Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Magnetic Resonance 163 (2003) 340–346
JMR Journal of Magnetic Resonance
www.elsevier.com/locate/jmr

Communication

# Accurate determination of small one-bond heteronuclear residual dipolar couplings by *F1* coupled HSQC modified with a G-BIRD$^{(r)}$ module

Krisztina Fehér,[a] Stefan Berger,[a,*] and Katalin E. Kövér[b]

[a] *Institute for Analytical Chemistry, University of Leipzig, Johnnisallee 29, D-04103, Leipzig, Germany*
[b] *Department of Inorganic and Analytical Chemistry, University of Debrecen, Debrecen, Hungary*

Received 18 December 2002; revised 7 March 2003

**Abstract**

We report a G-BIRD$^{(r)}$ modified coupled HSQC experiment for the accurate determination of one-bond heteronuclear residual dipolar couplings. The G-BIRD$^{(r)}$ module has been employed to refocus the long-range coupling evolution of the heteronucleus during the $t_1$ frequency labeling period. As a result, the crosspeaks obtained are split by only the direct one-bond coupling that can be extracted by measuring simple frequency differences between singlet maxima. Additionally the decoupling of long-range multiple bond splittings leads to considerable sensitivity enhancement. The modification also has been applied in a TROSY sequence resulting in a significant sensitivity and resolution improvement.


*Keywords:* Residual dipolar coupling constant; One-bond heteronuclear coupling constant; Coupled HSQC; G-BIRD; TROSY

## 1. Introduction

Residual dipolar couplings (RDC) provide unique long-range constraints for structure determination of molecules conveying information on the distance between nuclei in dipolar interaction and on the orientations of the corresponding internuclear bond vectors relative to the molecular alignment tensor. In recent years residual dipolar couplings have therefore been extensively applied for structural studies of proteins, nucleic acids and carbohydrates [1] in liquid state and proved to improve the precision of structures. Dipolar couplings are orientation dependent tensorial NMR parameters that are averaged to zero by the random tumbling of molecules in isotropic media. RDC's arise from the anisotropic tumbling of molecules resulting from their partial alignment in the applied medium. Intrinsic partial alignment is observed for molecules possessing sufficiently large magnetic susceptibility anisotropy such as nucleic acids, protein–nucleic acid complexes, and metal binding proteins in the presence of strong magnetic field. A tunable degree of alignment can also be achieved by dissolving the molecule in partially oriented media such as a dilute liquid crystalline phase. RDC's manifest themselves in the NMR spectra as an increase or decrease of the splittings due to scalar couplings between nuclei. Their magnitude can therefore be extracted by measuring changes of splittings in isotropic versus anisotropic sample conditions.

For small flexible molecules the RDC's are often small due to the effects of dynamics [2]. Spherical shape of the molecules may also result in low degree of alignment yielding only small RDC's [3–4]. In these cases special considerations have to be taken in the choice of the measurement technique.

Herein we propose a modification of the *F1*-coupled HSQC experiment where additional splittings caused by long-range multiple bond couplings are eliminated with the aid of G-BIRD$^{(r)}$ module inserted in the heteronucleus frequency labelling period. Thus one-bond heteronuclear RDC's can be determined from the resulting spectra by simply measuring the frequency difference between the peak maxima of singlets instead of the

* Corresponding author. Fax: +49-341-9736115.
*E-mail address:* stberger@rz.uni-leipzig.de (S. Berger).

1090-7807/$ - see front matter © 2003 Published by Elsevier Science (USA).
doi:10.1016/S1090-7807(03)00113-7

centers of complex multiplets. We demonstrate that decoupling of the heteronuclear multiple bond splittings leads to a significant enhancement of the signal intensities as well. Recently the G-BIRD module has been used by Pham et al. [5] for the same purpose in the analogous *J* modulated experiments. As a thorough description of the effect of the BIRD module can be found in [5,6], that will not be repeated here.

## 2. Experimental

All experiments were performed on Bruker DRX-400, 500, and 700 spectrometers (Bruker AG, Rheinstetten, Germany) equipped with TBI and TXI z-grad probes. All spectra were processed with XWINNMR 3.1 (Bruker AG, Karlsruhe, Germany). The isotropic dipeptide (**1**) sample was prepared by dissolving 25 mg of GlyVal in 700 μl of $D_2O$ resulting in 205 mM solution. The use of the proposed technique for the extraction of RDC's was demonstrated on a liquid crystal sample of 100 mM saccharose (**2**) in 700 μl anisotropic media. For the alignment, bicelles were formed from CHAPSO/DMPC (1/3.5, 7.5%). CHAPSO/DMPC was purchased from Sigma (Sigma–Aldrich) and used without further purification. The sample was prepared as described by Sanders et al. [24]. The temperature for measurements under isotropic conditions was set to 297 K while for anisotropic conditions to 308 K. The [$^{15}$N, $^{1}$H]-labeled TROSY spectra were recorded in 1.7 mM [U-$^{15}$N]ubiquitin (95%:5% = $H_2O$:$D_2O$), pH 4.7, purchased from VLI, Southeastern, PA) at 300 K.

All *F1*-coupled HSQC spectra were acquired with high spectral resolution of ca. 0.2–0.4 Hz for accurate measurement of small residual dipolar couplings.

## 3. Results and discussion

There are many possibilities among the classical NMR methods for the measurement of one-bond

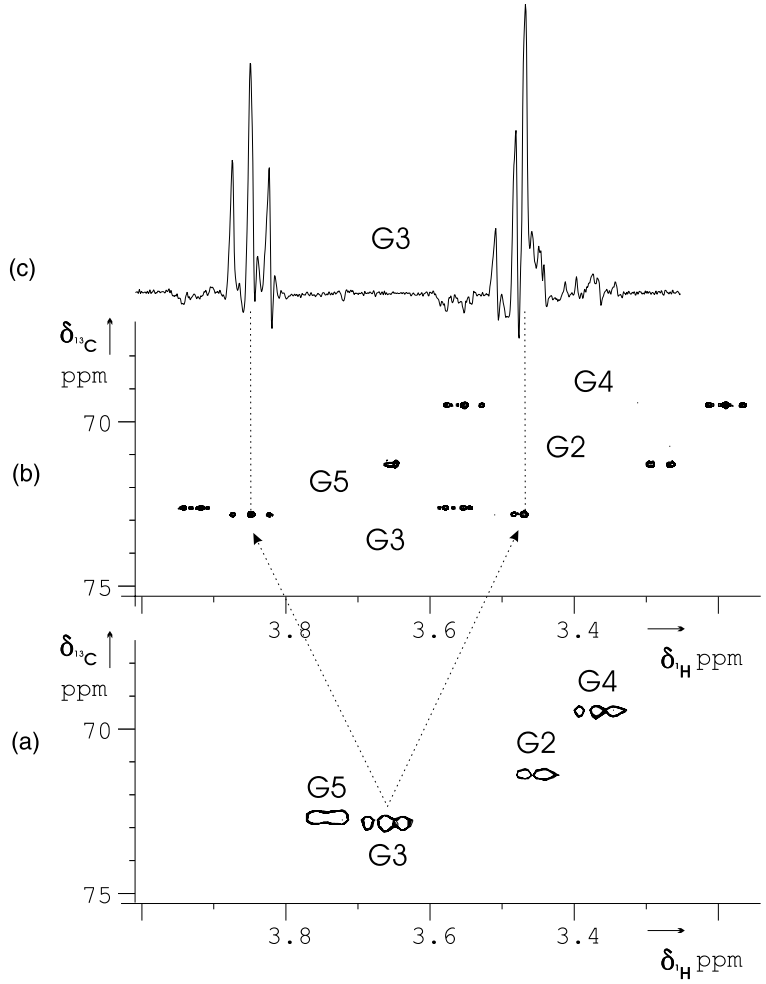



Fig. 1. Expansions of decoupled (a) and coupled (b) gradient enhanced, sensitivity improved HSQC spectra for saccharose **2** in liquid crystal medium. The inlet (c) is a $^{13}$C satellite subspectrum of Glc-H3 extracted as a row from the HSQC spectrum coupled during acquisition. In (c) due to strong coupling in the $^{13}$C-isotopomer spin system the Glc-H3 multiplet becomes asymmetric and cannot be analyzed as a first order spectrum to extract the value of the one-bond heteronuclear coupling constant.

heteronuclear coupling constants based on frequency difference such as coupled heteronuclear spectra e.g., INEPT, heteronuclear *J* resolved spectra or heteronuclear correlation spectra without decoupling. The most widely used approach is the HSQC experiment [7] without the 180° $^1$H refocusing pulse in the middle of the heteronucleus frequency labeling period (*F1*-coupled HSQC) [8]. The result is a X–$^1$H chemical shift correlation spectrum with peaks split in the indirect dimension by the $^1J_{X,H}$ coupling. Spectral crowding due to the increase of the number of crosspeaks is a problem in the case of macromolecules. To circumvent this complication the IPAP approach [9], spin-state selective methods [10–16], the TROSY technique [17–19] and the E-COSY principle [20] were proposed. Measurement in the indirect dimension is desirable due to reduction of additional splittings caused by homonuclear $^1$H, $^1$H couplings in the acquisition dimension and also because of favourable heteronuclear relaxation characteristics for macromolecules. Extraction of coupling constants from an HSQC experiment which is nondecoupled during the acquisition (*F2* coupled HSQC [21]) may also be complicated by strong coupling effects resulting in asymmetric multiplets as can be seen in Fig. 1. In these cases simulations have to be used to extract accurate values of coupling constants. Heteronuclear correlation experiments coupled in the indirect *F1* dimension are limited by the necessity of acquiring large number of $t_1$ points to achieve high digital resolution in a spectral window determined by the large chemical shift dispersion of the heteronucleus, therefore making the experiment rather time-consuming. *J* resolved spectroscopy can be employed to reduce the frequency span of the indirect dimension, this is however, often compromised by overlap in the acquisition dimension even in case of medium sized molecules.

Manifestation of any coupling different from the desired one-bond interaction further complicates the extraction of RDC's and reduces signal intensity. As mentioned before, this is in part the reason why *F2* coupled HSQC is preferably not used. There are additional splittings, however, even in *F1*-coupled HSQC spectra. During $t_1$ the antiphase X magnetization evolves not only due to the one-bond couplings, but due to the long-range heteronuclear couplings too. In the case of small molecules these couplings can readily be detected in high-resolution spectra as splittings, or as line broadening for macromolecules. Sensitivity is also degraded by splitting the resonance lines into multiplets. This is especially severe in the presence of several long-range couplings as seen for instance for Glc-5 in saccharose in Fig. 2b. The remote nuclei causing these additional splittings may also be dipolarly coupled to the heteronucleus, yielding additional dipolar contribu-

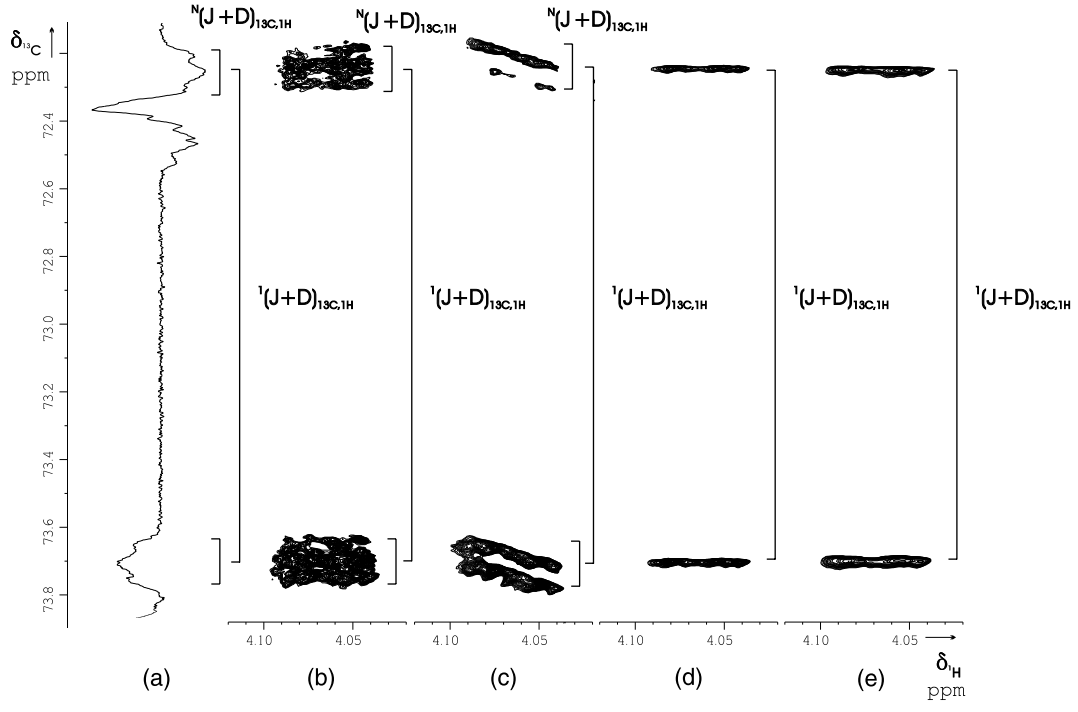



Fig. 2. Comparison of Glc-5 carbon multiplet of saccharose **2** in liquid crystal phase in nonrefocused, nondecoupled $^{13}$C INEPT (a), and in various gradient enhanced *F1*-coupled HSQC spectra without sensitivity improvement (b), with sensitivity improvement (c), with G-BIRD$^{(r)}$ module in the $t_1$ period (d), and with both sensitivity improvement and G-BIRD$^{(r)}$ module (e). In (a–c) the carbon signal is split by the one-bond $^1J_{13C,1H}$ coupling and by many long-range couplings resulting in a broad multiplet. The insertion of the G-BIRD$^{(r)}$ element (d, e) effectively removes the splittings caused by the long-range interactions.

tions of unknown magnitude to the multiplets in anisotropic media as demonstrated in Fig. 3. Multiplet patterns detected in isotropic and anisotropic media hence are different. For the precise value of coupling constants frequency difference has to be measured between multiplet centers, but not between peak maxima.

SCE HSQC [8] was suggested for proteins to decouple multiple bond heteronuclear couplings of backbone N to α and β protons by the application of selective pulses.

To solve the problem of line splittings caused by multiple bond couplings an *F1*-coupled HSQC sequence with

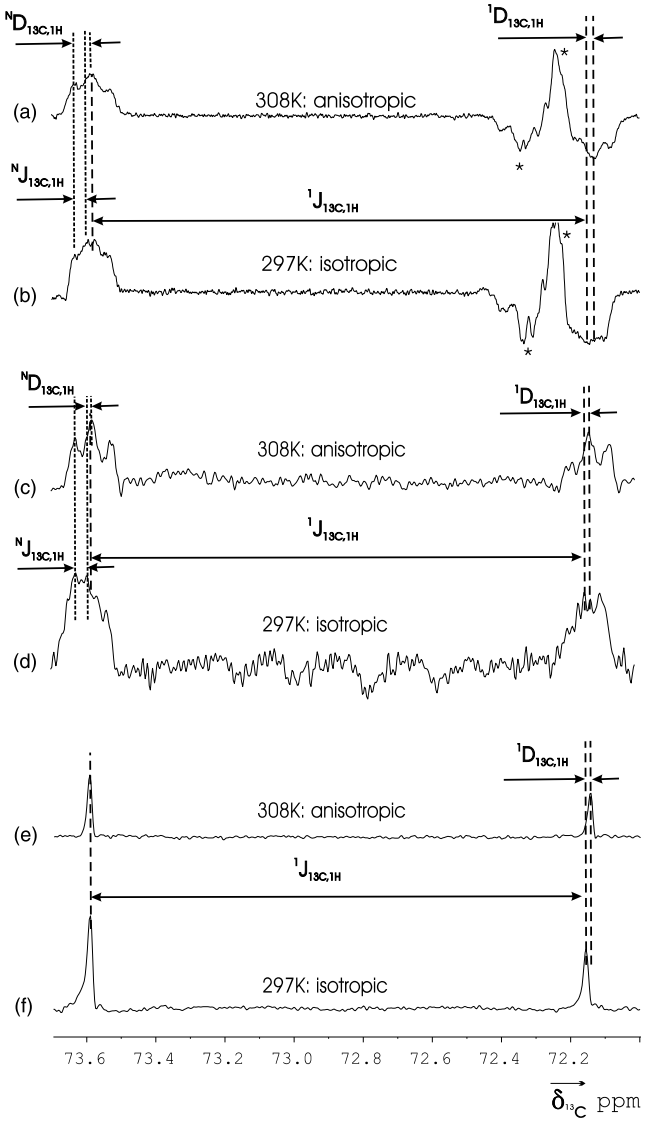



Fig. 3. Glc-5 carbon resonance of saccharose in nonrefocused, nondecoupled $^{13}C$ INEPT spectra (a and b) and the corresponding columns from gradient enhanced, sensitivity improved *F1*-coupled HSQC without (c and d) and with G-BIRD$^{(r)}$ module (e and f). Lower traces (b, d and f) are from spectra acquired in isotropic and upper traces (a, c and e) in anisotropic liquid crystalline media. Employing the G-BIRD$^{(r)}$ sequence long-range splittings disappear (e, f) allowing accurate and simple measurement of the one-bond dipolar contribution. Signals denoted with * are due to other overlapping carbons.

an inserted G-BIRD$^{(r)}$ [22] element is suggested here. The pulse sequence as depicted on Fig. 4 is actually a variation of the sensitivity enhanced, gradient selected HSQC experiment originally introduced by Kay et al. [23]. In place of the 180° $^{1}H$ refocusing pulse a G-BIRD$^{(r)}$ [24] sequence is inserted at midway of $t_1$. The G-BIRD$^{(r)}$ pulse inverts only the remote protons, and neither the directly attached nor the X nuclei are inverted. As a result, the chemical shift and heteronuclear one-bond coupling evolution of X nuclei are not affected during $t_1$, as they continue to evolve; whereas effective refocusing of heteronuclear long-range coupling evolution is achieved by the end of $t_1$ interval. The resulting correlation spectra have simplified crosspeaks with splittings due to only the desired one-bond couplings and with higher intensities. This allows the accurate determination of the scalar coupling constant and subsequently the dipolar contribution. Note that the two gradients of opposite signs purge out the fraction of magnetization that does not experience perfect rotation by the BIRD$^{(r)}$ pulse.

The method has been tested on small model compounds depicted in Scheme 1. Isotropic samples of a model dipeptide GlyVal **1** and partially oriented anisotropic sample of saccharose **2** have been used. In the peptide there is one single long-range coupling to the amide $^{15}N$ resulting in a clear doublet of doublet that can be seen on Fig. 3b. For a carbohydrate on the other hand, there are many long-range heteronuclear interactions that yield an unresolved, broad multiplet (Figs. 2a–c). The one-bond coupling constant is therefore extracted by measuring frequency difference between the multiplet centers which is a relatively easy task for the peptide, but problematical for the broad multiplet of the carbohydrate. As seen on Fig. 3 the Glc-5 carbon signal is split by the one-bond coupling to the directly bonded proton (an antiphase splitting in INEPT and an in-phase splitting in HSQC) and also by the long-range couplings to all the multiple bonded protons. Due to the large number of possible multiple bond interactions, the center of multiplets is ill-defined, as exemplified with the complex multiplet shown in Fig. 3d. The uncertainity in defining the accurate position of the multiplet center can lead to a significant error in the coupling constant measurement. However, with the use of the proposed G-BIRD$^{(r)}$ sequence, all the long-range splittings of the doublets disappear allowing accurate measurement of the desired one-bond coupling constants by simply measuring the frequency difference of the peak maxima. Furthermore, as the signal intensity is halved with each coupling partner, sensitivity gets degraded especially in carbohydrates. With the insertion of the G-BIRD$^{(r)}$ element to decouple the long-range interactions, sensitivity can significantly be improved. This is demonstrated in Fig. 5 by comparing the signal intensities of the columns extracted from the conventional and the G-BIRD$^{(r)}$ enhanced *F1*-coupled HSQC spectra.

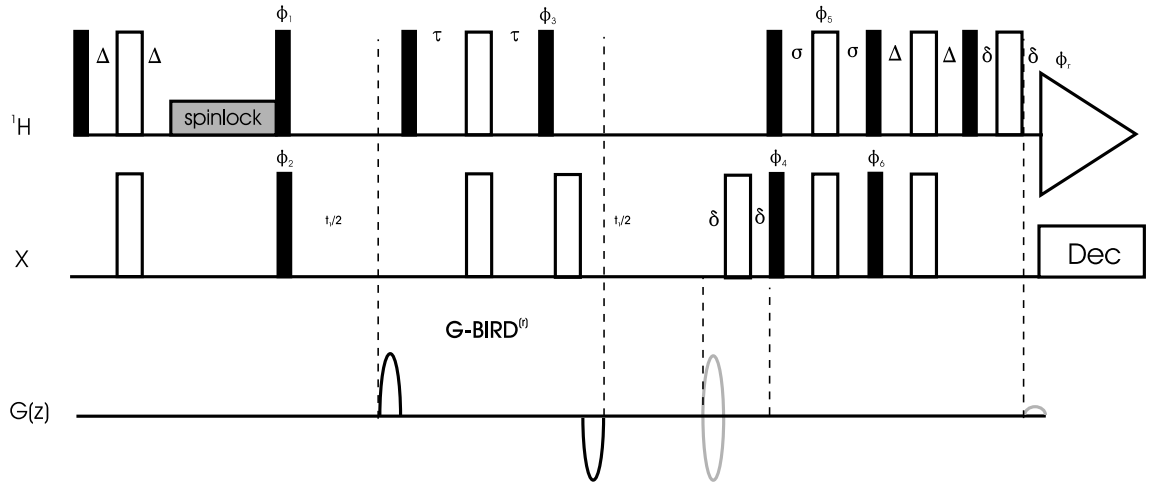
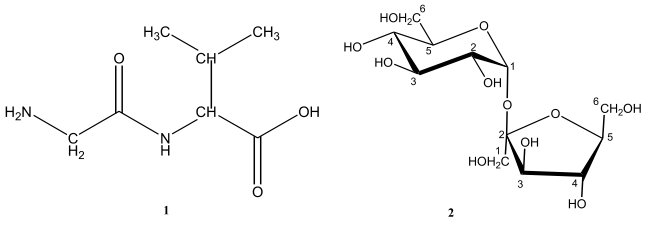



Fig. 4. Gradient enhanced, sensitivity improved HSQC sequence with G-BIRD$^{(r)}$ element in the $t_1$ interval to eliminate long-range coupling modulation of the evolving antiphase heteronucleus magnetization. Thin, filled bars represent 90° hard pulses, while thick, nonfilled bars 180° hard pulses. Phase cycling is as follows: phases for pulses without phase notation correspond to $x$, $\varphi_1 = y, -y$; $\varphi_2 = x,x,x,x,-x,-x,-x,-x$; $\varphi_3 = -x$; $\varphi_4 = x,x,-x,-x$; $\varphi_5 = y$; $\varphi_6 = y,y,-y,-y$; $\varphi_r = x,-x,-x,x,-x,x,x,-x$. The delay $\Delta$ is adjusted as $1/(4^1J_{X,H})$, $\tau$ is $1/(2^1J_{X,H})$, $\sigma$ is $1/(8^1J_{X,H})$ and $\delta$ is the duration of the shaped gradient pulses. Echo–antiecho selection is achieved by the shaded gradients.



Scheme 1. Formula of GlyVal and saccharose.

It should be noted that the mismatch of BIRD-delay, $\tau$, due to variations in the one-bond heteronuclear couplings can lead to partial inversion of the direct protons, yielding a residual "decoupled" signal in the center of doublets. The intensity of the center line depends on the difference between the actual value of the coupling constant and the one used for setting the duration of delay $\tau$. We found that the accuracy of the

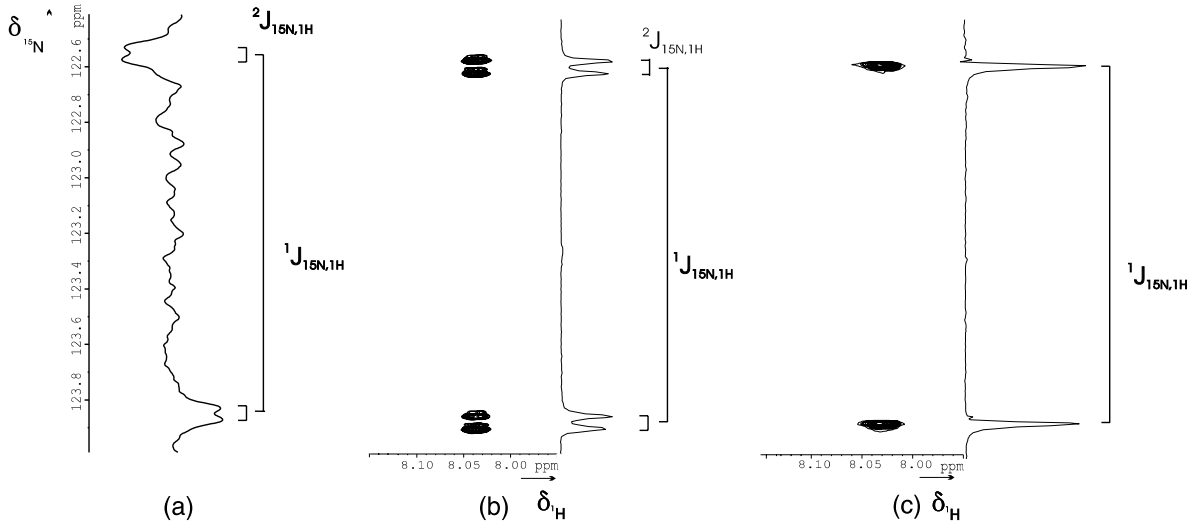



Fig. 5. Comparison of multiplicity and intensity of backbone $^{15}$N in a model dipeptide, GlyVal **1** in (a) nonrefocused, nondecoupled $^{15}$N INEPT, (b) gradient enhanced *F1*-coupled HSQC, and (c) gradient enhanced *F1*-coupled HSQC with G-BIRD$^{(r)}$ module in the $t_1$ period. In a and b the $^{15}$N signal is split by the one-bond $^1J_{15N,1H}$ coupling and by long-range coupling to Hα resulting in a well-resolved doublet. The insertion of the G-BIRD$^{(r)}$ element (c) effectively removes the splitting caused by the long-range interaction. Comparing the peak intensities in columns extracted from 2D spectra demonstrates that the decoupling of long-range interactions considerably improves the sensitivity.

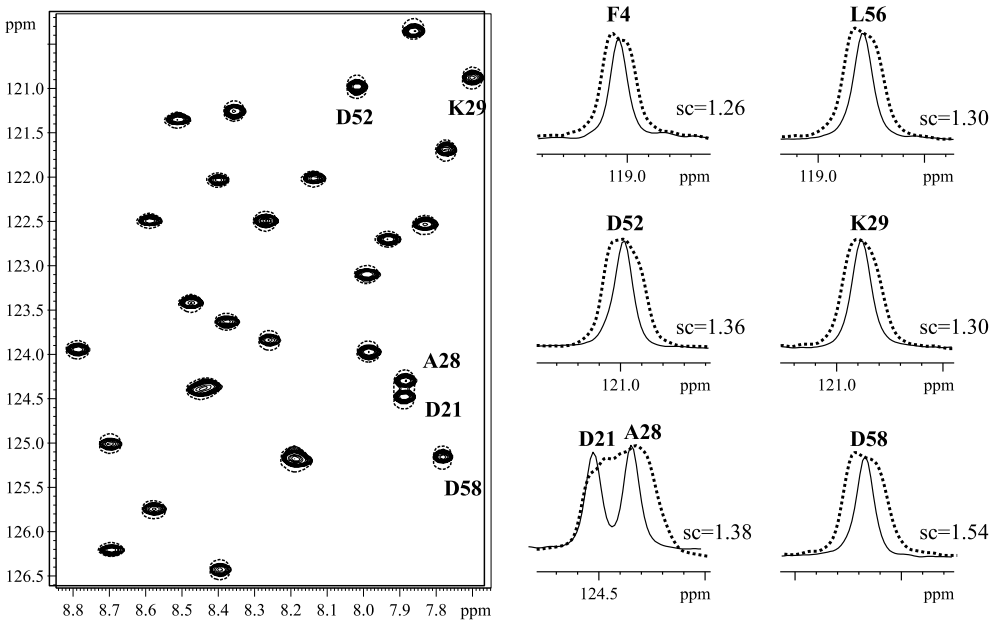



Fig. 6. Comparision of $^{15}N$, $^{1}H$ TROSY peak linewidths of ubiquitin in spectra obtained without (dashed line) and with (solid line) G-BIRD$^{(r)}$ decoupling of the long-range interactions. The advantage of the modification for macromolecules is clearly demonstrated by the obtained resolution and sensitivity enhancement. Scaling factors (sc) depicted above the traces indicate the achieved sensitivity improvement.

measured coupling constant is not degraded by the occurrence of this usually weak central signal.

In order to study the improvement that can be achieved for macromolecules, a TROSY experiment [17–19] incorporating the G-BIRD$^{(r)}$ in the $t_1$ evolution period has been carried out. As expected, long-range heteronuclear couplings cannot be resolved even in highly digitised spectra due to linewidths characteristic for macromolecules. With the application of the G-BIRD$^{(r)}$ module significant decrease in *F1* linewidths can be achieved. Decoupling of the long-range interactions yields sensitivity improvement and in certain cases may resolve overlap as can be seen for D21 and A28 residues of ubiquitin in Fig. 6. This allows accurate measurement of the heteronuclear one-bond coupling based on the appropriate TROSY-edited subspectra.

It should be noted that due to the long duration of BIRD-module the sensitivity is likely to be compromised for larger proteins. But, the arguments regarding the attainable resolution enhancement remain valid regardless of the size of the studied protein. In the case of perdeuterated proteins the BIRD-module becomes superfluous, because all the heteronuclear multiple bond couplings are eliminated by the deuteron substitution itself.

## 4. Conclusion

Small residual dipolar contributions between directly bonded nuclei can be accurately measured by means of an HSQC sequence modified with a G-BIRD$^{(r)}$ module which decouples all the long-range couplings of the heteronucleus while retaining the one bond coupling to the directly bonded proton. Summarizing the advantages of the proposed sequence: (i) allows simple frequency difference extraction between peak maxima, (ii) accurate measurement of small one-bond heteronuclear dipolar couplings without complications arising from other dipolar contributions or strong couplings, (iii) improved sensitivity due to simplification of multiplets, (iv) easy implementation of the G-BIRD$^{(r)}$ module in any variant of HSQC sequence. By incorporating the G-BIRD$^{(r)}$ element into the TROSY experiment a significant decrease in linewidth coupled with considerable sensitivity improvement can be achieved for macromolecules.

## Acknowledgments

Krisztina Fehér gratefully acknowledges the Marie Curie Training Site Fellowship grant HPMT-2000-00183. Financial support from the Hungarian National Research Foundation (OTKA T029089, and T034515) is gratefully acknowledged by K.E.K.

## References

[1] E. de Alba, N. Tjandra, NMR dipolar couplings for the structure determination of biopolymers in solution, Prog. Nucl. Magn. Reson. Spectrosc. 40 (2) (2002) 175–197.

[2] J.R. Tolman, J.M. Flanagan, M.A. Kennedy, J.H. Prestegard, Nuclear magnetic dipole interactions in field-oriented proteins – information for structure determination in solution, Proc. Natl. Acad. Sci. USA 92 (20) (1995) 9279–9283.

[3] M. Zweckstetter, A. Bax, Prediction of sterically induced alignment in a dilute liquid crystalline phase: aid to protein structure determination by NMR, J. Am. Chem. Soc. 122 (15) (2000) 3791–3792.
[4] M.X. Fernandes, P. Bernado, M. Pons, J.G. de la Torre, An analytical solution to the problem of the orientation of rigid particles by planar obstacles. Application to membrane systems and to the calculation of dipolar couplings in protein NMR spectroscopy, J. Am. Chem. Soc. 123 (2001) 12037–12047.
[5] T.N. Pham, T. Liptaj, K. Bromek, D. Uhrin, Measurement of small one-bond proton-carbon residual dipolar coupling constants in partially oriented carbon-13 natural abundance oligosaccharide samples: analysis of heteronuclear (1)J(CH)-modulated spectra with the BIRD inversion pulse, J. Magn. Reson. 157 (2) (2002) 200–209.
[6] D. Uhrin, T. Liptaj, K.E. Kover, Modified bird pulses and design of heteronuclear pulse sequences, J. Magn. Reson. Series A 101 (1) (1993) 41–46.
[7] G. Bodenhausen, D.J. Ruben, Natural abundance N-15 NMR by enhanced heteronuclear spectroscopy, Chem. Phys. Lett. 69 (1) (1980) 185–189.
[8] J.R. Tolman, J.H. Prestegard, Measurement of amide N-15-H-1 one-bond couplings in proteins using accordion heteronuclear-shift-correlation experiments, J. Magn. Reson. Series B 112 (3) (1996) 269–274.
[9] M. Ottiger, F. Delaglio, A. Bax, Measurement of *J* and dipolar couplings from simplified two-dimensional NMR spectra, J. Magn. Reson. 131 (2) (1998) 373–378.
[10] P. Andersson, J. Weigelt, G. Otting, Spin-state selection filters for the measurement of heteronuclear one-bond coupling constants, J. Biomol. NMR 12 (1998) 435–441.
[11] P. Addersson, K. Nordstrand, M. Sunnerhagen, E. Liepinsh, I. Turovskis, G. Otting, Heteronuclear correlation experiments for the determination of one-bond coupling constants, J. Biomol. NMR 11 (1998) 445–450.
[12] M.D. Sorensen, A. Meissner, O.W. Sorensen, Carbon-13 natural abundance (SE)-E-3 and (SCT)-C-3 experiments for measurement of *J* coupling constants between Carbon-13(alpha) or H- 1(alpha) and other protons in a protein, J. Magn. Reson. 137 (1) (1999) 237–242.
[13] P. Andersson, A. Annila, G. Otting, An $\alpha/\beta$ experiment for spin-selective editing of IS cross peaks, J. Magn. Reson. 133 (1998) 364–367.
[14] B. Brutscher, J. Boisbouvier, A. Pardi, D. Marion, J.P. Simorre, Improved sensitivity and resolution in H-1-C-13 NMR experiments of RNA, J. Am. Chem. Soc. 120 (46) (1998) 11845–11851.
[15] M.H. Lerche, A. Meissner, F.M. Poulsen, O.W. Sorensen, Pulse sequences for measurement of one-bond N-15-H-1 coupling constants in the protein backbone, J. Magn. Reson. 140 (1) (1999) 259–263.
[16] F. Cordier, A.J. Dingley, S. Grzesiek, A doublet-separated sensitivity-enhanced HSQC for the determination of scalar and dipolar one-bond *J*-couplings, J. Biomol. NMR 13 (2) (1999) 175–180.
[17] K. Pervushin, R. Riek, G. Wider, K. Wuthrich, Attenuated T-2 relaxation by mutual cancellation of dipole–dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution, Proc. Natl. Acad. Sci. USA 94 (1997) 12366–12371.
[18] J. Weigelt, Single scan, sensitivity- and gradient-enhanced TROSY for multidimensional NMR experiments, J. Am. Chem. Soc. 120 (1998) 10778–10779.
[19] M. Czisch, R. Boelens, Sensitivity enhancement in the TROSY experiment, J. Magn. Reson. 134 (1998) 158–160.
[20] C. Griesinger, O.W. Sorensen, R.R. Ernst, Practical aspects of the E-Cosy technique—measurement of scalar spin spin coupling-constants in peptides, J. Magn. Reson. 75 (3) (1987) 474–492.
[21] M. Ruckert, G. Otting, Alignment of biological macromolecules in novel nonionic liquid crystalline media for NMR experiments, J. Am. Chem. Soc. 122 (2000) 7793.
[22] J.R. Garbow, D.P. Weitekamp, A. Pines, Bilinear rotation decoupling of homonuclear scalar interactions, Chem. Phys. Lett. 93 (5) (1982) 504–509.
[23] L.E. Kay, P. Keifer, K. Saarinen, Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity, J. Am. Chem. Soc. 114 (26) (1992) 10663–10665.
[24] C.R. Sanders, J.H. Prestegard, Magnetically orientable phospholipid-bilayers containing small amounts of a bile-salt analog, chapso, Biophys. J. 58 (2) (1990) 447–460.