Expedited NMR Assignment of Small to Medium-Sized Molecules with Improved HSQC-CLIP-COSY Experiments

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Abstract

Resonance assignment is a pivotal step for any NMR analysis, such as structure elucidation or investigation of protein-ligand interactions. Both ^{1}H-^{13}C HSQC and ^{1}H-^{1}H COSY 2D experiments are invaluable for ^{1}H NMR assignment, by extending the high signal dispersion of ^{13}C chemical shifts onto the ^{1}H resonances, and by providing a high amount of through-bond ^{1}H-^{1}H connectivity information, respectively. The recently introduced HSQC-CLIP-COSY method combines these two experiments, providing COSY correlations along the high-resolution ^{13}C dimension with clean in-phase multiplets. However, two experiments need to be recorded to unambiguously identify COSY cross-peaks. Here, we propose novel variants of the HSQC-CLIP-COSY pulse sequence that edit cross-peak signs so that direct HSQC responses can be distinguished from COSY relay peaks, and/or the multiplicities of the ^{13}C nuclei are reflected, allowing assignment of all peaks in just a single experiment. The advanced HSQC-CLIP-COSY variants have the potential to accelerate and simplify the NMR structure elucidation process of both synthetic and natural products, and to become valuable tools for high-throughput computer-assisted structure determination.

Introduction

Structure verification and elucidation are day-to-day tasks at research institutes, pharmaceutical and other chemical industries. Nuclear magnetic resonance (NMR) spectroscopy is proven to be a central analytical tool for these purposes.[1] Resonance assignment is a fundamental step of any NMR investigation surrounding characterization of molecular structure and dynamics or protein-ligand interaction. NMR resonance assignment and structure elucidation of small and medium-sized molecules typically rest on a key set of classical 2D experiments including correlation spectroscopy (COSY),[2,3] total correlation spectroscopy (TOCSY),[4] nuclear Overhauser effect spectroscopy (NOESY)[5] / rotating-frame Overhauser effect spectroscopy (ROESY),[6] heteronuclear single quantum correlation (HSQC)[7] and heteronuclear multiple bond correlation (HMBC)[8] methods. The growing number of regulatory requirements in, for example, drug development increases the amount of individual experiments that must be recorded, creating a demand for approaches that provide a maximum information in the shortest possible time. For instance, NOAH-type (NMR by Ordered Acquisition using ^{1}H-detection) experiments,[9-12] which allow sequential recording of up to four 2D spectra with only one relaxation delay employed in the combined pulse sequence, offer significant time savings compared to the conventional data recording. Also considerable progress has been made in the development of non-uniform sampling (NUS) methods[13-16] for the reconstruction of multidimensional NMR spectra with high digital resolution using only a fraction of increments along the indirect dimension(s), cutting down the measurement time substantially. Furthermore, the resolution of ^{1}H-^{1}H COSY, NOESY or TOCSY experiments can be improved so that unambiguous spectral interpretation is greatly facilitated within a single spectrum. Pure shift methods enhance spectral resolution up to an order of magnitude by removing splittings from homonuclear couplings,[17-24] but these experiments often come with experimental restrictions or with extended measurement times relative to their parent experiment. An alternative strategy for reducing spectral crowding is to replace the indirect ^{1}H dimension with a ^{13}C dimension, which features a much wider dispersion in chemical shifts. For instance, HSQC-TOCSY[25] and HSQC-NOESY[26] experiments extend the parent HSQC experiment with a TOCSY or NOESY mixing step, revealing both one-bond ^{1}H-^{13}C correlations and subsequent ^{1}H-^{1}H connections. Multiple quantum based heteronuclear experiments, such as H2BC,[27,28] H2OBC[29] and HMQC-COSY,[30] were also introduced for the identification of neighboring protons using both ^{1}H and ^{13}C chemical shifts for distinction. However, an HSQC-based experiment would be preferred in many cases, as these generally achieve higher resolution in the ^{13}C dimension. We recently developed the HSQC-CLIP-COSY method,[31] which limits the ^{1}H-^{1}H correlations to those that are directly *J*-coupled, rendering the step-by-step assignment walk straightforward relative to the HSQC-TOCSY[25] experiment. Our method[31] is based on the CLIP-

COSY experiment,[32] which provides clean in-phase multiplets with full absorption mode lineshapes and enhanced cross-peak sensitivity relative to the standard COSY experiment for protons with small *J*-couplings. However, the original HSQC-CLIP-COSY experiment[31] has one major limitation, namely, a supplemental HSQC spectrum needs to be recorded for unambiguous distinction between the direct $^{13}C–^{1}H$ correlations and COSY-type $^{13}C–^{1}H–^{1}H$ peaks of the HSQC-CLIP-COSY spectrum.

Here, we show how the power of the HSQC-CLIP-COSY method can be further boosted by introducing simple heteronuclear spin-echo based building blocks into the original pulse sequence. The amended variants of HSQC-CLIP-COSY experiment allow differentiation of the direct and COSY responses and provide valuable carbon multiplicity information. This makes it possible to obtain unambiguously all one-bond ^{1}H-^{13}C correlations, ^{1}H-^{1}H COSY correlations, and ^{13}C multiplicities from just a single 2D experiment (i.e., one single 2D spectrum), significantly reducing the NMR measurement and analysis time needed for structure elucidation.

Experimental section

All experiments were performed on an Avance NEO 700 (^{1}H: 700.25 MHz; ^{13}C: 176.08 MHz) spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a 5 mm TCI prodigy probe. Data were processed with TopSpin 3.5 or 4.0.5 (Bruker Biospin GmbH, Karlsruhe, Germany). The pulse sequence specific experimental details are given in the corresponding figure legends.

For testing the proposed edited HSQC-CLIP-COSY heteronuclear correlation experiments, samples of 60 mg heparin-analog trisaccharide dissolved in 550 µl D2O and 7.55 mg octapeptide (amino acid sequence: A I K L S T V G) dissolved in 550 µl d_6-DMSO were used. For the measurements the nominal temperature was 298 K.

Results and discussion

Figure 1 illustrates the general scheme of the proposed editing options of the HSQC-CLIP-COSY experiment. On the one hand, the sign of signal amplitudes in the correlation spectra can be edited according to direct HSQC correlations versus COSY peaks (route I in Figure 1). The sign encoding of the direct and relay peaks facilitates tracking of the connectivity network of protonated carbons, making the assignment of both ^{1}H and ^{13}C resonances possible from a single spectrum without the necessity of recording an additional HSQC spectrum. On the other hand, even and odd

multiplicity of ^{13}C nuclei can be also distinguished on the basis of pertinent sign characteristics. Thus, peaks arising from CH_2 groups will appear as negative signals whereas those arising from CH and CH_3 groups will all be positive in the spectrum (route II/A). Optionally, ^{13}C multiplicity editing can be achieved by a recently proposed, alternative way,[33] where correlation peaks from CH_2 groups will appear as mirror images with reversed frequency offset and with negative amplitude in the spectra (route II/B). This latter approach changes the dispersion of the correlation peaks, reduces signal overlaps in severely crowded regions when the CH_2 correlation peaks can be reflected in a sparse spectral region. This also fully resolves potential sign interpretation ambiguities of combined (I and II) editing. The approach can be very useful in the case of peptides and also smaller proteins,[33] as will be demonstrated in the present article.

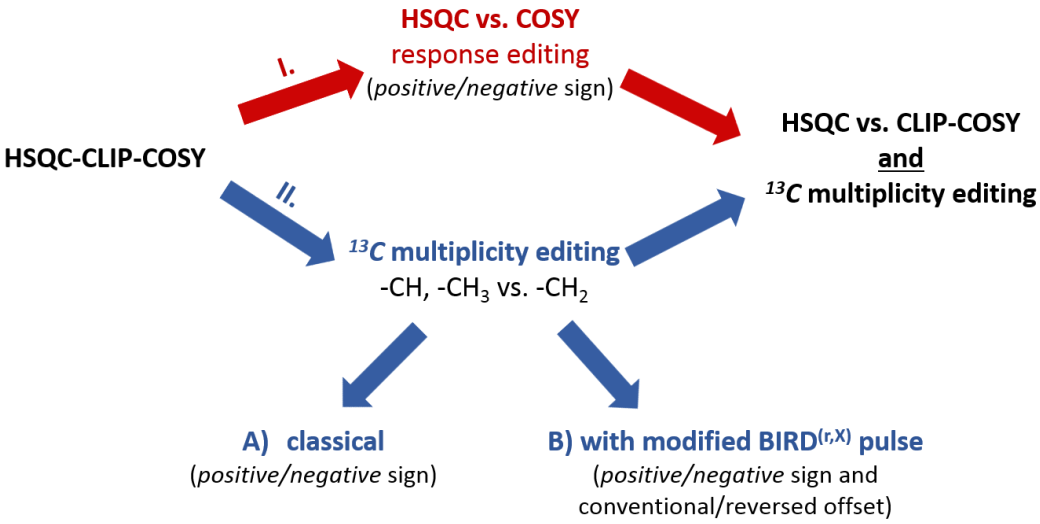

Figure 1. Schematic overview of the editing options of the HSQC-CLIP-COSY experiment. There are two major routes, which can be applied separately or simultaneously, depending on the complexity of the molecular structure investigated. On the one hand, signal amplitudes in the correlation spectra can be edited according to direct HSQC correlations versus COSY peaks. On the other hand, even and odd multiplicities of ^{13}C nuclei can be distinguished based on cross-peak signs (II/A), or by reflecting even multiplicities along F_1 (II/B).[33]

The pulse scheme of the novel, edited variants of the HSQC-CLIP-COSY experiment is shown in Figure 2 and is briefly discussed in the following. Initially, standard HSQC and CLIP-COSY sequences are merged to form the HSQC-CLIP-COSY as introduced and described in a recent publication of our group.[31] Importantly, for refocusing the antiphase proton magnetization generated in the first $^1H \rightarrow ^{13}C \rightarrow ^1H$ polarization transfer block of the sequence, a suitably positioned carbon 180° pulse is employed during the perfect-echo based COSY mixing segment. The HSQC *vs*. COSY response editing block of $(^1J_{CH})^{-1}$ duration (labeled with I in Figure 2) is inserted at the end of the original HSQC-CLIP-COSY sequence prior to ^1H detection. This spin-echo block allows reversing the sign of amplitude of direct (HSQC-type) peaks with respect to the relay (COSY-type) peaks due

to the $^{1}J_{CH}$ coupling evolution of the directly ^{13}C-coupled proton magnetization. Furthermore, to achieve the desired carbon multiplicity editing, an additional heteronuclear spin-echo block either with simultaneous ^{1}H and ^{13}C 180° pulses or with a modified BIRD$^{(r,X)}$ pulse applied in the center of the $(^{1}J_{CH})^{-1}$ echo[33] is incorporated into the pulse sequence after the t_1-evolution period. It is worth noting that the two different types of editing – HSQC vs. COSY peaks and ^{13}C multiplicity – can be employed either separately or together, as illustrated in Figure 1. To avoid signal overlap and/or accidental cancellation of signals with opposite phases due to the editing, high digital resolution in F_1 is recommended, which can be optimally achieved by applying non-uniform sampling (NUS) without increasing the measurement time.

Regarding previously published methods, the HMQC-based H2OBC experiment[29] provides similar spectral information to our HSQC-CLIP-COSY method. However, the H2OBC being a constant time (CT) experiment offers only limited resolution in F_1, while in HSQC-CLIP-COSY there is no such limit, so ultra-high ^{13}C resolution can be reached allowing separation of closely resonating overlapping signals. Moreover, in H2OBC/2BOB,[29] the direct and COSY cross-peaks have a 90° phase difference, which adversely affects spectral resolution in F_2. In contrast, all peaks in HSQC-CLIP-COSY possess pure absorption lineshapes. COSY correlations are also fully in-phase, offering increased sensitivity when homonuclear splittings are small, as discussed in an earlier publication.[32] Such favorable peak-shape characteristics of HSQC-CLIP-COSY also provide the best possible condition for automatic peak-picking procedures, central to computer-assisted structure elucidation.

Overall, the improved, edited variants of the HSQC-CLIP-COSY experiment allow differentiation of direct and COSY responses, and can provide useful carbon multiplicity information with only minor penalties in sensitivity from the use of more pulses, mismatch of echo-delays with respect to the pertinent ^{1}J(XH) value and resonance offset effects of carbon pulses. To minimize the latter adverse effect, utilization of adiabatic,[34-38] composite adiabatic,[39] or optimal control[40,41] broadband ^{13}C pulses is recommended, particularly at higher magnetic fields.

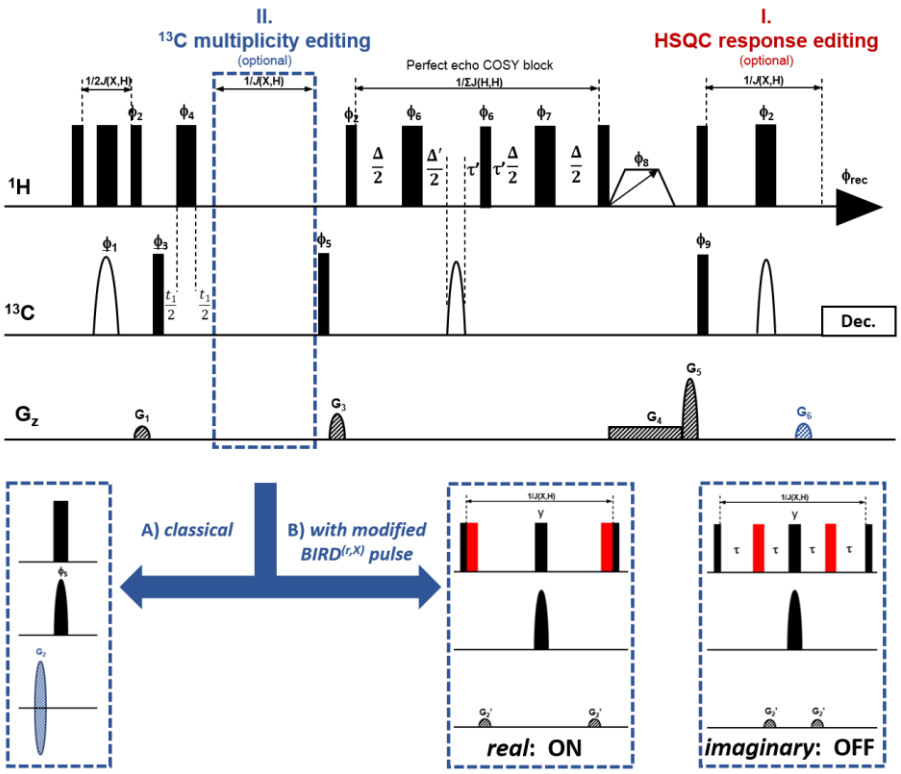

Figure 2. Pulse sequence scheme for HSQC-CLIP-COSY experiments. Narrow and wide filled bars correspond to 90° and 180° pulses, respectively, with phase x unless indicated otherwise. Broadband adiabatic inversion pulses (CHIRP) and shaped refocusing pulses applied to (composite CHIRP or BURBOP-180) ^{13}C are shown as open and filled half-ellipses. Phases are ϕ_1 = x; ϕ_2 = y; ϕ_3 = x, -x; ϕ_4 = $(x)_2$, $(-x)_2$; ϕ_5 = $(x)_4$, $(-x)_4$; ϕ_6 = $(-x)_2$, $(-y)_2$; ϕ_7 = -y; ϕ_8 = -x; ϕ_9 = x, -x; ϕ_{rec} = x, -x, -x, x. The minimum number of transients required by phase cycling is 2. Delays are set as follows: Δ is typically set to 20 – 25 ms, allowing sufficient transfer of magnetization between protons coupled with couplings ranging from 2 up to 14 Hz, and complying the preferred perfect-echo duration for in-phase coherence transfer between coupled spins; $\tau' = 1/(^1J_{(XH)}\times 4) - t_{\pi(X)/2}$. In case of classical editing route (A) coherence order selection and echo-antiecho phase sensitive detection in the ^{13}C dimension are achieved with gradient pulses G_2 and G_6 in the ratio 80:20.1 for ^{13}C. While in route (B) coherence order selection and phase sensitive detection are achieved according to States-TPPI protocol. Purging gradient pulses G_1, G_3, G_5 are set to 17%, 31%, -17.9% of maximum gradient strength (50 G/cm), typically with 1 ms duration followed by a recovery delay of 100 μs. Weak magnetic field gradient (G_4) used under the frequency-swept CHIRP pulse is adjusted for -7.5% of maximum gradient strength.

The heteronuclear correlation-based assignment strategy utilizing the HSQC *vs*. COSY response and the classical ^{13}C multiplicity editing is illustrated on the example of a heparin-analog trisaccharide. Based on the sign of correlation peaks – that is color-coded as positive-black and negative-red in the edited HSQC-CLIP-COSY spectrum shown in Figure 3 – the directly coupled ^{13}C-^{1}H (HSQC) pairs (black) and the indirectly coupled ^{13}C-^{1}H-^{1}H spin triples (red) can be easily and unambiguously identified, which makes a step-by-step walk along the spin system of each sugar residue possible. Note that D6, F6 and F7 CH_2-carbon correlation peaks show opposite sign pattern – red for HSQC- and black for COSY-type correlations – due to ^{13}C multiplicity editing. As a result, the ^{1}H-^{1}H coupled spin partners can be simply traced along the rows, while walking along the columns

identifies the neighboring carbons. In Figure 3 this assignment walk is indicated by dotted and solid lines for each residue of the trisaccharide. Thus, the HSQC-CLIP-COSY experiment delivering both HSQC-type one-bond heteronuclear correlation and COSY-type homonuclear connectivity information in one single spectrum allows rapid and accurate spin system identification in small to medium-sized molecules.

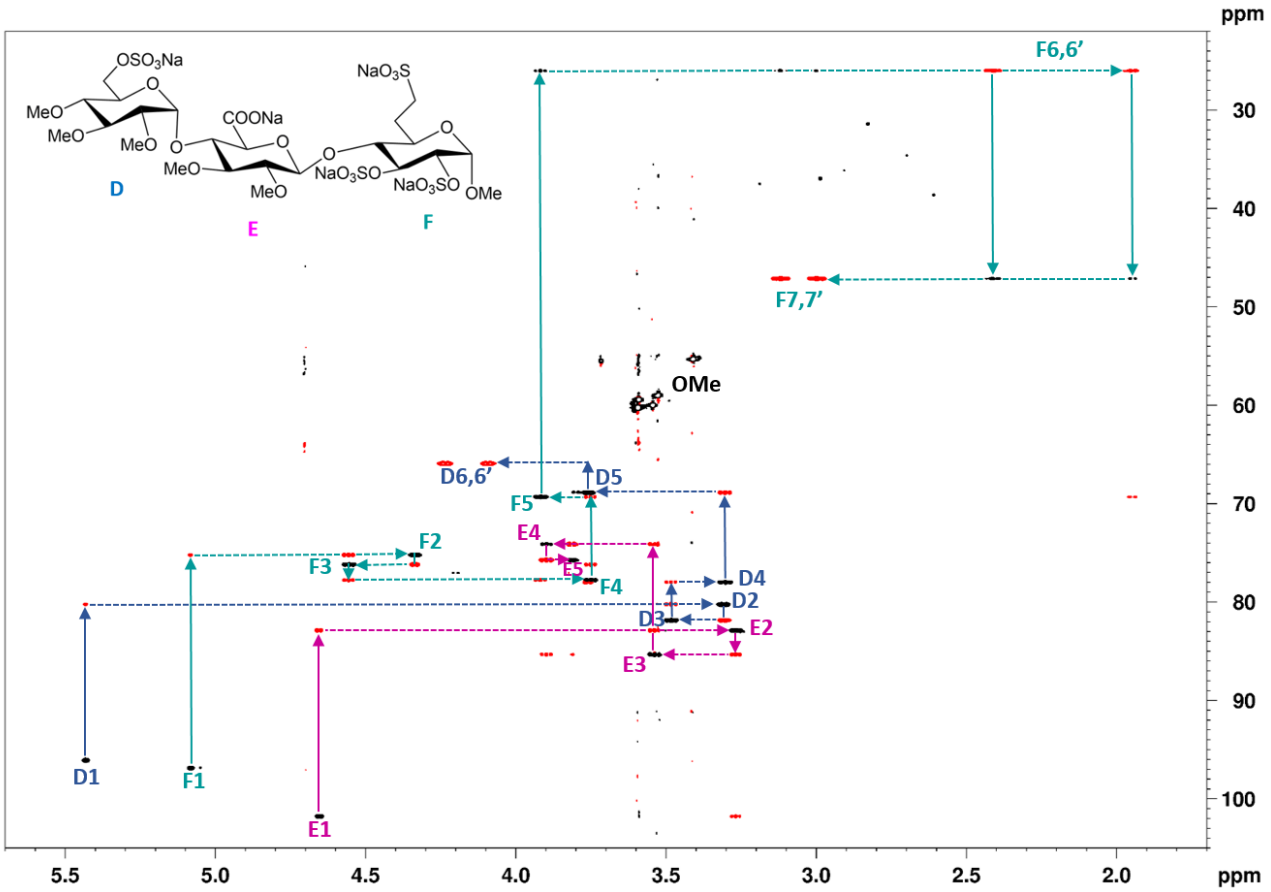

Figure 3. Edited HSQC-CLIP-COSY spectrum of a heparin-analog trisaccharide.[42] The assignment walks of D, E and F residues are labeled by colored solid and dotted lines along ^{13}C and ^{1}H dimension, respectively. All ring proton resonances of the trisaccharide can be unambiguously assigned by simply walking along the color-coded cross peaks of directly (black) and indirectly (red) coupled spins. For CH_2-correlations (D6, F6, F7) the color pattern is opposite due to carbon multiplicity editing. OMe label on the spectrum indicates the HSQC peaks of O-Methyl groups. Spectrum was recorded with spectral widths of 6.93 (120.0) ppm in ^{1}H (^{13}C) dimensions, a relaxation delay of 1.5 s, 1024 t_1 increments using 25% NUS, 2048 data points acquired in ^{1}H dimension and 2 scans per increment with an overall experimental time of 16 min.

In cases when the chemical shift distribution of resonances is small, and hence spectra contain severely crowded regions of carbons with different multiplicities (e.g. H_β/C_β, H_γ/C_γ and H_δ/C_δ regions of oligopeptides and proteins), the carbon-multiplicity editing approach, as proposed by Sakhaii and Bermel,[33] can be utilized as an additional, powerful tool to resolve the overlapping resonances. Inserting the modified, switchable (ON/OFF) BIRD$^{(r,X)}$ pulse cluster in the multiplicity editing module (Figure 2), the sign of carbon chemical shift evolution of the CH_2 groups is reversed relative to the CH/CH_3 groups, so that the corresponding CH_2 cross-peak positions are reflected relative to

the ^{13}C carrier frequency. When the latter is chosen judiciously, this repositions the CH_2 peaks into an empty region of the spectrum, avoiding overlap and cancellation between CH_2 and the oppositely-phased CH/CH_3 peaks. Hence, improved signal dispersion can be achieved without the need of increasing digital resolution in the F_1 dimension of the spectrum. In the doubly-edited HSQC-CLIP-COSY spectrum, positive peaks (black) represent direct CH and CH_3 connectivities or COSY-type relay peaks originating from CH_2 moieties, while negative peaks (red) represent direct CH_2 correlations or relay peaks arising from CH and CH_3 moieties. In addition, applying the BIRD-type carbon multiplicity editing,[33] correlation peaks from CH_2 groups appear as mirror images in the corresponding low-field (large ^{13}C chemical shift) region of the spectrum. The real ^{13}C chemical shifts (δ_{CH2}) of the reflected (mirror image) CH_2 peaks can be easily assessed from the values of the measured chemical shift ($\delta_{CH2\ measured}$) and the ^{13}C carrier offset frequency ($\delta_{13C\ offset}$) used for the measurement with the relation $\delta_{CH2} = 2\times\delta_{13C\ offset} - \delta_{CH2\ measured}$.

The proposed doubly-edited variant of HSQC-CLIP-COSY experiment relying on the frequency reversing multiplicity editing block provides an efficient tool for the identification of amino acid type in oligopeptides. The characteristic HSQC-CLIP-COSY peak patterns of α-amino acids with different spin topology in their side chains are shown in Figure 4 and Figures S1-S20 with illustration of the assignment walk along the spin system. Based on the peak pattern observable in the doubly-edited HSQC-CLIP-COSY spectrum, four types of amino acid topologies can be easily assigned and distinguished. Namely, Gly with CH_2 in α position gives only two peaks, the direct (Cα-Hα) and relay (Cα-Hα–HN) peaks at 'mirror image' carbon chemical shift in the spectrum (Figures 4/a and S1). The second group of amino acids with isolated CH_2 in β position (such as Asp, Asn, Cys, Ser, Phe, Tyr, Trp, His) can be easily identified following the assignment walk shown in Figures 4/b and S2-S9. Starting from the direct (Cα-Hα) peak and 'walking down' along the column, the neighboring Cβ(CH_2) can be found in the large carbon chemical shift (mirror image) region of the spectrum. Since CH_2-β protons have only Hα as coupling partner, the row of Cβ contains only one relay peak Cβ-Hβ-Hα. Alternatively, the amino acids with Cβ(CH_2) followed by protonated Cγ in their side chain (such as Pro, Leu, Met, Glu, Gln, Arg, Lys) show additional relay peaks Cβ-Hβ-Hγ in the row of their Cβ carbon (Figures 4/c and S10-S16). The assignment walk along the rest of the side chain – as demonstrated in Figures 4/c and S10-S16 – allows differentiation of the amino acids with different side chain topology. In the last group of amino acids (Ala, Val, Thr, Ile) with CH/CH_3 in β position the assignment walk starting from the direct (Cα-Hα) peak goes upward along the column, locating the adjoining Cβ in the high field (aliphatic) region of the spectrum (Figures 4/d and S17-S20). Again, completing the assignment walk along the side chain, the specific amino acids of the group can be easily identified.

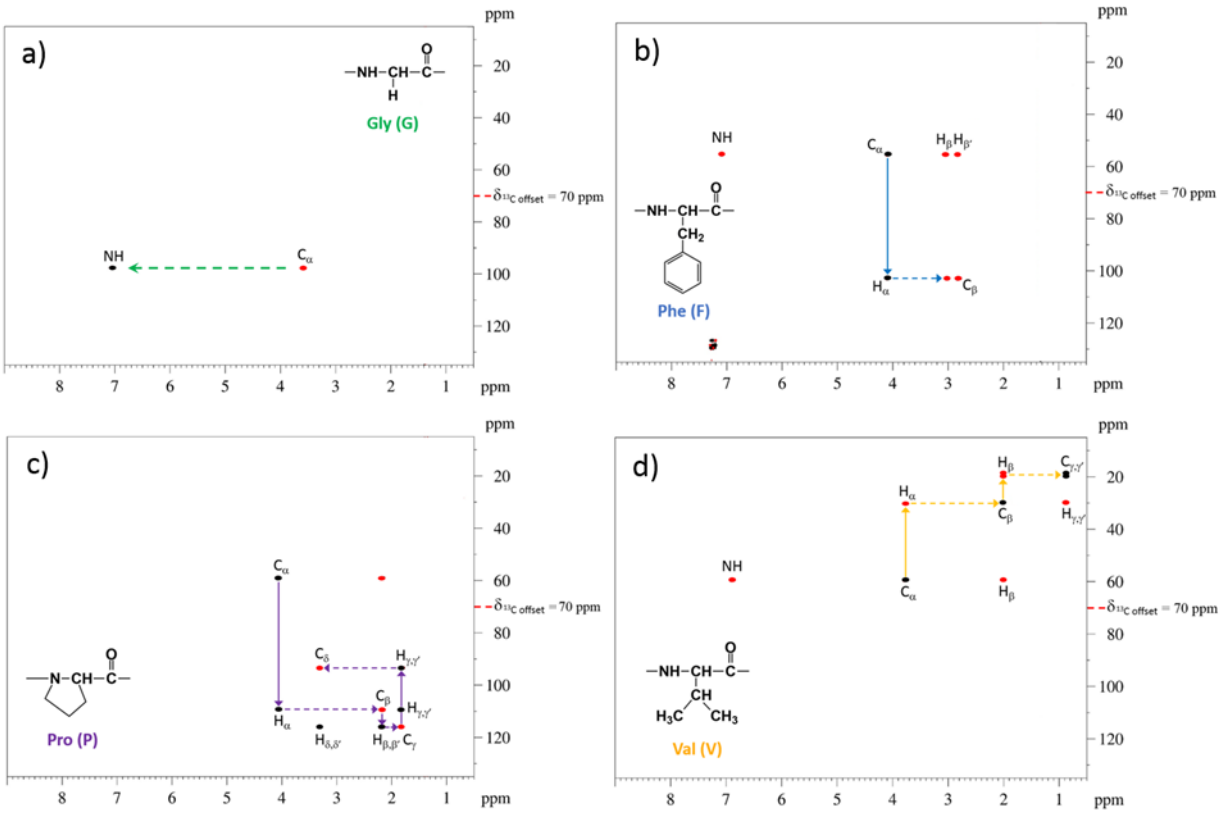

Figure 4. Characteristic doubly-edited HSQC-CLIP-COSY peak patterns of representative α-amino acids with different spin topology in their side chains. a) Glycine (containing only Cα); b) Phenylalanine (containing isolated CH_2 in β position); c) Proline (containing CH_2 in β position followed by protonated Cγ); d) Valine (containing CH/CH_3 in β position). Arrows on the spectra indicate the assignment walk along the spin systems.

The utility of this peak pattern-based assignment protocol is demonstrated on the example of an octapeptide containing at least one specific amino acid from each amino acid type sub-group. Accomplishing the assignment walk in the doubly-edited HSQC-CLIP-COSY spectrum as shown in Figure 5 – where different colors are used for coding the different amino acid types – a complete and unambiguous assignment of all ^{1}H and protonated ^{13}C resonances of individual amino acids could be attained. The mandatory sequential assignment of residues within bio-oligomers can next be accomplished by 2D ROESY, utilizing the shortrange ^{1}H–^{1}H distances between neighboring residues, or by 2D HMBC experiment, observing the interresidue three-bond ^{1}H–^{13}C connectivities as shown in Figures S21-S22.

Taken together, these examples demonstrate how the enhanced spectral information content of the edited HSQC-CLIP-COSY provides an ideal tool for quick and unambiguous assignment of ^{1}H and protonated ^{13}C resonances of small to medium-sized molecules, such as (oligo)saccharides and peptides.

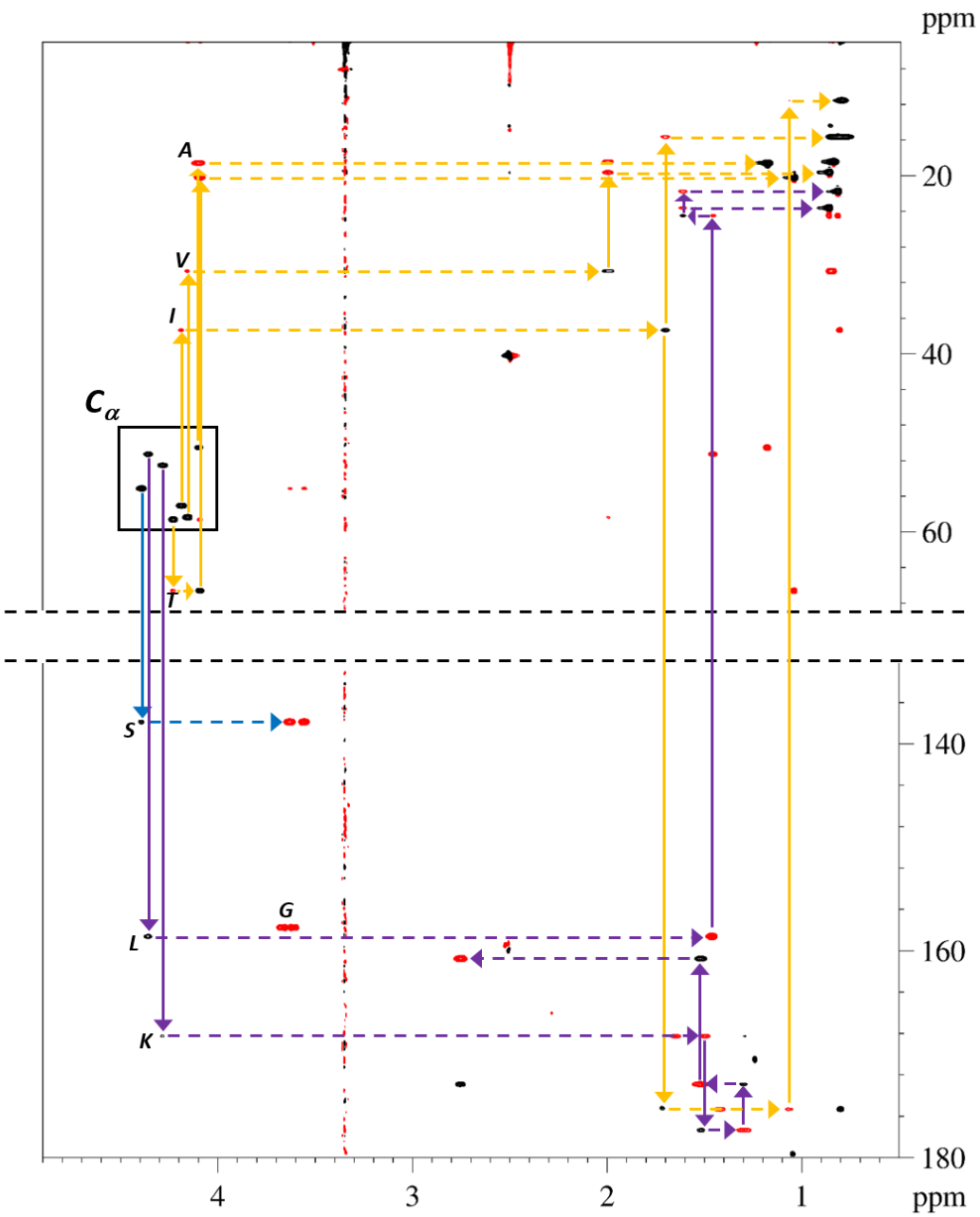

Figure 5. Doubly-edited HSQC-CLIP-COSY spectrum of an oligopeptide (amino acid sequence: A I K L S T V G), which contains at least one specific amino acid from each amino acid type subgroup. The different colors are used for coding the different amino acid types as described in the text. Starting from the direct Cα–Hα peaks (region is indicated by black box), all amino acid residues can be easily identified. The spectrum was recorded with spectral widths of 10.2 (190.0) ppm in ^{1}H (^{13}C) dimensions, a relaxation delay of 1.7 s, 1024 t_1 increments, 2048 total data points acquired in the ^{1}H dimension, 4 scans per increment.

Conclusions

Edited HSQC-CLIP-COSY experiments have been developed for making the ^{1}H and ^{13}C NMR assignment of small to medium-sized molecules simpler and faster. Sign editing of direct HSQC responses vs. CLIP-COSY peaks provides a straightforward approach for tracking down the

backbone of protonated ^{13}C nuclei step-by-step. This edited HSQC-CLIP-COSY spectrum offers the information content of HSQC and COSY spectra integrated in one data set, obviating the need for recording of separate experiments. The information content of an HSQC-CLIP-COSY spectrum can be further boosted by introduction of carbon multiplicity-editing. To simplify overcrowded spectral regions, the sign of carbon frequency of CH_2 signals can be also reversed utilizing the recently proposed BIRD$^{(r,X)}$ scheme for multiplicity editing. We believe that the proposed edited HSQC-CLIP-COSY experiments, providing high-quality, sign- and/or frequency-coded, well-resolved and pure absorption spectra, hold great promise to significantly accelerate and simplify NMR assignment of bio-oligomers and other types of synthetic molecules or natural products. Due to the high information content, excellent resolution and line shape characteristics of the resulting spectra, the edited HSQC-CLIP-COSY experiments have the potential to be valuable and indispensable for computer-assisted structure elucidation.

Associated Content

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/XXX.

Additional NMR spectra about the characteristic HSQC-CLIP-COSY peak pattern of α-amino acids, sequential assignment of the trisaccharide and the peptide, pulse sequence codes for Bruker spectrometers (PDF).

Notes

The authors declare no competing financial interest.

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Keywords: CLIP-COSY, HSQC, NMR spectroscopy, peptide, structure elucidation

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