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# Unequivocal location of sites of *N*-oxidation using natural abundance long-range $^{1}H,^{15}N$ GHNMQC two-dimensional NMR†

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ABSTRACT: A method is described for unequivocally establishing the site of *N*-oxidation which relies on the relatively large downfield shift of aliphatic nitrogen resonances following oxidation. The technique described is based on $^{15}N$ at natural abundance and does not require labelling. $^{15}N$ chemical shifts are established on the basis of long-range $^{1}H$–$^{15}N$ heteronuclear coupling pathways detected via two-dimensional NMR using the GHNMQC pulse sequence. The oxidized nitrogen in the two piperazine-containing systems studied was shifted downfield by approximately +68 ppm; the opposite, non-oxidized nitrogen in the piperazine ring was shifted upfield by an average of −6 ppm ($^{15}N$ chemical shifts are referenced to liquid ammonia with a chemical shift of 0 ppm). *N*-oxidation perturbations in $^{15}N$ chemical shifts were first parameterized using an oxazolidinone antibiotic (eperezolid) and its *N*-oxide as a model system. After the $^{15}N$ shift due to *N*-oxidation was determined, the method was used to establish unequivocally the site of *N*-oxidation in PNU-101387, a piperazine-containing anxiolytic agent. Conventional spectroscopic methods were equivocal and could not reliably establish the site of *N*-oxidation.


KEYWORDS: NMR; long-range $^{1}H$–$^{15}N$ correlation; natural abundance; GHNMQC; *N*-oxidation; $^{15}N$ chemical shifts

## INTRODUCTION

A series of recent papers has begun to explore the utility of long-range $^{1}H$–$^{15}N$ heteronuclear shift correlation experiments at natural abundance with a variety of alkaloids[1–9] and other nitrogenous compounds.[10–14] Correlations are established using a pulse sequence for which we recently proposed the mnemonic GHNMQC (gradient-enhanced hydrogen–nitrogen multiple quantum coherence).[14] The GHNMQC pulse sequence is essentially an amalgamation of the familiar GHMQC and GHMBC pulse sequences[15] where the gradient ratios have been modified to accommodate the gyromagnetic ratio of $^{15}N$ rather than $^{13}C$.[16,17] Both the BIRD pulse[18] from the G-/HMQC sequence and the low-pass *J*-filter[19] of the G-/HMBC are superfluous and omitted from the GHNMQC pulse sequence. Responses from protons not long-range coupled to nitrogen are effectively suppressed by gradients in conjunction with the GHMBC-derived phase-cycling scheme employed. Omission of the low-pass *J*-filter allows $^{1}H$–$^{15}N$ direct correlation responses to be observed in addition to the long-range responses in the same experiment. This approach obviates the need to obtain direct correlations from a separate experiment. There is an implicit assumption that the number and location of direct responses does not interfere with desirable long-range responses. In such instances as they do interfere, the low-pass *J*-filter should be reinserted into the pulse sequence. It is also important to note that the intensity of the direct responses will be modulated in the GHNMQC experiment and that some may, for some long optimizations, even be unobservable.[14]

In the course of a study of the $^{15}N$ chemical shifts and long-range $^{1}H$–$^{15}N$ coupling pathways of the HIV reverse-transcriptase inhibitor Delavirdine,[14] we noted an early $^{15}N$ NMR study by Städeli *et al.*[20] that dealt with the $^{15}N$ chemical shifts of a series of pyrimidine analogs. Their work suggested that $^{15}N$ chemical shift changes induced by *N*-oxidation might have little or only limited utility in locating the site of *N*-oxidation in the pyrimidine systems studied in their work. Reasoning that their observation, while undoubtedly correct for the systems they had studied, could also be a function of the hybridization state of the nitrogen in question, we elected to examine the problem further. Specifically, we were interested in developing a method capable of unequivocally determining the site of *N*-oxidation of aliphatic $sp^3$ nitrogens in candidate drug molecules subjected to oxidative stress, particularly in those cases

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† Dedicated to Professor John D. Roberts on the occasion of his 80th birthday.
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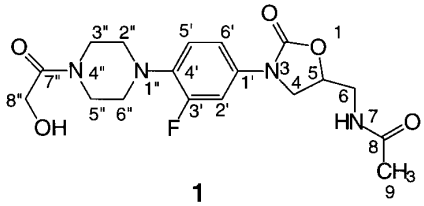

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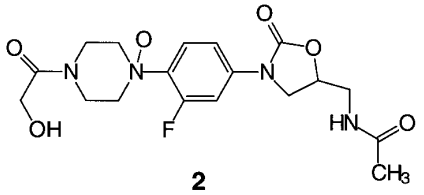

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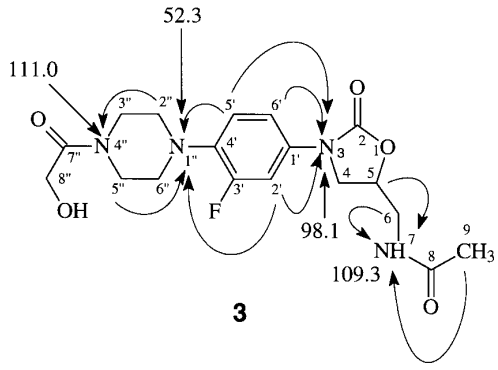
where there are potentially competing sites of *N*-oxidation. Our reasoning was based on the premise that the electronic properties of an $sp^3$ nitrogen *vs.* its *N*-oxidized counterpart would differ substantially more than the electronic properties of an $sp^2$ nitrogen and its *N*-oxidized counterpart. In this sense, we were optimistic that *N*-oxidation would afford a usable perturbation in the $^{15}N$ chemical shift of the oxidized nitrogen, thereby providing a diagnostically useful method. To pursue our hypothesis, our initial effort utilized a model oxazolidinone antibiotic eperezolid (**1**)[21] currently undergoing developmental evaluation, and its N1″-oxidized analog (**2**), whose structure was fixed by direct synthesis.[22]

## RESULTS AND DISCUSSION

### Effect of *N*-oxidation on $^{15}N$ chemical shifts of eperezolid

The natural abundance long-range $^{1}H$–$^{15}N$ 8 Hz optimized GHNMQC spectrum of eperezolid (**1**) is shown in the left panel of Fig. 1. The easiest point of entry into the assignment of the spectrum is provided by the N7 amide direct response, which appears as a 92 Hz doublet resonating at 109.3 ppm. In addition to the direct correlation, long-range couplings were also observed to N7 from the H5 methine proton, one of the H6 methylene protons and the 9Me singlet. Of the three remaining $^{15}N$ resonances, two were expected to exhibit chemical shifts near that of N7 since N3 is a carbamate and N4″ is also an amide. The remaining nitrogen, N1″, was expected to resonate the furthest upfield in the spectrum.

These expectations are supported by a cursory examination of the spectrum shown in the left panel of Fig. 1. A resonance was observed at a chemical shift of 111.0 ppm coupled solely to the H2″/H6″ methylene protons of the piperazine, allowing unequivocal assignment as N4″. The remaining downfield resonance at 98.1 ppm was long-range coupled to H2′ and H6′ and weakly to H5′. Based on these correlations, the nitrogen resonating at 98.1 ppm was assigned as N3 in the oxazolidinone ring. No correlations were observed for N3 in the 8 Hz optimized experiment from either of the anisochronous 4-methylene or H5 methine resonances. The remaining nitrogen resonance was observed at 52.3 ppm

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and was assigned at N1″ based on correlations from H5′ and the H3″/H5″ methylene resonances and a weak correlation from H2′. The long-range correlation pathways are summarized in structure **3**.

GHNMQC data were next acquired for the N1″ oxidized analog (**2**) of eperzolid, again using an 8 Hz optimization. These data are presented in the right panel of Fig. 1, plotted on an identical scale to that on the left. It is interesting that all of the nitrogen resonances are clustered in the range 100–125 ppm. This confirms our initial supposition that an aliphatic $sp^3$ nitrogen would have substantially different electronic properties following *N*-oxidation. The pronounced downfield shift of the oxidized nitrogen provides a diagnostically useful means of unequivocally determining the site of *N*-oxidation.

Again, the N7H direct correlation doublet provided an immediate basis for the assignment of N7 at 109.2 ppm. Long-range correlations were again observed from the H5 methine, the H6 methylene and 9Me resonances.

The N3 carbamate nitrogen resonance in the oxazolidinone ring was shifted downfield slightly to 101.7 ppm. The assignment was based on long-range correlations to the H2′ and H6′ phenyl protons and a correlation to one of the anisochronous H4 methylene protons. The weak correlation previously observed from H5′–N3 was not observed in this spectrum although this may merely be a function of signal-to-noise ratio since the sample of the *N*-oxide was more dilute than that used to acquire the data for **1**. The remaining amide nitrogen, N4″, was long-range coupled only to the H2″/H6″ methylene protons, confirming the assignment. The resonance was shifted upfield somewhat relative to the parent drug to 105.8 ppm.

Finally, the $sp^3$ aliphatic N1″ resonance, in contrast to the other resonances in the spectrum, experienced a significant downfield shift of +68.5 ppm following *N*-oxidation. The assignment of N1″ was confirmed on the basis of a long-range coupling to H5′ and correlations to the H3″/H5″ methylene resonances. Again, the weak correlation from H2′ was not observed, probably a function of sample concentration.

Correlation pathways for **2** are summarized in structure **4**; changes in chemical shift due to *N*-oxidation are given parenthetically with a + sign denoting a downfield shift of the resonance relative to the chemical shift of liquid ammonia, which is taken as 0.0 ppm. Perturbations in the $^{15}N$ chemical shifts of **2** merit brief

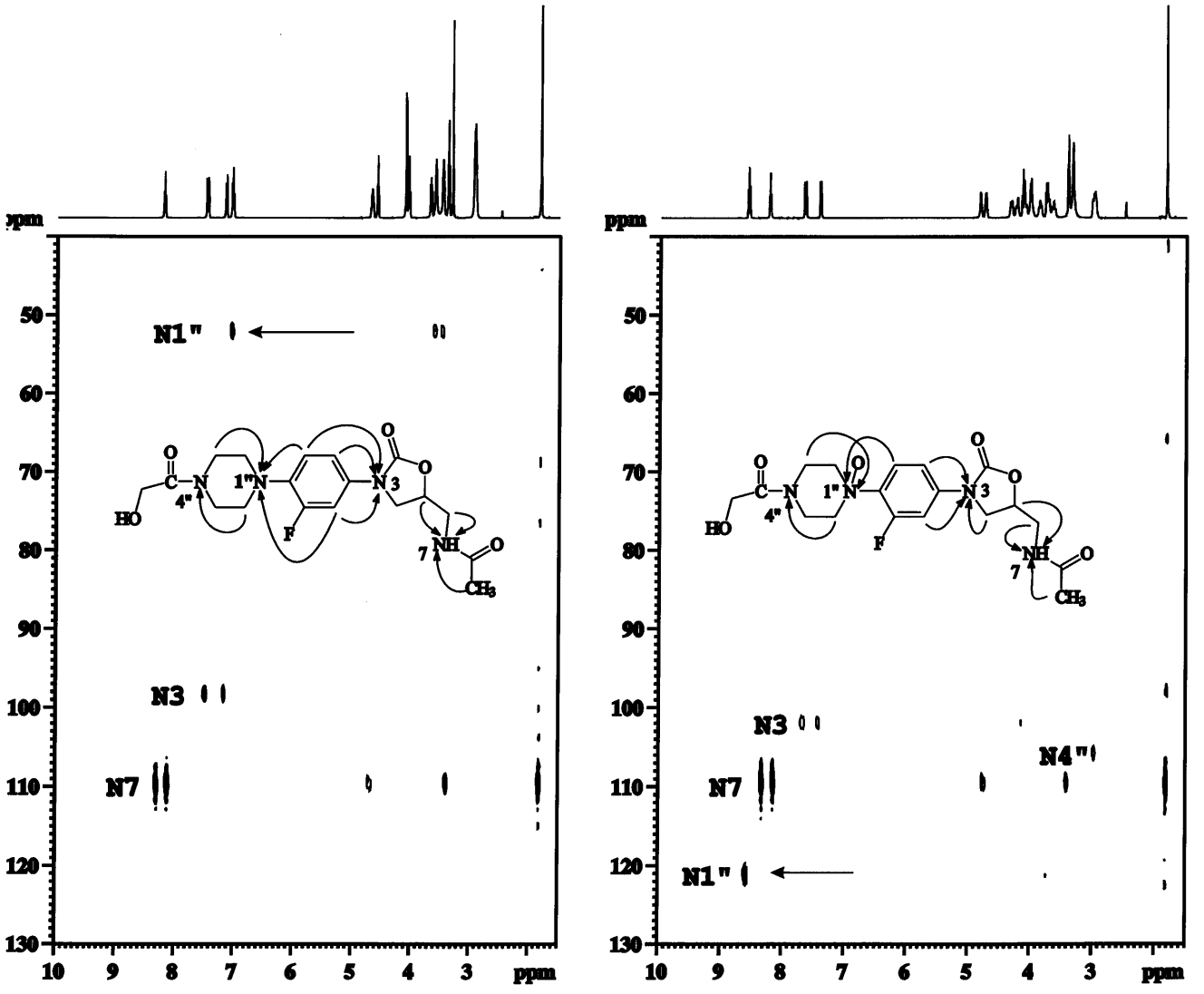
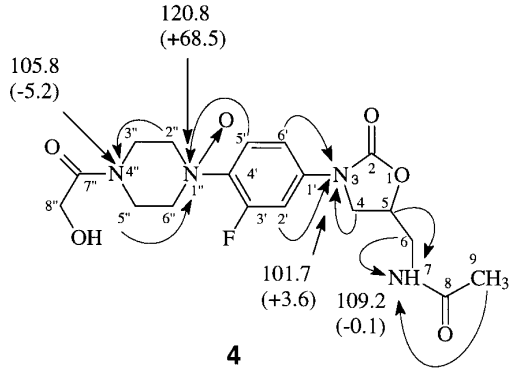



**Figure 1.** Long-range $^{1}$H–$^{15}$N GHNMQC spectra of the oxazolidinone antibiotic eperezolid (**1**) (left panel) and its N1″ *N*-oxidized analog (**2**) (right panel). Spectra were acquired and plotted identically (see Experimental). Following *N*-oxidation, the N1″ resonance (denoted by an arrow) is shifted downfield from 52.3 to 120.8 ppm, corresponding to a downfield shift of +68.5 ppm. The N4″ resonance is shifted upfield by −5.2 ppm (from 111.0 to 105.8 ppm); the N3 resonance in the oxazolidinone ring is shifted downfield by +3.6 ppm (from 98.1 to 101.7 ppm) after *N*-oxidation.

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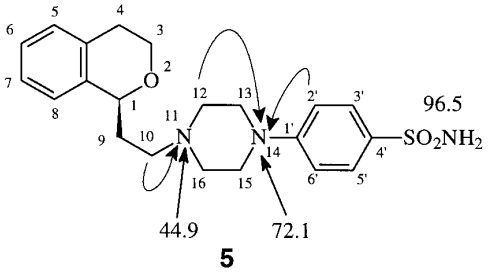
comment. Obviously, the downfield shift of N1″ relative to the parent drug can be directly ascribed to *N*-oxidation. It is reasonable to hypothesize that the downfield shift of the oxazolidinone N3 resonance relative to the parent molecule can be attributed to inductive effects. The now formal positive charge on N1″ presumably withdraws electron density from the phenyl ring, which is compensated for by N3, consequently inducing the downfield shift of N3. At this time, it is uncertain what causes the upfield shift of N4″. Possibilities might include transmitted electronic effects or a steric interaction between N4″ and the N1″ oxygen. The latter, although presumably less likely than the former, is suggested as a possibility since steric compression accounts for the differences in the methyl $^{13}$C chemical shifts of *E*- and *Z*-trisubstituted alkenes. Finally, there exists the possibility of synclinal interaction between the N4″ lone pair and the N1″ oxide. Until more extensive data are available, however, it is only possible to speculate on the origin of the upfield shift.

## Location of the site of *N*-oxidation in PNU-101387 based on $^{15}$N chemical shift perturbations

The piperazine-containing candidate drug PNU-101387 (**5**) is at present undergoing clinical trials as a potential antipsychotic agent. To gain insight into the stability of

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the molecule and an understanding of potential degradation pathways, it was subjected to peroxidative stress among other challenges. Mass spectral data suggested that the molecule had undergone *N*-oxidation at N14. The one-dimensional NMR data, in contrast, suggested that *N*-oxidation had occurred at N11. These conflicting data underscored the need for an unequivocal method to establish the site of *N*-oxidation. To make an irrefutable determination of the structure, PNU-101387 and its putative *N*-oxide were also studied using the $^{1}H$–$^{15}N$ long-range GHNMQC method described above for eperezolid (**1**) and its *N*-oxide (**2**).

The $^{1}H$–$^{15}N$ GHNMQC spectrum of **5** optimized for 8 Hz is shown in the left panel of Fig. 2. As expected, three distinct sets of nitrogen correlations were observed in the spectrum. The sulfonamido $^{15}N$ resonance was readily assigned on the basis of its 80 Hz doublet as the resonance at 96.5 ppm. This shift is also in good agreement with the reported $^{15}N$ shift of benzenesulfonamide at 93.8 ppm.[23]

Two $^{15}N$ resonances were observed upfield corresponding to the two piperazine nitrogens in the molecule, resonating at 44.9 and 72.1 ppm. The former was assigned to N11; the shift and assignment are reasonable. The model compound β-phenethyl-*N*-piperidine, for example, resonates at 44.9 ppm.[24] The remaining nitrogen resonance was assigned as N14 based on long-ranged correlations from the H2′/H6′ aromatic protons. An exact model for the $^{15}N$ shift of N14 was not avail-

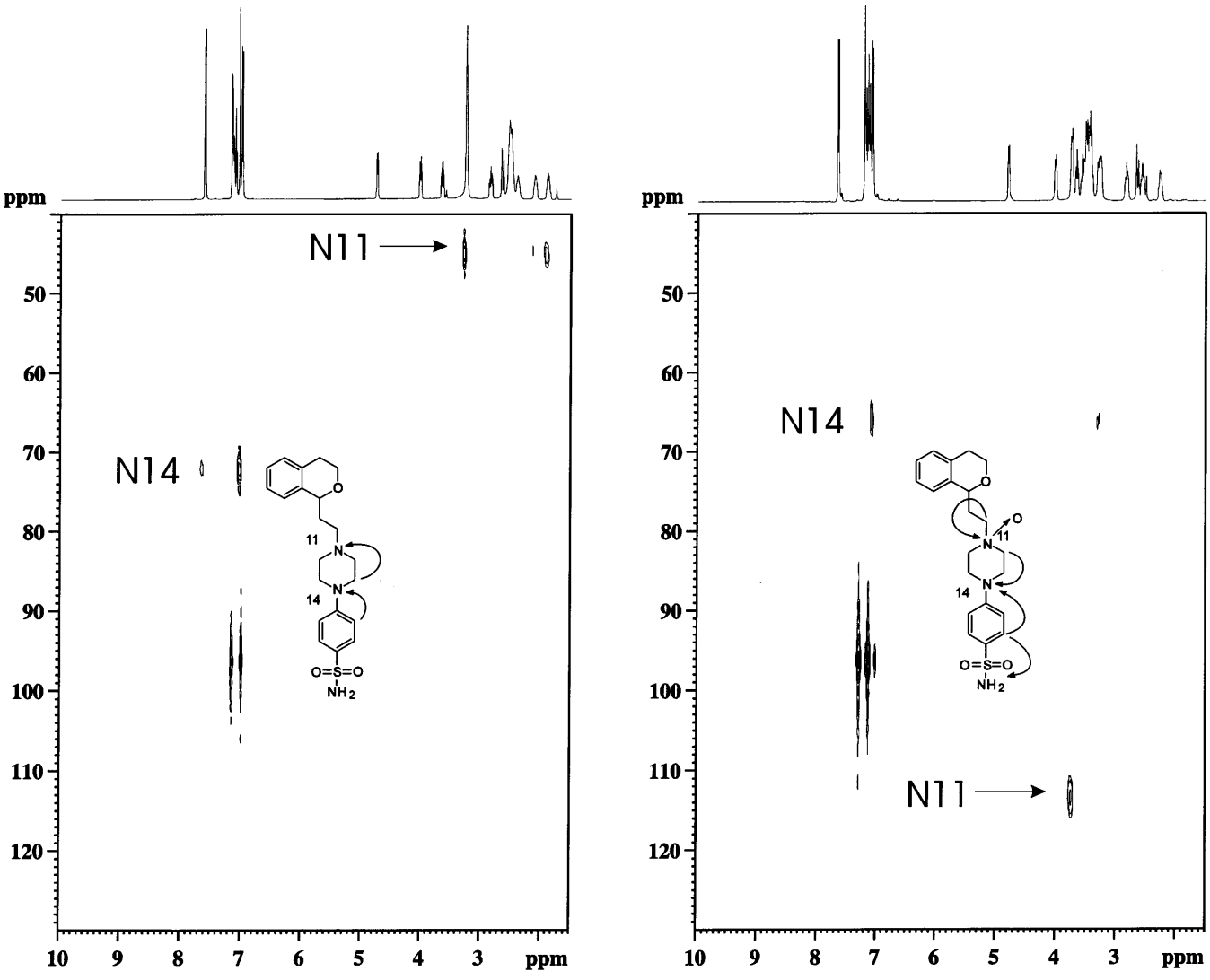



**Figure 2.** Long-range $^{1}H$–$^{15}N$ GHNMQC spectra of the antipsychotic agent (**5**) (left panel) and its N11 *N*-oxidized analog (**6**) (right panel). Spectra were acquired and plotted identically (see Experimental). Following *N*-oxidation, the N11 resonance is shifted downfield from 44.9 to 113.0 ppm, corresponding to a downfield shift of +68.1 ppm. The N14 resonance is shifted upfield by −7.1 ppm (from 72.1 to 65.0 ppm); the sulfonamido $NH_2$ resonance is shifted upfield slightly by −0.3 ppm (from 96.5 to 96.2 ppm) after *N*-oxidation.

able. A reasonable model is, however, afforded by *trans*-stilbene, in which one of the phenyl rings has been replaced by a piperidine; in that system, the piperidine nitrogen resonates at 75.9 ppm, in good agreement with the assigned shift of N14.[24] Finally, the order of assignments for N11 upfield of N14 is also reasonable from an electronic standpoint.

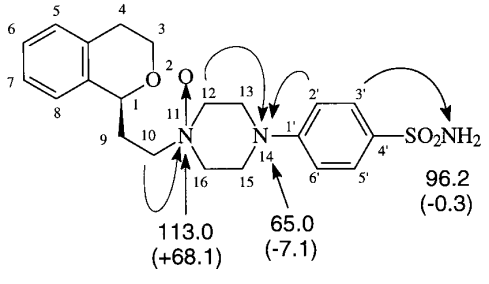
Following exposure of **5** to standard oxidative stress conditions with 3% hydrogen peroxide, a degradant was chromatographically isolated whose mass spectrum was consistent with *N*-oxidation. The peroxide-derived *N*-oxide was chromatographically and spectroscopically identical with authentic material. A 9 mg sample of the synthetically prepared material was analyzed by $^{1}$H–$^{15}$N GHNMQC; the 8 Hz long-range optimized spectrum of the *N*-oxide is presented in the right panel of Fig. 2, plotted on an identical scale to that on the left. Proton long-range coupled $^{15}$N resonances were observed at 65.0, 96.2 and 113.0 ppm.

Not surprisingly, the chemical shift of the sulfonamido nitrogen was essentially invariant, shifting upfield only −0.3 to 96.2 ppm; the direct response was again observed as an 80 Hz doublet. In addition to the direct response, a new long-range response was observed to this nitrogen from the H3′/H5′ aromatic methine protons.

The nitrogen resonance observed at 65.0 ppm was assigned as N14 on the basis of its long-range couplings. Couplings were observed from the H2′/H6′ aromatic methine protons and the piperazine H12/H16 methylene resonance. The observed shift is −7.1 ppm upfield from the parent molecule, similar in sense and magnitude to the upfield shift observed for N4″ in the *N*-oxide of eperezolid (see discussion above and **4**).

The remaining nitrogen resonating at 113.0 ppm was assignable as N11 based on its single long-range correlation from the H10 methylene as shown in structure **6**. Remarkably, the downfield shift of N11 after *N*-oxidation was + 68.1 ppm, almost identical to that observed for N1″ of eperezolid following *N*-oxidation.

The similar magnitude and sense of the shifts of the piperazine nitrogens of eperzolid (**1**) and PNU-101387 (**5**) after *N*-oxidation suggests that there may be reasonable generality associated with $^{15}$N chemical shift perturbations of piperazines following *N*-oxidation. Further work will be necessary to substantiate or refute this observation. It is also attractive to speculate that the roughly + 68 ppm downfield shift observed for the oxidized nitrogen in the two piperazine systems studied here can be applied to other alicyclic nitrogenous systems, although this also remains to be confirmed.

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## CONCLUSIONS

Two candidate drugs and their corresponding *N*-oxides were studied using the GHNMQC experiment to observe long-range $^{1}$H–$^{15}$N coupling pathways at natural abundance. In all cases, sufficient correlations were observed to make unequivocal assignments of all of the nitrogen resonances without having to resort to chemical shift arguments or comparisons. Furthermore, where model systems were available, assigned $^{15}$N shifts were in reasonable accord with the corresponding nitrogen shifts of the model systems.

In the present study, the first pair of compounds, eperezolid (**1**) and its synthetically prepared *N*-oxide (**2**), served as a model system for *N*-oxidation $^{15}$N chemical shift perturbation. Following oxidation, the subject nitrogen was shifted downfield by + 68.5 ppm, thereby providing a diagnostically useful basis for establishing sites of *N*-oxidation. The earlier suggestion of Städeli *et al.*,[20] based on pyrimidine systems, that *N*-oxidation does not afford a useful nitrogen shift perturbation, may therefore apply only to systems involving sp$^2$ nitrogens. Clearly, more work needs to be undertaken to determine the generality of the observations made in the present study to other alicyclic nitrogen-containing molecules.

Within the two piperazine systems studied, the nitrogen spared from oxidation shifted upfield from −5.2 to −7.1 ppm. Again, additional work will need to be undertaken to determine if this observation has general applicability. In the case of the oxazolidinone **1**, we have also made the suggestion that the effects of *N*-oxidation may be inductively experienced by other nitrogen substituents attached to a phenyl ring, the N3 resonance of **1** shifting downfield by +3.6 ppm after *N*-oxidation at N1″ (see **4**).

Finally, from the latter pair of examples, PNU-101387 (**5**) and its *N*-oxide (**6**), we were able to unequivocally establish the site of *N*-oxidation at N11. This work suggests that $^{15}$N shift perturbations caused by *N*-oxidation will provide a diagnostically useful method to establish the site of oxidation in alicyclic nitrogenous molecules.

With regard to the sensitivity of the method, although GHNMQC is not an especially sensitive method, more sensitive experimental alternatives exist than that afforded by the 5 mm gradient inverse triple resonance probe used for the present study. In particular, large gains in sensitivity can be obtained using a 3 mm gradient inverse dual (H, X) or triple resonance (H, C, N) probe in conjunction with a Shigemi NMR tube used to restrict the sample volume (from 130–150 μl for a standard 3 mm tube to *ca.* 70 μl for a 3 mm Shigemi tube *vs.* 500–600 μl for a standard 5 mm tube). Restriction of sample volume has a substantial impact on spectral measurement times.[25–28] It should be recalled that equivalent signal-to-noise ratios can be

obtained in one quarter the time by doubling the effective concentration or alternatively halving the volume for the same quantity of analyte. An example of the impact of sample size on long-range $^{1}H$–$^{15}N$ GHNMQC spectral quality using a 1 mg sample of the HIV RT inhibitor Delavirdine in standard 5 mm tubes, standard 3 mm tubes and 3 mm Shigemi sample tubes is described in a forthcoming paper.[29]

An optimum probe–sample configuration should make it possible to acquire GHNMQC spectra at 500 MHz on samples of *N*-oxidized material where the total available sample is limited to amounts of *ca.* 1 mg for molecules in the 300–500 Da molecular mass range. Samples of this size can be reasonably isolated from typical stability studies by preparative chromatographic methods. We hope to be able to demonstrate examples of this in forthcoming papers.

## EXPERIMENTAL

All of the experiments described here were performed on a Bruker AMX-500 three-channel spectrometer operating at 500.13 MHz for $^{1}H$ observation and equipped with single *z*-axis gradient capabilities and a Bruker 5 mm gradient inverse triple resonance probe. All data were acquired at 27 °C. Spectra were acquired using the pulse sequence described previously.[14] Samples were prepared by dissolving 28.2 mg of eperezolid (**1**), 37.0 mg of its *N*-oxide (**2**), 15 mg of PNU-101387 (**5**) and 9.1 mg of the synthetically prepared *N*-oxide (**6**) in a uniform volume of 600 μl of 99.996% DMSO-$d_6$ (Isotec) under a dry argon atmosphere in a glove-box.

Spectra were uniformly acquired as 2048 × 128 files with 256 transients accumulated per $t_1$ increment, except for the *N*-oxide of PNU-101387 (**6**), for which 1024 transients were accumulated per $t_1$ increment. A uniform interpulse delay of 1.5 s was employed. Spectra were optimized for an assumed 8 Hz (63 ms) long-range $^{1}H$–$^{15}N$ coupling. Spectral widths of 15 and 150 ppm were employed for $^{1}H$ and $^{15}N$, respectively, giving spectral widths of 7575 and 7600 Hz for $F_2$ and $F_1$, respectively. The proton spectral window was set from 0 to 15 ppm; the $^{15}N$ spectral window was set from 0 to 150 ppm relative to liquid ammonia (0.0 ppm, which is +379.5 ppm upfield relative to nitromethane). Thus, $^{15}N$ chemical shifts are reported in ppm downfield (+) from liquid ammonia rather than in ppm upfield (also + in that convention) relative to nitromethane. The 90° pulse widths were 13.2 and 34.0 μs for $^{1}H$ and $^{15}N$, respectively.

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