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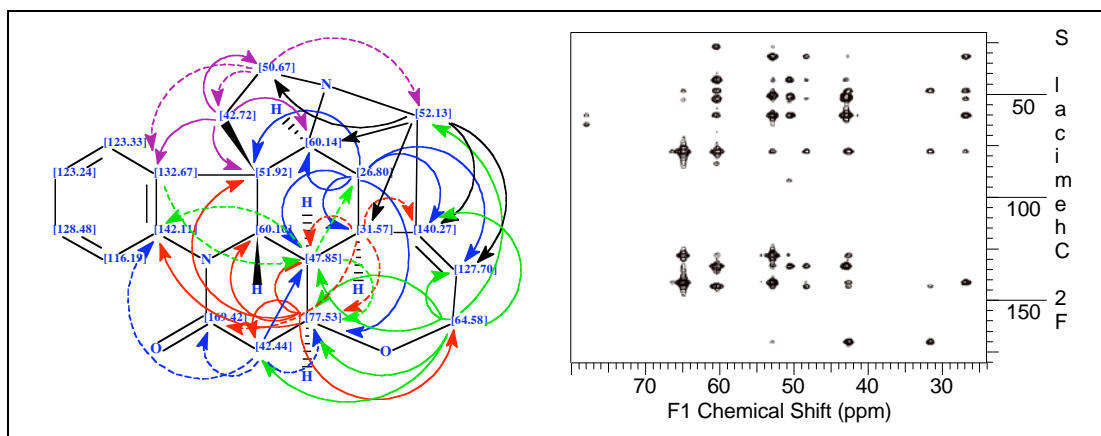
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Recent reports have demonstrated the unsymmetrical indirect covariance combination of discretely acquired 2D NMR experiments into spectra that provide an alternative means of accessing the information content of these spectra. The method can be thought of as being analogous to the Fourier transform conversion of time domain data into the more readily interpreted frequency domain. Hyphenated 2D-NMR spectra such as GHSQC-TOCSY, when available, provide an investigator with the means of sorting proton-proton homonuclear connectivity networks as a function of the  ${}^{13}\text{C}$  chemical shift of the carbon directly bound to the proton from which propagation begins. Long-range heteronuclear chemical shift correlation experiments establish proton-carbon correlations *via* heteronuclear coupling pathways, most commonly across three bonds ( ${}^3J_{\text{CH}}$ ), but in more general terms across two ( ${}^2J_{\text{CH}}$ ) to four bonds ( ${}^4J_{\text{CH}}$ ). In many instances  ${}^3J_{\text{CH}}$  correlations dominate GHMBC spectra. We demonstrate in this report the improved visualization of  ${}^2J_{\text{CH}}$  and  ${}^4J_{\text{CH}}$  correlations through the unsymmetrical indirect covariance processing of GHSQC-TOCSY and GHMBC 2D spectra.

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## INTRODUCTION

Recent investigations of the possibilities provided by unsymmetrical indirect covariance (UIDC) processing algorithms [1] have shown that it is possible to combine  ${}^1\text{H}$ - ${}^{13}\text{C}$  heteronuclear 2D NMR experiments with various

homonuclear 2D NMR experiments to produce the equivalent of hyphenated 2D NMR spectra [2-4]. In a similar fashion,  ${}^1\text{H}$ - ${}^{13}\text{C}$  direct and  ${}^1\text{H}$ - ${}^{13}\text{C}$  long-range correlation experiments can be co-processed to afford the equivalent of *m,n*-ADEQUATE spectra [5,6].  ${}^1\text{H}$ - ${}^{13}\text{C}$  and  ${}^1\text{H}$ - ${}^{15}\text{N}$  heteronuclear 2D NMR spectra can also be co-

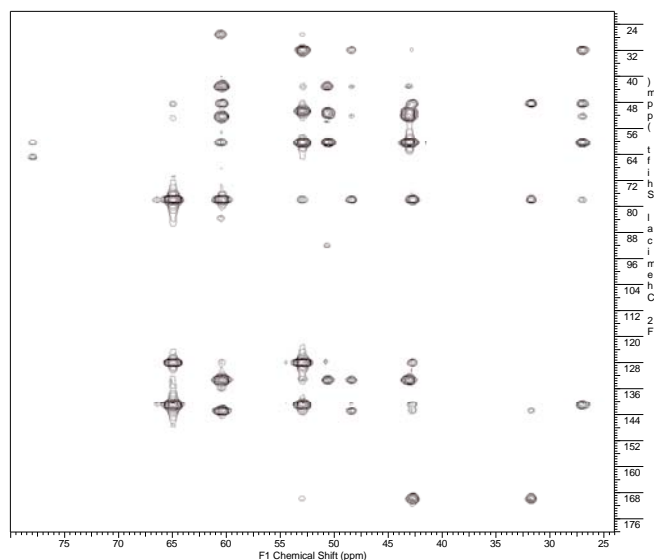
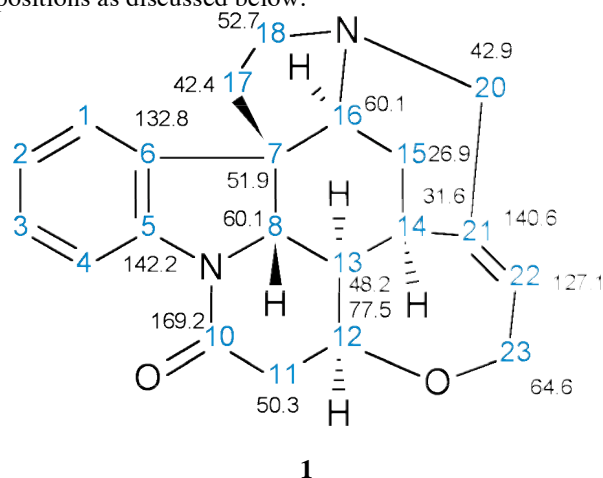
processed to afford various types of  $^{13}\text{C}$ - $^{15}\text{N}$  heteronuclear correlation spectra [7-9]. Most recently, we have demonstrated the utility of indirect covariance processing of GHSQC spectra as a means of identifying artifact responses in various indirect and unsymmetrical indirect covariance processed spectra [9,10]. Care should be taken in segments of the spectra with overlapped resonances due to the possibility of artifacts potentially arising in association with those resonances.

Modern structure elucidation relies on the establishment of atom-to-atom connectivity networks through the identification of either homo- or heteronuclear coupling pathways or, in some cases, *via* through-space dipole-dipole interactions. In most instances homonuclear correlations are observed using COSY or TOCSY experiments across three or four bonds. Direct  $^1\text{H}$ - $^{13}\text{C}$  heteronuclear correlation establishes proton-carbon pairings; long-range  $^1\text{H}$ - $^{13}\text{C}$  correlation experiments establish those correlations across typically two to four bonds, with three-bond correlations being by far the most prevalent. Generally, these experiments are employed to assemble the carbon skeleton of a molecule being characterized, and are supplemented, when necessary, by direct or long-range  $^1\text{H}$ - $^{15}\text{N}$  heteronuclear shift correlation data. In most cases these correlation data, when interpreted in concert, allow an investigator to successfully assemble the structure of an unknown molecule. There are, however, cases in which access to what would normally be weak long-range correlations can be very beneficial. There also may be instances when a correlation to a remote proton (*e.g.*  $^4J_{\text{CH}}$  or  $>^4J_{\text{CH}}$ ) will resolve an ambiguity due to the overlap of less distant proton resonances. Examples of both of these types of problems are regularly encountered in the characterization of complex natural product structures [11-13]. It is in this vein that we were interested in exploring the possibility of using unsymmetrical indirect covariance processing methods to combine the proton-proton connectivity networks that can be accessed in a GHSQC-TOCSY experiment with long-range heteronuclear coupling pathways from a GHMBC experiment.

## RESULTS AND DISCUSSION

The processed IDR-(Inverted Direct Response)-GHSQC-TOCSY and GHMBC spectra were subjected to unsymmetrical indirect covariance co-processing using ACD/SpecManager. The resulting icv-(indirect covariance calculated)-HSQC-TOCSY-HMBC spectrum derived from the discretely acquired IDR-HSQC-TOCSY and GHMBC spectra is shown in Figure 1. The assigned  $^{13}\text{C}$  chemical shifts of strychnine are shown by **1**. Overlap of the C8 and C16 carbon resonances at 60.1 ppm can hamper the extraction of information from the HSQC-

TOCSY-HMBC spectrum shown in Figure 1 for these positions as discussed below.

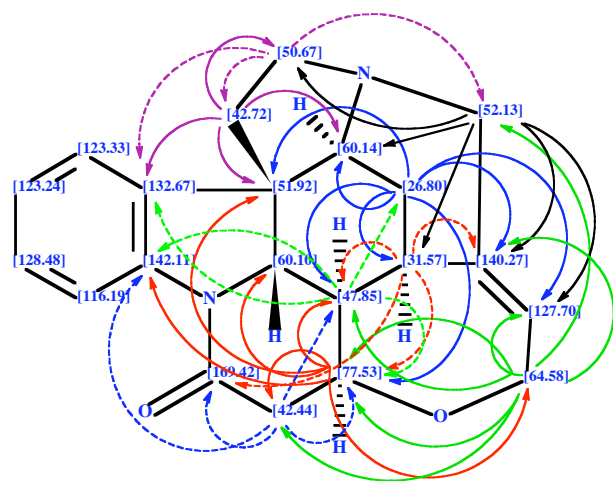


**Figure 1.** HSQC-TOCSY-HMBC spectrum calculated from a 16 ms IDR-GHSQC-TOCSY spectrum and a 6 Hz optimized GHMBC spectrum using unsymmetrical indirect covariance processing.

Connectivity information was individually extracted for the aliphatic portion of strychnine from the icv-HSQC-TOCSY-HMBC and GHMBC spectra as shown in Figures 2 and 3, respectively. The overlap of C8 and C16 (60.10 and 60.14 ppm, respectively) precludes the extraction of long-range correlation information for these positions from the icv-HSQC-TOCSY-HMBC spectrum shown in Figure 1. In contrast, the H8 and H16 proton resonances are resolved at 500 MHz, allowing long-range  $^1\text{H}$ - $^{13}\text{C}$  connectivity information to be derived for these positions from the 6 Hz optimized GHMBC spectrum.

The utility of an unsymmetrical indirect covariance co-processed spectrum such as the icv-HSQC-TOCSY-HMBC spectrum shown in Figure 1 derives from information content less readily accessed from the

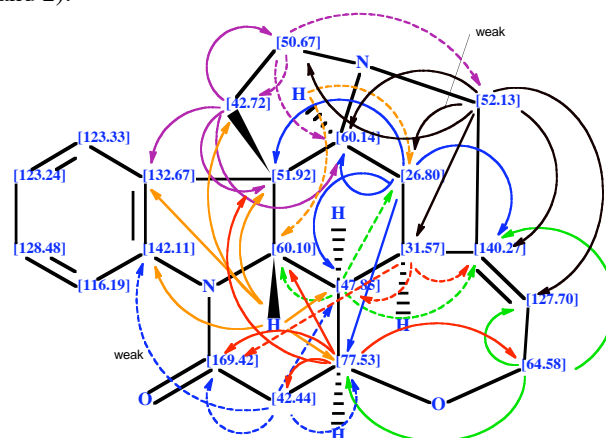
discretely acquired spectra that were co-processed. Conversely, a limitation is imposed when there is  $^{13}\text{C}$  resonance overlap as in the case of the C8 and C16 resonances which both resonate at  $\sim 60.1$  ppm. To evaluate the correlation information obtained from the icv-HSQC-TOCSY-HMBC spectrum shown in Figure 1, the correlations shown in Figures 2 and 3 were compared. Correlations contained in both the icv-HSQC-TOCSY-HMBC and conventional HMBC spectra were eliminated, and the remaining correlation information unique to the unsymmetrical indirect covariance co-processed spectrum is shown in Figure 4. In comparison, long-range  $^1\text{H}$ - $^{13}\text{C}$  connectivity information contained only in the 6 Hz optimized GHMBC spectrum is shown in Figure 5.



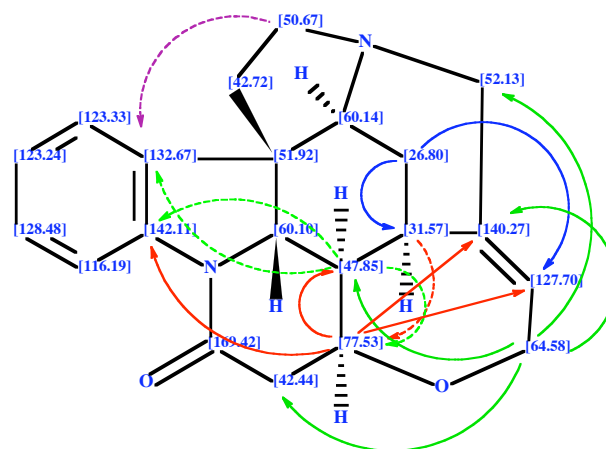
**Figure 2.** Long-range connectivity information extracted from the aliphatic portion of the unsymmetrical indirect covariance processed GHSQC-TOCSY-HMBC spectrum of strychnine (1) derived by co-processing 16 msec IDR-GHSQC-TOCSY and 6 Hz optimized GHMBC spectra. Correlations for the C8 (60.10 ppm; refer to structure 1 for numbering scheme) and C16 (60.14 ppm) cannot be extracted from the spectrum shown in Figure 1 because of the overlap of these resonances at 125 MHz. (Correlation pathways are color-coded identically for specific positions in Figures 2 and 3 to facilitate comparison.)

Inspection of the correlations unique to the icv-HSQC-TOCSY-HMBC spectrum shown in Figure 4 shows that the majority of the correlations not observed in the GHMBC spectra are two- or four-bond correlations that will generally be much weaker than the more prevalent three-bond correlations typically observed in the GHMBC spectrum. In this sense, the co-processed spectrum is effectively enhancing the visibility of weaker correlation responses relative to the noise threshold of the spectrum. Two- and four-bond correlations can also be accessed in accordion-optimized long-range heteronuclear shift correlation experiments that have been reviewed [15]. In contrast, apart from the correlations from the H8 and H16 resonances shown in Figure 5, there are only four other correlations observed in the GHMBC spectrum that are not observed in the unsymmetrical indirect covariance

processed HSQC-TOCSY-HMBC spectrum (Figures 1 and 2).



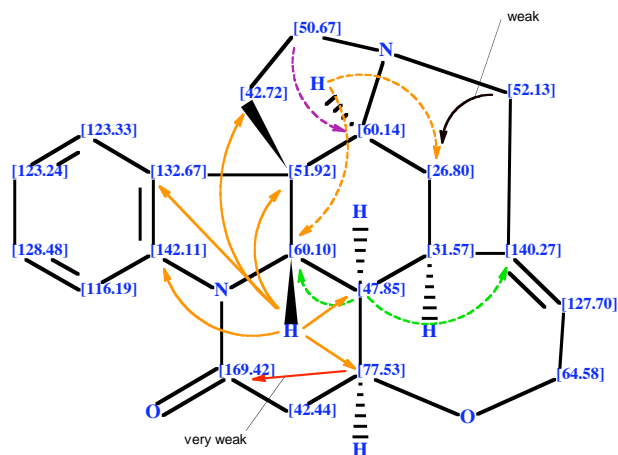
**Figure 3.** Long-range  $^1\text{H}$ - $^{13}\text{C}$  correlations extracted from the 6 Hz optimized GHMBC spectrum of strychnine. Since the H8 and H16 protons (refer to structure 1 for numbering scheme) are resolved, unlike their corresponding carbons, which are overlapped, connectivity information can be obtained for these positions from the GHMBC spectrum. (Correlation pathways are color-coded identically for specific positions in Figures 2 and 3 to facilitate comparison.)



**Figure 4.** Correlations obtained from the unsymmetrical indirect covariance processed HSQC-TOCSY-HMBC spectrum not observable in the GHMBC spectrum at the common threshold level used to prepare the plot shown in Figure 1. Correlation arrows are color coded for individual positions in the structure in a manner identical to Figures 2 and 3 to facilitate direct comparison. New, long-range connectivity information has not been created by unsymmetrical indirect covariance co-processing of the IDR-GHSQC-TOCSY and GHMBC spectra – rather, very weak correlations below the identical thresholds of the IDR-GHSQC-TOCSY and GHMBC spectra are rendered more visible as a result of the processing, making the utilization of this information viable whereas the very weak correlations in the discrete experiment might be considered suspect.

The unsymmetrical indirect covariance processed icv-HSQC-TOCSY-HMBC spectrum shown in Figure 1 should not be misconstrued as providing connectivity information that is not contained in the discretely acquired spectra that have been processed together. Rather, as demonstrated in several instances with calculated icv-

HSQC-COSY spectra [2,3] the co-processing algorithm provides higher relative s/n ratios in the same fashion as Fourier transforming a normal 2D NMR spectrum [16].



**Figure 5.** Long-range  $^1\text{H}$ - $^{13}\text{C}$  connectivity information unique to the 6 Hz optimized GHMBC spectrum. While the correlations for H8 (C8, 60.10 ppm; refer to structure **1** for numbering scheme) and H16 (C16, 60.14 ppm) may well be contained in the HSQC-TOCSY-HMBC spectrum, these correlations cannot be reliably differentiated and used because of the overlap of their respective carbon resonances. In contrast, these correlations are available in the GHMBC spectrum and complement the information content of the HSQC-TOCSY-HMBC plot.

In this sense, legitimate connectivity information contained in the spectra being co-processed that is weak or near the 2D contour plot threshold is observed after co-processing with higher apparent s/n allowing this information to be reliably used rather than question the legitimacy of the response(s) in question.

## CONCLUSIONS

Using unsymmetrical indirect covariance processing to co-process IDR-GHSQC-TOCSY and GHMBC spectra to produce an icv-HSQC-TOCSY-HMBC spectrum leads to the reliable visualization of weaker two- and four-bond correlations that would be expected to be contained in the discretely acquired GHMBC spectrum used in the co-processing. In most instances, it should be possible to solve a structure from the available GHSQC-TOCSY and GHMBC spectra without resorting to the use of unsymmetrical indirect covariance processing. There may, however, be instances when there are proton resonance overlaps where having the connectivity information spread as a function of  $^{13}\text{C}$  shifts on both axes will be beneficial. Alternatively, in those cases where reliable access to two- and or four-bond correlations is necessary to confirm a structure, the icv-HSQC-TOCSY-HMBC presentation may be a useful adjunct to the interpretation of the spectra and the assignment of a given structure.

## EXPERIMENTAL

All of the NMR data employed in this study were acquired using a sample of ~5 mg of strychnine dissolved in ~180  $\mu\text{L}$  deuteriochloroform (CIL) which was then transferred to a 3 mm NMR tube (Wilmad) using a flexible Teflon needle and a gas-tight syringe (Hamilton). Data were acquired using a Varian 500 MHz two channel NMR instrument equipped with a Varian 3 mm gradient inverse detection probe; the sample temperature was regulated at 26  $^{\circ}\text{C}$ . The pulse sequences for the experiments performed were used directly from the vendor-supplied pulse sequence library without further modification. The GHSQC-TOCSY data were acquired with inversion of the direct responses (IDR) and a 16 ms mixing time [13]. The long-range delay in the GHMBC experiment was optimized for 6 Hz.

2D NMR spectra were acquired with identical spectral widths in the  $F_2$  frequency domain although this is not specifically required for the ACD/SpecManager v10.02 software used in this study to perform the co-processing of the data. The spectral width in the  $F_1$  frequency domain was set as appropriate for each experiment. Weighting functions were optimized for each experiment and the data were processed *via* a combination of linear prediction and zero-filling in the second frequency domain to afford data matrices for both experiments of 2048 x 512 points. The processed spectra were subjected to unsymmetrical indirect covariance processing using ACD/SpecManager software v10.02. The processing was done on a Dell laptop computer with a 1.7 GHz processor and 1 Gb of RAM. The unsymmetrical indirect covariance processing required ~5 sec.

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[16] In several reports (see refs. 2-4) it has been feasible to perform the experiment comparable to the indirect covariance or unsymmetrical indirect covariance processed data allowing direct s/n comparison of the experimental and calculated spectra to be made. In contrast, in the present case, there is no experimental equivalent to the spectrum derived by unsymmetrical indirect covariance processing shown in Figure 1. Consequently, we can only express the results of unsymmetrical indirect covariance processing vs. the initial GHMBC

spectrum in relative terms. The weaker  $^2J_{CH}$  and  $^4J_{CH}$  correlations are present in the GHMBC spectrum but were below the threshold used to prepare contour plots of those data. In contrast, these responses are observed above the threshold used to prepare the contour plot shown in Figure 1. A direct comparison would entail plotting  $^1H$  spectral slices from the GHMBC spectrum at a given  $^{13}C$  shift vs.  $^{13}C$  "spectra" at a given  $^{13}C$  chemical shift from the spectrum shown in Figure 1, which the authors did not feel was a legitimate comparison.