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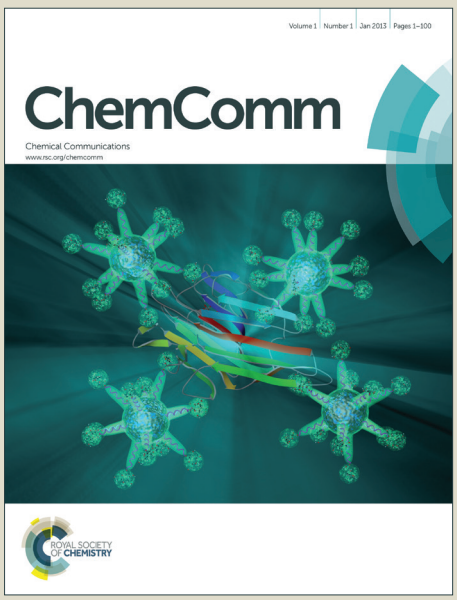





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ARTICLE TYPE

# Simultaneous $^{1}$H and $^{13}$C NMR enantiodifferentiation from highly-resolved pure shift HSQC spectra

**Míriam Pérez-Trujillo,[1] Laura Castañar,[1] Eva Monteagudo,[1] Lars T. Kuhn,[2] Pau Nolis,[1] Albert Virgili,[1] R. Thomas Williamson,[3] and Teodor Parella[1,*]**

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**NMR enantiodifferentiation studies are greatly improved by the simultaneous determination of $^{1}$H and $^{13}$C chemical shift differences through the analysis of highly resolved cross-peaks in Spectral Aliased Pure Shift (SAPS) HSQC spectra.**

The determination of enantiomeric purity can be accomplished by NMR spectroscopy using a great variety of auxiliary chiral sources.[1] Of these, chiral solvating agents (CSAs), such as the so-called Pirkle alcohol (PA) or cyclodextrins (CDs), have been widely used. They do not typically introduce significant line-broadening, the sample is easily prepared and the analysis is quickly performed by observing chemical shift differences (ΔΔδ) between the resulting diasteromeric complexes in conventional $^{1}$H NMR spectra. However, signal enantiodifferentiation using CSAs is not uniform for all protons and in many cases, low ΔΔδ values and signal overlap caused by complex multiplets lead to the lack of spectral signal dispersion that preclude a straightforward analysis. Alternatively, enantiodifferentiation using $^{13}$C NMR spectroscopy can be more advantageous because singlet signals are analyzed although its routine use is limited by its low sensitivity.[2] Another strategy to deconvolute these enantiodifferentiated data is to take advantage of improved signal dispersion offered by multidimensional spectra as shown for instance in the chiral recognition of camphor and α-pinene enantiomers with CDs made through HSQC spectra.[3] Recently, pure shift NMR spectroscopy has emerged as a promising tool to simplify the typical J(HH) multiplicity pattern of $^{1}$H signals to singlets.[4-8] This affords a general improvement on signal dispersion that allows an improved analysis of complex and overcrowded resonances. Recently, this concept has proved its usefulness in the detection of ΔΔδ values between diastereosiomeric complexes involving CSAs.[8]

In this study we utilized a racemic mixture of compound (**1**), a precursor for a series of diarylether lactams as cancer chemotherapeutic agents,[9] complexed with *R*-PA as a CSA. Its $^{1}$H NMR spectrum (Fig. 1A) does show some well-differentiated signals (for instance, H12 appears around 7.10-7.15 ppm as two triplet signals separated by 20.9 Hz or 34.8 ppb), but most of the signals cannot be individually distinguished. For example, H13 is hidden under the stronger H2 signal from *R*-PA (6.6ppm), and the splitting in signals resonating in the congested aliphatic area at 1.4-1.8 ppm cannot be clearly observed due to spectral overlap. Other protons present complex multiplet patterns (H4a or H6b) or are poorly resolved (H7a), hindering their direct analysis. On the other hand, up to 9 signals appear split in the conventional $^{13}$C spectrum of racemic **1** acquired after 9 hours, with a maximum ΔΔδ($^{13}$C) of 84.1 ppb (Fig. S3). As an alternative to the acquisition and analysis of 1D $^{13}$C NMR data, pure shift 1D $^{1}$H NMR experiments can be employed to simplify the analysis and provide a much more sensitive approach in the determination of small ΔΔδ($^{1}$H) values (Fig. 1B). In this 1D pure shift $^{1}$H spectrum acquired in 9 min. using the pseudo-2D Zangger-Sterk (ZS) method,[4] the separation of each individual signal can be visualized allowing the accurate measurement of ΔΔδ($^{1}$H) as small as 2 Hz (3.3 ppb), even for signals that would exhibit very complex multiplets and serious overlapping in a standard 1D $^{1}$H NMR (Fig. 1C vs 1D).

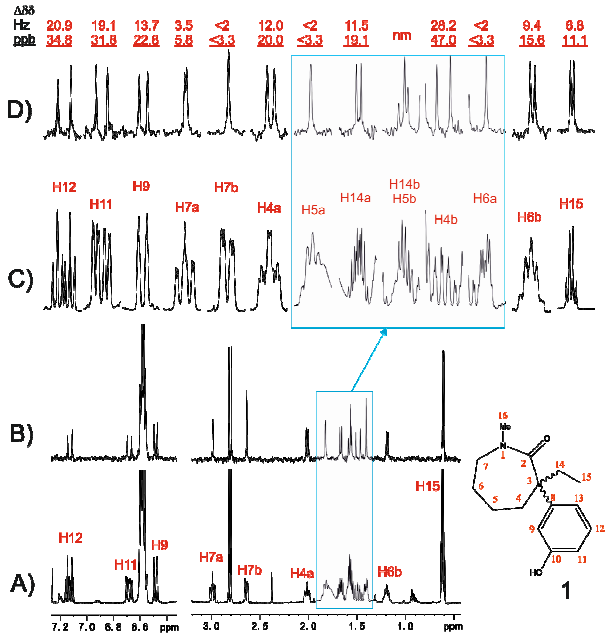


Figure 1: 600.13 MHz A) conventional and B) pure shift $^{1}$H NMR spectra of 2 mM racemic compound **1** complexed with 9.6 equiv. of *R*-PA in $CDCl_3$. C-D) Comparison of expanded $^{1}$H multiplets for determining accurate ΔΔδ($^{1}$H) values (shown in Hz and ppb).

In this communication, we show how highly resolved 2D HSQC spectra can be an efficient tool for enantiodifferentiation

studies and also for the detection and accurate quantification of very small ΔΔδ values. Traditionally, attempts to obtain highly resolved HSQC spectra over the entire $^{13}C$ spectral width involved an enormous investment in instrument time. Our method is based on the concerted leveraging of several approaches to improve signal dispersion in 2D HSQC spectra. First, spectral aliasing (SA) is incorporated to improve resolution along the indirect dimension by one or two orders of magnitude without increasing the total experimental time by using a reduced $^{13}C$ spectral width.[10] Secondly, a sensitivity-improved version[11] of the pure shift (PS) HSQC experiment (Figure 2A),[6] referred to as psHSQCsi, is applied to enhance the resolution in the alternate $^{1}H$ dimension. This experiment applies 180º($^{1}H$)-BIRD modules for homodecoupling and also heteronuclear decoupling during the τ acquisition periods to obtain fully decoupled $^{1}H$ singlet signals. Diastereotopic protons belonging to methylene AB spin systems appear as doublets because geminal $^{2}J(HH)$ magnetization is inverted together during the BIRD filter and is therefore not decoupled.[5] Finally, resolution can be further improved using non-uniform sampling[12] in combination with zero-filling and linear prediction during data processing.

Fig. 2B compares a portion of the SAPS-HSQC vs SA-HSQC spectra of **1**, in order to evaluate multiplet simplification, signal dispersion and relative sensitivity. These data, acquired using a reduced $^{13}C$ spectral width of 2.5 ppm in a 600 MHz spectrometer with 256 $t_1$ increments per 2046 points each, provides a digital resolution around 2-3 Hz/pt in both dimensions. It is shown that improved signal dispersion due to the combined effects of $^{1}H$ and $^{13}C$ δ differentiation is further enhanced by the multiplet pattern simplification provided by homo- and heteronuclear decoupling. The pure shift approach can afford a general sensitivity enhancement by 10-40% through collapse of the multiplet structure. As expected, the proposed psHSQCsi version affords a substantial SNR improvement for CH cross-peaks when compared to the psHSQC (Fig. S4). In terms of spectral quality, homodecoupling during acquisition in psHSQC/psHSQCsi experiments generates small sidebands at specific frequencies around the main signal and a minimum broadening of the signal (~3 Hz vs ~3.5 Hz) when compared to traditional experiments (Fig. S4).[6] In practice, this does not affect the ΔΔδ determination, and signal discrimination less than 0.5 Hz (0.8 ppb for $^{1}H$ and 3.3 ppb for $^{13}C$, respectively) can typically be achieved (Table 1), even for NMR signals with no apparent splitting in the $^{13}C$ spectrum.

The analyzed sample contains several examples that illustrate the power of the proposed method which, *a priori*, could detect enantiodifferentiated signals even in the case that $\Delta\Delta\delta(^{1}H)$ or $\Delta\Delta\delta(^{13}C)$ is close to 0, whenever one of the two are sufficiently dispersed. In the example shown, of the 16 available proton signals, 5 are detected as enantiodifferentiated in the $^{1}H$ spectrum, 10 in the 1D ZS and 15 in the psHSQCsi. In addition, of the 11 signals of protonated carbons, 6 are detected as enantiodifferentiated in the 1D $^{13}C$ spectrum and 10 in the psHSQCsi (Table 1). A new parameter $\Delta\Delta\delta(CH)^2=\Delta\Delta\delta(^{1}H)^2+\Delta\Delta\delta(^{13}C)^2$ is defined to describe mathematically the signal dispersion in HSQC cross-peaks (Fig. S5). In general, we can say that both $\Delta\Delta\delta(^{1}H)$ and $\Delta\Delta\delta(^{13}C)$ values can be measured when $\Delta\Delta\delta(CH)>5$ ppb (Table 1). For instance, the two singlets corresponding to the NMe group in **1** (H16) are well resolved in the regular $^{1}H$ spectrum (27.0 ppb) whereas the corresponding C16 carbon is not resolved in the $^{13}C$ spectrum (<13.2 ppb). From the HSQC cross-peak an accurate value of $\Delta\Delta\delta(C16)$=9.9 ppb can be obtained. Similar analysis can be made for the H9/C9 and H13/C13 pairs where the carbon signals do not appear split in the 1D $^{13}C$ spectrum but values of 4.6 ppb and 9.2 ppb, respectively, can be extracted from the 2D analysis (Fig. 2B and S8). Another challenging analysis involves the H7a/H7b protons and their C7 carbon which present very low resolution. A very small $\Delta\Delta\delta(H7b)$=1.2 ppb which is not distinguishable in the ZS $^{1}H$ spectrum can be measured from the highly resolved 2D cross-peak.

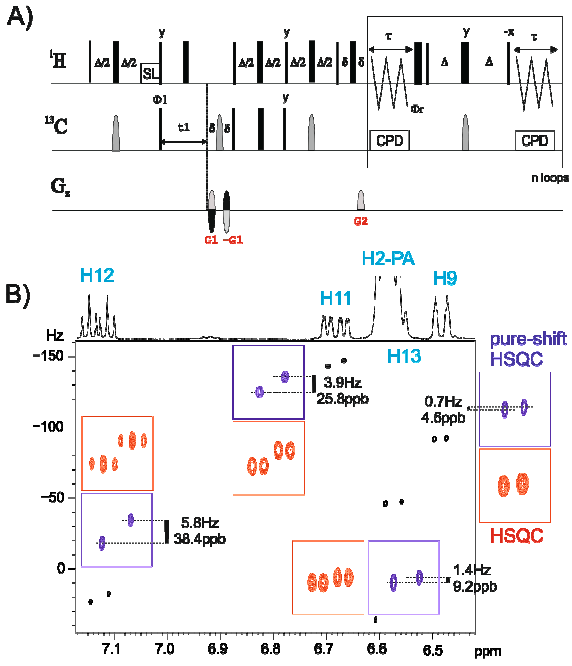


Figure 2: A) Pulse scheme of the pure shift sensitivity-improved HSQC experiment (Δ=1/(2*$^{1}J(CH)$); B) Expanded areas comparing some cross-peaks in SA- (red) and SAPS-HSQC (blue) spectra of the racemic compound **1**/*R*-PA mixture acquired with a reduced $^{13}C$ spectral width of 2.5 ppm.

Table 1: $^{1}H$ and $^{13}C$ NMR chemical shift differences ($\Delta\Delta\delta(^{1}H)$ and $\Delta\Delta\delta(^{13}C)$) of the racemic compound 1 (29 mM) enantiodifferentiated with *R*-PA (9.6 equiv.) measured at 600 MHz and 298K.

| Label | ΔΔδ($^{1}H$) [in ppb] | | | ΔΔδ($^{13}C$) [in ppb] | | | ΔΔδ (CH) |
|---|---|---|---|---|---|---|---|
| | 1D $^{1}H$ | 1D ZS-$^{1}H$ | Pure shift HSQC[c] | 1D $^{13}C$ | Pure shift HSQC[c] | HSQMBC[b] | [in ppb] |
| 2 | - | - | - | 14.5 | - | 14.5 | 30.3[b] |
| 3 | - | - | - | <13.2 | - | 3.3 | 12.4[b] |
| 4a/4b | x[a]/x[a] | 20.0/47.0 | 20.8/46.1 | 72.9 | 73.5 | - | 76.4/86.7 |
| 5a/5b | x[a]/x[a] | <3.3/ x[a] | 2.5/21.0 | <13.2 | 5.3 | - | 5.9/21.7 |
| 6a/6b | x[a]/x[a] | <3.3/15.6 | <3.3/16.7 | <13.2 | <3.3 | - | <4.7/17.0 |
| 7a/7b | x[a]/x[a] | 5.8/<3.3 | 4.6/1.2 | 13.9 | 13.2 | - | 14.0/13.2 |
| 8 | - | - | - | 84.1 | - | 84.1 | 90.9[b] |
| 9 | 22.4 | 22.8 | 23.3 | <13.2 | 4.6 | | 23.7 |
| 10 | - | - | - | 43.7 | - | 44.4 | 56.2[b] |
| 11 | 30.8 | 31.8 | 30.6 | 27.1 | 25.8 | - | 40.0 |
| 12 | 34.4 | 34.8 | 34.5 | 39.0 | 38.4 | - | 51.6 |
| 13 | x[a] | x[a] | 32.0 | <13.2 | 9.2 | - | 33.3 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 14a/14b | $x^a/x^a$ | 19.1/ $x^a$ | 18.8/9.8 | 30.4 | 29.8 | - | 35.2/31.3 |
| 15 | 11.5 | 11.1 | 12.0 | 21.2 | 18.5 | - | 22.0 |
| 16 | 27.0 | 27.1 | 26.6 | <13.2 | 9.9 | - | 28.4 |

[a] Not determined
[b] Only relevant data on quaternary carbons is shown
[c] digital resolution of ±0.5 and ±2.6 ppb for $^1$H and $^{13}$C, respectively.

The same strategy can be followed to determine ΔΔδ on quaternary carbons from an aliased non-refocused HSQMBC experiment[13]. Unfortunately, broadband homodecoupling in a similar manner as that described for the psHSQC experiment cannot be achieved because the detected signals correspond to $^1$H-$^{12}$C magnetization that is homonuclear J coupled to other protons with the same $^1$H-$^{12}$C topology. Although at least one pure-shift HMBC approach has been reported, this technique requires long acquisition times and a complex processing protocol.[7] Fig. S9 shows some HSQMBC cross-peaks for the four quaternary carbons of **1** where a very small value of ΔΔδ(C3)= 3.3 ppb can be measured.

In all these spectra, each aliased $^{13}$C peak appears without sign inversion at a position that is exactly a multiple of the spectral width ($SW_c$) from its real position ($\delta_r$) that can be determined from the relationship $\delta_r=\delta_{obs}+(K*SWc)$, where $\delta_{obs}$ is the experimental δ(13C) measured in the aliased spectra using a given $^{13}$C offset $\Omega_c$ and K is the fold number which can be determined from a reference non-aliased HSQC or HSQMBC spectra using a moderate number of $t_1$ increments (Fig. S10). Several automated strategies that have been proposed to determine the correct $^{13}$C $\delta_r$ values and to reconstruct the entire spectrum could also be applied here.[10] The enantiodifferentiation from high resolved HSQC spectra allows the unambiguous $^1$H and $^{13}$C chemical shift assignment that is not available from the exclusive use of 1D spectra and, in addition, the pure shift nature of cross-peaks makes the proposed technique highly suitable for the quantitative determination of enantiomeric excess by 2D volume integration, because equivalent signals from both diastereoisomers have practically similar J(CH) coupling and $T_2$ relaxation values.

## Conclusions

In summary, the combination of spectral aliasing and pure-shift HSQC experiments represents an excellent routine tool for NMR enantiodifferentiation studies, yielding simultaneous $^1$H and $^{13}$C enantiodifferentiated data (ΔΔδ($^1$H) and ΔΔδ($^{13}$C)) in short times and with high digital resolution and signal dispersion for both $^1$H and $^{13}$C nuclei. Its use increases significantly the probability to detect an enantiodifferentiated nucleus since more signals are observed ($^1$H and $^{13}$C nuclei), overlapping problems of common 1D $^1$H experiments are overcome, and poor enantiodifferentiation in 1D experiments can be now detected, allowing the study of cases abandoned in the past for reasons of poor enantioresolution and/or long experimental times. Alternatively, aliased long-range heteronuclear correlation experiments can be used to measure accurately such ΔΔδ values for quaternary carbons. The method is compatible with other heteronuclei and with the use of other chiral auxiliaries, and it can be of special interest for chiral metabonomic studies, where chiral molecules in complex mixtures are enantiodifferentiated and small chemical shifts need to be resolved in overcrowded spectra.[14]

## Notes and references

[1]*Servei de Ressonància Magnètica Nuclear and Departament de Química, Universitat Autònoma ed Barcelona, E-08193 Bellaterra, Barcelona, Spain. Tel: +34 935812291; E-mail: teodor.parella@uab.cat.*
[2]*DFG Research Center Molecular Physiology of the Brain (CMPB) & European Neuroscience Institute Göttingen (ENI-G), Göttingen,Germany.*
[3]*NMR Structure Elucidation, Process and Analytical Chemistry, Merck & Co. Inc., 126 E. Lincoln Avenue, Rahway, NJ 07065, USA*

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