Assignment of the NMR Spectra of a Potent Ribonucleotide Reductase Inactivator - Utilization of HMQC-TOCSY to Establish Connectivities in Exchange Broadened Spectra

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Inverted and Suppressed Direct Response (IDR and SDR, respectively) HMQC-TOCSY experiments are evaluated relative to the conventional HMQC-TOCSY experiment in the assignment of the congested proton and carbon spectra of a 2-acetylpyridine thiocarbonohydrazone used to potentiate the antiviral drug acyclovir. Spectra with moderate overlap can be most expeditiously interpreted using the inverted direct response HMQC-TOCSY variant; spectra with severe overlap can be more readily interpreted if direct responses are suppressed.

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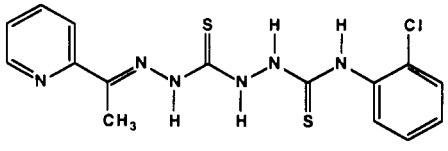
Introduction.

Recurrent labial and perioral herpes simplex virus type 1 infections (HSV-1), the common cold sore or fever blister, are the most frequent cutaneous infections encountered in immunocompetent patients [1]. Ribonucleotide reductase inactivators have been studied in these laboratories as potentiators of the antiviral drug acyclovir (**2**) (ACV) for the treatment of herpes virus infections [2,3]. We recently reported a series of 2-acetylpyridine thiocarbonohydrazones that have potent activity against the ribonucleotide reductase encoded by HSV-1 infected cells, and are relatively selective versus the human enzyme [4]. Studies with several virus strains in *in vivo* models showed that thiocarbonohydrazone BW 348U87 (**1**) significantly potentiated the antiviral activity of ACV [5]. Compound **1** also inactivates varicella zoster virus (VZV) and HSV-2 ribonucleotide reductases, in addition to the HSV-1 enzyme [6]. These studies led to the selection of BW 348U87 (**1**) for development as combination topical therapy with ACV for treatment of cutaneous herpes virus infections.



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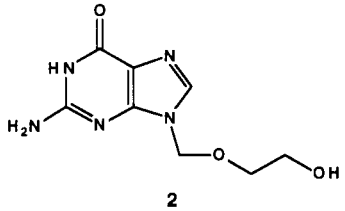

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Structural characterization of **1** for regulatory submission, in part, required the unequivocal ^{1}H- and ^{13}C-nmr resonance assignment of the 2-pyridyl and 2-chloroaniline rings. While some resonances in the proton spectrum of **1** were quite sharp, intermediate exchange amongst various conformers resulted in a spectrum in which a number of resonances were significantly broadened. Attempting to establish proton-proton connectivities using the COSY experiment in such cases, particularly when the spectrum is further complicated by near resonance overlap, is difficult, at best. In contrast, sorting proton-proton connectivities as a function of the well resolved carbon chemical shifts using the HMQC-TOCSY [7,8] experiment is a much more facile process. HMQC-TOCSY, as demonstrated in the present report, is also useful to establish connectivities even when resonances are broadened due to restricted rotation. When both proton and carbon spectral regions of interest are congested, recently reported modifications of the HMQC-TOCSY experiment can be used to either invert [9] or completely suppress direct responses [10]. The comparative utility of the available HMQC-TOCSY experiments in the total assignment of the congested proton and carbon resonances of the anilino-derived phenyl system of **1** is reported in this study.

NMR Spectroscopy.

Direct proton-carbon heteronuclear correlations necessary to begin assigning the spectra of **1** were obtained from an HMQC spectrum recorded using the pulse sequence of Bax and Subramanian [11]. As noted above, a COSY spectrum provided little information due to a combination of the considerable exchange broadening in the spectrum coupled with the near overlap of many of the broadened resonances. The HMQC-TOCSY experiment [7,8], however, provides comparable proton-proton connectivity information but with the advantage that correlations are sorted in the second frequency domain as a function of the resolved carbon chemical shifts.

The conventional HMQC-TOCSY spectrum of the aro-

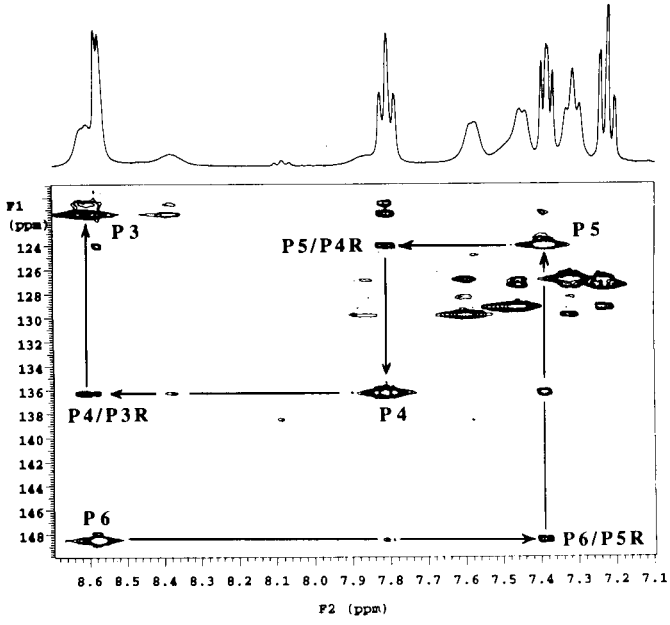


Figure 1. Conventional HMQC-TOCSY spectrum of **1** recorded in d_6-DMSO at an observation frequency of 400 MHz. The pulse sequence used was that of Lerner and Bax [7] except that broadband heteronuclear decoupling was initiated at the beginning of the acquisition period [8]. Connectivities in the 2-pyridyl system are labeled. Direct responses are labeled by origin, *e.g.* "P6". Relayed responses are labeled using the format "P6/P5R" indicating that magnetization was relayed from P6 to P5 and that the response denoted is the relay response.

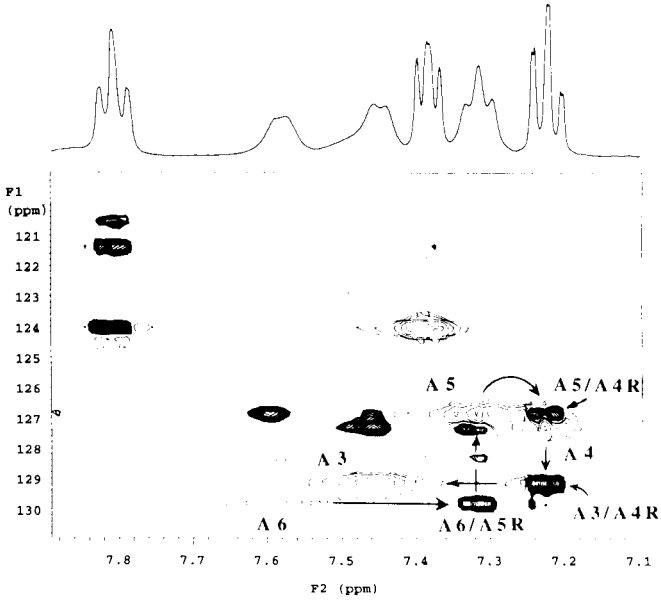


Figure 3. Expansion of the aromatic region of the IDR-HMQC-TOCSY (Inverted Direct Response) [9] spectrum of **1**. The direct responses are observed with negative intensity and are denoted by open contours. Direct responses are labeled as to their origin, *e.g.* "A6". Relayed responses are positive in intensity and are denoted by closed (black) responses in the contour plot. Relayed responses used to assemble the connectivity network are labeled, *e.g.* "A6/A5R". Not all single relay responses are labeled; none of the double relay responses are labeled. The region presented is identical to that shown in Figure 2.

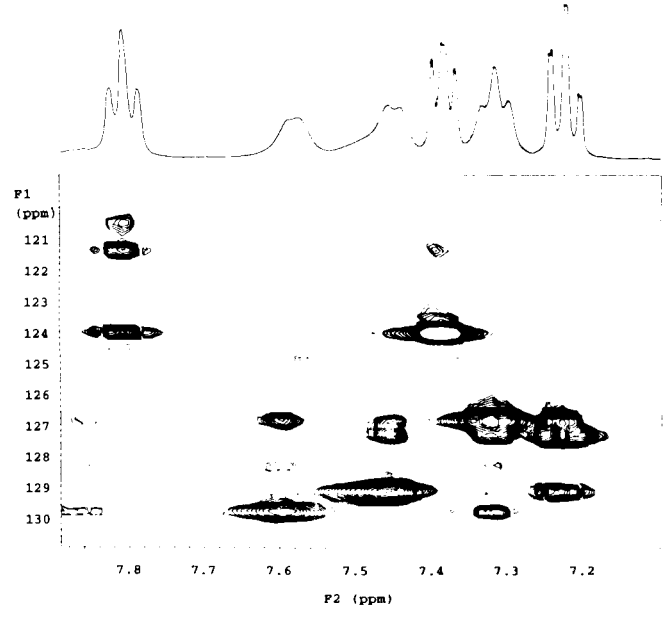


Figure 2. Expansion of a portion of the aromatic region of the HMQC-TOCSY spectrum shown in Figure 1. Connectivities in this region of the spectrum correspond to the four-spin system of the chloroaniline-derived portion of **1**.

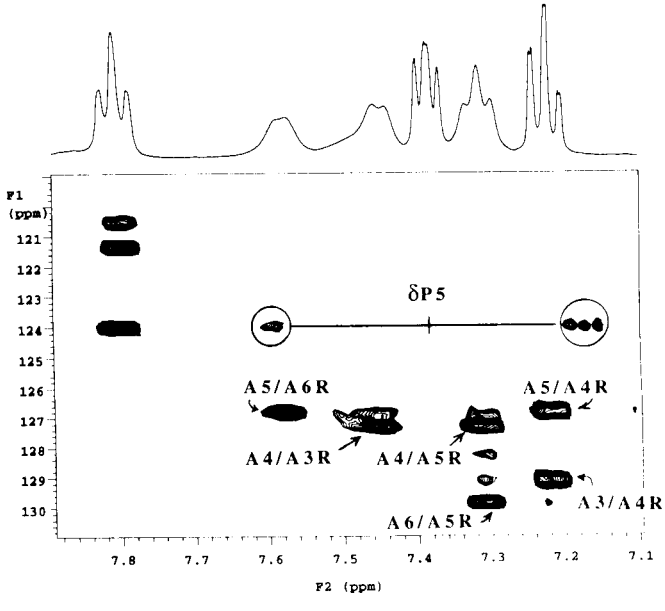


Figure 4. Expansion of the aromatic region of the SDR-HMQC-TOCSY (Suppressed Direct Response) [10] spectrum of **1**. The region presented is identical to that shown in Figure 2. Mismatch of the delays used flanking the $^1H/^{13}C$ 180°/90° pulse pair leads to incomplete suppression of the direct response. Incomplete suppression is shown by the circled, satellite responses of the P5 resonance. All single relay responses are labeled using the convention described in Figures 1 and 3. None of the double relayed responses are labeled in the figure.

matic region of **1** is shown in Figure 1; an expansion of a portion of the aromatic region is shown in Figure 2. The data presented were acquired with broadband heteronuclear decoupling initiated at the beginning of the acquisition period [8]. Lerner and Bax [7] suppressed direct responses in the HMQC-TOCSY spectrum by delaying the onset of broadband heteronuclear decoupling by $\frac{1}{2}(^1J_{CH})$. More recently, Domke [9] suggested a method for inverting direct responses in the HMQC-TOCSY experiment by applying simultaneous 180° $^1H/^{13}C$ pulses $\frac{1}{2}(^1J_{CH})$ after the completion of the isotropic mixing period followed by a second refocusing interval of equal duration. The IDR-HMQC-TOCSY (Inverted Direct Response HMQC-TOCSY) spectrum of **1** is shown in Figure 3. Extending Domke's method [10] we have demonstrated that the application of a 180° ^1H/90° ^{13}C pulse pair in the same fashion affords a convenient means of suppressing direct responses. The SDR-HMQC-TOCSY (Suppressed Direct Response HMQC-TOCSY) spectrum of a portion of the aromatic region of **1** is shown in Figure 4.

Interpretation of the HMQC-TOCSY spectrum shown in Figure 1, in conjunction with HMQC data, allows all of the vicinal proton-proton connectivities in the 2-pyridyl system to be readily established. Assignments can be made directly since the P6 resonance (resonances from the two four-spin systems are denoted as either "P" for those in the 2-pyridyl system or "A" for the anilino derived system) can be unequivocally assigned on the basis of chemical shift and coupling considerations as the sharp multiplet resonating at 8.597 ppm. It is also worth noting, first, that the proton assigned as P3, resonating at 8.620 ppm, is shifted considerably downfield of the normal chemical shift for a proton at the 3-position of a pyridine system and, second, that the P3 resonance is significantly broadened while the other pyridyl resonances are reasonably sharp.

Assignment of the proton and protonated carbon resonances of the chloroaniline-derived ring of **1** is a more challenging undertaking. There is no unequivocal starting point for proton resonance assignment. The proton resonances in this four-spin system may, however, be sequenced using HMQC-TOCSY. Referring in the expansion of the aromatic region shown in Figure 2 to the proton/carbon pair resonating at 7.59/129.8 ppm, we observe a relay to the apparent proton "triplet" resonating at 7.32 ppm. The direct proton/carbon correlation is observed at 7.32/126.9 ppm (HMQC) in a rather congested region of the HMQC-TOCSY spectrum. Correlations from the 7.32/-126.9 ppm direct response are observed redundantly to the proton resonating at 7.59 ppm and to the proton resonating at 7.22 ppm. The proximity of the 7.32 and 7.22 ppm vicinal protons and their directly bound carbons, which resonate at 126.9 and 127.4 ppm, respectively, afford a region of the HMQC-TOCSY spectrum which is difficult to interpret. Before completing the sequencing of the resonances of the chloroaniline-derived ring, recently reported alternatives to the conventional HMQC-TOCSY spectrum will be considered.

Contrasting the region of the HMQC-TOCSY spectrum shown in Figure 2 with the same region in the IDR-HMQC-TOCSY (Inverted Direct Response) [9] spectrum shown in Figure 3, the latter is clearly more interpretable. Direct responses are inverted (negative response intensity in the phase sensitive data matrix) in the experiment and are denoted in Figure 3 by contours which are "open" (defined by widely spaced contour levels). Relayed responses, which have positive response intensity, are denoted by "closed" contours (nearly solid black responses in the contour plot). The interpretability of the direct and relayed responses associated with the 7.32/126.9 and 7.22/-127.4 ppm resonant pairs is considerably greater in the IDR-HMQC-TOCSY spectrum (Figure 3) than in the conventional HMQC-TOCSY experiment (Figure 2), in which the direct and relayed responses have the same phase.

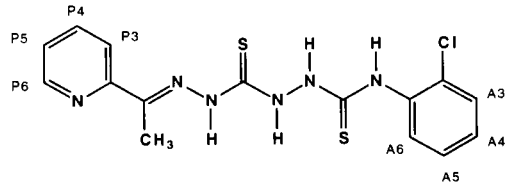
A further contrast can be drawn between the data shown in Figures 2 and 3 with the SDR-HMQC-TOCSY (Suppressed Direct Response) [10] spectrum shown in Figure 4. Suppression of direct responses removes any possibility of obscuring direct responses arising from proximity of vicinally coupled protons attached to carbons with similar chemical shifts. The utility of the experiment is, however, somewhat lower since constant referral must be made between the SDR-HMQC-TOCSY and HMQC spectra for the former to be interpreted. Thus, Domke's IDR-HMQC-TOCSY experiment [9] is preferable in this and probably will be in many applications. The SDR-HMQC-TOCSY [10] or DEPT-HMQC based analogue should probably be reserved for molecules such as steroids, *e.g.* digoxin [12] or other congested aliphatic molecules where the likelihood of relayed responses being obscured by more intense direct responses is higher.

Sequencing of the protonated carbon resonances of the chloroaniline-derived portion of **1** is completed referring to Figure 3. Relay of magnetization from the 7.22/127.4 ppm proton/carbon pair to the proton resonating at 7.46 ppm, which is directly bound to the carbon resonating at 129.1 ppm, completes the sequencing.

The sole task remaining in the assignment of the chloroaniline-derived portion of **1** requires the orientation of the four-spin system relative to the two quaternary carbons which resonate at 136.8 and 130.3 ppm. An HMBC spectrum optimized for 63 msec (8 Hz) was acquired using the pulse sequence of Bax and Summers [13]. The protons of the chloroanilino four-spin system resonating at 7.45 and 7.32 ppm were long-range coupled to the nitrogen bearing quaternary carbon resonating at 136.8 ppm. The protons

resonating at 7.59 and 7.22 ppm were long-range coupled to the chloro-bearing quaternary carbon resonating at 130.3 ppm. On this basis, the proton resonating at 7.45 ppm is assignable as the A3 resonance and, from the sequencing of the four-spin system done using the HMQC-TOCSY spectrum, the assignments of the remaining three resonances of the chloroanilino-derived four-spin system follow. From responses contained in the HMBC spectrum, the remaining quaternary carbon resonances of **1** were also assigned. Complete resonance assignments for **1** are contained in Table 1.

Table 1. Proton and carbon nmr resonance assignments for **1** in d_6-DMSO at 400 MHz.



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Position	Chemical Shift (ppm) ^1H	^{13}C
P2	-	154.4
P3	8.61	121.4
P4	7.81	136.3
P5	7.39	124.1
P6	8.59	148.4
A1	-	136.8
A2	-	130.3
A3	7.45	129.2
A4	7.22	127.4
A5	7.32	126.9
A6	7.59	129.8
imine CH_3	2.44	12.3
imine quaternary		149.3
C=S	-	180.1
C=S	-	181.1
NH	9.3 - 11.1	

EXPERIMENTAL

All nmr studies were performed on a sample prepared by dissolving 20 mg of **1**, prepared according to the procedure described by Blumenkopf, *et al.* [4], in 0.7 ml d_6-DMSO (99.96% Merck) and filtering through cotton into a 5 mm NMR tube. All nmr studies were performed on a Varian Unity 400, operating at 399.952 MHz for ^1H observation equipped with a 5 mm Z-Spec® indirect detection probe obtained from Nalorac Cryogenic Corp., Martinez, CA. The HMQC spectrum was acquired using the pulse sequence of Bax and Subramanian [11] as 1024 x 40 data points. Acquisition time was 30 minutes. The data were zero-filled to 2048 x 256 points and were processed using Gaussian multiplication prior to the first Fourier transformation and cosine multiplication prior to the second. All of the HMQC-TOCSY data reported in this study were acquired using identical digitization of 1536 x 96 points with a mixing period of 18 msec duration. The data were zero-filled to 2048 x 256 points using Gaussian multiplication prior to the first Fourier transform and cosine multiplication prior to the second. The conventional HMQC-TOCSY spectrum was acquired using the pulse sequence of Lerner and Bax [7] except that broadband heteronuclear decoupling was initiated at the beginning of the acquisition period; the data are shown in Figures 1 and 2. The IDR-(Inverted Direct Response) HMQC-TOCSY data were acquired using the pulse sequence recently reported by Domke [9]. The region of the IDR-HMQC-TOCSY spectrum shown in Figure 3 is identical to the expansion of the conventional HMQC-TOCSY spectrum shown in Figure 2. The SDR-(Suppressed Direct Response)HMQC-TOCSY data were acquired using the recently reported pulse sequence of Martin, *et al.* [10]. The region of the SDR-HMQC-TOCSY spectrum shown in Figure 4 is identical to the expansions shown in Figures 2 and 3. All three of the HMQC-TOCSY spectra were acquired in a single overnight time period.

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