

Efficient Chemoenzymatic Synthesis of N-Glycans with a β 1,4-Galactosylated Bisecting GlcNAc Motif

Michael Weiss,^[a] Dimitri Ott,^[a] Theodoros Karagiannis,^[a] Markus Weishaupt,^[a] Mathäus Niemiets,^[a] Steffen Eller,^[a] Marie Lott,^[a] Mónica Martínez-Orts,^[b] Ángeles Canales,^[b] Nahid Razi,^[c] James C. Paulson,^[c] and Carlo Unverzagt^{*[a]}

Dedicated to Professor Horst Kessler on the occasion of his 80th birthday.

In human serum immunoglobulin G (IgG), a rare modification of biantennary complex N-glycans lead to a β 1,4-galactosylated bisecting GlcNAc branch. We found that the bisecting GlcNAc on a biantennary core-fucosylated N-glycan was enzymatically galactosylated under stringent reaction conditions. Further optimizations led to an efficient enzymatic approach to this particular modification for biantennary substrates. Notably, tri- and tetra-antennary complex N-glycans were not converted by bovine galactosyltransferase. An N-glycan with a galactosylated bisecting GlcNAc was linked to a lanthanide binding tag. The pseudo-contact shifts (PCS) obtained from the corresponding Dy-complex were used to calculate the conformational preferences of the rare N-glycan. Besides two extended conformations only a single folded conformation was found.

from an unexpected side reaction during the enzymatic galactosylation of a synthetic bisected N-glycan we became aware that galactosylated bisecting GlcNAc moieties are present in human serum IgG. The Gal- β 1,4-bisecting GlcNAc motif was initially discovered by Nishimura^[2] and later confirmed by Rudd^[3] (Scheme 1). A similar motif was also found on bisected hybrid N-glycans of the 19 A glycoprotein derived from a lectin-resistant HEK cell line.^[3] In cells lacking GlcNAc-transferase II the truncated bisected N-glycans leads to a Gal- β 1,4-bisecting GlcNAc motif, which can be further modified.^[4] An enzymatic galactosylation of a single GlcNAc β 1,2-linked to the central β -mannose was found for a synthetic N-glycan bearing the LEC 14 antigen.^[5]

Introduction

Immunoglobulins are one of the best-studied classes of glycoproteins due to their high therapeutic value. The N-glycans of immunoglobulins are known to affect the biological activity of antibodies, for example, by modulating the interactions of the Fc part.^[1] Thus, the glycosylation pattern of therapeutic antibodies is thoroughly characterized. Originating

Results and Discussion

As part of a collaboration with the Consortium for Functional Glycomics (CFG), we synthesized various bisected complex-type N-glycans and functionalized them with a bifunctional spacer for glycan microarray printing.^[6] The bisected N-glycans^[7] were synthesized chemically following a modular approach and deprotected to the free N-glycans.

In the case of the core fucosylated bisected compound **4**, precursor **1** was obtained from three building blocks^[8] and was subsequently fucosylated using thiofucoside **2**^[9] (Scheme 2). The low stereoselectivity (α/β ratio 5.4:1) required the separation of the unnatural β -anomer **3b** by preparative HPLC. Subsequently, the protecting groups of nonasaccharide **3** were removed in a multistep procedure. After treatment with ethylenediamine in *n*-butanol an acetylation/deacetylation sequence yielded a benzylated nonasaccharide azide intermediate, which

[a] M. Weiss, Dr. D. Ott, T. Karagiannis, Dr. M. Weishaupt, Dr. M. Niemiets, Dr. S. Eller, M. Lott, Prof. C. Unverzagt
University of Bayreuth, Bioorganic Chemistry
Universitätsstraße 30, 95447 Bayreuth (Germany)
E-mail: carlo.unverzagt@uni-bayreuth.de

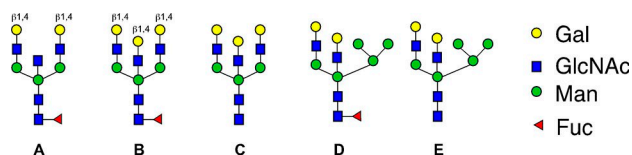
[b] M. Martínez-Orts, Dr. Á. Canales
Dpto. Química Orgánica I, Fac. Ciencias Químicas
Universidad Complutense de Madrid
Avd. Complutense s/n, 28040 Madrid (Spain)

[c] Dr. N. Razi, Prof. J. C. Paulson
Depts. of Molecular Medicine, and Immunology and Microbiology
The Scripps Research Institute
10550 N. Torrey Pines Road, La Jolla, CA 92037 (USA)

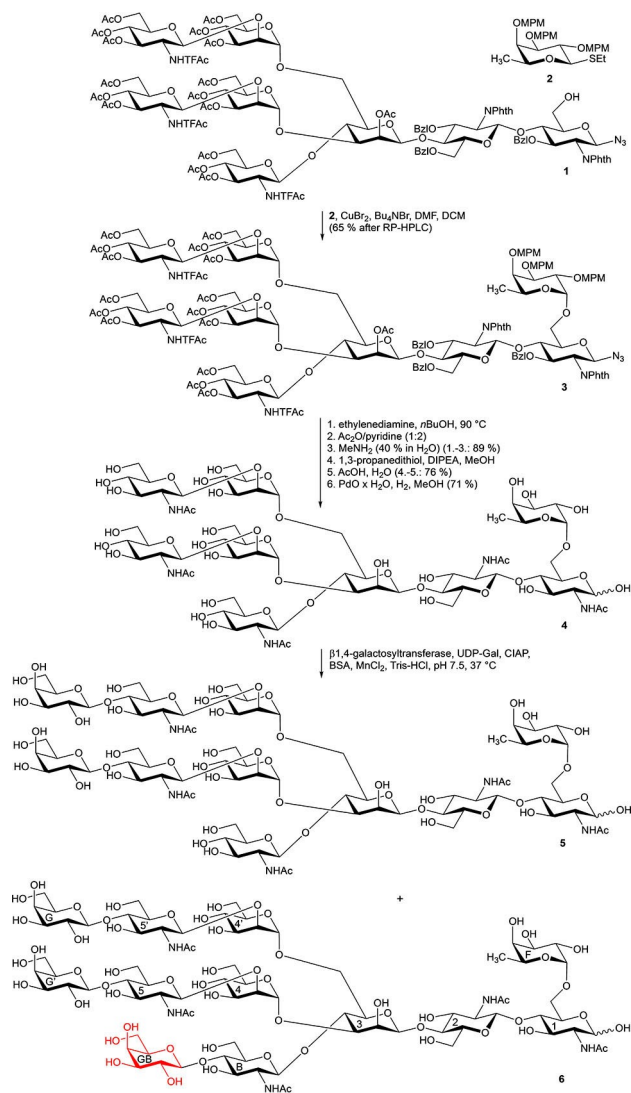
Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cbic.202000268>

This article is part of a Special Collection on the occasion of Horst Kessler's 80th birthday. To view the complete collection, visit our homepage

© 2020 The Authors. ChemBioChem published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.



Scheme 1. Biantennary N-glycan with unmodified bisecting GlcNAc (A); complex N-glycans found on human serum IgG bearing a β 1,4-galactosylated bisecting GlcNAc motif (B, C); hybrid N-glycans from 19 A glycoprotein with a galactosylated bisecting GlcNAc motif (D, E).



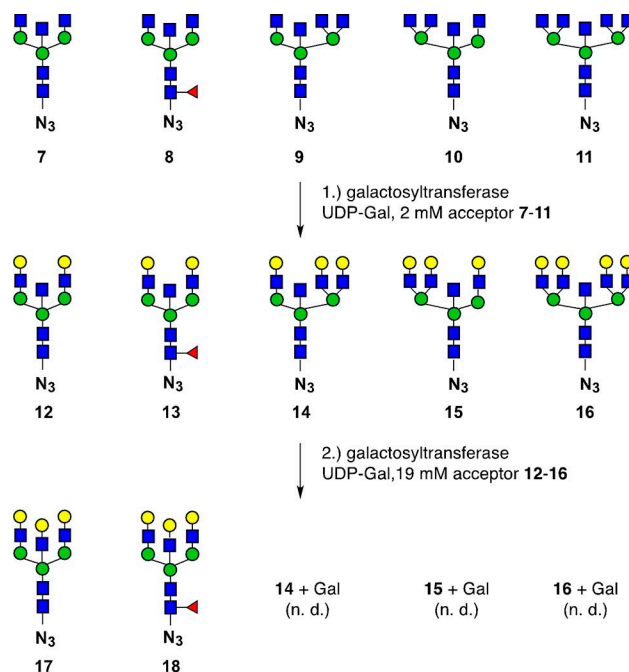
Scheme 2. Synthesis of N-glycan **3**, deprotection to hemiacetal **4** and enzymatic galactosylation to **5** and **6**. The numbering of the residues for NMR assignments is shown for **6**.

was reduced to the anomeric amine using propanedithiol.^[10] Complete removal of residual thiol was crucial prior to acidic hydrolysis of the glycosylamine. The final catalytic hydrogenation gave the free N-glycan **4** in good overall yield. The enzymatic galactosylation of **4** was carried out using UDP-Gal, bovine galactosyltransferase and calf alkaline phosphatase (CIAP).^[11] Analysis of the reaction mixture after three days by MALDI-TOF-MS showed the desired galactosylated N-glycan **5** but also a side product (ca. 23%) with the mass of an additional galactose unit and we assumed that the additional galactose might be attached to the bisecting GlcNAc (**6**). We noticed that relative to previous galactosylations of biantennary bisected N-glycan-conjugates^[12] the concentration of the N-glycan acceptor (**4**) was higher (4x) as well as the relative amount of galactosyltransferase (2x). Using even higher concentrations of N-glycan acceptor **4** and repeated addition of galactosyltransferase gave nearly complete conversion to the trigalactosylated

product **6**. On a preparative scale **4** (3.7 mg; 19 mM) was incubated with 800 mU of galactosyltransferase added in three portions over 9 days, which led to complete conversion according to MALDI-TOF-MS. The trigalactosylated compound **6** was isolated by gel filtration in a yield of 74%. Using a lower concentration of **4** in combination with lower amounts of galactosyltransferase furnished the selectively digalactosylated compound **5** in 78% yield.

Both compounds **5** and **6** were characterized by a set of 2D NMR experiments revealing that the third galactose was indeed transferred to O-4 of the bisecting GlcNAc moiety. This was evident from the ¹H signal of H-4^B shifting from 3.06 to 3.3 ppm and the ¹³C signal of C-4^B moving from 72.7 to 81.7 ppm indicative for a glycosylation at C4^B. The glycans **5** and **6** were equipped with a bifunctional linker and added to the repertoire of the CFG glycan array (data not shown).

Next the scope of the enzymatic elongation of a bisecting GlcNAc was investigated on an analytical scale using a series of fully synthetic bisected N-glycan azides (Scheme 3). The panel of potential substrates consisted of the unsubstituted bisected N-glycan **7**,^[7] the corresponding core-fucosylated glycan **8**^[8] and bisected compounds with three or four antennae (**9–11**).^[7] When attempting to apply the stringent conditions of galactosylation directly to the acceptors (19 mM **7–11**), strong substrate inhibition was found, which only led to sluggish and incomplete galactosylation after several days (HPLC data not shown). In contrast, dilute conditions (2 mM acceptor **7–11**) gave complete galactosylation of the GlcNAc termini within 1 day (**12–16**). The galactosylated compounds were submitted

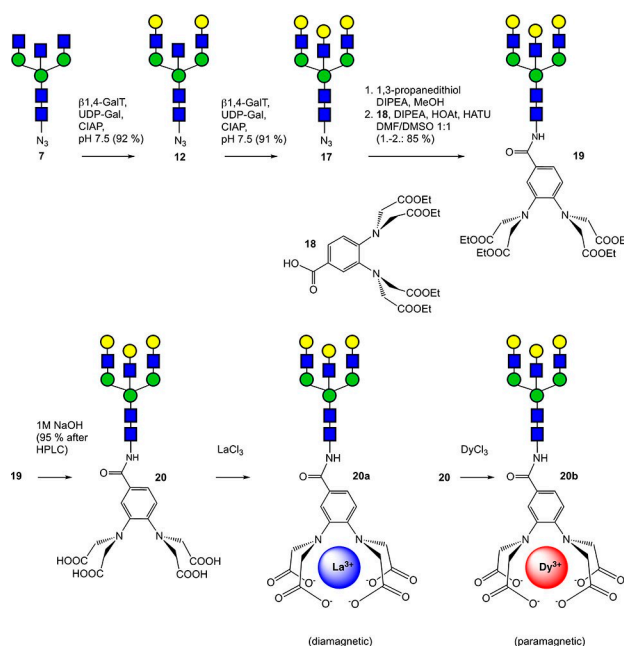


Scheme 3. The panel of bisected N-glycan azides (**7–11**) was enzymatically galactosylated under dilute (1.) and subsequently under stringent (2.) conditions. Transfer of an additional galactose to the bisecting GlcNAc was observed only in the case of the biantennary N-glycans **17** and **18**. (n. d. = not detected).

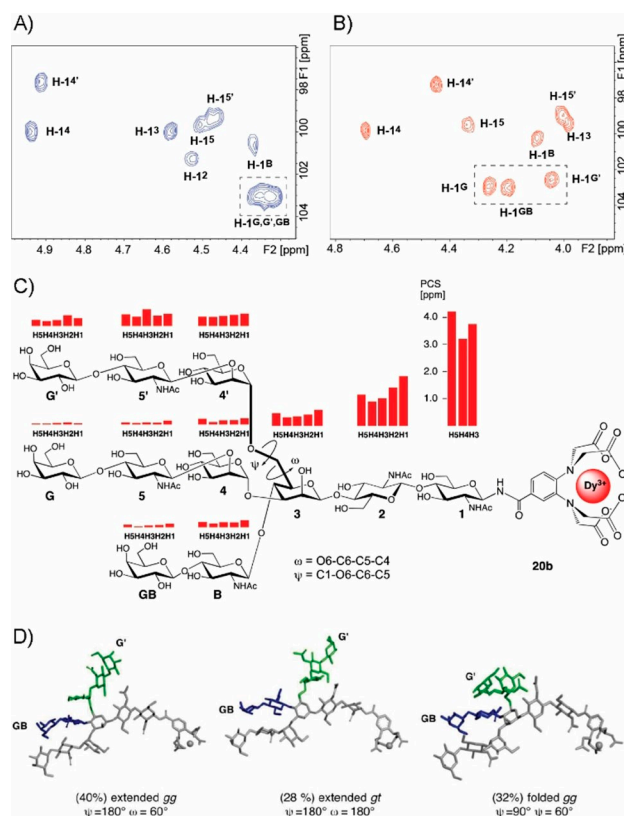
to stringent galactosylation conditions (19 mM acceptor **12**–**16**). Out of the five compounds only the biantennary N-glycans **12** and **13** gave the corresponding product with a galactosylated bisecting GlcNAc (**17**–**18**) whereas no conversion according to LC–MS was found for the compounds with three or four antennae (**9**–**11**). The galactosylation of a bisecting GlcNAc appears to be particularly sensitive to additional steric hindrance and might not be possible in the case of complex N-glycans with more than two antennae. According to the HPLC peak areas of the galactosylations after 3 days, the transfer of the third galactose occurs significantly more slowly ($\sim 1/5$) in the presence of a core fucose (**13**) compared with the unsubstituted bisected compound (**12**) (see S4 in the Supporting Information).

Because only the biantennary digalactosylated N-glycan azides were accepted by galactosyltransferase, we assumed that the galactosylated bisecting GlcNAc residue might affect the conformational freedom of the neighboring antennae. We decided to investigate the conformational preferences of the N-glycan **17** by attaching a suitable lanthanide binding tag (**19**) and subsequently measure the pseudo contact shifts of paramagnetic N-glycan complexes to calculate the preferred conformations.^[13] On a preparative scale the bisected octasaccharide azide **7** was first converted to the digalactosylated compound **12** (92% yield) and subsequently to the trigalactosylated undecasaccharide **17** (91% after RP-HPLC). The azide of **17** was reduced and coupled with the lanthanide binding tag (**18**). Purification of the conjugate **19** by RP-HPLC was important at this stage since a small percentage ($\sim 12\%$) of α -configured product also formed. The ethyl esters of **19** were saponified in 1 M NaOH followed by a purification of **20** by RP-HPLC and complexation with either LaCl₃ or DyCl₃ in a D₂O-imidazole buffer (Scheme 4.^[13b])

The conformational behavior of the trigalactosylated bisected N-glycan **17** was characterized by using paramagnetic NMR.^[14] We have previously implemented this approach to determine the conformation of a non-bisected biantennary N-glycan and the same methodology has been employed in this work.^[13a] The good HSQC-signal dispersion obtained for the paramagnetic dysprosium complex **20b** allowed full resolution of the anomeric signals of GlcNAc residues 5 and 5' and the three galactoses (G, G' and GB), which overlapped in the diamagnetic complex **20a** (Scheme 5A, B). In total 58 PCSs were obtained and used for conformation analysis (Scheme 5C). The PCS depends on the distance and the orientation of the NMR nuclei relative to the paramagnetic metal ion and thus provides structural information. The experimental PCs were correlated with the back-calculated values from the different minimum energy conformations of the N-glycan by using Mspin software.^[15] Iterative calculations were carried out with Mspin to obtain the number of conformers and the population of each conformer that better fit the experimental PCS values. The best correlation between experimental and calculated data was obtained considering three interconverting main conformations for the Man^{4'} α 1,6-Man³ linkage, extended *gauche-gauche* (*gg*), extended *gauche-trans* (*gt*) and folded *gg* conformation, with populations of 40, 28, and 32%, respectively (Scheme 5D).



Scheme 4. Synthesis of the trigalactosylated bisected N-glycan azide **17**, conjugation with lanthanide binding tag **18**, deprotection and formation of the diamagnetic complex **20a** and the paramagnetic complex **20b**.



Scheme 5. A, B) HSQC sections of the anomeric region of the diamagnetic (**20a**) and paramagnetic complex (**20b**); the three galactose signals are within the dotted lines. C) PCS of paramagnetic complex **20b**. D) Relative percentages of the main conformations calculated from PCS.

The main conformations are the extended *gg* and *gt* conformers as also found in the corresponding non-bisected biantennary N-glycan.^[13a] However, a higher population (32%) of a single folded conformation ($\psi = 90^\circ$) was obtained for the galactosylated bisected glycan compared with the non-bisected biantennary structure, where two folded conformations were found (10% $\psi = 60^\circ$ and 10% $\psi = 90^\circ$). Notably, the galactosylated bisected branch affects the preferred conformations of the N-glycan leading to a threefold increase of a single folded geometry of the $\alpha 1,6$ arm. The extended *gt* conformation served as a starting point for a preliminary model where the bisecting GlcNAc of **12** could be placed in the acceptor site of bovine galactosyltransferase (PDB ID: 1TW5)^[16] with only minor adjustments of the 1,3 arm (see Schemes S7 and S8 in the Supporting Information). We are currently investigating the conformational preferences of complex N-glycans with the typical bisecting GlcNAc-modification lacking the additional galactose unit.

In summary a rare N-glycan identified in human serum IgG could be synthesized by using stringent conditions for the enzymatic galactosylation of the bisecting GlcNAc moiety. This particular conversion appears to be limited to biantennary-N-glycans. The conformational analysis of a conjugate of the glycan bearing the galactosylated bisecting GlcNAc motif using paramagnetic NMR revealed two extended conformers but only a single folded geometry.

Acknowledgements

We are grateful for the funding from the Deutsche Forschungsgemeinschaft and Bavaria California Technology Center (BaCaTeC). We are also grateful to the Agencia Estatal de Investigacion and MINECO of Spain for grant CTQ2016-76263-P, and support from grant GM62116 from the US National Institutes of Health. We thank Prof. Javier Pérez Castells from Universidad San Pablo-CEU for providing the protected lanthanide tag.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: N-glycans · glycosylation · glycobiology · immunoglobulin · paramagnetic NMR spectroscopy

- [1] a) T. Shinkawa, K. Nakamura, N. Yamane, E. Shoji-Hosaka, Y. Kanda, M. Sakurada, K. Uchida, H. Anazawa, M. Satoh, M. Yamasaki, N. Hanai, K. Shitara, *J. Biol. Chem.* **2003**, *278*, 3466–3473; b) P. Sondermann, A. Pincetic, J. Maamary, K. Lammens, J. V. Ravetch, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 9868–9872; c) T. Li, D. J. Di Lillo, S. Bournazos, J. P. Giddens, J. V. Ravetch, L. X. Wang, *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 3485–3490; d) R. M. Anthony, F. Nimmerjahn, D. J. Ashline, V. N. Reinhold, J. C. Paulson, J. V. Ravetch, *Science* **2008**, *320*, 373–376.
- [2] Y. Takegawa, K. Deguchi, H. Nakagawa, S. Nishimura, *Anal. Chem.* **2005**, *77*, 6062–6068.
- [3] D. J. Harvey, M. Crispin, C. Scanlan, B. B. Singer, L. Lucka, V. T. Chang, C. M. Radcliffe, S. Thobhani, C. T. Yuen, P. M. Rudd, *Rapid Commun. Mass Spectrom.* **2008**, *22*, 1047–1052.
- [4] Y. Wang, J. Tan, M. Sutton-Smith, D. Ditto, M. Panico, R. M. Campbell, N. M. Varki, J. M. Long, J. Jaeken, S. R. Levinson, A. Wynshaw-Boris, H. R. Morris, D. Le, A. Dell, H. Schachter, J. D. Marth, *Glycobiology* **2001**, *11*, 1051–1070.
- [5] I. Prah, C. Unverzagt, *Angew. Chem. Int. Ed.* **2002**, *41*, 4259–4262; *Angew. Chem.* **2002**, *114*, 4434–4437.
- [6] O. Blixt, T. Mondala, C. Scanlan, M. E. Hufleit, R. Alvarez, M. C. Bryan, F. Fazio, D. Calarese, J. Stevens, N. Razi, D. J. Stevens, J. J. Skehel, I. van Die, D. R. Burton, I. A. Wilson, R. Cummings, N. Bovin, C. H. Wong, J. C. Paulson, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 17033–17038.
- [7] M. Mönnich, S. Eller, T. Karagiannis, L. Perkams, T. Luber, D. Ott, M. Niemietz, J. Hoffman, J. Walcher, L. Berger, M. Pischl, M. Weishaupt, C. Wirkner, R. G. Lichtenstein, C. Unverzagt, *Angew. Chem. Int. Ed.* **2016**, *55*, 10487–10492; *Angew. Chem.* **2016**, *128*, 10643–10648.
- [8] T. Luber, M. Niemietz, T. Karagiannis, M. Mönnich, D. Ott, L. Perkams, J. Walcher, L. Berger, M. Pischl, M. Weishaupt, S. Eller, J. Hoffman, C. Unverzagt, *Angew. Chem. Int. Ed.* **2018**, *57*, 14543–14549.
- [9] D. Ott, J. Seifert, I. Prah, M. Niemietz, J. Hoffman, J. Guder, M. Mönnich, C. Unverzagt, *Eur. J. Org. Chem.* **2012**, 5054–5068.
- [10] C. Unverzagt, *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 2350–2353; *Angew. Chem.* **1996**, *108*, 2507–2510.
- [11] C. Unverzagt, H. Kunz, J. C. Paulson, *J. Am. Chem. Soc.* **1990**, *112*, 9308–9309.
- [12] a) 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; b) S. André, T. Kozar, R. Schuberth, C. Unverzagt, S. Kojima, H. J. Gabius, *Biochemistry* **2007**, *46*, 6984–6995.
- [13] a) A. Canales, A. Mallagaray, J. Pérez-Castells, I. Boos, C. Unverzagt, S. André, H. J. Gabius, F. J. Cañada, J. Jiménez-Barbero, *Angew. Chem. Int. Ed.* **2013**, *52*, 13789–13793; *Angew. Chem.* **2013**, *125*, 14034–14038; b) A. Canales, I. Boos, L. Perkams, L. Karst, T. Luber, T. Karagiannis, G. Domínguez, F. J. Cañada, J. Pérez-Castells, D. Häussinger, C. Unverzagt, J. Jiménez-Barbero, *Angew. Chem. Int. Ed.* **2017**, *56*, 14987–14991; *Angew. Chem.* **2017**, *129*, 15183–15187.
- [14] a) S. Yamamoto, T. Yamaguchi, M. Erdelyi, C. Griesinger, K. Kato, *Chem. Eur. J.* **2011**, *17*, 9280–9282; b) T. Yamaguchi, Y. Sakae, Y. Zhang, S. Yamamoto, Y. Okamoto, K. Kato, *Angew. Chem. Int. Ed.* **2014**, *53*, 10941–10944; *Angew. Chem.* **2014**, *126*, 11121–11124.
- [15] a) <http://www.mestrelab.com> Program Mspin; b) A. Navarro-Vazquez, *Magn. Reson. Chem.* **2012**, *50*, 73–9.
- [16] B. Ramakrishnan, E. Boeggeman, P. K. Qasba, *Biochemistry* **2004**, *43*, 12513–12522.

Manuscript received: April 30, 2020
Revised manuscript received: June 26, 2020
Accepted manuscript online: June 28, 2020
Version of record online: August 19, 2020