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Boosting the Resolution of ^{1}H NMR Spectra by Homonuclear Broadband Decoupling

N. Helge Meyer and Klaus Zangger*[a]

Broadband homonuclear decoupling of proton spectra, that is, the collapse of all multiplets into singlets, has the potential of boosting the resolution of ^{1}H NMR spectra. Several methods have been described in the last 40 years to achieve this goal. Most of them can only be applied in the indirect dimension of multi-dimensional NMR spectra or special data processing is necessary to yield decoupled 1D proton spectra. Recently, complete decoupling of proton spectra during acquisition has been introduced; this not only significantly reduced the experimental time to record these spectra, but also removed the need for any sophisticated processing schemes. Here we present an introduction and overview of the techniques and applications of broadband proton-decoupled proton experiments.

1. Introduction

NMR spectroscopy is probably the most frequently used technique for the structural investigation of small to medium-sized organic and biological molecules. Due to its high sensitivity and widespread occurrence the most frequently measured nucleus is ^{1}H. In contrast to its favourable sensitivity, the resolution of proton NMR spectra is rather poor. This results in part from the limited proton chemical shift range (~10 ppm), but also often from extensive signal splitting due to scalar coupling to nearby protons. The size of the splitting, the coupling constant J, corresponds to the energy difference of a nucleus with its coupling partner being in the α or β state. While the multiplet pattern caused by scalar coupling contains important structural information, it also significantly increases signal overlap, particularly in ^{1}H NMR spectra. In contrast, ^{13}C NMR spectra show well-resolved singlets in the spectrum (Figure 1).

The heteronuclear couplings to attached protons are removed by broadband ^{1}H decoupling and at natural abundance (~1.1% for ^{13}C) the chances of finding a carbon coupling partner are too low; typically, ^{13}C–^{13}C coupling doublets are hidden in the noise of the spectra. For overlapped proton signals it would sometimes also be desirable to remove the homonuclear coupling and obtain singlet-only proton spectra,[1] similar to the simulated spectrum in Figure 1c. Continuous decoupling can be achieved by a repetitive perturbation of the coupling partner magnetization with a period substantially shorter than 1 J.[2] Heteronuclear decoupling (e.g. proton broadband decoupling of carbon spectra) can be easily achieved using composite pulse decoupling, such as WALTZ[3] or GARP.[4] It is, however, not possible to carry out proton broadband decoupling by these sequences when acquiring proton spectra as all the desired magnetization is destroyed by the decoupling sequence. In contrast to broadband homonuclear decoupling, frequency-selective decoupling of individual signals has been used since the early days of NMR spectroscopy (the first report was published 40 years ago).[2] It has mainly been used to identify coupling partners, but also to locally simplify proton NMR spectra. While in the last decades the sensitivity of NMR hardware, and thus the signal to noise ratio, improved significantly, for example, by cryogenically cooled probes, the resolution, especially of small molecule spectra can solely be enhanced by going to ever higher magnetic field strength. The gain in resolution of broadband homonuclear decoupled proton spectra (also called pure-shift NMR) can be quite significant.[5] For example, a proton which is coupled to two methyl groups with coupling constants of 10 Hz gives a septet, which covers ~60 Hz in the spectrum. If the linewidth is on the order of 2 Hz this means that decoupling of this signal would reduce the width of this signal by a factor of 30. In other words, for such a signal, the resolution of a broadband decoupled spectrum recorded at 300 MHz is equivalent to the theoretical resolution of a regular (coupled) proton spectrum at 9 GHz. This factor would be even larger for narrower signals and higher order multiplets. Due to this significant resolution enhancement there have been a large number of attempts towards homonuclear broadband decoupling. Decoupling is achieved by only manipulating the passive spins (nuclei that give rise to the coupling of an observed signal) while leaving the active spins (the observed spins which show the splitting) unperturbed. This is not possible for all proton spins at the same time. Therefore, broadband homonuclear decoupling can only be implemented indirectly, either by methods that are able to separate scalar coupling from chemical shift evolution, typically in the indirect dimension of two-dimensional experiments, or

[a] Dr. N. H. Meyer, Prof. K. Zangger
Institute of Chemistry/Organic and Bioorganic Chemistry
University of Graz, Heinrichstrasse 28, 8010 Graz (Austria)
Fax: (+43) 316 380-9840
E-mail: klaus.zangger@uni-graz.at

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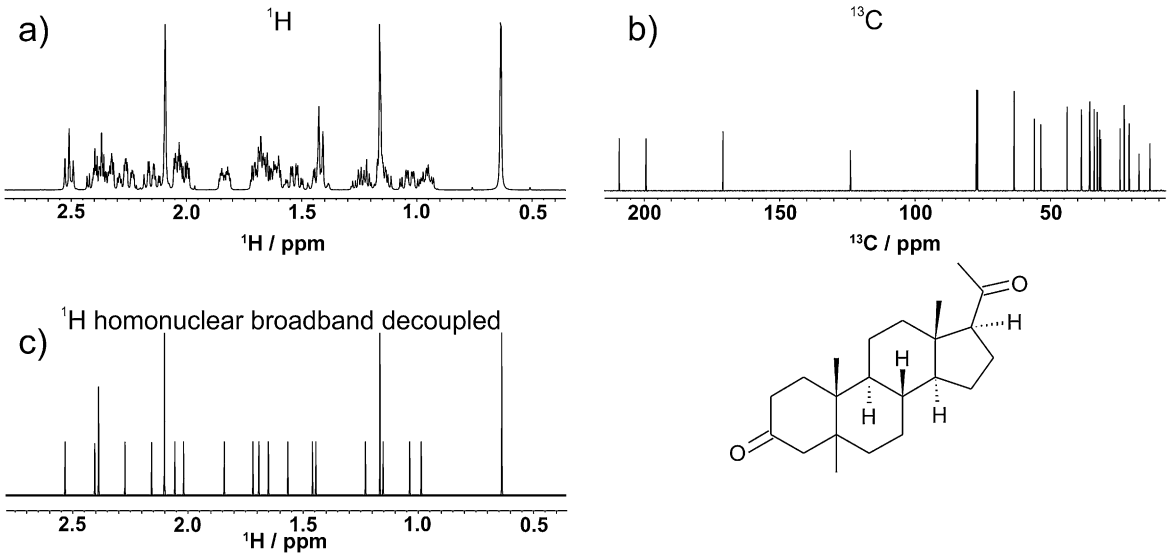

Figure 1. Experimental ^{1}H (a) and ^{13}C (b) spectra of progesterone. c) Simulated broadband proton-decoupled ^{1}H spectrum.

by transforming a homonuclear system into a pseudo heteronuclear system. The latter can be done spatially selective (separating the individual signals locally) or through the attached carbon polarization (separating ^{13}C from ^{12}C-bound protons). In this overview, the methods developed for broadband homonuclear decoupling are divided into techniques which can only be used in an indirect or pseudo decoupling dimension and direct methods that can also be used during the acquisition of a single scan.

2. Broadband Homonuclear Decoupling in the Indirect Dimension of Multi-Dimensional Spectra

Projections of *J*-Resolved Spectra

The first techniques reported for broadband homonuclear decoupling could only be applied in the indirect dimensions of two- and multi-dimensional experiments or yielded decoupled 1D spectra through the use of an additional decoupling dimension. The first such experiment was the 2D *J*-resolved spectrum, described by Ernst et al. in 1976.[6] It relies on the fact that in the spin-echo experiment the signal intensity as a function of the echo delay is only modulated by the scalar coupling. In a 2D *J*-resolved spectrum, the indirect dimension yields the scalar coupling pattern only, while both scalar coupling and chemical shift evolution are present in the direct (acquisition) dimension. This leads to a tilted multiplet pattern in the 2D spectrum. A broadband homodecoupled 1D spectrum can be obtained by a suitable projection of the 2D *J*-resolved spectrum onto the direct dimension. A problem with the original approach was the so-called "phase-twisted" line-shape of the peaks in *J*-resolved spectra. Only absolute value mode spectra could be projected which results in broad signals in the decoupled spectra. Several solutions to this problem have been described[7] and broadband homonuclear decoupling by projection of *J*-resolved spectra has been implemented in various types of NMR experiments.[7,8]

Constant-Time Experiments

In a two-dimensional experiment, evolution of the frequency information in the indirect dimension is achieved by a stepwise incrementation of the evolution time t_1. On the contrary, in a constant-time experiment,[9] the evolution time is embedded in a constant-time delay T. Instead of incrementing t_1, the position of a 180° pulse is shifted within T. The 180° pulse is positioned after a time $^{1}/_{2}(T+t_1)$ followed by a delay $^{1}/_{2}(T-t_1)$. Thus, the chemical shift evolves only during t_1. Homonuclear scalar coupling is not influenced by the 180° pulse and therefore evolves during the total delay T for each experiment. Since scalar coupling is not modulated as a function of t_1 it is not effective in the indirect dimension. Although scalar coupling does not give rise to a splitting in the constant-time dimension, it still leads to an evolution of anti-phase magnetization. If the coupling constants for different signals vary, this leads to a variation of signal intensities in the spectrum and, in the worst case, evanescence of peaks. For this reason constant-time experiments have not become very popular for proton spectra. On the contrary ^{13}C–^{13}C coupling constants are much more uniform and constant-time HSQC-type spectra are used frequently on uniformly ^{13}C-labelled proteins for homonuclear broadband decoupling in the indirect carbon dimension.[10]

Time-Reversal Experiments

Broadband decoupling is achieved by inverting the passive spins while leaving the active ones unperturbed. For homonuclear systems this is not possible for all spins simultaneously by a single set of pulses, but the combination of spectra ac-

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quired with small flip-angle pulses of different pulse-angles or phases leads to the same result. This type of broadband homonuclear decoupling is implemented in the indirect dimension of 2D experiments by placing a pulse of variable tilt angle β and phase ϕ in the middle of the evolution time.[11] The single pulse of angle β can also be replaced by two 90° pulses with phases $90°(\beta+\phi)$–$90°(\pi+\phi)$. A series of experiments with different values for β are then summed up with weighting factors determined by the maximum number of coupling partners to yield broadband homonuclear decoupling during t_1. This approach does not distort the signal intensities and could therefore be used, for example, in NOESY experiments. However, the number of scans per increment, which are necessary for coherence selection, drastically increases for higher number of coupling partners.

Diagonal Projection of Anti z-COSY Spectra

The use of small flip-angle pulses to separate active from passive spins by coherence selection also lies at the heart of another method for broadband homonuclear decoupling—the projection of the diagonal signals in anti z-COSY spectra.[12] The second 90° pulse of a basic 2D COSY experiment is replaced by a $(\beta+\pi)$–τ–β element, in which β is a small flip-angle pulse and τ is a short delay. This leads to a 2D spectrum where the remaining components of the diagonal peaks are the ones where the passive spins have the opposite spin state of the active ones. Thus the remaining components lie on a line that is perpendicular to the diagonal. A projection perpendicular to the diagonal yields a pure absorptive broadband decoupled spectrum. The integrals of the signals are not distorted, which allows a quantitative evaluation of compound mixtures. It is of course important to use only diagonal peaks and no cross peaks for the projection as they would reintroduce the coupling. In other words, strongly coupled peaks cannot be decoupled. Due to the use of small flip-angle pulses (typically 10–20°) the signal intensity is also reduced to for example, ~6% for $\beta=20°$. Some intensity is regained by the collapse of multiplets into singlets. Similar to all previously mentioned methods, the projection of anti z-COSY spectra can only be applied in the indirect dimension of two- or multi-dimensional NMR experiments. To obtain 1D spectra a 2D or pseudo-2D spectrum has to be acquired and processed accordingly, in order to obtain the 1D spectrum. Consequently, in order to obtain a 2D spectrum, which is homonuclear broadband decoupled in the direct dimension, the acquisition of a de facto 3D spectrum is necessary, in which one dimension is used exclusively to obtain the decoupled spectrum. With the above-mentioned techniques it is not possible to decouple during the acquisition. However, there are two methods available which are suitable for decoupling during acquisition in order to obtain single scan broadband homodecoupled spectra. Both were first described in a pseudo-2D mode.

BIRD-Based Decoupling

One way to separate chemical shift evolution from scalar coupling is the bilinear rotation decoupling (BIRD) sequence,[13] which takes advantage of the low natural abundance of ^{13}C (~1.1%) in order to decouple ^{13}C-bound protons from ^{12}C-bound ones. The BIRD pulse element selectively inverts the spins of ^{12}C bound protons (passive spins) leaving the ^{13}C bound protons (active spins) unaffected[14] (see Figure 2).

INEPT-filtering is then used to suppress the signals arising from ^{12}C-bound protons, which are not decoupled. In the original experiment BIRD was performed in the middle of a chemical shift evolution period t_1, which was incremented after each experiment in order to record a full FID in a pseudo-2D experiment.[14] For each experiment only one point was recorded in the acquisition dimension. However, scalar coupling is in the order of 10 Hz and is thus evolving much slower than chemical shift. Thus, it is in principle possible to record data for 10–20 ms of the FID during acquisition, which is virtually free of coupling. In order to record a full, typically 1 s long FID several of these data chunks are recorded in individual experiments and subsequently concatenated.

Frequency and Spatially Selective Decoupling

Selective decoupling of individual proton resonances has been used since the early days of NMR (see above).[2] It is possible to extend this concept and to selectively decouple all signals at once by using a weak magnetic field gradient to spread the signals into different slices along the z direction of the NMR sample tube.[15] If a linear z gradient is applied along the sample volume, different regions of the sample experience a different magnetic field strength, that is, a location-dependent frequency shift $\Delta\omega=\gamma G^*s$ across the sample volume over a length of s is established, with the gyromagnetic ratio of the observed nucleus γ and the gradient strength G.[15–17] A selective excitation pulse during this gradient excites the whole spectrum, but each signal is excited in a defined region of the sample. Therefore, the spatial encoding allows the manipulation of individual spins. Particularly, a slice-selective spin echo, which refocuses homonuclear coupling but not chemical shift evolution, can be achieved simply by placing a slice selective 180° pulse followed by a non-selective (hard) 180° pulse in the middle of a delay. The slice-selective spin echo can be easily used as a chemical shift evolution period of a homonuclear 2D experiment for homonuclear broadband decoupling of the indirect dimension.[15,18] In analogy to the BIRD experiment the spatially selective decoupling can also be utilized to decouple the acquisition dimension in a pseudo-2D fashion. The spatially and frequency selective homonuclear broadband decoupling (also called Zangger–Sterk method) was actually the first application of "data chunking" to record pure-shift spectra. As an example a slice-selectively decoupled proton spectrum of 2-butanol is shown in Figure 3.

While some of the original concepts of this technique were reported about 15 years ago,[15] this method became much more useful with improvements introduced in the last ~5

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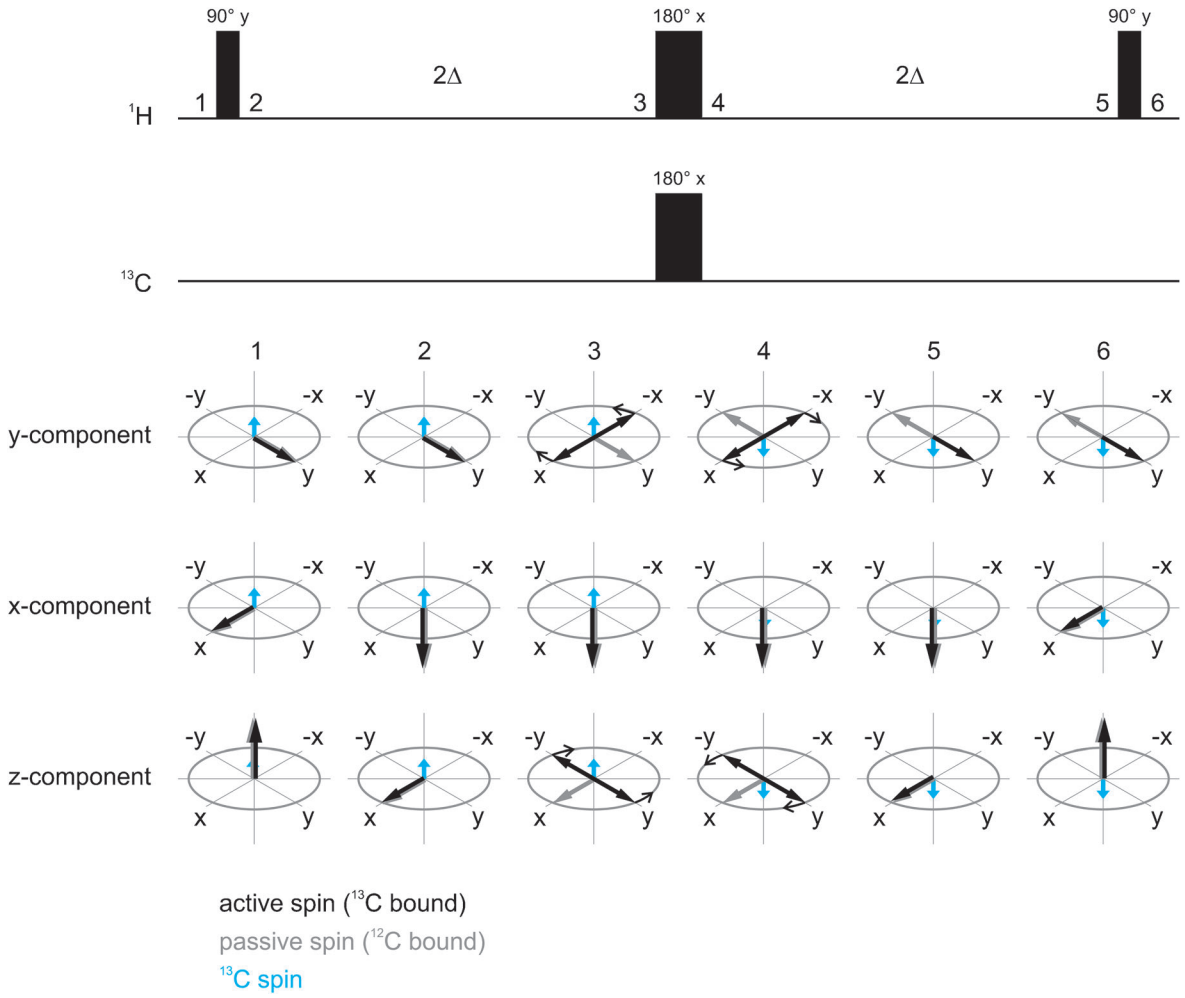

Figure 2. Top: Pulse sequence used for BIRD. Bottom: Representation of spin evolution during the BIRD element using the vector model (*x*, *y* and *z* component of the proton spins are treated separately). Heteronuclear coupling, $^{1}J_{CH} = 1/(4\Delta)$, between active spin (black) and ^{13}C spin (blue), is represented as a splitting of the active spin. Coupling between active (black) and passive (grey) spins is considered to be much smaller than the heteronuclear coupling and is thus neglected in this representation.

years by Morris et al.[5,18,19] As far as the measurement time of indirect broadband decoupling is concerned, all indirect methods are far more time-consuming compared to a 1D proton spectrum, as they are de facto 2D experiments. Further reductions in sensitivity are found especially for BIRD decoupling (1% of a regular spectrum is observed), slice-selective decoupling (~1–10%, depending on the spectral width and signal separation) and anti z-COSY (~5–10%). The sensitivity loss of time-reversal spectra depends mainly on the expected number of coupling partners and constant-time experiments show largely varying signal reductions, which depend on the number of coupling partners and size of coupling constants. Projections of *J*-resolved spectra lead to highest sensitivity of indirect decoupling methods.

3. Broadband Homonuclear Decoupling During Acquisition

Although pure-shift spectra have been on the wish-list of many people who use NMR spectra for structural analysis they have not been employed very often. Only constant-time multidimensional experiments are used routinely for ^{13}C-labelled biomolecules to decouple the indirect carbon dimension(s). In contrast broadband decoupled proton spectra, especially 1D versions, which are constructed from pseudo-2D data sets, have not been used for routine small molecule analysis. Besides the typically low sensitivity, the need for special processing of NMR data (e.g. projection of 2D spectra or concatenation of data chunks) has kept many potential routine users from using these techniques. Very recently it has been shown that homonuclear decoupling can also be achieved during acquisition of a single FID, without any special data processing using BIRD[20] or slice-selective decoupling.[21] These are the only two techniques which can be used for this purpose, as they actually physically decouple the proton coupling partners and do not per se rely on special data processing. Instead of recording each fraction of the FID in an individual experiment, the acquisition is interrupted after every ~$1/3(^{3}J_{HH})$ to perform either slice-selective or BIRD decoupling. The pulse-sequence as well as the magnetizations of the observed and the decou-

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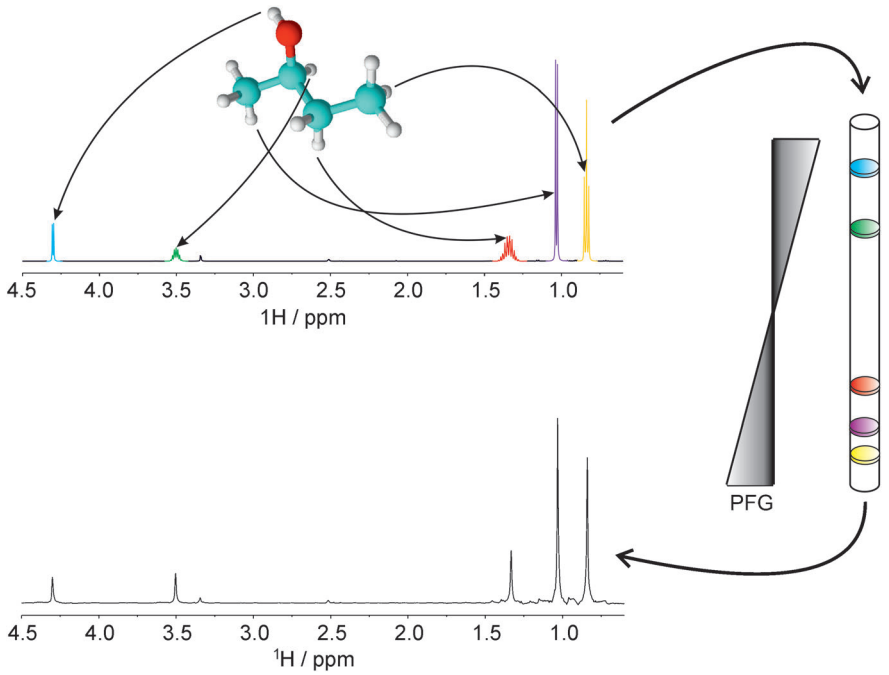

Figure 3. A slice-selectively decoupled ^{1}H spectrum of 2-butanol is obtained by selective decoupling of each proton signal in a separate slice of the NMR tube. This is achieved by selective excitation during a weak pulsed field gradient (PFG).

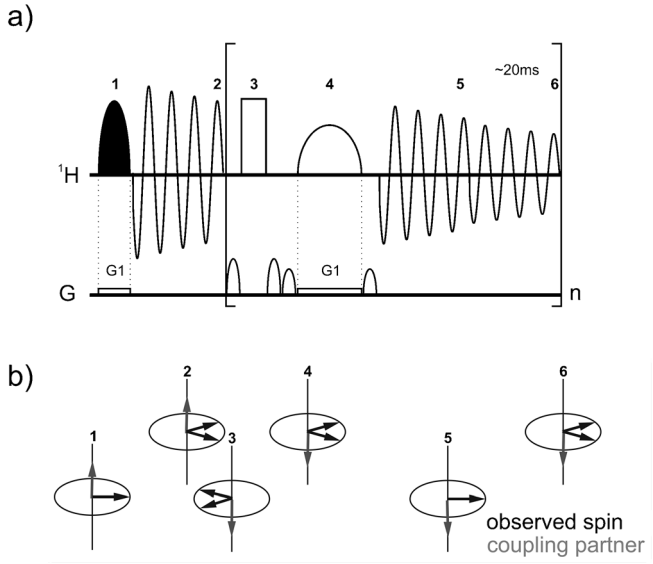

Figure 4. Pulse-sequence a) and magnetization vectors b) for slice-selective decoupling during acquisition.

pled spins is shown in Figure 4. Since the FID is recorded in a single experiment, additional processing of the FID becomes obsolete. It has to be taken into consideration that decoupling during acquisition is only possible if no chemical shift evolution occurs while the acquisition is interrupted. Any delay occurring during the decoupling block has to be refocused carefully. However, relaxation during decoupling cannot be avoided. It is therefore of foremost importance to keep the interruptions as short as possible, especially for larger molecules which have shorter T_2 relaxation times. Otherwise discontinuities in the FID may lead to artefacts in the spectrum. However, restriction of the pulse length of the slice selective refocusing may compromise the decoupling of close signals. The length of the BIRD-composite pulse largely depends on the heteronuclear *J*-coupling constants and cannot be optimized. An example of a directly, slice-selectively decoupled proton spectrum of the macrolide antibiotic azithromycin is shown in Figure 5. Both methods suffer from some disadvantages. BIRD decoupling works only for carbon-bound protons and reduces the integrated sensitivity down to 1% of a regular proton spectrum. While the sensitivity of slice-selective decoupling is also reduced significantly, it can be tuned to the desired spectral width. The thickness of the slice excited during the weak gradient corresponds to the ratio $\Delta\omega_{ex}/\Delta\omega$, where $\Delta\omega_{ex}$ is the excitation width of the selective pulse and $\Delta\omega$ is the frequency shift range induced by the weak gradient in the detected sample volume of length *s*. Therefore, the reduction in signal-to-noise Δ(S/N) of a spatially selectively excited spectrum compared to a regular one is given by Equation (1):

$$\Delta\frac{S}{N} = \frac{\Delta\omega_{ex}}{\gamma G s} \quad (1)$$

For a typical excitation bandwidth of 100 Hz and a gradient of 1 G cm^{-1} this leads to a reduction of the sensitivity down to ~3% of a regular spectrum.[16] Besides lower signal intensities, problems also arise for slice-selective decoupling if the coupled signals are too close in the spectrum. They must be clearly distinguishable by the selective pulses. If the pulse has to be more selective, the excited slice is narrower which further reduces the sensitivity. For both methods it is important that the acquisition is not interrupted too long and that the molecule under study does not relax too fast, as this would lead to relaxation losses between the individual FID blocks and yields artefacts around the singlets in the resulting spectrum.

4. Applications

Probably the most often used approach for broadband homonuclear decoupling has been constant-time carbon–carbon decoupling of uniformly labelled biomolecules.[10] These experiments are easy to set up and provide better resolution if the number of increments in the corresponding dimension is high

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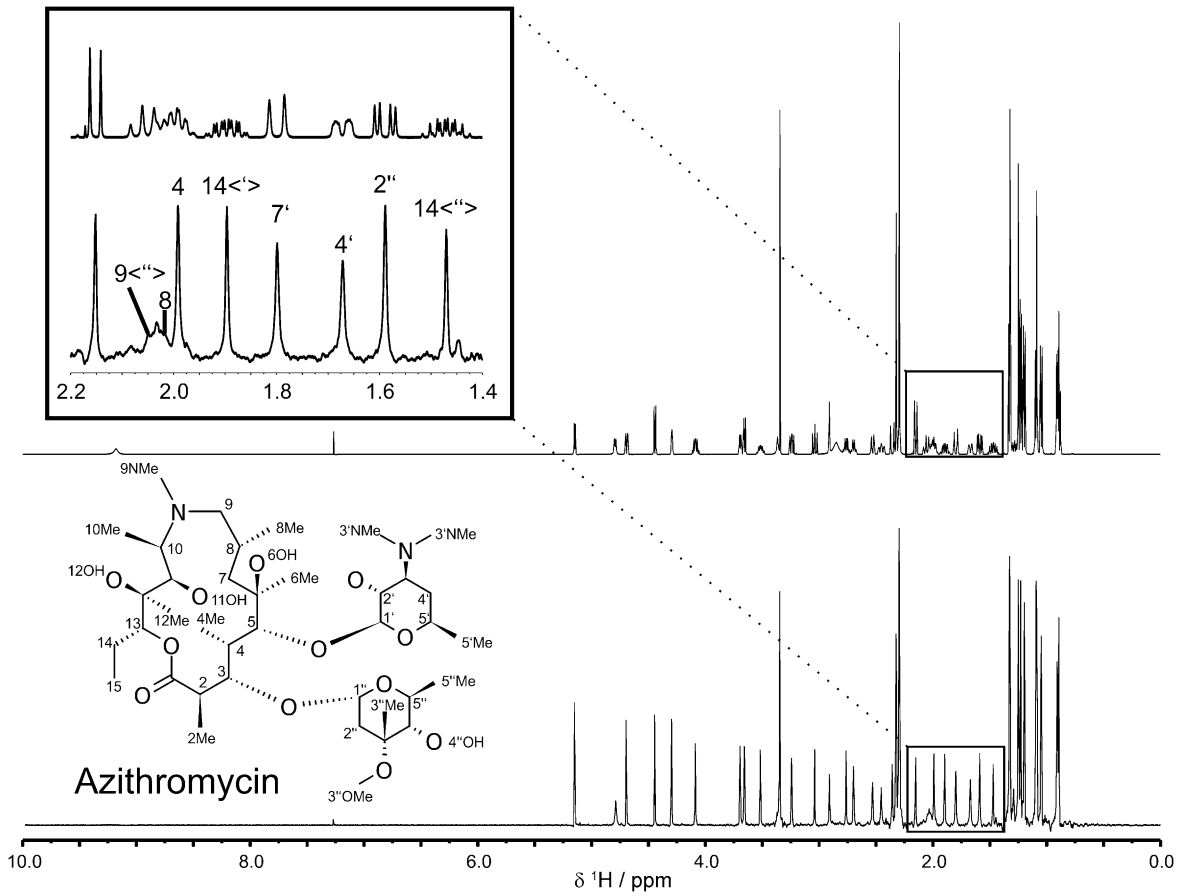

Figure 5. Regular and slice-selectively decoupled (during acquisition) ^{1}H spectra of azithromycin. Reproduced with permission from Wiley-VCH.[21].

enough. Proton–proton broadband decoupling has so far been less frequently used due to its huge loss in sensitivity and the often quite sophisticated processing schemes. The low sensitivity is less severe considering advances in spectrometer hardware, in particular the advent of cryogenically cooled probes in the last decades. The introduction of decoupling during acquisition comes along with a big boost in sensitivity per time, and maybe even more advantageously, it completely eliminates any special processing. The FIDs can be treated like regular 1D NMR experiments. The resulting pure-shift spectra are especially useful for the analysis of mixtures of several similar organic compounds, which often arise in organic synthesis. The group of Morris studied tetraallyl calix[4]arenes[5] which can occur as a mixture of cone and partial cone conformation. In this particular case a modified version of the original slice-selective homodecoupling experiment resulted in an average tenfold gain in resolution and therefore allowed an unambiguous identification of proton signals. High resolution is also important for DOSY spectra as the analysis of diffusion coefficients is much more error prone for overlapped peaks.[19] Several decoupling schemes have been used in order to record 2D spectra decoupled in the indirect as well as the direct dimension. The range of 2D spectra that have been decoupled in one or both dimensions encompasses TOCSY,[15,18] NOESY,[22] HMBC[23] and HSQC[24,25] experiments. A major breakthrough for homodecoupling of organic molecules at natural abundance may be the development of the directly BIRD-decoupled HSQC,[24] which at the same time achieves sensitivity and resolution improvement (see Figure 6).

For $^{1}H–^{13}C$ correlations BIRD decoupling is the optimal choice as it does not impose any additional sensitivity loss and

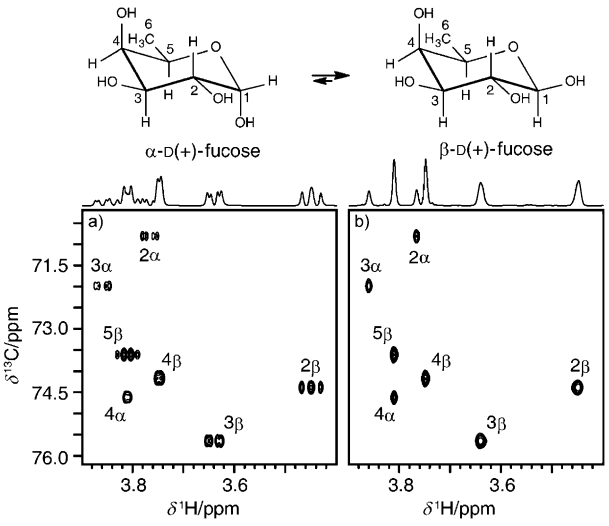

Figure 6. Selected regions of a regular HSQC (a) and a real-time BIRD-decoupled HSQC (b) of D(+)-fucose. Reproduced with permission from Wiley-VCH.[24]

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all observed signals can be decoupled during acquisition. However, it should been mentioned that BIRD decoupling is not able to remove scalar coupling of geminal protons as they are both bound to the same ^{13}C nucleus. While homonuclear broadband proton decoupling has only been reported on small molecules it also holds great promise for intrinsically disordered proteins. Because of the missing stable tertiary structure, these proteins suffer from particularly strong signal overlap and in addition their transverse relaxation is relatively slow. Both features make pure-shift spectra particularly useful. We have recently adapted 2D and 3D experiments (HSQC and HCCONH) for broadband homonuclear decoupling in the direct and/or indirect dimension using the slice-selective decoupling and were able to assign many more signals compared to regular (non-decoupled) spectra.[26] In order to keep the sensitivity loss acceptable, the spectral width was tuned to the desired region for decoupling, for example, methyl or H_α region.

5. Conclusions and Outlook

Homonuclear broadband decoupling of protons has been on the radar of NMR spectroscopists for more than 40 years[1] and several indirect methods have been developed to achieve this goal. Until recently all these approaches could merely be applied in the indirect dimension of multi-dimensional NMR experiments or required special data processing in order to extract a fully decoupled 1D ^1H spectrum. In addition, they all reduce the overall sensitivity of the spectra compared to regular ^1H experiments. In the last 5 years renewed interest in pure-shift spectra resulted in several improvements which increased both the sensitivity and user-friendliness of these experiments. The broadband decoupling during acquisition using either BIRD or slice-selective decoupling completely eliminates any special data processing and yields significant sensitivity gains per instrument time. The very recently introduced BIRD–HSQC experiment is particularly interesting as it not only provides increased resolution but also a sensitivity gain compared to a regular HSQC experiment of molecules at natural abundance. Unfortunately, BIRD decoupling is not suitable for HMBC-type spectra and slice-selective decoupling during acquisition would make it unacceptably insensitive. For fully decoupled 1D spectra both BIRD and slice-selective decoupling can be used without sophisticated data processing, but both have some shortcomings. While BIRD decoupling cannot remove the splittings of protons bound to nuclei other than carbon and geminal protons and its sensitivity is always restricted to 1% of a regular proton spectrum, slice-selective decoupling does not work well for coupling partners which are too close in the spectrum. Therefore, depending on the problem to be solved one or the other technique can be employed. Overall, the quest for singlet-only proton spectra has gone a long way and novel pulse-sequence improvements together with much higher sensitivity NMR hardware should finally make pure-shift NMR a useful experiment for routine NMR users in the structural investigation of small molecules.

Acknowledgements

Financial support to K.Z. by the Austrian Science Foundation (FWF) under the project number P24742 is gratefully acknowledged.

Keywords: homonuclear broadband decoupling · HSQC · NMR spectroscopy · pure-shift spectra · structure elucidation

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Received: September 17, 2013
Published online on December 12, 2013

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