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Communication

3D H2BC: A novel experiment for small-molecule and biomolecular NMR at natural isotopic abundance

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ABSTRACT

3D H2BC is introduced for heteronuclear assignment on natural abundance samples even for biomolecules up to at least 10 kDa in low millimolar concentrations as an overnight experiment using the latest generation of cryogenically cooled probes. The short pulse sequence duration of H2BC is maintained in the 3D version due to multiple use of the constant-time delay. Applications ranging from a small lipid to a non-recombinant protein demonstrate the merits of 3D H2BC and the ease of obtaining assignments in chains of protonated carbons.

1. Introduction

Over the last couple of decades NMR spectroscopy has established itself as a powerful approach for detailed studies of structure and function of biomolecules in solution. An arsenal of multidimensional experiments combined with uniform or selective isotope labeling with ^{13}C and/or ^{15}N has continued to increase the size limit of biomolecules amenable to investigation by NMR.

Whilst isotope labeling in many cases has become routine for recombinant proteins, there are other large molecules where such labeling is not common, costly or too time consuming. Hence it is of interest to explore an extension of the suite of NMR experiments for natural abundance samples using the latest generation of sensitive cold probes. Such an experiment is introduced in this Communication starting from the H2BC NMR experiment designed for obtaining two-bond correlations in work with small molecules without any form of isotope labeling.

2. Results and discussion

The easy part of heteronuclear natural abundance NMR is to correlate pairs of, e.g., ^{13}C and ^{1}H nuclei covalently bound to each other and hence NMR-wise connected by a large one-bond coupling constant. In isotope-labeled molecules such spin pairs can be correlated with neighboring spin pairs via other relatively large one-bond coupling constants that effectively are absent in natural abundance work. Thus correlating spin pairs with each other in natural abundance has to occur via smaller long-range coupling constants.

Time required for coherence transfer or correlation of two spins is roughly proportional to the inverse of the coupling constant between them, which is why coherence transfer via long-range coupling constants requires relatively long time. That in turn is a problem for larger molecules because the transverse relaxation time generally decreases with increasing molecular size and hence makes coherence transfer inefficient.

H2BC originally introduced as a two-dimensional (2D) experiment for two-bond correlation in small molecules [1–3] is also well-suited for larger molecules because the pulse sequence is extraordinarily short considering that it includes coherence transfer via long-range ^{1}H–^{1}H couplings. The two spectra of HSQC (one-bond correlation) and H2BC combined allow full assignment of chains of protonated carbons [1–3], but the novel experiment in Fig. 1 combines these features in a single 3D experiment while maintaining the short duration of H2BC with the added bonus of improved resolution due to the third dimension.

3D H2BC is a very compact experiment with the constant-time delay, *T*, exploited for multiple purposes. The ^{13}C evolution part is of the one-bond HMQC type with heteronuclear decoupling whilst the ^{1}H evolution part and coherence transfer between ^{1}H spins is of the constant-time COSY type. The final part of the pulse sequence

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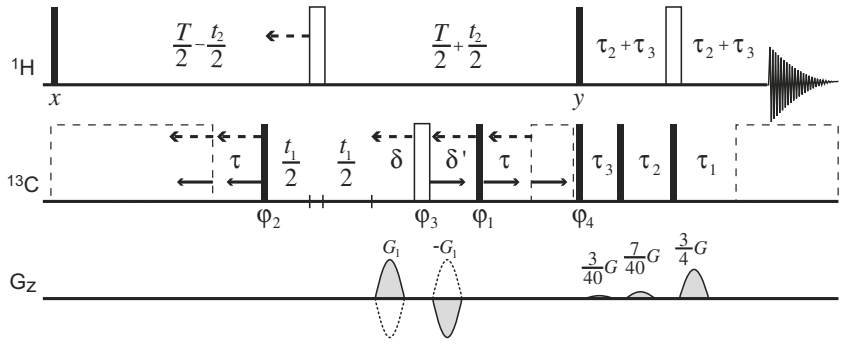

Fig. 1. Pulse sequence for 3D H2BC with a 3rd order low-pass J filter. Filled and open bars represent $\pi/2$ and π pulses, respectively, and dashed boxes indicate ^{13}C decoupling. T denotes the constant time delay of 15–25 ms. $G_1 = (\gamma_H/4\gamma_C)G$ for echo and $G_1 = -(\gamma_H/4\gamma_C)G$ for antiecho. The filter delays are $\tau_1 = 1/2[{}^1J^{min} + 0.07({}^1J^{max} - {}^1J^{min})]^{-1}$, $\tau = \tau_2 = ({}^1J^{max} + {}^1J^{min})^{-1}$ and $\tau_3 = 1/2[{}^1J^{min} - 0.07({}^1J^{max} - {}^1J^{min})]^{-1}$ [8], while $\delta' = \delta + t(\pi^H)$, where δ is the gradient delay. J^{min} and J^{max} were set to 135 and 165 Hz, respectively, for all the spectra shown. The recommended phase cycle is an even number of steps out of $\varphi_1 = \{x, -x, -x, x\}$, $\varphi_2 = \{x, x, 4(-x), x, x\}$, $\varphi_3 = \{4(x), 4(y), 4(-x), 4(-y)\}$ and $\varphi_4 = \{16\{x\}, 16(-x)\}$ with receiver phase $\{x, -x\}$. A pulse program for 3D H2BC is available at www.crc.dk/nmr.

represents a 3rd order low-pass J filter that suppresses magnetization that would correspond to the uninformative diagonal in 2D COSY spectra. These features make a significant difference over an older rudimentary version of HMQC–COSY [4].

^{13}C evolution (the t_1 period) is monitored by the element between and including the first two $\pi/2(^{13}C)$ pulses independent of where this element is placed between the two $\pi/2(^1H)$ pulses, where the latter monitors ^1H evolution (the t_2 period). The full and dashed arrows in Fig. 1 indicate how decoupling and pulses are moved in the pulse sequence according to t_1 and t_2, respectively. The evolution is of the constant-time type with T typically in the range 15–25 ms.

Fig. 2 shows an application of 3D H2BC to the 58 amino acid protein aprotinin (BPTI) in a non-recombinant preparation. For the spectral assignment it is convenient to represent the 3D spectrum as 2D COSY planes split according to ^{13}C chemical shifts. As can be seen, this results in a spectral appearance that is much simplified compared to a conventional 2D COSY spectrum (Fig. 3). Sequential walks through three amino acid side chains are illustrated in Fig. 3. For example, at $(\omega_1, \omega_2) = (\Omega(C^\beta),(\Omega(H^\beta))$ there can be found $\omega_3 = \Omega(H^\alpha)$ and $\omega_3 = \Omega(H^\gamma)$, which connects the spins of H^α, H^β and H^γ. Fig. 4 represents an application of 3D H2BC to an organic molecule outlining the complete assignment of all CH_n groups in cholic acid from the 3D H2BC spectrum alone. Other applications of 3D H2BC not shown here include assignment of complex oligosaccharides where isotopic enrichment is not possible and especially ^1H chemical shift dispersion is moderate. The 3D H2BC spectrum generally offers significant simplifications for unambiguous assignments as compared to alternative assignment strategies based on combining data out of different spectra. The capability to obtain assignments from a single experiment greatly facilitates spectral interpretation in all the examples shown, with-

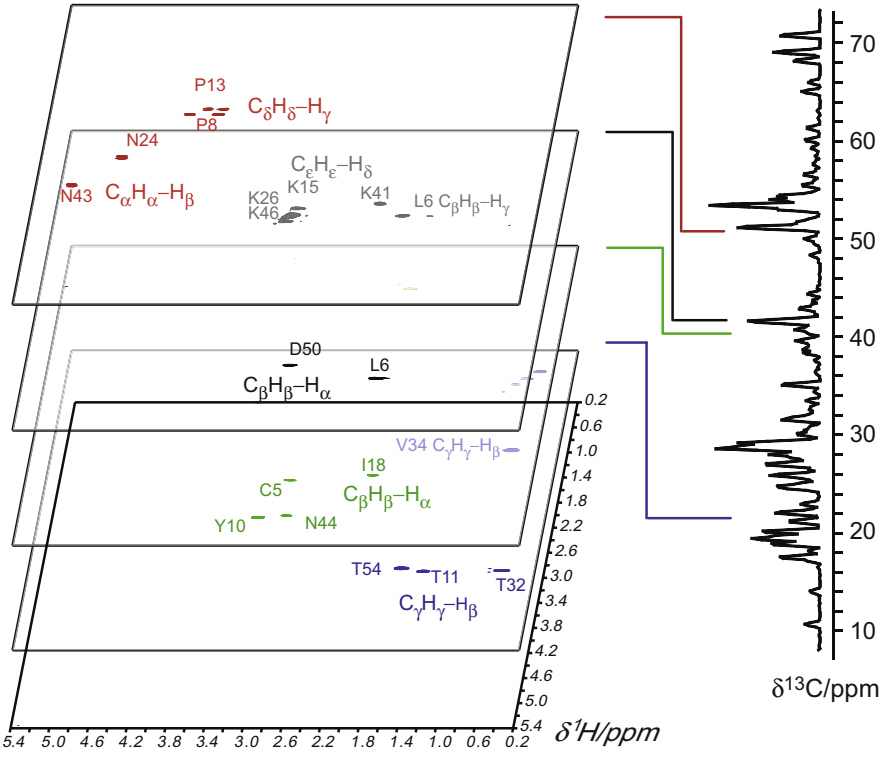

Fig. 2. 3D H2BC spectrum of 3.5 mM BPTI in ^2H_2O at pH 5.5 and 310 K (22 h experiment time). ^1H–^1H correlations are shown for four different ^{13}C planes that are substantially simplified compared to a ^1H–^1H COSY spectrum (see Fig. 3).

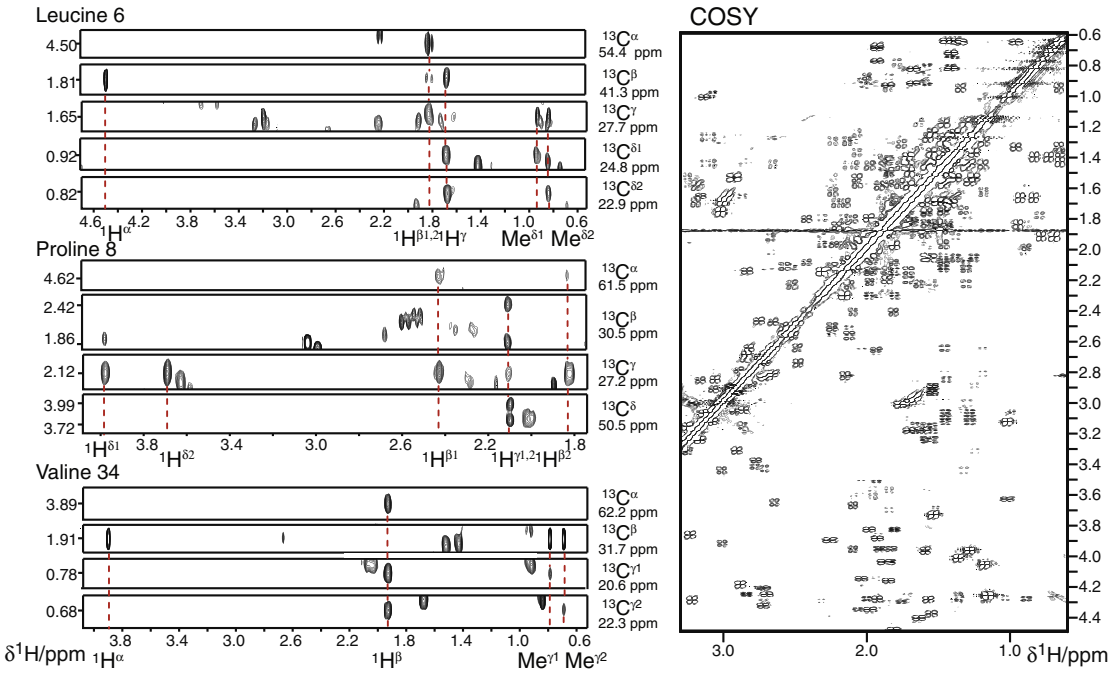

Fig. 3. Strip plots from the 3D H2BC spectrum shown in Fig. 2. Full side chain assignments are obtainable from a single 3D H2BC experiment as exemplified for residues Leu 6, Pro 8 and Val 34. A section of the two-dimensional COSY spectrum of the same sample is shown for comparison on the right.

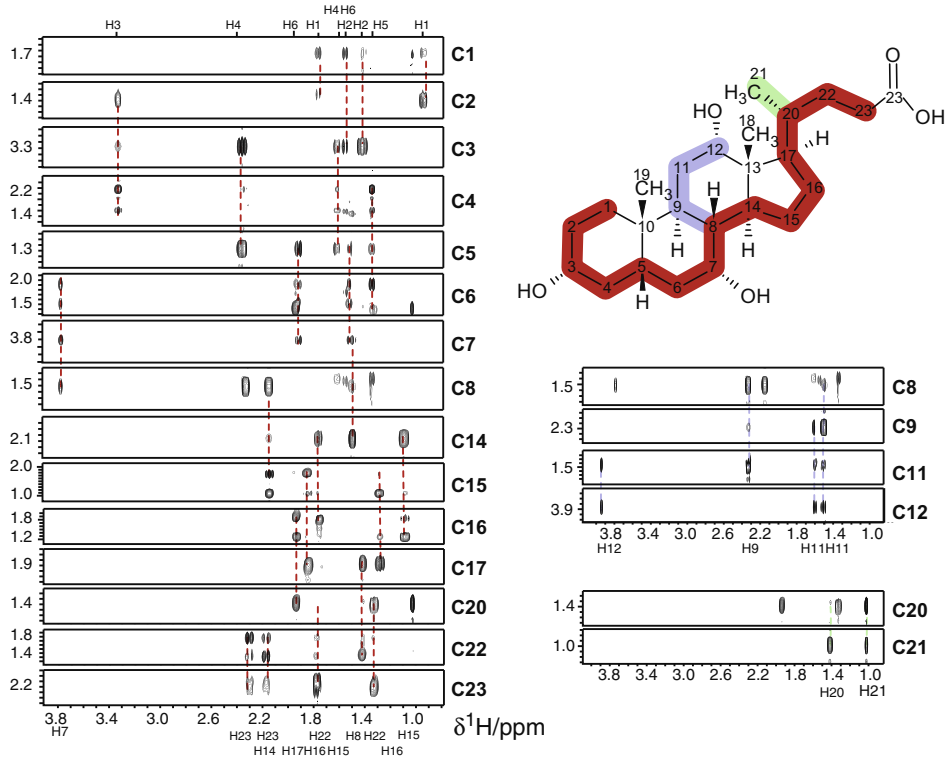

Fig. 4. Strip plots from a 3D H2BC spectrum recorded on a 15 mM sample of cholic acid (6 h experiment time) in d_6-acetone at 298 K. ^{1}H and ^{13}C resonances are assigned by a sequential walk along the paths indicated in red, blue and green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

out the need to transfer peak lists between several different data sets [5].

The literature on H2BC has described the complementarity of H2BC and HMBC leading to the general recommendation to record both of them in routine applications. As 3D H2BC essentially spreads an H2BC 2D spectrum out in a third dimension there is no change in the complementarity to HMBC, and hence no change to the recommendation to still perform HMBC in addition to 3D

H2BC. The only modification to earlier literature on H2BC is that an HSQC spectrum no longer is necessary, because that information is contained in the 3D H2BC spectrum.

3. Conclusion

In conclusion, we introduce a novel NMR pulse sequence, 3D H2BC, for spectral assignment of biomolecules and other complex organic compounds or natural products without isotopic enrichment. A most prominent characteristic of the new experiment is the short duration that is the key to its workability on biomolecules with short transverse relaxation times. With the latest generation of cryoprobes the experiment is doable overnight even at low millimolar concentrations. We expect 3D H2BC to extend the applicability of NMR in studies of all classes of natural products and biomacromolecules up to at least 10 kDa at natural isotopic abundance.

4. Experimental

Aprotinin (BPTI) and cholic acid were purchased from Sigma. BPTI was dissolved to 3.5 mM in ^2H_2O without any further addition of buffer or salt, yielding a sample of pH 5.5. Cholic acid was dissolved in d_6-acetone without further modification to the composition of the sample. BPTI spectra were recorded at 310 K and the cholic acid spectrum was recorded at 298 K, both on an 800 MHz Bruker AVANCE spectrometer equipped with a TCI cryoprobe. A constant-time delay of $T = 20$ ms (Fig. 1) was used in all experiments. The data set recorded on the aprotinin sample consisted of $512(^1H) \times 64(^1H) \times 64(^{13}C)$ complex data points corresponding to acquisition times of 65 (^1H), 10 (^1H) and 4 (^{13}C) milliseconds. For the cholic acid sample an experiment was performed with $512(^1H) \times 30(^1H) \times 50(^{13}C)$ complex data points sampling 92 (^1H), 6 (^1H) and 4 (^{13}C) milliseconds. Data were processed with nmrPipe [6] and analyzed with PIPP [7].

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