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Establishing the carbon skeleton of pharmaceutical agents using HSQC-ADEQUATE spectra

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ABSTRACT

Two-dimensional NMR methods are the cornerstone of modern structure elucidation methods. When the ensemble of 1D and 2D NMR experiments normally employed for structure assignment fails, investigators typically resort to successively more complex 2D NMR experiments for structure determination and/or spectral assignment. Unsymmetrical Indirect Covariance (UIC) NMR data processing methods provide a convenient and highly efficient means of accessing the connectivity information embodied in more complex experiments such as HSQC-TOCSY spectra. Using Unsymmetrical Indirect Covariance (UIC) or General Indirect Covariance (GIC) processing to mathematically combine multiplicity-edited GHSQC and 1,1-ADEQUATE 2D NMR spectra affords an HSQC-ADEQUATE spectrum that offers a new method for establishing the carbon skeleton of a molecule. The application of this technique is demonstrated for a novel cyclin-dependant kinase inhibitor, Dinaciclib™ (SCH 727965).

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1. Introduction

Structural characterization of pharmaceutical agents and related molecules is generally undertaken using a combination of mass spectrometry and NMR spectroscopy [1,2]. Ultra high resolution mass measurements can now elegantly define the empirical formula of most molecules in a short period of time with extremely high sensitivity [3,4]. Fragmentation pathways derived from MS/MS experiments supplement the molecular formula and, in some instances, are sufficient to define chemical structures. NMR spectroscopy, while much lower in sensitivity than mass spectrometric methods can provide the atom-to-atom connectivity information that is crucial to define a chemical structure when mass spectrometry is incapable of doing so. Some of the sensitivity limitations of NMR spectroscopy are ameliorated by resorting to higher magnetic field strengths, smaller diameter NMR probes [5,6], and cryogenically cooled NMR probes [7,8]. Furthermore, NMR methods can also be used to define stereochemical features of a molecule that are beyond the capability of mass spectrometric methods.

Two-dimensional NMR methods are undeniably the cornerstone of modern structure elucidation methods and have been for many years [9]. GCOSY, ^{1}H–^{13}C multiplicity-edited GHSQC, and ^{1}H–^{13}C GHMBC experiments are a frequently used ensemble of experiments for determining molecular structures. As molecular complexity increases, the likelihood of spectral overlap, predominantly in the proton NMR spectrum, correspondingly increases. Structural ambiguity can be introduced due to resonance overlaps in the proton spectrum thereby complicating the interpretation of the GCOSY data. Less commonly, resonance overlaps may be encountered in ^{13}C spectra. In such cases, experiments such as GHSQC-TOCSY [10,11] can be employed to sort proton–proton connectivity information as a function of the greater chemical shift dispersion of the carbon frequency domain. Alternatively, structural ambiguity can also be introduced during the structure elucidation process due to the inability to differentiate $^{2}J_{CH}$ from $^{3}J_{CH}$ or longer-range heteronuclear correlations in ^{1}H–^{13}C GHMBC spectra.

To circumvent resonance overlap problems in the proton spectrum, hyphenated 2D NMR experiments such as GHSQC-TOCSY [10,11] can be employed. Likewise, variants of the GHMBC experiment such as 2J3J-HMBC [12], H2BC [13,14], and HAT-HMBC [15] have been developed to differentiate $^{2}J_{CH}$ correlations from longer-range ($^{n}J_{CH}$, $n>2$) heteronuclear correlations. Unfortunately, the 2J3J-HMBC, H2BC, and HAT-HMBC experiments only work with protonated adjacent carbon pairs. In contrast, the 1,1-ADEQUATE experiment provides an unequivocal means of establishing adjacent (*via* $^{1}J_{CC}$) carbon–carbon connectivity *via*

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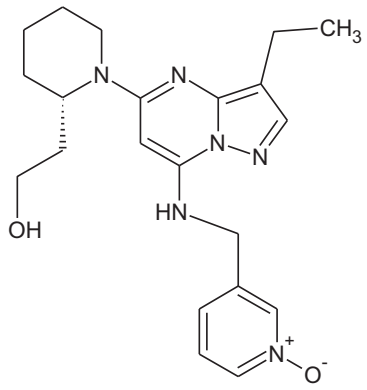
an out and back magnetization transfer with proton-detection [16–18].

Using Unsymmetrical Indirect Covariance (UIC) [19,20] or General Indirect Covariance (GIC) [21] processing to combine 2D NMR experiments that share a common frequency domain provides an alternative means of accessing the information content of, for example, hyphenated 2D NMR techniques such as GHSQC-TOCSY [10,11] that are generally lower in sensitivity than the component 2D NMR experiments from which they are derived. Another powerful combination for determining molecular structures is afforded by the concatenation of multiplicity-edited GHSQC and 1,1-ADEQUATE spectra to afford a diagonally symmetric HSQC-ADEQUATE spectrum that establishes the carbon–carbon connectivity of a molecule [22]. The application of the method is illustrated using the cyclin-kinase dependant inhibitor, Dinaciclib™ (SCH-727965, **1**) [23,24].



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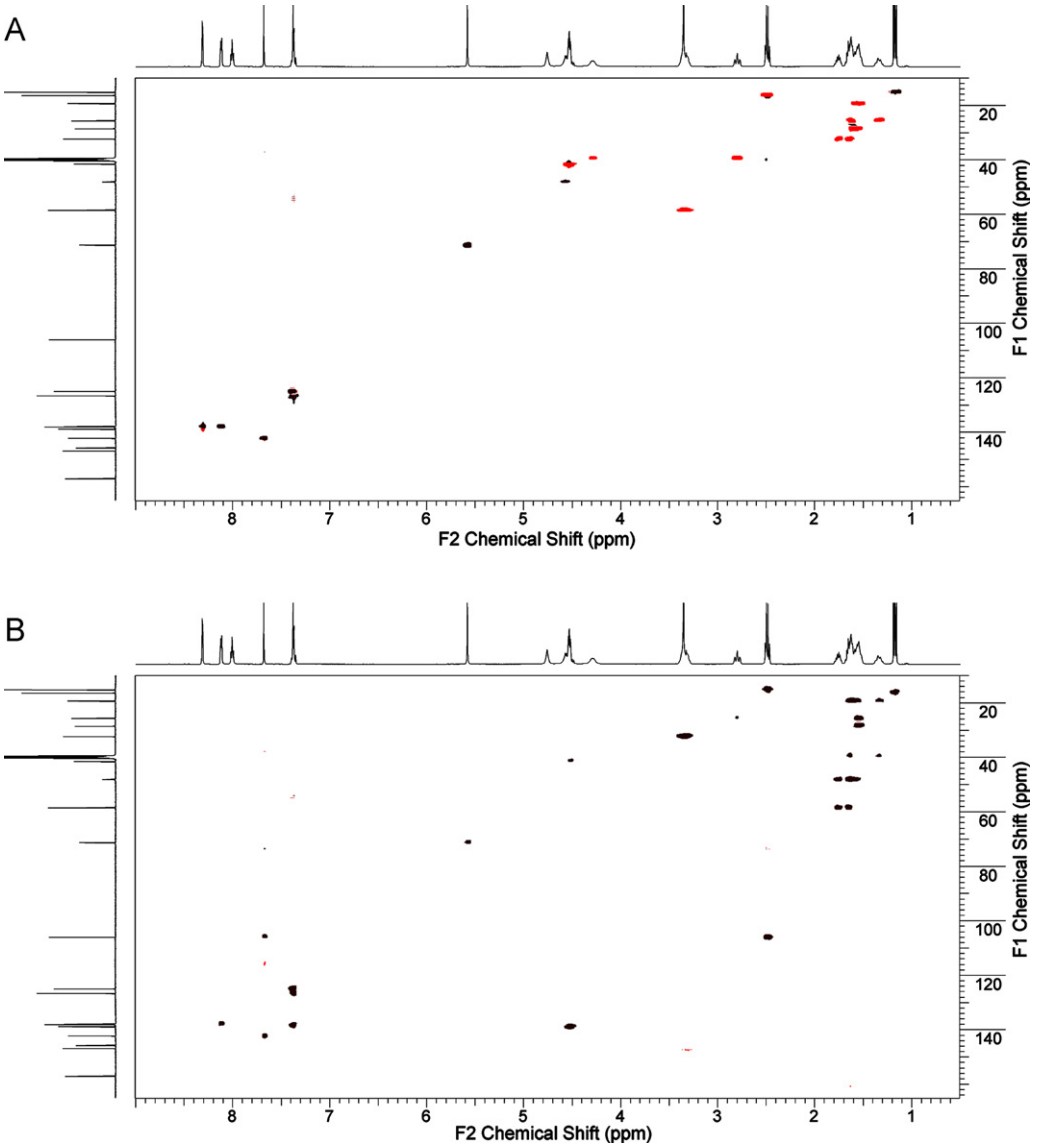

Fig. 1. (A) Multiplicity-edited GHSQC spectrum of **1** acquired as 2048 × 160 points and processed to 1*K* × 1*K* points. Methine and methyl correlations have positive phase and are plotted in black; methylene resonances have negative phased and are plotted in red. Partial overlap of the resonances of the piperidine ring could lead to ambiguous assignments when conventional resonance assignment strategies are employed. (B) 40 Hz optimized 1,1-ADEQUATE spectrum of **1** acquired as 2048 × 160 points and processed to 1*K* × 1*K* points. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

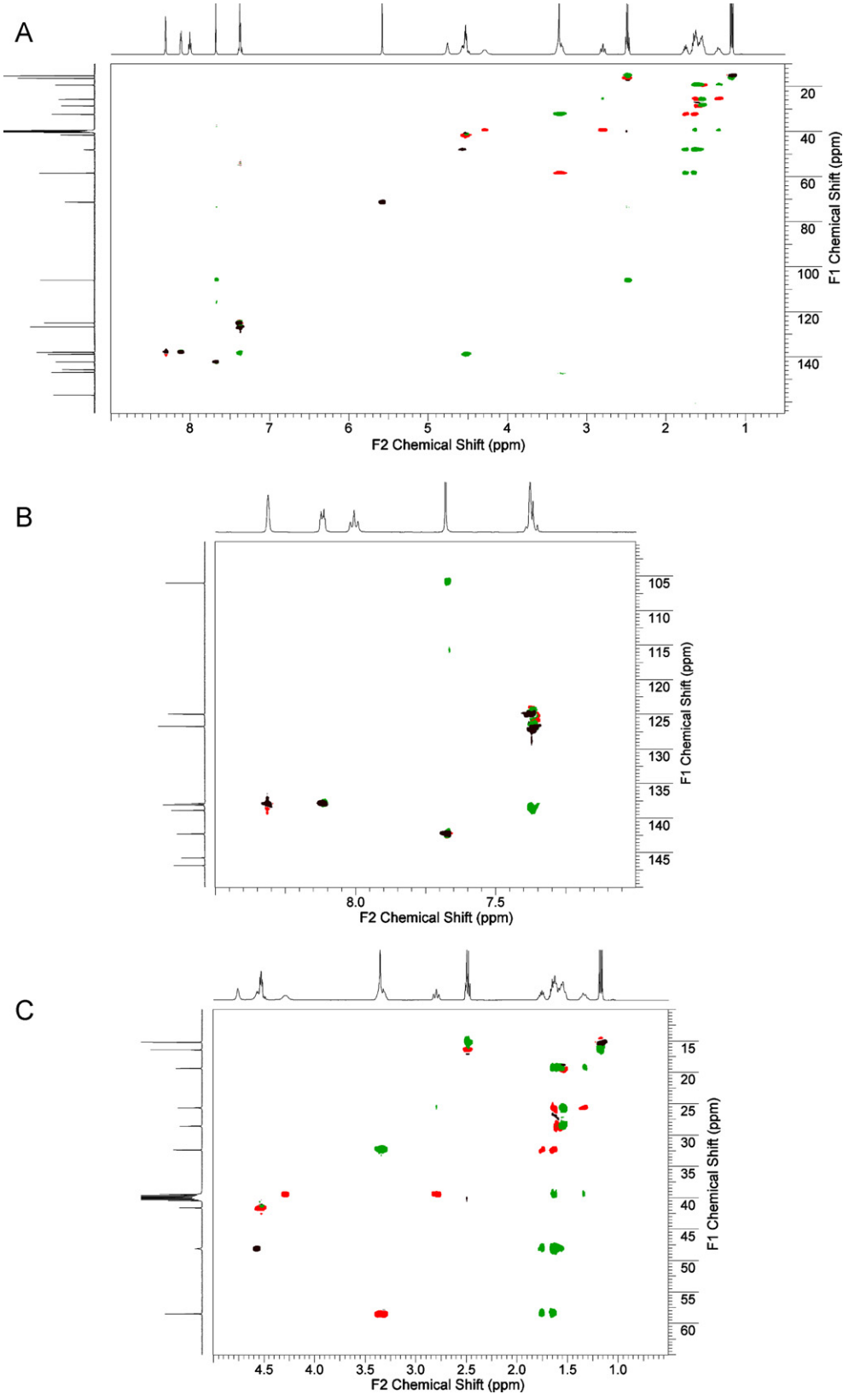

Fig. 2. (A) Overlaid multiplicity-edited GHSQC and 40 Hz optimized 1,1-ADEQUATE spectra. The responses from the multiplicity-edited GHSQC spectrum uses the same color convention as shown in Fig. 1A; responses from the overlaid 40 Hz optimized 1,1-ADEQUATE spectrum are plotted in green. (B) Expansion of the aromatic region of the overlaid spectra. (C) Expansion of the aliphatic region of the overlaid spectra. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

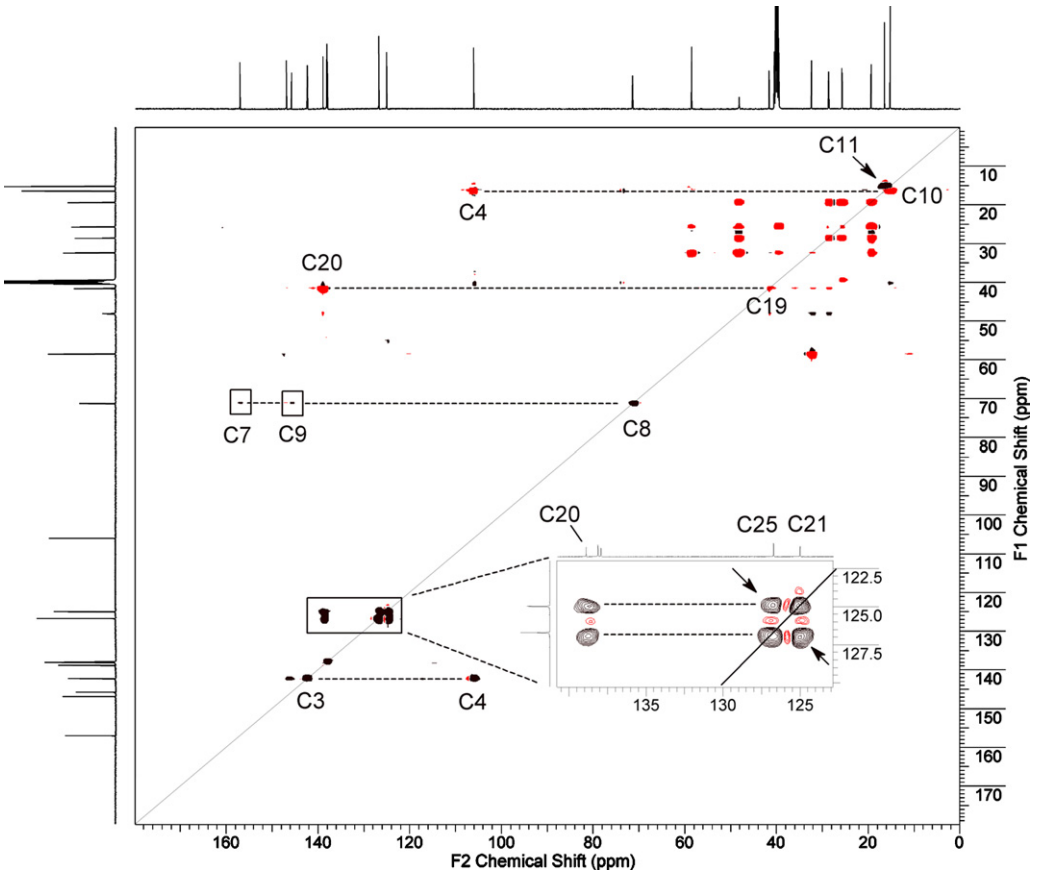

Fig. 3. HSQC-ADEQUATE spectrum calculated using GIC processing [21] with power = 0.5. The spectrum is diagonally symmetric analogous to the familiar COSY spectrum. Adjacent protonated carbons (correlated *via* $^{1}J_{CC}$) are diagonally symmetric as shown in the expansion of the aliphatic region of the spectrum shown in Fig. 4. Resonance multiplicity information that was phase encoded in the multiplicity-edited GHSQC spectrum is retained in the HSQC-ADEQUATE spectrum. Correlations between protonated and non-protonated adjacent carbons are diagonally asymmetric and are observed at the F_1 shift of the protonated carbon at the F_2 frequency of the non-protonated resonance. The correlations between protonated and non-protonated adjacent carbons are designated by dashed horizontal black lines. The boxed inset shows correlations to the carbons of the pyridine ring. The sensitivity of the HSQC-ADEQUATE spectrum is governed by the much higher sensitivity GHSQC experiment rather than the lower sensitivity of the 1,1-ADEQUATE experiment [22,32].

2. Materials and methods

All experiments were performed using a Bruker 500 MHz NMR spectrometer equipped with a 5 mm $^{1}H/^{19}F$-^{13}C TCI triple resonance probe. A sample of 20 mg of the cyclin-dependant kinase inhibitor dinaciclib (SCH 727965, **1**) was dissolved in 550 μL of DMSO-d_6 (Cambridge Isotope Laboratories) and transferred to a 5 mm NMR tube (Wilmad) using a flexible Teflon™ and a Hamilton gas-tight syringe. The pulse sequences employed in the study for the multiplicity-edited GHSQC (hsqcedetgp) and 1,1-ADEQUATE (adeq1letgprdsp) spectra were those taken directly from the Bruker pulse sequence library and were used without any modification. Data were acquired as 2048 × 160 point matrices and were processed by linear predicting to 512 points in the second dimension followed by zero-filling to afford final data matrices that were $1K \times 1K$ points. Spectral widths of 0–9 and 5–180 ppm were used for all experiments for ^{1}H and ^{13}C, respectively. The multiplicity-edited GHSQC spectrum was acquired using two transients/t_1 increment giving an acquisition time of ~7 min. The CHIRP 1,1-ADEQUATE spectrum was optimized for 40 Hz with 96 transients accumulated per t_1 increment giving an acquisition time of 11 h 15 m. Data were processed using the Spectrus 2011 program package provided by Advanced Chemistry Development Laboratories [25]. The HSQC-ADEQUATE spectrum [22] was calculated using the general indirect covariance processing option [21] with power = 0.5.

3. Results and discussion

Structure characterization protocols normally utilize proton–proton connectivity networks as a first step, with directly bound carbon identities defined by a multiplicity-edited GHSQC spectrum (Fig. 1A). Quaternary carbons can be linked to structural fragments defined in the first step of the process *via* long-range ^{1}H–^{13}C heteronuclear correlations observed in a GHMBC experiment [26]. Various segments of the chemical structure spanning heteroatoms, etc., are likewise linked together *via* long-range ^{1}H–^{13}C heteronuclear correlations. In most cases, the process concludes with the self-consistent establishment of a chemical structure based on the NMR data that is also consistent with the empirical formula derived by exact mass measurements and the fragmentation pathways deduced from the mass spectrometric data. Structural ambiguities can arise when there are proton resonance overlaps or when there are alternative structures that can be drawn based on the inability to differentiate $^{2}J_{CH}$ correlations in the GHMBC data from $^{n}J_{CH}$ correlations where $n \geq 3$.

When 1,1-ADEQUATE data are available (Fig. 1B), carbon–carbon correlations between adjacent protonated carbon pairs and between a protonated and adjacent non-protonated carbon(s) are defined. Correlations between pairs of non-protonated carbons cannot be accessed *via* 1,1-ADEQUATE data. The interpretation of 1,1-ADEQUATE data typically employs either the side-by-side interpretation of the GHSQC and 1,1-ADEQUATE data

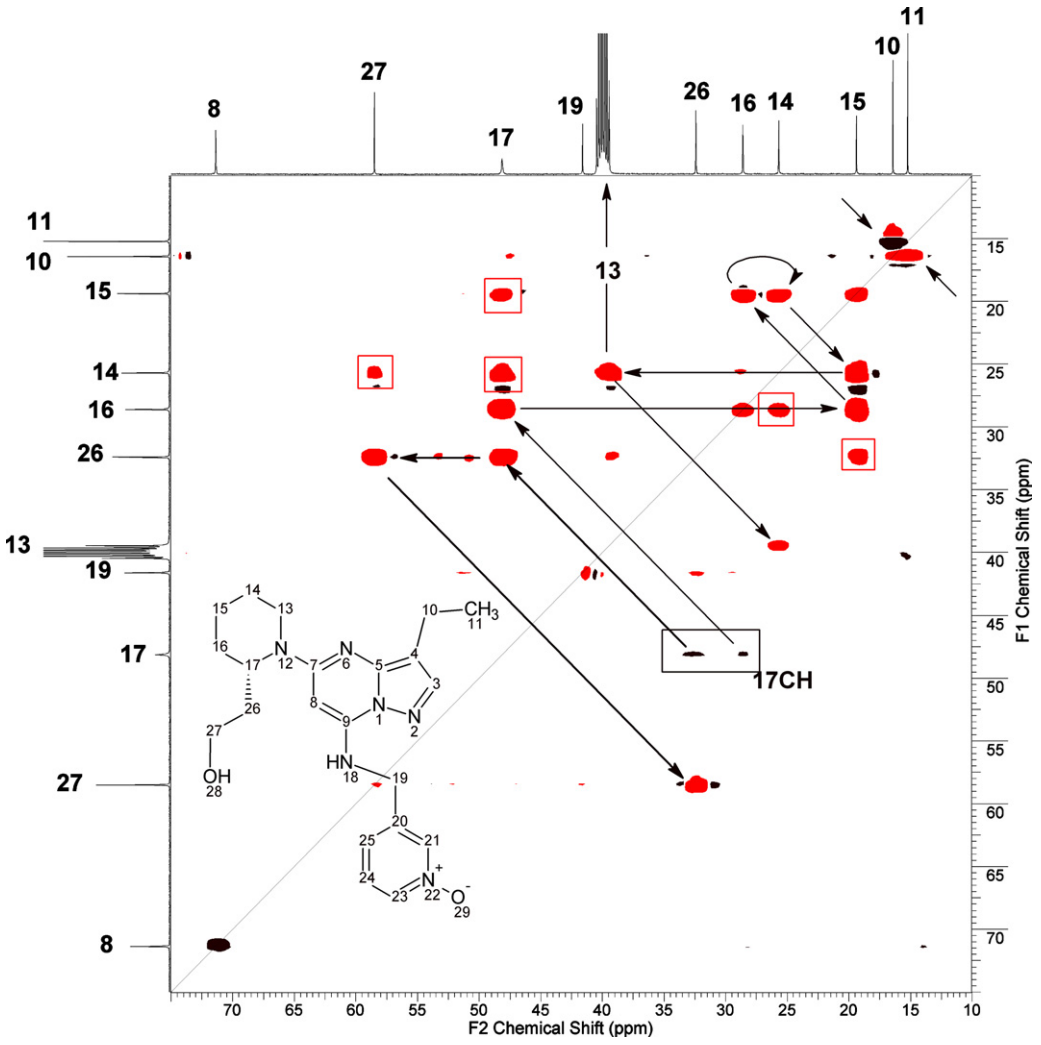

Fig. 4. Annotated expansion of the aliphatic region of the 40 Hz HSQC-ADEQUATE spectrum of **1**. Although a starting point for the interpretation of the spectrum may be arbitrarily chosen, a convenient starting point is provided by the piperidine methine carbon resonance at ~47 ppm enclosed in the black box. Two correlation pathways can be traced beginning from the 17CH resonance. One coupling pathway defines the pendant β-hydroxy ethyl moiety (C26, C27). The other connectivity pathway sequences and assigns the resonances of the remainder of the piperidine (C16–C13). Potentially ambiguous resonance assignments that might arise due to proton resonance overlaps are circumvented through the establishment of the carbon–carbon connectivity network defined by the HSQC-ADEQUATE spectrum. Responses enclosed in red boxes are artifacts that arise due to resonance overlaps proton spectrum in the multiplicity-edited GHSQC [33,34] and 1,1-ADEQUATE spectra used in the GIC calculation of the HSQC-ADEQUATE spectrum. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

or proceeds with the two spectra overlaid as shown in Fig. 2. Regardless of which approach is employed, it is still necessary for starting points to be identified and assignments made from the GHSQC data before the structural assignment can progress. In contrast, when the multiplicity-edited GHSQC and 1,1-ADEQUATE spectra are subjected to covariance processing, the resulting HSQC-ADEQUATE spectrum provides a diagonally symmetric carbon–carbon correlation plot akin to the familiar homonuclear COSY spectrum that allows fragments of a molecular skeleton to be conveniently defined.

Covariance NMR processing methods have been the subject of a recent, brief chapter [27]. Indirect covariance NMR spectra can be calculated according to Eq. (1)

$$C_{\text{indirect}} = (F \cdot F^T)^{1/2} \tag{1}$$

affording a symmetric matrix whose frequency axes are defined by the indirectly determined NMR frequency (ω_1) of F. Indirect covariance processing methods were extended by the work of Martin and co-workers [19,20] and by Kupče and Freeman [28,29] to the reconstruction of nonsymmetric spectra from pairs of spectra, F and G, according to Eq. (2)

$$C = F \cdot G^T. \tag{2}$$

Snyder and Brüschweiler [21] subsequently reported a further modification of unsymmetrical indirect covariance processing that they termed General Indirect Covariance processing, which is also represented by Eq. (2), except that the resulting data matrix is symmetric and can be raised to a matrix power, λ, as denoted by Eq. (3), eliminating artifacts in some cases

$$C = (F \times G^T)^{\lambda}. \tag{3}$$

In the present example, a multiplicity-edited GHSQC spectrum (F) was multiplied by the transpose of a 40 Hz optimized 1,1-ADEQUATE spectrum (G^T) to afford the HSQC-ADEQUATE spectrum raised to the matrix power, λ = 0.5.

Subjecting the multiplicity-edited GHSQC and 40 Hz optimized 1,1-ADEQUATE spectra of 1 to general indirect covariance processing, λ = 0.5, affords the HSQC-ADEQUATE spectrum shown in Fig. 3. Diagonally symmetric correlations between adjacent (correlated *via* $^1J_{CC}$) protonated carbons are denoted by solid black

lines perpendicular to the diagonal. Correlations between protonated and non-protonated adjacent carbons are observed at the F_2 frequency of the non-protonated carbon and the F_1 frequency of the protonated carbon; these correlations are denoted by horizontal dashed lines. An expansion of the aliphatic region of the spectrum is shown in Fig. 4 that allows the complete framework of the β-hnydroxyethyl piperidine moiety to be conveniently traced out despite partial overlaps of the aliphatic proton resonances that could be problematic if COSY or GCOSY data were being used to establish proton–proton connectivity networks to assemble and/or assign the structure.

Although the present study was conducted using a 20 mg sample of **1**, it is important to note that the 500 MHz 5 mm cryoprobe in which the study was performed was optimized for ^{13}C detection rather than an inverse-detection probe. Comparable data can be efficiently recorded on considerably smaller samples when an standard gradient, inverse detection cryoprobe is employed [30] and submilligram samples can be studied if an investigator has access to a spectrometer equipped with a 1.7 mm TCI Micro CryoProbe™ [31,32].

In principle, any correlation can be used as a point of entry into an HSQC-ADEQUATE spectrum. In the present example, a convenient starting point is provided by the piperidine methine, from which the carbons of the β-hydroxyethyl side chain and the contiguous methylenes of the remainder of the piperidine ring can be assigned by working outward in both directions.

4. Conclusions

Two-dimensional NMR methods provide an invaluable means of establishing the structure of pharmaceuticals as well as their impurities, degradation products, and metabolites. Ideally, the simplest and highest sensitivity methods should be employed to characterize a given structure, but when ambiguities or spectral overlaps are encountered, more sophisticated methods should be available when simpler methods fail. Structural ambiguities arising due to an inability to differentiate $^2J_{CH}$ from $^nJ_{CH}$ correlations, where $n \geq 3$, can be circumvented using HSQC-ADEQUATE as described in this study. By subjecting multiplicity-edited GHSQC and 1,1-ADEQUATE spectra to UIC or GIC processing data acquisition times for the 1,1-ADEQUATE spectrum can be significantly reduced as has been previously shown [32] making HSQC-ADEQAUTE spectra a more viable alternative than the 1,1-ADEQUATE spectrum itself. While the calculation of the HSQC-ADEQUATE spectrum in the present report was not done using data recorded for a mass limited sample, the acquisition of 1,1-ADEQUATE data for submilligram samples of several compounds has been reported by one of the authors [31,32], with one study showing that these data are amenable to the calculation of an HSQC-ADEQUATE spectrum [32]. A report demonstrating the characterization of an isolated degradant of a pharmaceutical will be the subject of a forthcoming report.

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