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# H2BC: a new technique for NMR analysis of complex carbohydrates

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**Abstract**—It is demonstrated that the H2BC NMR pulse sequence (*J. Am. Chem. Soc.* **2005**, *127*, 6154, *Magn. Reson. Chem.* **2005**, *43*, 971–974) offers unambiguous assignments and significant simplification of NMR spectra of large and complex carbohydrates compared to other techniques for the establishment of correlations over more than one bond. H2BC almost exclusively correlates protons and proton-bearing carbon spins separated by two covalent bonds and is independent of occasionally vanishing $^{2}J_{CH}$ coupling constants, which alleviates the problem of missing two-bond correlations in HMBC spectra. H2BC also solves the problem of distinguishing two- and three-bond correlations in HSQC–TOCSY or HMBC. It is a further asset of H2BC that the experiment is significantly shorter than HMBC and HSQC–TOCSY, and hence less sensitive to transverse relaxation. The H2BC experiment is demonstrated on an approximately 30-residue oligosaccharide from *Francisella victoria*.


*Keywords:* Oligosaccharide; NMR; $^{1}$H; $^{13}$C; H2BC

Structure assignment of complex carbohydrates, oligo- or polysaccharides is a prerequisite for understanding their biological function and biosynthesis. One of the most powerful methods for this purpose has proven to be nuclear magnetic resonance (NMR) in combination with, for example, mass spectrometry or chemical analysis methods.

NMR structure determination of large carbohydrates at natural $^{13}$C isotopic abundance is primarily faced with two problems: low sensitivity and spectral overlap caused by inherently low chemical shift dispersion of the sugar residues and low diversity even at high field. In contrast to NMR of proteins and other biomolecules, where $^{13}$C and $^{15}$N enrichment is used routinely, isotopic enrichment is currently not widespread for large carbohydrates and hence NMR work on carbohydrates is normally performed on natural abundance samples. Two-dimensional NMR techniques, and especially such involving $^{13}$C together with $^{1}$H and thereby combining the chemical shift dispersions of the two nuclei, are indispensable for the assignment of large and complex oligo- or polysaccharides. Occasionally, also three-dimensional NMR techniques are used.

This paper demonstrates, through application to a challenging complex oligosaccharide consisting of about 30 residues, that the recently published pulse sequence H2BC[1,2] (heteronuclear 2-bond correlation) is very useful for the assignment of carbohydrates. The key advantage of H2BC is that it pinpoints the two-bond correlations, in contrast to alternative pulse sequences for establishing multiple-bond correlations, HMBC[3] and HSQC–TOCSY, which usually yield no indication of the number of bonds between the correlated $^{1}$H and $^{13}$C nuclei. Needless to say, the simplicity of H2BC spectra is another asset in work with large carbohydrates. Thus H2BC is expected to find its place in the standard protocol of carbohydrate NMR experiments.[4]

As described in detail elsewhere,[1] the H2BC experiment initially excites proton magnetization and then,

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in a delay $T$ of typically 15–25 ms, establishes an anti-phase character with respect to another coupled proton via $^{3}J_{HH}$. The constant-time delay $T$ is also used for temporary excitation of $^{13}C$ coherence via $^{1}J_{CH}$ so that $^{13}C$ chemical shifts evolve in the indirect dimension of the 2D experiment. Next, the proton magnetization anti-phase with respect to $^{3}J_{HH}$ is transferred for detection by a $\pi/2$(H) pulse to the coupled proton three bonds away from the original proton and two-bonds away from the $^{13}C$ monitored in the $F_1$ dimension.

The largest carbohydrate structures determined by NMR so far are, to the best of our knowledge, composed of 23 and 25 monosaccharide residues.[5,6] The full structure of the oligosaccharide used for demonstration in this paper will be published separately. Briefly, the purification of lipopolysaccharide from the bacteria *Francisella victoria* afforded a large complex oligosaccharide of about 30 residues as the main component along with a couple of minor components of similar size.

Figure 1 shows the spectral region of the $^{1}H$ anomeric signals of this glycan, and the correlations to C1 and C2. In grey are shown the one-bond C1–H1 anomeric correlations and C5–H5 fucose correlations obtained by a gradient-enhanced HSQC experiment. Overlaid in black is the plot of the two-bond correlations involving the anomeric protons obtained by an H2BC experiment. For the main component, this is the complete set of C2–H1 correlations and nothing else.

The alternative to H2BC for obtaining the C2–H1 correlations would be HSQC–TOCSY, but such a spectrum would also contain correlations between the anomeric protons and further intra-ring $^{13}C$, that is, it would not be possible to unequivocally assign the C2 carbons from such a spectrum. In HMBC spectra, as

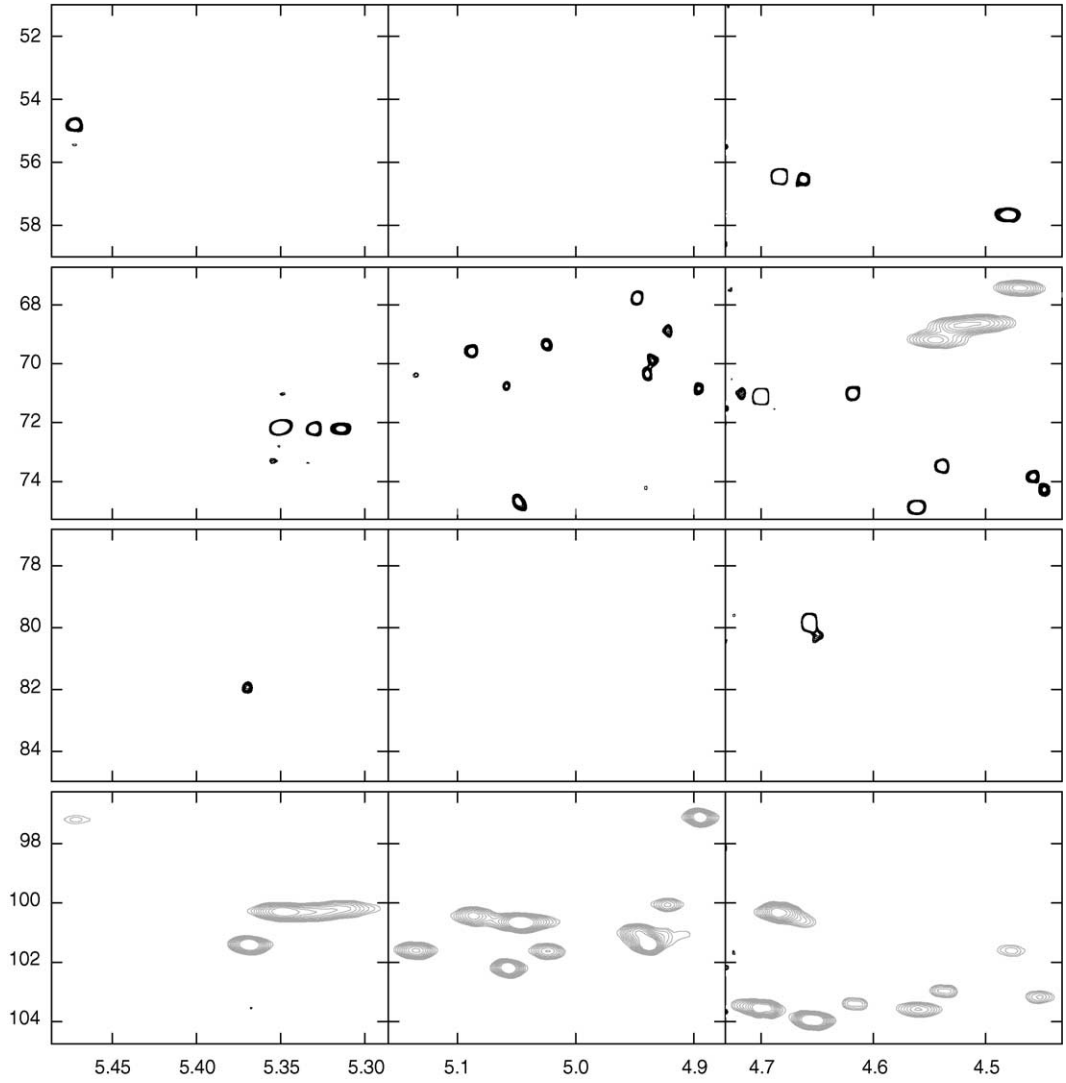


**Figure 1.** $^{1}H$ anomeric region of overlaid HSQC (grey) and H2BC (black) 800 MHz NMR spectra of the about 30-residue oligosaccharide from *Francisella victoria*.

an alternative for establishing C2–H1 correlations, some of the correlations might be missing due to vanishing $^{2}J$(C2–H1) coupling constants.[7–9] Both H2BC and HSQC–TOCSY are independent of these $J$ coupling constants as they rely on $^{3}J$(H1–H2) for establishing C2–H1 correlations. An example of such an HMBC-unfriendly and H2BC-friendly situation is in sugar residues with an anti-periplanar arrangement of H1 and H2 like β-glucopyranosides or β-galactopyranosides, which have large $^{3}J$(H1–H2) couplings and small $^{2}J$(H1–C2). The opposite occurs in, for example, β-mannopyranosides with a small $^{3}J$(H1–H2) and a relatively large $^{2}J$(H1–C2),[8,9] that is, a stronger C1–H2 two-bond correlation can be expected in HMBC than in H2BC. Other examples include 3-deoxy-D-manno-octulosonic acid (Kdo) residues as illustrated below.

Another example of the usefulness of H2BC as shown in Figure 2 involves not starting the assignment from the anomeric end but from the opposite end of the spin system. Similar to Figure 1, the alternatives of HSQC–TOCSY and HMBC would not uniquely identify H5 for glucose units or H7 for Kdo units, as their spectra possibly would also contain correlations between H4 and C6 or H6 and C8, respectively. The H2BC spectrum was recorded with the edited version of H2BC[2] with $^{13}$CH+$^{13}$CH$_3$ signals in one subspectrum (plotted in red) and $^{13}$CH$_2$ signals in another (plotted in fade red). These H2BC spectra are overlaid with edited HSQC spectra showing CH$_2$ (plotted in green) and CH+CH$_3$ (plotted in fade green) one-bond correlations. The editing pulse sequences are useful for solving overlap involving hydroxymethyl groups of several monosaccharide residues, especially when glycosylation of these gives rise to a $^{13}$C downfield shift to about 66–72 ppm into the bulk region of CH signals as is evident in the lower part of Figure 2. Also illustrated in Figure 2 is another key aspect of H2BC, namely effective suppression of one-bond correlations by a third-order low-pass $J$ filter.[10] Without this filter, the leftmost C8,H7 peak could easily have been obscured by a dominating C8,H8a one-bond correlation peak.

As has been demonstrated above, the pinpointing of two-bond correlations in H2BC is a valuable help in spectral assignments but concomitantly there is also the fact of spectral simplification in H2BC, as these spectra ideally contain only two-bond correlations, that is, there are fewer peaks than in HSQC–TOCSY or HMBC spectra. This is illustrated in Figure 3a with the bulk region of the H2BC spectrum. The number of possible two-bond correlations is about double the number of one-bond correlations and less than the number of peaks in HMBC or HSQC–TOCSY spectra. In fact, the bulk spectral region of this carbohydrate is sufficiently well resolved to allow numerous assignments in combination with a one-bond correlation spectrum. For comparison, the same region from the HMBC spectrum is shown in Figure 3b. The H2BC and HMBC spectra are quite complementary as can be seen in the expanded frame in Figure 3c with the two spectra overlaid, and hence the combination of the two is most helpful for assigning this crowded spectral region of carbohydrate spectra. The interplay of the two is essen-

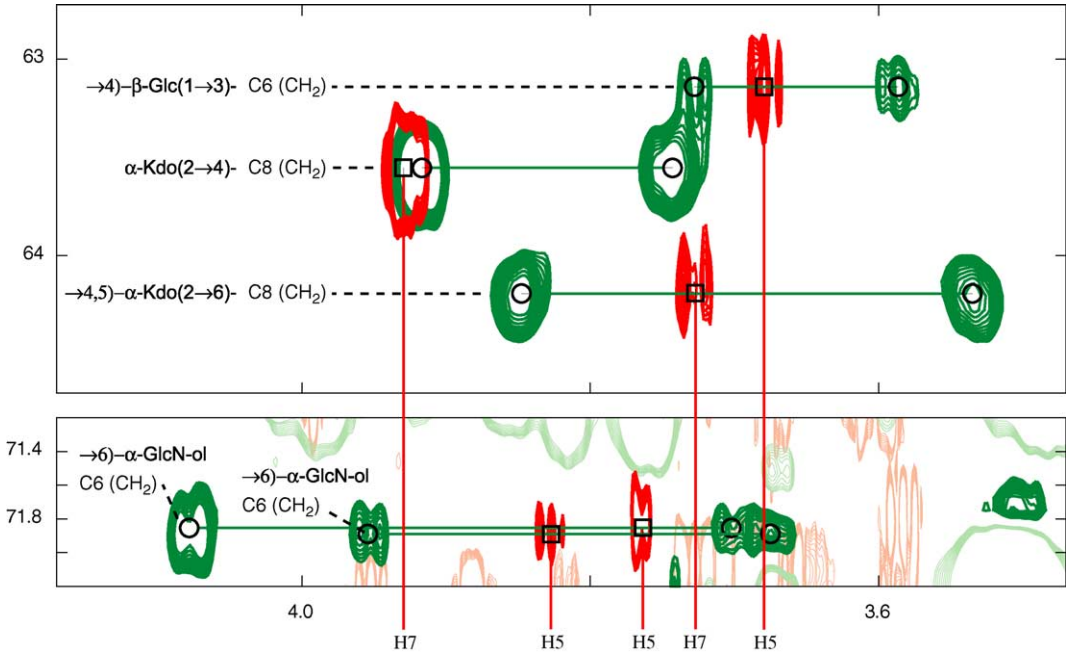


**Figure 2.** Excerpt from an edited HSQC (green colour representing CH$_2$ groups and fade green representing CH+CH$_3$ groups) spectrum showing one-bond correlations from H6a/H6b to C6 (or from H8a/H8b to C8) overlaid with an edited H2BC (red representing CH+CH$_3$ groups and fade red representing CH$_2$ groups) spectrum giving the correlation to H5 or H7 for five of the sugar residues.

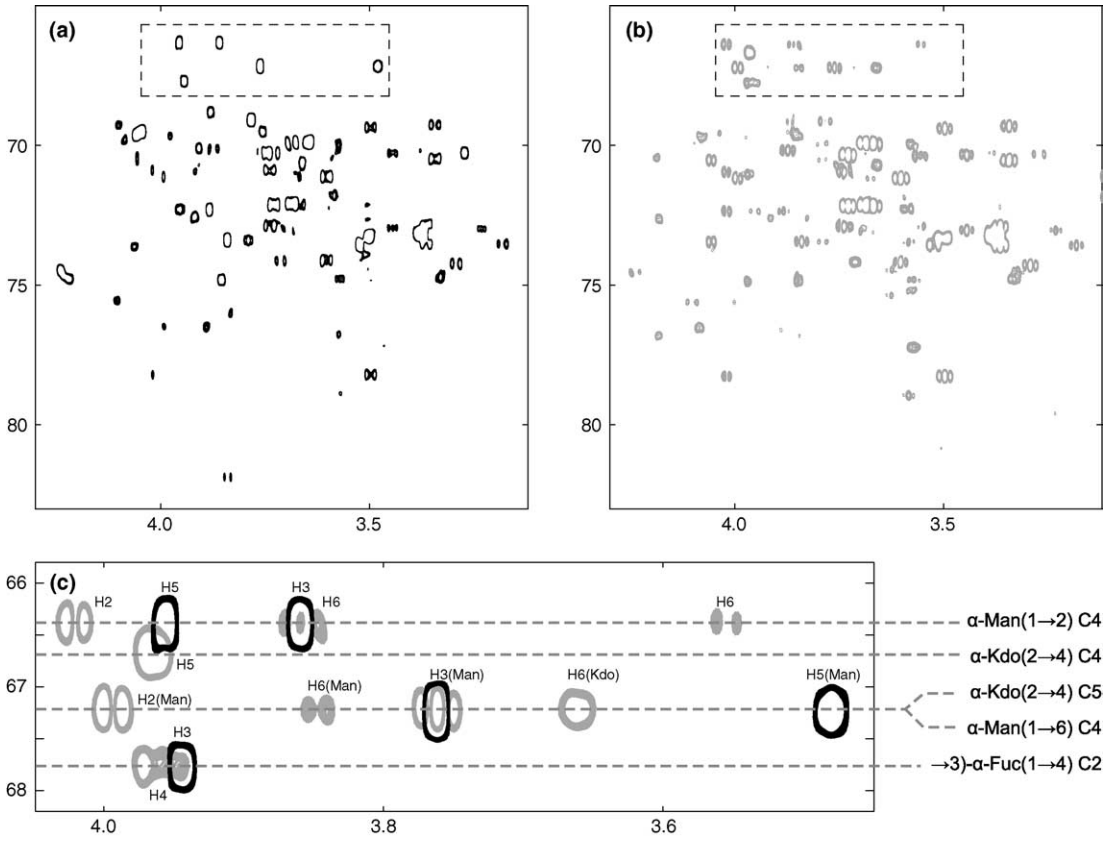


**Figure 3.** The crowded bulk region of (a) H2BC, (b) HMBC NMR spectra of the studied large oligosaccharide and (c) expansion of the framed region in (a) and (b).

tial when $^{3}J_{HH}$ vanishes and at least one of the corresponding $^{2}J_{CH}$ does not. The expansion in Figure 3c shows all H2BC peaks to be expected for C2 in the fucopyranose and C4 in the two mannopyranose units while the mannopyranose C4–H5 two-bond correlations are missing in the HMBC spectrum due to vanishing $^{2}J$(C4–H5) coupling constants. For the Kdo units, the C4–H5 and C5–H6 correlations are missing or very weak in the H2BC spectrum due to vanishing $^{3}J$(H4–H5) and $^{3}J$(H5–H6) coupling constants, respectively, whilst the C4 correlation to the deoxy H3 protons are present but outside of the spectral region of Figure 3c.

Note that H2BC peaks for molecules of this size often do not exhibit multiplet structure because the phase-sensitive spectra are phased to pure dispersion in the $F_2$ dimension,[2] which concentrates the intensity in the centre of the peaks.

Sugar units with a configuration corresponding to the configuration of C3, C4 and C5 in galactopyranose represent an unusual case of long-range correlations. In this category, the oligosaccharide studied contains several fucose residues and a few Kdo residues where C5 corresponds to C4 in galactopyranose. The following was observed in these Kdo residues. No C5–H6 two-bond correlations were present in the H2BC spectrum due to vanishing $^{3}J$(H5–H6) coupling constants, but all these correlations were observed in the HMBC spectrum where the coherence transfer pathway relies on $^{2}J$(C5–H6). No C5–H7 three-bond correlations were observed in the HMBC spectrum probably due to vanishing $^{3}J$(C5–H7) coupling constants in the Kdo configuration. C5–H4 two-bond correlations were observed in both spectra with the stronger ones in the HMBC spectrum where all of them were present. In the HMBC spectrum, only few C5–H3 three-bond correlations were observed. Finally, the spectral features around C4 in the fucose residues were similar to the observations around C5 in Kdo.

Figure 4 shows an excerpt from the overlaid HSQC (plotted in green) and H2BC (plotted in red) spectra showing how the entire intra-ring assignment of a 3-substituted β-QuiN (2-amino-2,6-dideoxyglucose) sugar unit from C1,H1 to C6,H6 can be traced out. This kind of assignment walks hinge on the two-bond correlation exclusivity of H2BC that is the key feature of the technique. Similar unequivocal assignments can be obtained by the INADEQUATE experiment that currently is not feasible with the amount of material available in this study.

H2BC supplements the experiments in the standard protocol of carbohydrate NMR experiments rather than replacing any of them. H2BC can be considered equivalent to HSQC–TOCSY with a cut-off after the first

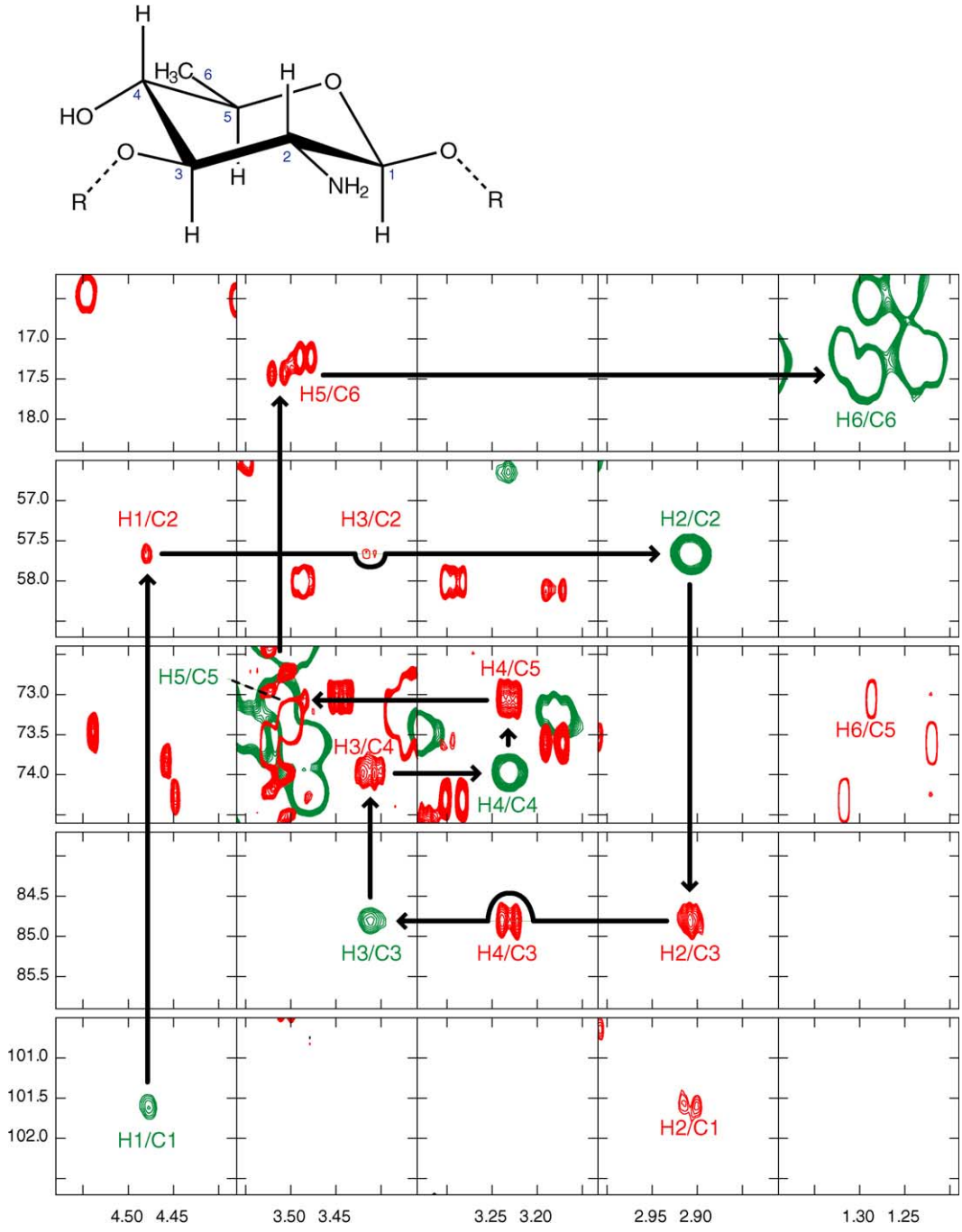


**Figure 4.** Example of the assignment of the complete spin system for a 3-substituted β-QuiN residue based on the overlaid HSQC (green) and H2BC (red) spectra starting from the anomeric proton/carbon 4.478 ppm/101.6 ppm correlation in the HSQC spectrum via H2BC to H2/C2 2.908 ppm/57.7 ppm and all the way to H6/C6 1.288 ppm/17.4 ppm.

$^{1}$H–$^{1}$H TOCSY transfer, that is, no further transfer than two-bonds away from the pertinent carbon, but all H2BC correlations can usually also be found in HSQC–TOCSY spectra. The complementarity between HMBC and H2BC spectra is based on the fact that the strongest correlations in HMBC spectra often are over three bonds while some two-bond correlations are absent. Furthermore, HMBC is usually superior to H2BC and HSQC–TOCSY in bridging across the glycosidic bond in carbohydrates.

The following is a practical procedure for the interpretation of heteronuclear correlation spectra over more than one bond. A priori, the assumption is that all peaks in H2BC spectra represent two-bond correlations. Error signals in H2BC spectra, that is, correlations over more than two bonds only occur to the extent that four-bond

or higher $J_{HH}$ coupling constants[11] do not vanish. Hence it must be kept in mind that there is a chance that the weakest peaks in H2BC spectra can represent three-bond or higher correlations, although that is unlikely in applications to large carbohydrates. All correlations in H2BC spectra will normally also occur in HSQC–TOCSY spectra, which then tentatively allows all peaks present in the HSQC–TOCSY spectrum and absent in the H2BC spectrum to be interpreted as correlations over more than two bonds. A similar rule applies to the HMBC spectrum compared to the H2BC spectrum, although it must be kept in mind that a two-bond correlation can be stronger in the HMBC spectrum (relying on $^2J_{CH}$) than in the H2BC spectrum (relying on $^3J_{HH}$) when $^2J_{CH}$ is larger than $^3J_{HH}$. In the extreme case of a vanishing $^3J_{HH}$, a two-bond correlation can be absent in the H2BC spectrum and only be observed in the HMBC spectrum as discussed for C4–H5 correlations in the galactopyranose configuration above.

In conclusion, H2BC is useful as a general technique for assigning heteronuclear two-bond correlations and for tracing out the proton-bearing carbon skeleton of the molecule together with an HSQC spectrum. HSQC–TOCSY is useful for delineating the intra-residue $^1$H spin systems on the anomeric carbons. HMBC is unique for bridging the glycosidic bond. In addition, HMBC is a valuable support for H2BC in the bulk region and for both H2BC and HSQC–TOCSY in the region of the anomeric carbons. The key assets of H2BC in work with large carbohydrates is (1) that the peaks observed in H2BC spectra in general can be relied on as representing correlations over two bonds, and (2) that two-bond correlation peaks are observed in H2BC spectra that are absent in HMBC spectra due to vanishing $^2J_{CH}$ coupling constants.

## 1. Experimental

### 1.1. Sample preparation

*F. victoria* was initially isolated as the predominant Gram-negative pathogen from the kidney of a moribund, apparently wild *Tilapia* sp., *Oreochromis niloticus*. *F. victoria* grew slowly at <30 °C on cysteine heart agar and was positively speciated as a *Francisella* sp. by cross reactivity with rabbit antisera to three well-known *Francisella* sp. and as a unique species by 16S rRNA gene sequencing. Oligosaccharide preparation will be described in a future publication.

### 1.2. NMR spectroscopy

All NMR spectra of about 30-residue oligosaccharide from *F. victoria* were recorded at 25 °C on a Varian Unity Inova 800 instrument at 799.96 MHz for proton and 201.12 MHz for carbon using a 5 mm cold probe. The sample contained 3 mg of freeze-dried material dissolved in 120 μL $D_2O$ in a 3 mm NMR tube. The parts per million scales are calibrated to acetone for proton (2.225 ppm) and to 1,4-dioxane for carbon (67.4 ppm).

**1.2.1. HSQC experiments.** The delays of the gradient-enhanced pulse sequence were tuned for a one-bond coupling of 140 Hz. The number of scans was 32, the acquisition time 0.21 s with WURST adiabatic decoupling with $B_1$ field strength of 3100 Hz as limited by the cold probe, and the relaxation delay 1.5 s. The data matrix (784, 2$k$) covering 30,000 Hz in $F_1$ and 5000 Hz in $F_2$ were treated with a cosine window function in $t_1$ and $t_2$ prior to Fourier transformation.

**1.2.2. HMBC experiments.** The gradient-enhanced pulse sequence employed an excitation delay of 63 ms and a third-order low-pass $J$ filter set for $140 < J < 170$ Hz for suppression of one-bond correlations. The number of scans was 64, the acquisition time 0.21 s and the relaxation delay 1.5 s. The data matrix (784, 2$k$) covering 30,000 Hz in $F_1$ and 5000 Hz in $F_2$ were treated with a cosine window function in $t_1$ and a $\pi/4$ shifted sine in $t_2$ prior to Fourier transformation.

**1.2.3. H2BC experiments.** Both the standard H2BC and edited H2BC spectra were obtained with $T = 21.8$ ms, a relaxation delay of 1.5 s, 64 scan. The third-order low-pass filter was set for $125 < {}^1J_{CH} < 170$ Hz for the standard experiment and $140 < J < 170$ Hz for the editing pulse sequences, as the focus in the latter was the bulk region without the methyl groups. An acquisition time of 0.21 s was used in the standard H2BC experiment with WURST adiabatic decoupling with $B_1$ field strength of 1100 Hz. The data matrix (784, 2$k$) covering 30,000 Hz in $F_1$ and 5000 Hz in $F_2$ were treated with a cosine window function in $t_1$ and a $\pi/4$ shifted sine in $t_2$ prior to Fourier transformation. In the editing H2BC experiment, the parameters were the same as in the standard H2BC experiment apart from an acquisition time of 0.41 s doubling the size of the data matrix. Addition and subtraction of the standard and up-down pulse sequence data sets yielded the $CH+CH_3$ and $CH_2$, subspectra, respectively.

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## References

1. Nyberg, N. T.; Duus, J. Ø.; Sørensen, O. W. *J. Am. Chem. Soc.* **2005**, *127*, 6154–6155.
2. Nyberg, N. T.; Duus, J. Ø.; Sørensen, O. W. *Magn. Reson. Chem.* **2005**, *43*, 971–974.
3. Bax, A.; Summers, M. *J. Am. Chem. Soc.* **1986**, *108*, 2093–2094.
4. Duus, J. Ø.; Gotfredsen, C. H.; Bock, K. *Chem. Rev.* **2000**, *100*, 4589–4614.
5. Vinogradov, E.; Petersen, B. O.; Sadovskaya, I.; Jabbouri, S.; Duus, J. Ø.; Helander, I. M. *Eur. J. Biochem.* **2003**, *270*, 3036–3046.
6. du Penhoat, C. H.; Gey, C.; Pellerin, P.; Perez, S. *J. Biomol. NMR* **1999**, *14*, 253–271.
7. Bock, K.; Pedersen, C. *J. Chem. Soc., Perkin Trans. 2* **1974**, 293–297.
8. Oikawa, M.; Adachi, S.; Kusumoto, A. *Org. Lett.* **2004**, *7*, 661–664.
9. Podlasek, C. A.; Wu, J.; Stripe, W. A.; Bondo, P. B.; Serianni, A. S. *J. Am. Chem. Soc.* **1995**, *117*, 8635–8644.
10. Kogler, H.; Sørensen, O. W.; Bodenhausen, G.; Ernst, R. R. *J. Magn. Reson.* **1983**, *55*, 157–163.
11. Otter, A.; Bundle, D. R. *J. Magn. Reson.* **1995**, *B109*, 194–201.
12. Kjær, M.; Andersen, K. V.; Poulsen, F. M. *Methods Enzymol.* **1994**, *239*, 288–307.