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Four-in-one: HSQC, HSQC-TOCSY (or H2BC), TOCSY, and enhanced HMBC spectra integrated into a single NO Relaxation Delay (NORD) NMR experiment

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ABSTRACT

The NMR pulse sequence design strategy of NORD (NO Relaxation Delay) is extended to design of two new three-module experiments, NORD {HMBC}-{HSQC-TOCSY}-{TOCSY} and NORD {HMBC}-{2BOB}-{TOCSY}, each delivering four spectra – HMBC, HSQC, TOCSY, and either HSQC-TOCSY or H2BC. Compared to individual recording of these spectra particularly the sensitivity of the least sensitive module, HMBC, is enhanced by designing the homonuclear TOCSY module to allow buildup of magnetization pertinent to HMBC during its execution. Effectively, the sensitivity of the heteronuclear modules is boosted at the expense of the inherently much higher TOCSY sensitivity, thus resulting in a significant saving in spectrometer time.

1. Introduction

A small-molecule NMR analysis typically includes heteronuclear $^{1}H–^{13}C$ one-bond and long-range correlation spectra along with a homonuclear $^{1}H–^{1}H$ correlation spectrum. In each of these categories there is a number of pulse sequences to choose from depending on the particular application, and there is an obvious interest in getting the spectra recorded with adequate signal-to-noise ratio (S/N) in the shortest possible time on the spectrometer.

The first aspect of this is optimization of individual pulse sequences delivering the various spectra, something that has always enjoyed attention in the history of NMR, and will continue to do so. The second aspect is concerned with possible advantages in the form of saving time by concatenating different pulse sequences. An early example of this was the so-called COCONOESY experiment [1] where the COSY acquisition time is part of the NOESY mixing time.

Another more recent advance is related to the fact that different experiments in some cases rely on different pools of magnetization, and if such pools can be manipulated independently they can be exploited for spectrum generation sequentially while spin-system recovery or relaxation toward thermal equilibrium or a steady state occur simultaneously [2]. In other words in its simplest form, two concatenated experiments are sharing a single relaxation delay.

The NO Relaxation Delay (NORD) strategy [3] takes this a step further by designing concatenated experiments that do not require a relaxation delay at all. This has been accomplished in analogy to the Ernst angle concept [4] of saving magnetization for succeeding scans and by having the first pool of magnetization relax while a second pool is used and vice versa. It needs some kind of synergy to make NORD concatenation of experiments worthwhile compared to sequential NORD execution of individual experiments. The synergy is typically of the one-way type where the sensitivity of a low-sensitivity module is enhanced at the expense of a higher-sensitivity module in a concatenated experiment resulting in an overall saving of spectrometer time.

2. Results and discussion

In this paper, we present two new three-module NORD experiments, NORD {HMBC}-{HSQC-TOCSY}-{TOCSY} and NORD

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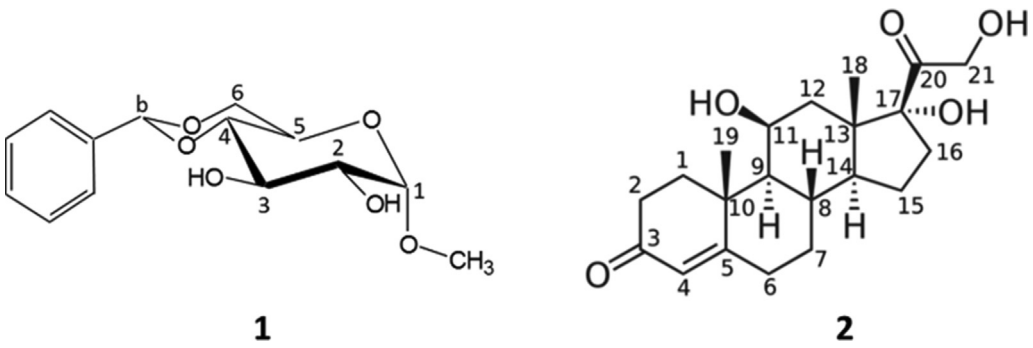

Scheme 1. Structure of 4,6-benzylidene-1-methoxy-α-D-glucose (**1**) and hydrocortisone (**2**).

{HMBC}-{2BOB}-{TOCSY}, each delivering four spectra covering the needs of most small-molecule NMR analyses. They are outlined in Fig. 1 and the middle modules of both (i.e. {HSQC-TOCSY} or {2BOB}) include two editing steps where the first separates odd and even ^{13}C multiplicities whilst the second separates one-bond and longer-range correlations. The upper parts in the middle modules represent standard versions where odd and even ^{13}C multiplicities have the same phase whilst the lower parts represent updown versions where odd and even ^{13}C multiplicities have opposite phases. In the second editing step, the dashed adiabatic ^{13}C inversion pulse right before acquisition is off in half the scans and on in the other half where the former and latter result in like or opposite phases of one-bond and longer-range correlations. The use of a *J*-compensated adiabatic ^{13}C inversion pulse in this type of editing provides compensation for a spread in $^{1}J_{CH}$ coupling constants, yielding higher sensitivity in the one-bond spectrum than in the earlier approach with a regular π(C) pulse [5].

The NORD {HMBC}-{HSQC-TOCSY}-{TOCSY} and NORD {HMBC}-{2BOB}-{TOCSY} experiments essentially deliver the same spectra apart from the first having a general heteronuclear long-range correlation spectrum (HSQC-TOCSY) and the second having a two-bond correlation spectrum (H2BC) instead.

Clearly, TOCSY, relying only on ^{1}H magnetization is the most sensitive of the modules, so it is fine to sacrifice some of its sensitivity if that comes along with an increase for the other modules that rely on the natural-abundance level of ^{13}C.

The pool of protons not attached to ^{13}C (the I pool) is the one relevant for HMBC and TOCSY, whilst it is the pool of protons attached to ^{13}C (the IS pool) that is pertinent to 2BOB or HSQC-TOCSY in the middle module. The pulse sequence elements of BANGO [6], BIG-BIRD [7], and TIG-BIRD [8] manipulate the two pools of magnetization independently to various degrees of flexibility and they are all used in the new experiments.

BANGO performs arbitrary combinations of flip angles with a common phase in the two pools of magnetization. BIG-BIRD adds arbitrary combinations of pulse phases in the two pools to BANGO. However, only TIG-BIRD has the full flexibility of BIG-BIRD plus the feature of manipulating the two doublet components in the IS pool independent of each other.

BANGO replaces the conventional excitation pulse in HMBC, inverting the IS pool and exciting the I pool by a rotation angle of β^I that for the current applications is $\frac{\pi}{2}$ or slightly larger. BIG-BIRD replaces the conventional excitation pulse in 2BOB, acting as a $\left(\frac{\pi}{2}\right)_y$ and a $\left(\frac{\pi}{2}\right)_v$ rotation in the I and IS pools, respectively. TIG-BIRD is used as preparation sequence in HSQC-TOCSY, where it inverts the I pool and one of the I spin doublet resonances in the IS pool while leaving the other one untouched. All these features of the pulse sequence elements can be verified by a vector model analysis.

The excitation pulse in TOCSY is usually $\frac{\pi}{2}$, but in NORD applications it is typically in the range of $10^{\circ} < \alpha < 30^{\circ}$, so that a fraction proportional to $cos(\alpha)$ is saved for succeeding scans in both pools of magnetization. This is key to the NORD feature of continuously replenishing longitudinal magnetization during the concatenated pulse sequence. The first and second modules do not compete with each other for the benefit of the sacrifice in TOCSY S/N but in fact both gain from it, because they rely on different pools of magnetization.

These features are evident in comparing the signal-to-noise ratio, S/N, in the heteronuclear spectra from the earlier two-module NORD {HMBC}-{2BOB} [3a] and the new three-module NORD {HMBC}-{2BOB}-{TOCSY} experiment recorded with the same number of scans on the monosaccharide **1** (Scheme 1) shown in Fig. 2. The two-module experiment already enjoys the NORD benefit but appending the homonuclear TOCSY module to the end provides additional time for recovery of magnetization needed for the two heteronuclear modules, which is reflected in a higher effective sensitivity.

The optimum β^I in BANGO and υ in BIG-BIRD were determined experimentally to be 120° and 20°, respectively, in the two-module experiment whilst they for the three-module experiment were 90° and 0°, respectively. The resulting higher steady state longitudinal magnetization from buildup of longitudinal magnetization during the TOCSY module in the three-module experiment leads to an average relative S/N gain obtained in HMBC with the three-module experiment of 14 %, with a range of 0–41 %. The corresponding average sensitivity improvement in the 2BOB spectrum was 13 %. Fluctuation in the sensitivity enhancements most likely reflects the variation in proton T_1. Protons with shorter T_1 benefit more from the longitudinal relaxation during the added TOCSY sequence. In general, it is recommended to determine the optimum pulse sequence parameters for one out of a group of similar molecules and then use them for the whole group. The spectra shown are representative of the variation in sensitivity enhancement to expect within a molecule or within a group of similar molecules.

Fig. 3 illustrates comparison between NORD {HMBC}-{HSQC-TOCSY}-{TOCSY} and the three individually optimized experiments NORD {HMBC}, NORD {HSQC-TOCSY}, NORD {TOCSY} on the same monosaccharide **1**.

The average relative S/N gain obtained with the three-module experiment is 82 % in the least sensitive spectrum, i.e. HMBC, whilst it is 13 % in average in the HSQC-TOCSY spectrum. For these welcome enhancements the most sensitive experiment, TOCSY, pays the price with an average signal intensity drop of 45 %. This loss is in the context of the homonuclear TOCSY signals being about two orders of magnitude larger than the signals in the heteronuclear HSQC-TOCSY correlation spectrum. Hence the loss in the homonuclear TOCSY spectrum has no practical conse-

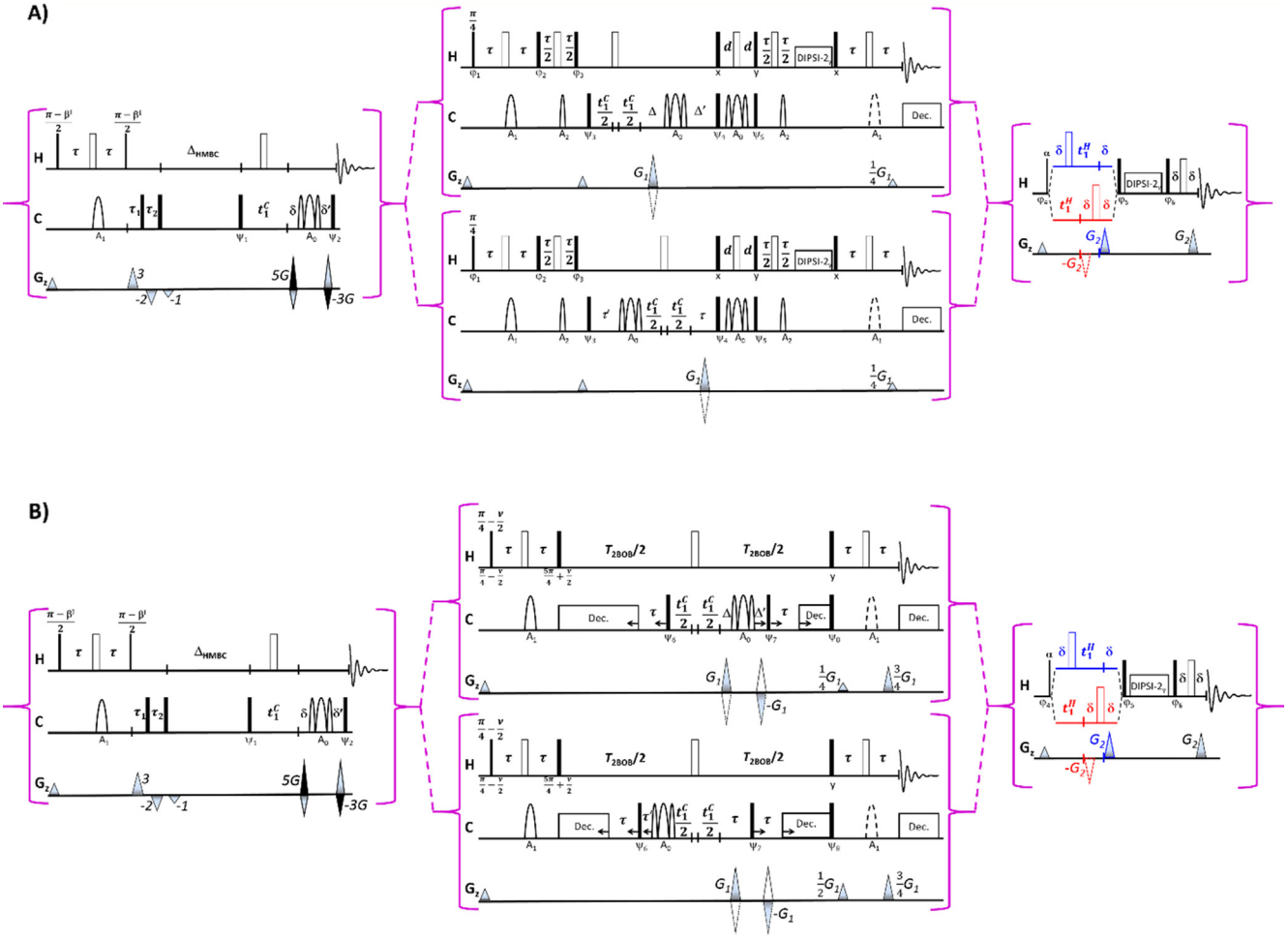

Fig. 1. A) NORD {HMBC}-{HSQC-TOCSY}-{TOCSY} and B) NORD {HMBC}-{2BOB}-{TOCSY} pulse sequences including two editing steps. The upper parts in the middle modules represent standard versions where odd and even ^{13}C multiplicities have the same phase whilst the lower parts represent updown versions where odd and even ^{13}C multiplicities have opposite phases. Standard and updown data are additively and subtractively combined to yield even and odd multiplicity subspectra, respectively. The second editing step is right before acquisition in the middle modules where the dashed adiabatic ^{13}C inversion pulse is off in half the scans and on in the other half. Additive and subtractive combination of these data delivers one-bond and long-range correlation spectra, respectively. Narrow filled, wider filled and open bars refer to π/4, π/2 and π pulses, respectively, whilst α represents a 10-30° angle and the BANGO excitation pulse β^I in the first module is π/2 (or slightly larger). The angle ν in the BIG-BIRD excitation element of the 2BOB (Two-Bond and One-Bond) module is typically 0-30°. In 2BOB T_{2BOB} is a constant-time delay usually in the range 15–25 ms and the decoupling periods with the arrows are decremented as t_1^C is incremented. A_1 and A_2 are *J*-compensated adiabatic ^{13}C CAWURST-20 inversion pulses (240 ppm (or 121 ppm for sugars), 1.92 and 0.97 ms, respectively) with low-to-high frequency sweep. A_0 is a composite 20 % smoothed CHIRP (2 ms, 80 kHz sweep; Crp80comp.4). The delay $\tau = 0.5(^1J)^{-1}$ is set according to a ^1J of 113 Hz. A 2nd order low-pass *J* filter is employed with $\tau_1 = 0.5[^1J_{min} + 0.146(^1J_{max} - {}^1J_{min})]^{-1}$ and $\tau_2 = 0.5[^1J_{max} - 0.146(^1J_{max} - {}^1J_{min})]^{-1}$. δ and Δ are gradient delays with $\delta' = \delta + t_{180(H)}$, $\Delta' = \Delta + t_{180(H)}$, and $\tau' = \tau + t_{180(H)}$,. d is set to 1.86 ms as a compromise for all carbon multiplicities. The delay Δ_{HMBC} is for evolution under heteronuclear long-range couplings. The DIPSI-2 sequence is used for mixing in TOCSY. The amplitude of the purging gradients (triangles without labelling) and the low-pass *J* filter gradients in the HMBC module can be set an order of magnitude lower than the amplitudes of the other ones selecting coherence transfer echo or antiecho. The receiver phases in all three modules always alternate between × and –x. For the spectra presented only 2 scans were recorded in each module with the first two steps of the following phase cycles. HMBC: $\psi_1 = \{x,-x,-x,x\}$, $\psi_2 = \{x,x,-x,-x\}$; echo/antiecho selection occurs with the gradients (5G,-3G)/(-3G,5G). For antiecho selection in HSQC-TOCSY: $\varphi_1 = \{\frac{\pi}{4},\frac{\pi}{4},\frac{\pi}{4},\frac{\pi}{4},-\frac{\pi}{4},-\frac{\pi}{4},-\frac{\pi}{4},-\frac{\pi}{4}\}$, $\varphi_2 = \{\frac{5\pi}{4},\frac{5\pi}{4},\frac{5\pi}{4},\frac{5\pi}{4},\frac{3\pi}{4},\frac{3\pi}{4},\frac{3\pi}{4},\frac{3\pi}{4}\}$, $\varphi_3 = \{y,y,y,y,-y,-y,-y,-y\}$, $\psi_3 = \{x,-x,-x,x\}$, $\psi_4 = \{-x,-x,x,x,x,x,-x,-x\}$, $\psi_5 = \{y,y,-y,-y,-y,-y,y,y\}$; for echo selection phase ψ_5 is inverted. TOCSY: $\varphi_4 = \{x,-x\}$, $\varphi_5 = \{x,x,-x,-x\}$,$\varphi_6 = \{-x,-x,x,x\}$ and the upper and lower panels applicable for echo and antiecho, respectively. In the 2BOB module $\psi_6 = \{x,-x,-x,x\}$, $\psi_7 = \{x,x,-x,-x\}$ and $\psi_8 = \{x,x,x,x-x,-x,-x,-x\}$. In HSQC-TOCSY and TOCSY the echo is selected with the positive gradients G_1 and G_2, respectively, prior to mixing and the antiecho with the corresponding negative dashed gradients. In 2BOB the echo/antiecho is selected by the pair of filled/open gradients after the evolution period. Before standard processing the obtained combined dataset is separated into three blocks, corresponding to HMBC, HSQC-TOCSY or 2BOB, and TOCSY data, using the Bruker au-program *splitx*. The HSQC-TOCSY or 2BOB data block is further divided into four data sets with the au-program *split* prior to linear combinations. The F_1 chemical shift scale in TOCSY is corrected with au-program *fixF1*. Then the data are processed as in their stand-alone experiments. The pulse sequence codes for Bruker spectrometers can be found in ESI.

quences – only the sensitivity improvement for the other modules counts.

The proposed three-module NORD experiments have also been tested on hydrocortisone **2** (Scheme 1) (45.7 mg in 550 µl DMSO d_6), with several overlapping resonances in both the proton and carbon spectra. Fig. 4 illustrates is shown the overlay of the edited 2BOB spectra from NORD {HMBC}-{2BOB}-{TOCSY}, and the edited HSQC-TOCSY spectra from NORD {HMBC}-{HSQC-TOCSY}-{TOCSY} are shown in Fig. 5 that fully and unambiguously assign the proton coupling networks and the corresponding attached carbons in **2**. The 4-step editing makes feasible the separation of even completely overlapping correlations, as demonstrated in the excerpts of Figs. 4 and 5. The overlapping cross peaks shown in blue and red (e.g. C15-H15a and C15-H14) or in green and purple (e.g. C14-H14 and C14-H15a) can only be separated, and so distinguished, in the edited subspectra. Without this information there is a risk of misinterpretation and thus misassignment of the peaks.

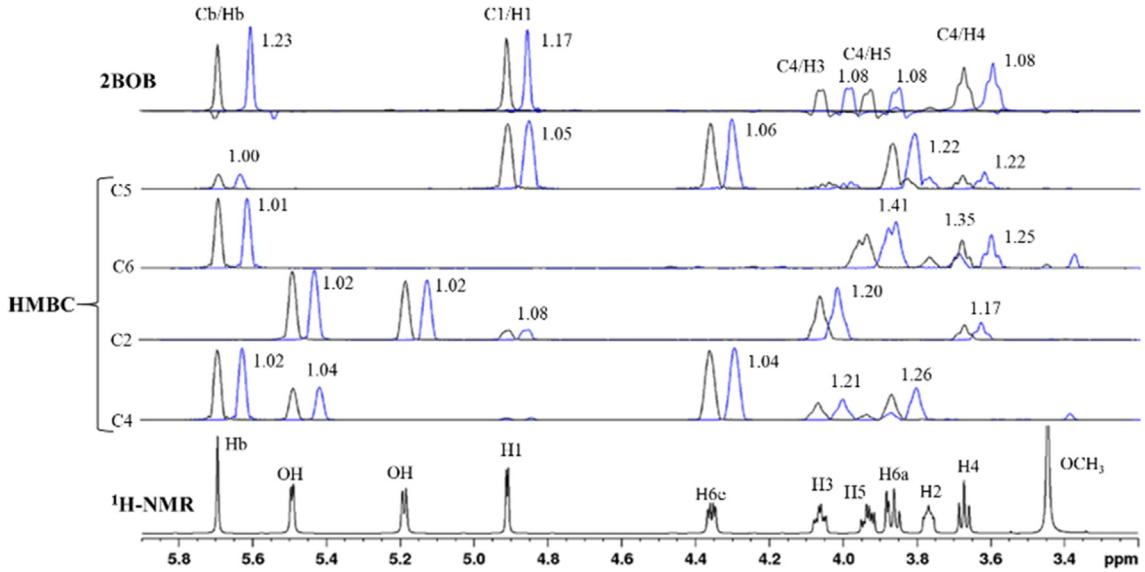

Fig. 2. Sensitivity comparison between the novel three-module NORD {HMBC}-{2BOB}-{TOCSY} (blue, shifted for better visualization) and the earlier two-module NORD {HMBC}-{2BOB} (black) experiments performed on the monosaccharide **1** (60 mg in 550 µl DMSO d_6/C_6D_6). The ^{1}H spectrum at the bottom shows the resonance assignment, whilst the measured relative S/N ratios are given above the extracted F_2-sections of the respective 2D spectra. Two scans were acquired per increment with 1024 points recorded in both t_1 and t_2 in all modules. The spectral widths were 5.1 ppm and 100 ppm in the ^{1}H and ^{13}C dimension, respectively. The direct acquisition time (t_2) was 143.4 ms and the constant-time 2BOB delay T_{2BOB} = 23 ms. CAWURST-20 adiabatic ^{13}C inversion pulses (121 ppm, 1.94 ms; H2L) were used in the BANGO and BIG-BIRD elements to compensate for the spread in $^1J_{CH}$. The two- and three-module NORD experiments were optimized separately: β^I for the BANGO pulse was set to 120° in the two-module and 90° in the three-module experiment, while the υ angle of BIG-BIRD was set to 20° and 0°, respectively. For the TOCSY block of the three-module experiment, an excitation pulse (α) of 20° and a mixing time of 80 ms were used. All spectra were recorded on a Bruker Avance NEO 700 MHz spectrometer equipped with a TCI z-gradient prodigy probe at 298 K.

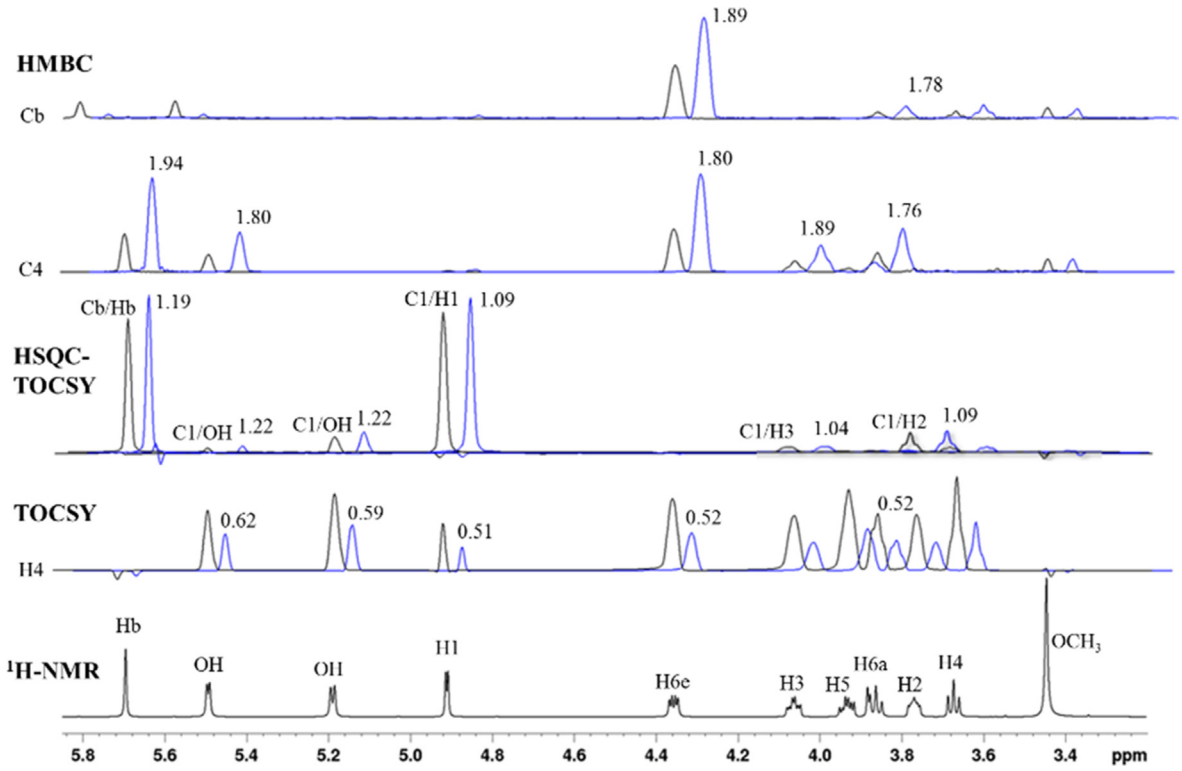

Fig. 3. Sensitivity comparison between the novel three-module NORD {HMBC}-{HSQC-TOCSY}-{TOCSY} (blue, shifted for better visualization) and the three individually optimized single-module NORD (black) experiments performed on the monosaccharide **1** (60 mg in 550 µl DMSO d_6/C_6D_6). The measured relative S/N ratios are given above the extracted F_2-sections of the respective 2D spectra. Two scans were acquired per increment with 1024 points recorded in both t_1 and t_2 in all modules. The spectral widths were 5.1 ppm and 100 ppm in the ^{1}H and ^{13}C dimension, respectively. The direct acquisition time (t_2) was 143.4 ms. In the BANGO and BIG-BIRD sequences CAWURST-20 (121 ppm, 1.92 ms; H2L), whereas in the INEPT part CAWURST-20 (121 ppm, 0.97 ms; H2L) adiabatic ^{13}C inversion pulses were used to compensate for the spread in $^1J_{CH}$. In the optimized three-module NORD {HMBC}-{HSQC-TOCSY}-{TOCSY} experiment β^I for the BANGO pulse was set to 90° and the υ angle of BIG-BIRD to 0°. 80 ms DIPSI-2 sequence was used in both the HSQC-TOCSY and TOCSY modules. The latter module started with α excitation pulse of 20°. The optimized single-module NORD experiments were run with β^I = 130° for BANGO in HMBC, with υ = 0° for BIG-BIRD in HSQC-TOCSY and with α = 60° excitation pulse in TOCSY. 80 ms mixing time was used for DIPSI-2 in each case.

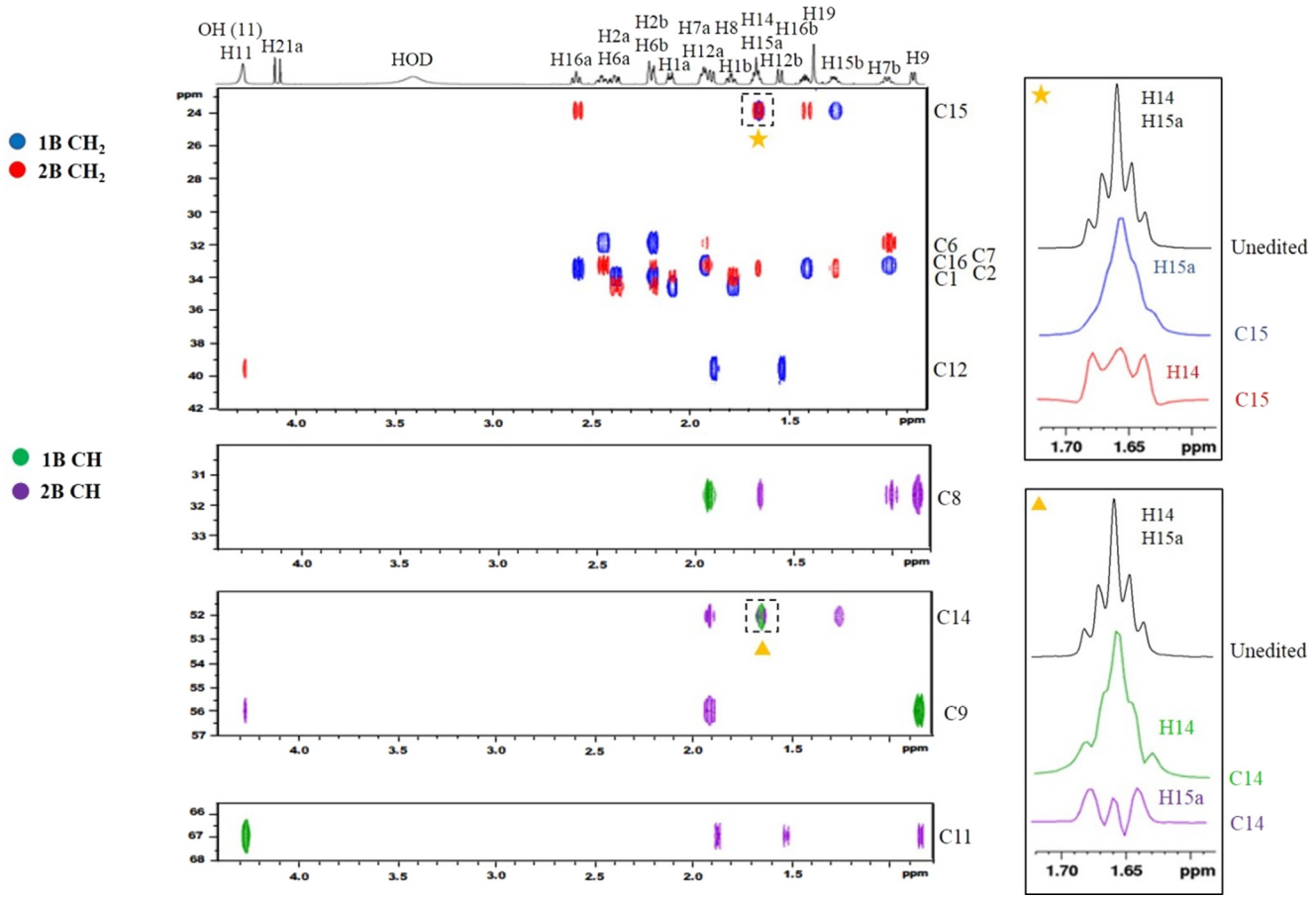

Fig. 4. Overlay of excerpts of fully edited 2BOB spectra of hydrocortisone (**2**) recorded using the NORD {HMBC}-{2BOB}-{TOCSY} experiment (shown in Fig. 1B) on a Bruker Avance NEO 700 MHz spectrometer. One-bond (1B) correlations of CH_2 and CH carbons are plotted in blue and green, respectively, whereas the corresponding two-bond (2B) correlations are shown in red and purple, respectively. The F_2-sections (right) extracted at the marked cross peaks of the respective 2BOB subspectra illustrate the resolving power of the four-step editing. The excitation pulses, BANGO for HMBC, BIG-BIRD for 2BOB and α for TOCSY, were used as given in the Fig. 2 caption. The spectra were acquired with the following parameters: spectral widths of 6.5 ppm (^{1}H) and 200 ppm (^{13}C), 512 points in t_1 with 2 scans per increment in the HMBC and TOCSY modules and 128 points with 8 scans in the 2BOB module due to the four-step time-shared editing cycle. For all three modules 2048 data points were acquired in t_2 amounting to direct acquisition time of 225 ms. The total measurement time was 35 min. CAWURST-20 adiabatic ^{13}C inversion pulses (240 ppm, 1.92 ms; H2L) were used in the BANGO and BIG-BIRD elements. The delay τ was set to ^1J = 113 Hz. Δ_{HMBC} = 83 ms, T_{2BOB} = 23 ms, DIPSI-2 mixing time = 80 ms, $^1J_{min}$ = 125 Hz and $^1J_{max}$ = 165 Hz for the low-pass filter in HMBC. Before standard processing the combined data set is separated into three blocks, corresponding to HMBC, 2BOB and TOCSY, using the Bruker au-program *splitx*. The 2BOB data block is divided into four data sets prior to linear combination using the au-program *split*.

To complete the assignment of carbon resonances with the identification of the quaternary carbons the HMBC spectrum (Fig. 6A) recorded in the first block of the three-module NORD experiments provides the required information on heteronuclear multiple-bond correlations. The homonuclear TOCSY correlation map (Fig. 6B) offers additional support for the assignment of the proton coupling networks. Moreover, it is an option to use different mixing times in the HSQC-TOCSY and TOCSY modules, so that shorter and longer range connectivities can be distinguished and thus further help the 'assignment walk' along the homonuclear ^{1}H coupled spin systems. Finally, it is also an option to replace the HMBC module in both experiments by a corresponding SEA XLOC [9] module if it is desirable to distinguish between two- and three-bond correlations in the $^nJ_{CH}$-based heteronuclear spectrum.

Both NORD {HMBC}-{2BOB}-{TOCSY} and NORD {HMBC}-{HSQC-TOCSY}-{TOCSY} can each be truncated in three different ways to include only any two of the three modules. These five two-module pulse sequences along with the four individual one-module NORD experiments are all outlined in the Supporting Information.

In conclusion, two new three-module NORD experiments, NORD {HMBC}-{2BOB}-{TOCSY} and NORD {HMBC}-{HSQC-TOCSY}-{TOCSY}, each delivering four spectra, have been introduced. The addition of the TOCSY module to the corresponding two-module experiments can be valuable in its own right as an additional spectrum easing assignment but the key benefit of synergy is that it enhances the spectra generated by the first two modules. In other words, the three-module NORD experiments constitute a sensitive full-package approach in small-molecule NMR.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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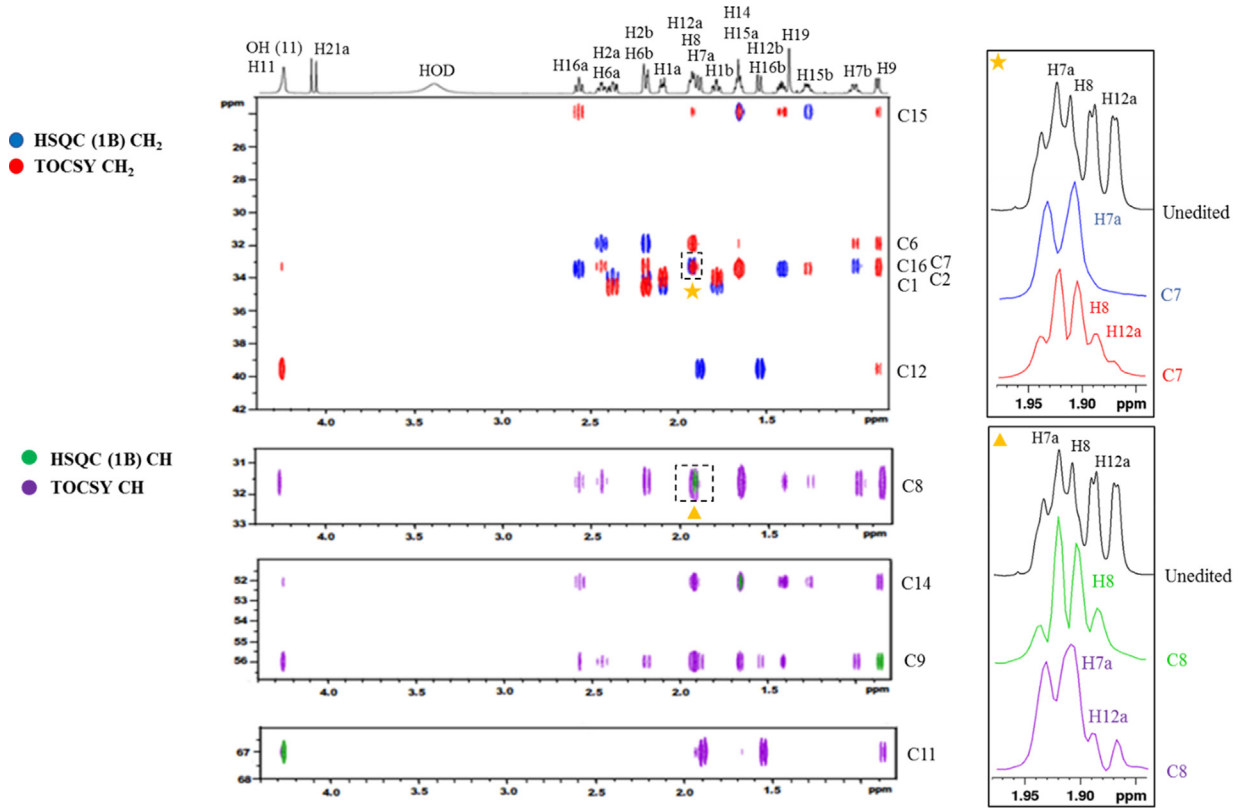

Fig. 5. Overlay of excerpts of fully edited HSQC-TOCSY spectra of hydrocortisone **2** recorded using the NORD {HMBC}-{HSQC-TOCSY}-{TOCSY} experiment (shown in Fig. 1A) on a Bruker Avance NEO 700 MHz spectrometer. Direct HSQC correlations of CH_2 and CH carbons are plotted in blue and green, respectively, whereas the longer range TOCSY correlations are shown in red and purple, respectively. F_2-sections (right) extracted at the marked cross peaks of the respective HSQC-TOCSY subspectra illustrate the crucial role of the four-step editing. The excitation pulses, BANGO for HMBC, TIG-BIRD for HSQC-TOCSY and α for TOCSY, were used as given in Fig. 3. The spectra were acquired with the following parameters: spectral widths of 6.5 ppm (^{1}H) and 200 ppm (^{13}C), 512 points in t_1 with 2 scans per increment in the HMBC and TOCSY modules and 128 points with 8 scans in the HSQC-TOCSY module due to the four-step time-shared editing cycle. For all three modules 2048 data points were acquired in t_2 amounting to direct acquisition time of 225 ms. The total measurement time was 38 min. CAWURST-20 adiabatic ^{13}C inversion pulses (240 ppm, 1.92 ms; H2L) were used in the BANGO and BIG-BIRD elements, whereas it was in the INEPT part CAWURST-20 (240 ppm, 0.97 ms; H2L). The delay τ was set to $^1J = 113$ Hz. DIPSI-2 mixing time of 80 ms was used in both HSQC-TOCSY and TOCSY blocks. $^1J_{min} = 125$ Hz and $^1J_{max} = 165$ Hz for the low-pass filter and $\Delta_{HMBC} = 83$ ms for evolution of heteronuclear long-range couplings in HMBC. Before standard processing the combined data set is separated into three blocks, corresponding to HMBC, HSQC-TOCSY and TOCSY, using the Bruker au-program *splitx*. The HSQC-TOCSY data block is divided into four data sets prior to linear combination using the au-program *split*. Excerpts from the HMBC and TOCSY spectra are shown in Fig. 6.

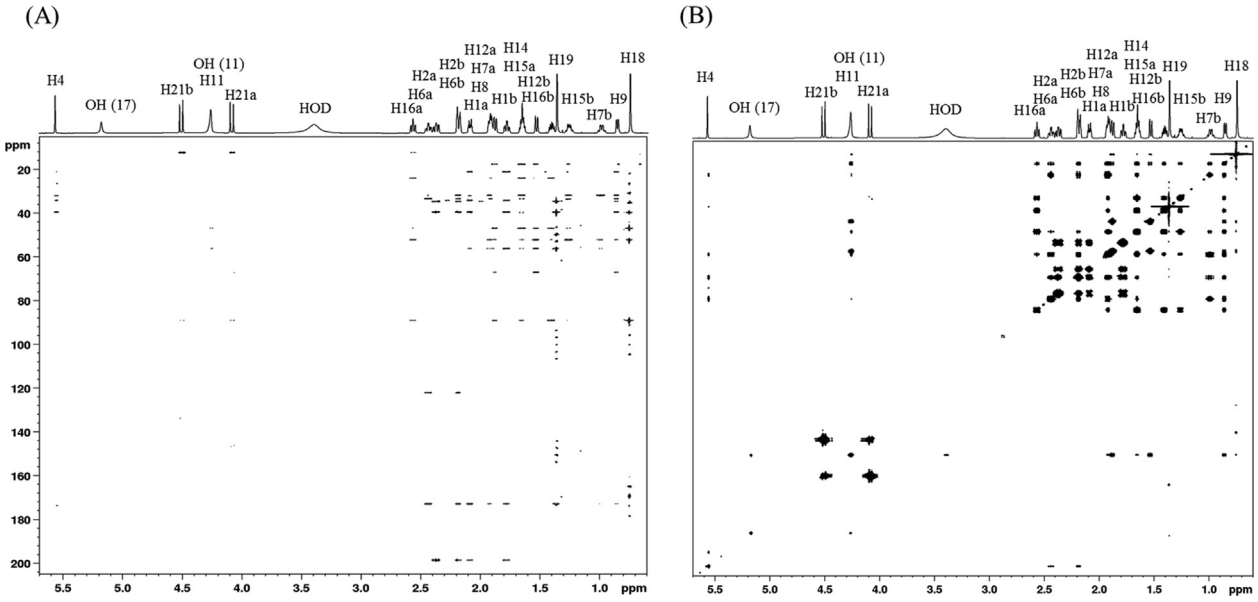

Fig. 6. Excerpts of HMBC and TOCSY spectra of hydrocortisone **2** acquired in 38 min with the NORD {HMBC}-{HSQC-TOCSY}-{TOCSY} sequence (shown in Fig. 1A). The spectra were recorded with the parameters given in Fig. 5.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmr.2022.107297.

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