NMR Spectroscopy

DOI: 10.1002/anie.201408615

Multiple Paramagnetic Effects through a Tagged Reporter Protein**

*Aldo R. Camacho-Zarco, Francesca Munari, Melanie Wegstroth, Wei-Min Liu, Marcellus Ubbink, Stefan Becker, and Markus Zweckstetter**

***Abstract:** Paramagnetic effects provide unique information about the structure and dynamics of biomolecules. We developed a method in which the lanthanoid tag is not directly attached to the protein of interest, but instead to a "reporter" protein, which binds and then transmits paramagnetic information to the target. The designed method allows access to a large number of paramagnetic restraints and residual dipolar couplings produced from independent molecular alignments in high-molecular-weight proteins with unknown 3D structure*

Lanthanoid tags are used in diverse areas such as NMR spectroscopy,[1] X-ray crystallography,[2] and luminescence-based measurements.[3] Unpaired electrons in f orbitals of lanthanoids cause paramagnetic effects. When the lanthanoid is located in a target molecule, its anisotropic magnetic susceptibility results in weak alignment of the molecule in the magnetic field and affects the signals of its NMR spectrum in a distance-, orientation-, and orientation/distance-dependent way.[4] Since the magnetic moment of electrons is almost three orders of magnitude greater than that of protons, the perturbation is transmitted over long distances.[5] The long-range nature of paramagnetic restraints allows unique insights into the structure and dynamics of biomolecules.[1a,b,6]

In order to obtain information about the distance between the solvent and the nucleus and thereby structural information about the biomolecule, lanthanoids can be added as soluble complexes to the sample.[7] However, for access to orientation information through residual dipolar couplings (RDCs) and intra- or intermolecular distance information, lanthanoids have to be attached to a specific site in the molecule.[1c,8] In the case of Ca^{2+}- or Mg^{2+}-containing metalloproteins, this can be achieved by replacement of the natural metal by lanthanoid ions,[9] while for most proteins, lanthanoids have to be attached to the target protein by methods such as the insertion of lanthanoid-binding peptides into protein loops,[10] the attachment of lanthanoid-binding motifs to the N- or C-terminus,[11] and the covalent tagging of a small organic molecule to a surface-reactive cysteine.[1c,12] In addition, diamagnetic proteins lacking native metal-binding sites might be fused with entire protein domains such as zinc finger proteins,[13] EF hand motifs,[14] or a calmodulin-binding peptide and then treated with calmodulin loaded with paramagnetic lanthanoids to transmit molecular alignment.[4a]

Several aspects have to be considered when using lanthanoid tags. Tagging should not perturb the structure and dynamics of the protein. Covalent attachment to reactive cysteines also requires one or a pair (in case of double-legged tags) of unique surface-accessible cysteines. In addition, it can be difficult to insert a lanthanoid-binding peptide into a loop or select a surface-accessible site when the 3D structure of the protein is unknown. Acquisition of complementary sets of paramagnetic data even requires attachment of the lanthanoid to multiple sites, which then requires multiple cysteine mutants of the protein. Here we present a strategy that alleviates many of the problems and was motivated by the early approaches of fusion with a paramagnetic domain or peptide that binds to a paramagnetic domain.[4a,13a,14] The central idea is to attach the lanthanoid tag not to the target protein itself, but instead to a reporter protein, which in turns binds and hence transmits the paramagnetic effects to any target that contains a specific recognition sequence (Figure 1). The reporter protein can be tagged in a separate reaction before its mixture/binding with the target protein and can thus be applied to proteins with multiple accessible cysteines, as long as disulfide exchange does not play a significant role.

The reporter protein that was chosen is the Erbin PDZ domain (Figure S1).[18] It is a stable, 103-residue, well-characterized protein that does not contain any cysteine residues and binds with high affinity to the sequence TGWETWV when it is located at the C-terminus of another protein (Table S1).[19] To evaluate whether independent alignments can be transferred by systematic point mutations on PDZ,[20] six different double-cysteine mutants of the Erbin PDZ domain (PDZ-1 to PDZ-6) were designed (Figure S1). The mutants were then tagged with the lanthanoid tag CLaNP-5,[21] which had been preloaded with the paramagnetic lanthanoid Tm^{3+} or diamagnetic Lu^{3+}. CLaNP-5 attaches to two cysteine residues, resulting in increased rigidity of the tag. Even though the tagging of all PDZ mutants was optimized, tagging efficiency greatly varied (Table S2). In addition, PDZ-4 turned out to be unstable and was excluded from further analysis.

---

[*] A. R. Camacho-Zarco, Dr. F. Munari, M. Wegstroth, Dr. S. Becker, Prof. M. Zweckstetter
Max Planck Institute for Biophysical Chemistry
Am Fassberg 11, 37077 Göttingen (Germany)
and
German Center for Neurodegenerative Diseases (DZNE)
Göttingen (Germany)
and
Center for the Molecular Physiology of the Brain
University Medical Center, Göttingen (Germany)
E-mail: markus.zweckstetter@dzne.de
Dr. W.-M. Liu, Prof. M. Ubbink
Leiden Institute of Chemistry, Leiden University
Einsteinweg 55, 2333 CC Leiden (The Netherlands)

[**] This work was supported by the ERC (ERC grant agreement number 282008 to M.Z.) and the Netherlands Organisation for Scientific Research, VICI grant 700.58.441 (W.-M.L. and M.U.).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201408615.

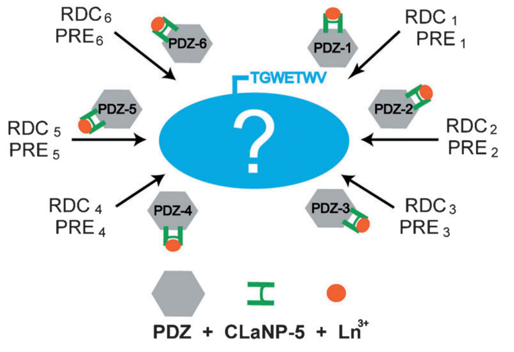


Figure 1. Representation of the method. The lanthanoid tag is attached to a reporter protein (gray) that binds and transmits paramagnetic effects to the target protein (blue), which contains a specific recognition sequence (TGWETWV). By attaching the lanthanoid tag at different positions in PDZ, multiple independent alignments of the target protein are obtained.

The strategy was tested on the 8 kDa protein ubiquitin and the 42 kDa maltose-binding protein[22] (MBP), both with the C-terminal TGWETWV extension. Binding of wild-type PDZ to ubiquitin and MBP was slow on the NMR timescale (Figures S2 and S3a). In line with the high affinity of the PDZ–TGWETWV interaction (Figure S2), binding to MBP was saturated at a 2:1 excess of PDZ. In addition, excess PDZ tagged with CLaNP-5 did not perturb signal intensities in ubiquitin (Figure S4). Chemical shifts in ubiquitin^{TGWETWV}/MBP^{TGWETWV} bound to PDZ were similar to those in wild-type ubiquitin/MBP, indicating that PDZ binding does not perturb the structure of the protein of interest (Figures S3b and S6). Next we added the PDZ mutants, tagged with CLaNP-5, to ^{15}N-labeled ubiquitin^{TGWETWV}, as well as to ^{15}N/^{2}H-labeled and ^{15}N/^{13}C/^{2}H-labeled MBP^{TGWETW}. ^{1}H-^{15}N HSQC spectra were of high quality, allowing quantitative analysis of RDCs, PCSs, and PREs (Figure 2; Figures S5, S7, and S8). For MBP, we also obtained 3D HNCO-based experiments to measure RDCs (Figure S8g). Thus, the increase in molecular weight due to PDZ binding (Figures S9 and S10) did not interfere with the accurate measurement of RDCs and PCSs (Figure 2b; Figure S5d).

At a proton Larmor frequency of 900 MHz, RDCs induced by PDZ-1 in ubiquitin ranged from −7.3 to +5.8 Hz (Figure 2c). The fit of 57 experimental RDCs to the 3D structure of ubiquitin was of very high quality with a Pearson's correlation coefficient of 0.99, that is, comparable to that achieved by steric alignment. At 600 MHz, the RDCs decreased according to the change in field strength by a factor 2.25, but the correlation coefficient to the 3D structure remained at 0.98 (Figure S5b). In the case of MBP, experimental RDCs induced by PDZ-2 were in the range from −11.2 to 7.9 Hz (Figure 2d; Table S3). The fit to the 3D structure of MBP resulted in Pearson correlation coefficients of 0.95 for the RDCs and 0.97 for PCSs (Figure 2d,e). The lower quality of the RDC fit when compared to ubiquitin is due to the lower precision of the RDC measurement at the increased molecular weight as well as the lower resolution of the 3D structure of MBP. The magnitude of alignment transmitted to MBP varied between different PDZ mutants (Table S3). PDZ-3 and PDZ-5 induced a smaller degree of alignment, likely due to different contributions from dynamics with respect to MBP and a higher flexibility of the CLaNP-5 tag in these PDZ variants (Figures S11–S13).

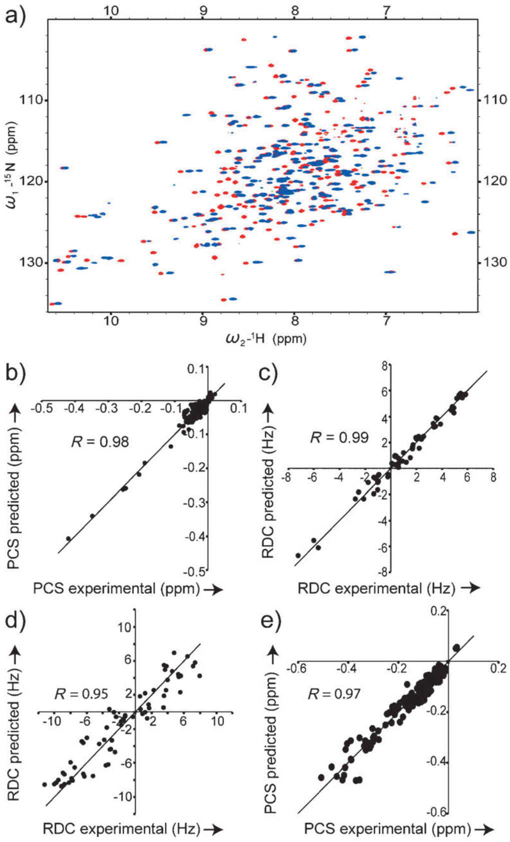
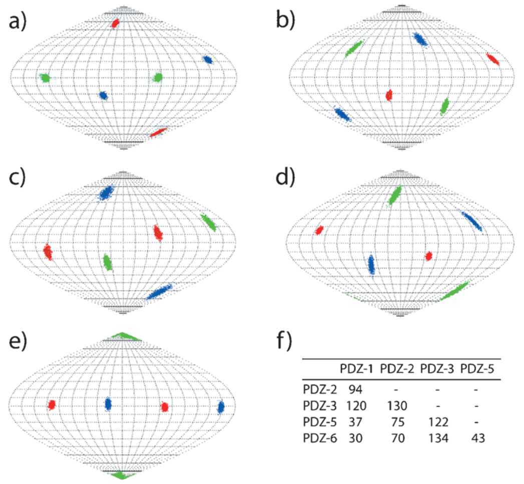


Figure 2. RDCs and PCSs transmitted by PDZ variants to ubiquitin^{TGWETWV} and MBP^{TGWETWV}. a) Superposition of ^{1}H-^{15}N HSQC spectra of MBP^{TGWETWV} bound to PDZ-1, which had been tagged with CLaNP-5 preloaded with Lu^{3+} (red) or Tm^{3+} (blue). b) Correlation between two independent PCS sets (recorded in identical experimental conditions at 600 MHz) induced by PDZ-1 in ubiquitin^{TGWETWV}. c) RDCs induced in ubiquitin^{TGWETWV} by PDZ-1 binding (^{1}H Larmor frequency of 900 MHz; 37 °C; acquired using BSD-IPAP HSQC[15]) were fitted to the 3D structure of ubiquitin (PDB code: 1D3Z). d,e) RDCs and PCSs induced in MBP^{TGWETWV} at 900 MHz by PDZ-2 binding were fitted to the 3D structure of MBP (PDB code: 1DMB) using PALES[16] and Numbat,[17] respectively. Only cross-peaks not strongly affected by signal overlap were analyzed.

The experimental RDCs were used to determine the alignment tensors induced in MBP by the five PDZ variants. In agreement with the high quality of the experimental RDCs, the orientations of the three tensor axes were well defined (Figure 3a–e). Moreover, the orientations of the axes differed largely between the PDZ variants. Quantification using a generalized angle in the five-dimensional space spanned by the Saupe matrix revealed that each alignment tensor differed by more than 30° from any of the other four (Figure 3f). For some combinations, 5D angles of 90–130° were found.

In addition to orientational restraints, the lanthanoid-loaded PDZ variants caused relaxation of the NMR signals of nearby nuclei (Figure S14). For example, when PDZ-1 was



	PDZ-1	PDZ-2	PDZ-3	PDZ-5
PDZ-2	94	-	-	-
PDZ-3	120	130	-	-
PDZ-5	37	75	122	-
PDZ-6	30	70	134	43

Figure 3. Orientations of the alignment tensor transmitted to the 42 kDa protein MBP^{TGWETWV} by CLaNP-5-tagged PDZ mutants 1, 2, 3, 5, and 6 (a–e). *z*, *y* and *x* axis are shown in red, blue, and green in Sanson–Flamsteed projections, respectively. The orientation of the alignment tensors was obtained by fitting the experimental RDCs to the 3D structure of MBP (PDB code: 1DMB).[22a] Uncertainties were evaluated by 1000 cycles of the "structural noise Monte-Carlo method".[16,23] f) 5D angles between the alignment tensors induced in MBP.[24]

added to MBP^{TGWETWV}, the signals of MBP residues 46 to 55 were broadened beyond detection (Figure S14a). The location of these residues in the 3D structure of MBP is close to the C-terminus, where PDZ binds to the TGWETWV extension (Figure S14a–d,f). For metal ions with an anisotropic g-tensor and a short electron relaxation time such as Tm^{3+}, NMR signal broadening is primarily due to Curie spin relaxation. Back-calculation of the Curie spin relaxation induced broadening from the position of the metal ion (determined on the basis of the experimental PCSs) resulted in less line broadening as observed experimentally in MBP^{TGWETWV} (Figure S14a,e). The difference is caused by cross-correlations with other relaxation mechanisms as well as exchange contributions arising from conformational dynamics and highlights the difficulty in using PRE broadening induced by metal ions with an anisotropic g-tensor for structural analysis.[25] When PREs are used for structural analysis in the current approach, CLaNP-5 can be loaded with a metal ion having an isotropic g-tensor such as Gd^{3+} and subsequently attached to PDZ.

The transmission of paramagnetic effects from the CLaNP-5-carrying PDZ to MBP/ubiquitin is facilitated by hydrodynamic coupling between the two proteins.[26] The degree of transmission is therefore influenced by the motion of PDZ with respect to MBP/ubiquitin. For example, RDCs observed directly within PDZ-1 at 900 MHz were in the range from −31.2 Hz to 18.0 Hz (Figure S15, Table S4), while the RDCs transmitted upon binding to MBP^{TGWETWV} were decreased due to interdomain dynamics to −9.4 to 7.3 Hz (Figure S8a). According to ^{15}N relaxation rates in ubiquitin^{TGWETWV} bound to PDZ (Figure S16), the last three residues of ubiquitin and the first two residues of the TGWETWV sequence are flexible. This suggests that the shorter the linker, the stronger the coupling and the more pronounced the transmission of paramagnetic effects. In line with this hypothesis, shortening the connection from ubiquitin to the PDZ domain by three residues (including the removal of G76 from ubiquitin) increased the general magnitude of the alignment tensor from $4.6\,e^{-4}$ in ubiquitin^{TGWETWV} (Figure 2c) to $6.3\,e^{-4}$ in ubiquitin^{WETWV} (Figure S17). Noteworthy, the decrease in alignment due to interdomain dynamics has the advantage that cross-peaks in the paramagnetic spectrum of ubiquitin/MBP are only slightly shifted when compared to the diamagnetic spectrum (Figure 2a; Figures S5a and S7), simplifying sequence-specific assignment.

In summary, we have presented a robust method that allows access to a large number of RDCs and PCSs produced from independent molecular alignments. Although in this study a PDZ domain with the lanthanoid tag CLaNP-5 was used, other lanthanoid tags can be attached,[12,27] as long as the probe mobility is low enough to provide sufficient alignment in the protein of interest. Lanthanoid-binding motifs might be engineered into the PDZ domain[3,10] or proteins other than PDZ might be employed, such as calmodulin, which binds with high affinity to short peptide sequences.[4a] Moreover, the present set of PDZ mutants are stable, thus avoiding instability problems that might occur when a mutation is introduced directly into the protein of interest. The tagged PDZ mutants can be added to any target protein, as long as the TGWETWV peptide has been fused. Importantly, the method can be applied to proteins of unknown 3D structure and no internal modification of the protein of interest is required, which otherwise might affect its structure and dynamics.

Received: August 27, 2014

Keywords: lanthanoids · NMR spectroscopy · paramagnetism · proteins · structural biology

[1] a) G. Otting, *Annu. Rev. Biophys.* **2010**, *39*, 387–405; b) I. Bertini, C. Luchinat, *Curr. Opin. Chem. Biol.* **1999**, *3*, 145–151; c) P. M. Keizers, M. Ubbink, *Prog Nucl Mag Res. Sp* **2011**, *58*, 88–96; d) W. M. O. Liu, M. Ubbink, *Coord. Chem. Rev.* **2013**, *273-274*, 2–12.
[2] N. R. Silvaggi, L. J. Martin, H. Schwalbe, B. Imperiali, K. N. Allen, *J. Am. Chem. Soc.* **2007**, *129*, 7114–7120.
[3] K. N. Allen, B. Imperiali, *Curr. Opin. Chem. Biol.* **2010**, *14*, 247–254.
[4] a) J. Feeney, B. Birdsall, A. F. Bradbury, R. R. Biekofsky, P. M. Bayley, *J. Biomol. NMR* **2001**, *21*, 41–48; b) X. F. Xu, P. H. J. Keizers, W. Reinle, F. Hannemann, R. Bernhardt, M. Ubbink, *J. Biomol. NMR* **2009**, *43*, 247–254; c) P. H. J. Keizers, B. Mersinli, W. Reinle, J. Donauer, Y. Hiruma, F. Hannemann, M. Overhand, R. Bernhardt, M. Ubbink, *Biochemistry* **2010**, *49*, 6846–6855; d) A. N. Volkov, J. A. R. Worrall, E. Holtzmann, M. Ubbink, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 18945–18950; e) I. Bertini, A. Donaire, B. Jimenez, C. Luchinat, G. Parigi, M. Piccioli, L. Poggi, *J. Biomol. NMR* **2001**, *21*, 85–98; f) T. Saio, K. Ogura, M. Yokochi, Y. Kobashigawa, F. Inagaki, *J. Biomol. NMR* **2009**, *44*, 157–166.
[5] M. Blackledge, *Prog. Nucl. Magn. Reson. Spectrosc.* **2005**, *46*, 23–61.

[6] a) C. Tang, C. D. Schwieters, G. M. Clore, *Nature* **2007**, *449*, 1078 – U1012; b) Y. Hiruma, M. A. Hass, Y. Kikui, W. M. Liu, B. Olmez, S. P. Skinner, A. Blok, A. Kloosterman, H. Koteishi, F. Lohr, H. Schwalbe, M. Nojiri, M. Ubbink, *J. Mol. Biol.* **2013**, *425*, 4353 – 4365.

[7] T. Madl, W. Bermel, K. Zangger, *Angew. Chem. Int. Ed.* **2009**, *48*, 8259 – 8262; *Angew. Chem.* **2009**, *121*, 8409 – 8412.

[8] a) G. Pintacuda, A. Y. Park, M. A. Keniry, N. E. Dixon, G. Otting, *J. Am. Chem. Soc.* **2006**, *128*, 3696 – 3702; b) M. John, G. Pintacuda, A. Y. Park, N. E. Dixon, G. Otting, *J. Am. Chem. Soc.* **2006**, *128*, 12910 – 12916.

[9] a) L. Lee, B. D. Sykes, *Biochemistry* **1981**, *20*, 1156 – 1162; b) J. R. Tolman, J. M. Flanagan, M. A. Kennedy, J. H. Prestegard, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 9279 – 9283; c) I. Bertini, I. Gelis, N. Katsaros, C. Luchinat, A. Provenzani, *Biochemistry* **2003**, *42*, 8011 – 8021.

[10] K. Barthelmes, A. M. Reynolds, E. Peisach, H. R. A. Jonker, N. J. DeNunzio, K. N. Allen, B. Imperiali, H. Schwalbe, *J. Am. Chem. Soc.* **2011**, *133*, 808 – 819.

[11] a) L. W. Donaldson, N. R. Skrynnikov, W. Y. Choy, D. R. Muhandiram, B. Sarkar, J. D. Forman-Kay, L. E. Kay, *J. Am. Chem. Soc.* **2001**, *123*, 9843 – 9847; b) L. J. Martin, M. J. Hahnke, M. Nitz, J. Wöhnert, N. R. Silvaggi, K. N. Allen, H. Schwalbe, B. Imperiali, *J. Am. Chem. Soc.* **2007**, *129*, 7106 – 7113.

[12] X. C. Su, G. Otting, *J. Biomol. NMR* **2010**, *46*, 101 – 112.

[13] a) V. Gaponenko, A. Dvoretsky, C. Walsby, B. M. Hoffman, P. R. Rosevear, *Biochemistry* **2000**, *39*, 15217 – 15224; b) T. Saio, M. Yokochi, H. Kumeta, F. Inagaki, *J. Biomol. NMR* **2010**, *46*, 271 – 280; c) J. Wöhnert, K. J. Franz, M. Nitz, B. Imperiali, H. Schwalbe, *J. Am. Chem. Soc.* **2003**, *125*, 13338 – 13339.

[14] C. Ma, S. J. Opella, *J. Magn. Reson.* **2000**, *146*, 381 – 384.

[15] L. S. Yao, J. F. Ying, A. Bax, *J. Biomol. NMR* **2009**, *43*, 161 – 170.

[16] M. Zweckstetter, *Nat. Protoc.* **2008**, *3*, 679 – 690.

[17] C. Schmitz, M. J. Stanton-Cook, X. C. Su, G. Otting, T. Huber, *J. Biomol. NMR* **2008**, *41*, 179 – 189.

[18] N. J. Skelton, M. F. T. Koehler, K. Zobel, W. L. Wong, S. Yeh, M. T. Pisabarro, J. P. Yin, L. A. Lasky, S. S. Sidhu, *J. Biol. Chem.* **2003**, *278*, 7645 – 7654.

[19] R. P. Laura, A. S. Witt, H. A. Held, R. Gerstner, K. Deshayes, M. F. T. Koehler, K. S. Kosik, S. S. Sidhu, L. A. Lasky, *J. Biol. Chem.* **2002**, *277*, 12906 – 12914.

[20] L. S. Yao, A. Bax, *J. Am. Chem. Soc.* **2007**, *129*, 11326 – 11327.

[21] P. H. J. Keizers, A. Saragliadis, Y. Hiruma, M. Overhand, M. Ubbink, *J. Am. Chem. Soc.* **2008**, *130*, 14802 – 14812.

[22] a) A. J. Sharff, L. E. Rodseth, F. A. Quiocho, *Biochemistry* **1993**, *32*, 10553 – 10559; b) K. H. Gardner, X. C. Zhang, K. Gehring, L. E. Kay, *J. Am. Chem. Soc.* **1998**, *120*, 11738 – 11748.

[23] M. Zweckstetter, A. Bax, *J. Biomol. NMR* **2002**, *23*, 127 – 137.

[24] J. Sass, F. Cordier, A. Hoffmann, A. Cousin, J. G. Omichinski, H. Lowen, S. Grzesiek, *J. Am. Chem. Soc.* **1999**, *121*, 2047 – 2055.

[25] a) G. Pintacuda, A. Kaikkonen, G. Otting, *J. Magn. Reson.* **2004**, *171*, 233 – 243; b) G. M. Clore, J. Iwahara, *Chem. Rev.* **2009**, *109*, 4108 – 4139.

[26] a) I. Bertini, C. Del Bianco, I. Gelis, N. Katsaros, C. Luchinat, G. Parigi, M. Peana, A. Provenzani, M. A. Zoroddu, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 6841 – 6846; b) N. Rezaei-Ghaleh, F. Klama, F. Munari, M. Zweckstetter, *Angew. Chem. Int. Ed.* **2013**, *52*, 11410 – 11414; *Angew. Chem.* **2013**, *125*, 11621 – 11625.

[27] a) T. Ikegami, L. Verdier, P. Sakhaii, S. Grimme, B. Pescatore, K. Saxena, K. M. Fiebig, C. Griesinger, *J. Biomol. NMR* **2004**, *29*, 339 – 349; b) W. M. Liu, P. H. J. Keizers, M. A. S. Hass, A. Blok, M. Tirnmer, A. J. C. Sarris, M. Overhand, M. Ubbink, *J. Am. Chem. Soc.* **2012**, *134*, 17306 – 17313; c) D. Häussinger, J. R. Huang, S. Grzesiek, *J. Am. Chem. Soc.* **2009**, *131*, 14761 – 14767.