψ 180), gt (ψ 180), gg (ψ 60), and gg (ψ 90)).

Then, combinations of the different geometries were considered and their corresponding fittings were calculated by MSpin. A very good correlation with the experimental data (Q factor of 0.10, Figure 3c) was obtained when four conformers were included, with the "extended gg" and "extended gt" as the major contributors (about 45% and 35%, respectively). The presence of the half-backfolded and backfolded conformations (10% each) gave the best fit. The employed protocol enables to experimentally define the conformational behavior in solution of the A and B arms of the complex-type N-glycan (**1**) in an independent manner.

We thus applied the differentiation of the NMR signals of the A and B arms by PCS to biorecognition. As proof-of-principle, we tested the carbohydrate recognition domain (CRD) of human galectin-3 (hGal3), a galactose-binding protein with affinity to N-glycans.[22] Addition of hGal3 to a sample containing paramagnetic **1b** (protein:**1b** = 1:8) induced further line broadening of the carbohydrate signals (Figure S1a). Clear changes were detected for the H1 and H4 protons of the Gal and GlcNAc units at the termini of both arms (positions 5, 6, 5′, and 6′). In addition, the CH_3 groups of the GlcNAc 5 and 5′ also displayed clear line broadening.

HSQC spectra of both samples were then acquired for further analysis of those signals that overlapped in the 1D NMR spectra. The analysis of the HSQC peaks allowed confirming the specific interaction of hGal3 with the Gal and GlcNAc residues at both ends of the A and B arms. This result is in line with data from precipitation analysis of N-glycans with a related receptor from the galectin family.[23] The signals of the β-Man unit of the core were not affected by the presence of the protein (Figure S1b).

In conclusion, the use of the tagged nonasaccharide derivative **1** loaded with a paramagnetic ion (**1b** Dy^{3+}) has allowed to unequivocally defining the conformational behavior of this flexible molecule in solution for the first time. The use of the lanthanide tag has permitted to break the inherent pseudo-symmetry of the NMR spectra of the identical branches, revealing that the T-shaped gg rotamer at the Manα1-6Man junction is the major one in solution, with minor contributions of other backfolded geometries. This approach has permitted the characterization of the binding epitopes of the symmetrical N-glycan **1**, showing that both arms are involved in the recognition of human galectin-3. Our approach provides new tools to further pinpoint the relevance of multiantennary N-glycans as biomedically important receptors.

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[1] H.-J. Gabius, S. André, J. Jiménez-Barbero, A. Romero, D. Solís, *Trends Biochem. Sci.* **2011**, *36*, 298–313.
[2] a) *The Sugar Code. Fundamentals of glycosciences* (Ed.: H.-J. Gabius), Wiley-VCH, Weinheim, **2009**; b) V. Roldós, F. J. Cañada, J. Jiménez-Barbero, *ChemBioChem* **2011**, *12*, 990–1005.
[3] a) S. André, T. Kozár, S. Kojima, C. Unverzagt, H.-J. Gabius, *Biol. Chem.* **2009**, *390*, 557–565; b) C. Zuber, J. Roth in *The Sugar Code. Fundamentals of glycosciences* (Ed.: H.-J. Gabius), Wiley-VCH, Weinheim, **2009**, pp. 87–100; c) M. Amano, H. Eriksson, J. C. Manning, K. M. Detjen, S. André, S.-I. Nishimura, J. Lethiö, H.-J. Gabius, *FEBS J.* **2012**, *279*, 4062–4080; d) A. Ardá, D. V. Silva, V. Schubert, S. André, M. Bruix, F. J. Cañada, H.-J. Gabius, C. Unverzagt, J. Jiménez-Barbero, *J. Am. Chem. Soc.* **2013**, *135*, 2667–2675.
[4] a) M. Aebi, R. Bernasconi, C. Clerc, M. Molinari, *Trends Biochem. Sci.* **2010**, *35*, 74–82; b) A. Antonopoulos, S. J.

North, S. M. Haslam, A. Dell, *Biochem. Soc. Trans.* **2011**, *39*, 1334 – 1340.
[5] a) S. André, C. Unverzagt, S. Kojma, X. Dong, C. Fink, K. Kayser, H.-J. Gabius, *Bioconjugate Chem.* **1997**, *8*, 845 – 855; b) C. Unverzagt, S. André, J. Seifert, S. Kojima, C. Fink, G. Srikrishna, H. H. Freeze, K. Kayser, H.-J. Gabius, *J. Med. Chem.* **2002**, *45*, 478 – 491; c) S. André, C. Unverzagt, S. Kojima, M. Frank, J. Seifert, C. Fink, K. Kayser, C.-W. von der Lieth, H.-J. Gabius, *Eur. J. Biochem.* **2004**, *271*, 118 – 134; d) S. André, T. Kozár, R. Schuberth, C. Unverzagt, S. Kojima, H.-J. Gabius, *Biochemistry* **2007**, *46*, 6984 – 6995.
[6] S. W. Homans, R. A. Dwek, T. W. Rademacher, *Biochemistry* **1987**, *26*, 6571 – 6578.
[7] M. R. Wormald, A. J. Petrescu, Y. L. Pao, A. Glithero, T. Elliott, R. A. Dwek, *Chem. Rev.* **2002**, *102*, 371 – 386.
[8] a) D. H. Jozaisse, W. E. C. M. Schiphorst, D. H. van den Eijnden, J. A. van Kuik, H. van Halbeek, J. F. G. Vliegenthart, *J. Biol. Chem.* **1985**, *260*, 714 – 719; b) A. M. Barb, E. K. Brady, J. H. Prestegard, *Biochemistry* **2009**, *48*, 9705 – 9707; c) V. Ramasamy, B. Ramakrishnan, E. Boeggeman, D. M. R, P. H. Seeberger, P. K. Qasba, *J. Mol. Biol.* **2005**, *353*, 53 – 67; d) S. Guo, T. Sato, K. Shirane, K. Furukawa, *Glycobiology* **2001**, *11*, 813 – 820; e) Y. Kaneda, R. F. Whittier, H. Yamanaka, E. Carredano, M. Gotoh, H. Sota, Y. Hasegawa, Y. Shinohara, *J. Biol. Chem.* **2002**, *277*, 16928 – 16935.
[9] T. H. Nguyen, K. Ozawa, M. Stanton-Cook, R. Barrow, T. Huber, G. Otting, *Angew. Chem.* **2011**, *123*, 718 – 720; *Angew. Chem. Int. Ed.* **2011**, *50*, 692 – 694.
[10] G. Pintacuda, M. John, X. C. Su, G. Otting, *Acc. Chem. Res.* **2007**, *40*, 206 – 212.
[11] M. Erdélyi, E. d'Auvergne, A. Navarro-Vázquez, A. Leonov, C. Griesinger, *Chem. Eur. J.* **2011**, *17*, 9368 – 9376.
[12] A. Mallagaray, A. Canales, G. Dominguez, J. Jimenez-Barbero, J. Perez-Castells, *Chem. Commun.* **2011**, *47*, 7179 – 7181.
[13] S. Yamamoto, T. Yamaguchi, M. Erdelyi, C. Griesinger, K. Kato, *Chem. Eur. J.* **2011**, *17*, 9280 – 9282.
[14] S. Yamamoto, Y. Zhang, T. Yamaguchi, T. Kameda, K. Kato, *Chem. Commun.* **2012**, *48*, 4752 – 4754.
[15] Y. Zhang, S. Yamamoto, T. Yamaguchi, K. Kato, *Molecules* **2012**, *17*, 6658 – 6671.
[16] G. Otting, *J. Biomol. NMR* **2008**, *42*, 1 – 9.
[17] I. Bertini, C. Luchinat, G. Parigi, R. Pierattelli, *Dalton Trans.* **2008**, 3782 – 3790.
[18] V. Ullmann, M. Rädisch, I. Boos, J. Freund, C. Pöhner, S. Schwarzinger, C. Unverzagt, *Angew. Chem.* **2012**, *124*, 11734 – 11738; *Angew. Chem. Int. Ed.* **2012**, *51*, 11566 – 11570.
[19] a) W. Nishima, N. Miyashita, Y. Yamaguchi, Y. Sugita, S. Re, *J. Phys. Chem. B* **2012**, *116*, 8504 – 8512; b) S. Re, N. Miyashita, Y. Yamaguchi, Y. Sugita, *Biophys. J.* **2011**, *101*, L44 – 46.
[20] F. Mohamadi, N. G. J. Richards, W. C. Guida, R. Liskamp, M. Lipton, C. Caufield, G. Chang, T. Hendrickson, W. C. Still, *J. Comput. Chem.* **1990**, *11*, 440 – 467.
[21] http://www.mestrelab.com Program MSpin.
[22] a) H. Sanchez-Ruderisch, C. Fischer, K. M. Detjen, M. Welzel, A. Wimmel, J. C. Manning, S. André, H. J. Gabius, *FEBS J.* **2010**, *277*, 3552 – 3563; b) M. Krzeminski, T. Singh, S. André, M. Lensch, A. M. Wu, A. M. J. J. Bonvin, H.-J. Gabius, *Biochim. Biophys. Acta Gen. Subj.* **2011**, *1810*, 150 – 161; c) H. Dawson, S. André, E. Karamitopoulou, I. Zlobec, H.-J. Gabius, *Anticancer Res.* **2013**, *33*, 3053 – 3060.
[23] a) D. Gupta, H. Kaltner, X. Dong, H.-J. Gabius, C. F. Brewer, *Glycobiology* **1996**, *6*, 843 – 849; b) H. Kaltner, H.-J. Gabius, *Histol. Histopathol.* **2012**, *27*, 397 – 416.

