^{15}N NMR STUDY OF TOXOFLAVIN AND FERVENULIN

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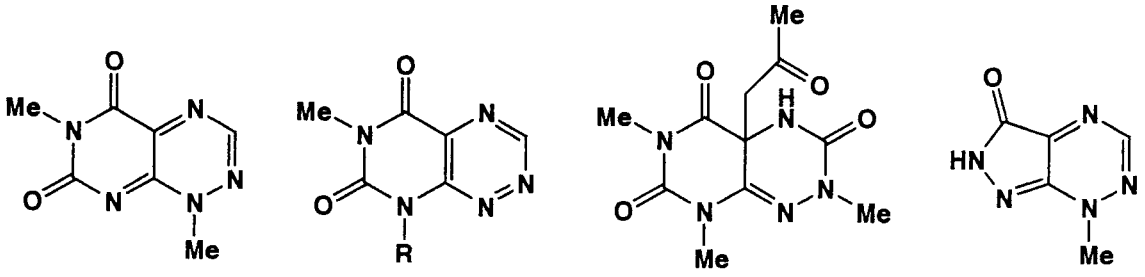
Abstract - ^{15}N NMR chemical shifts and the long-range ^{1}H-^{15}N coupling constant values of toxoflavin and fervenulin are reported. Complete ^{15}N NMR assignments were established by 2D ^{1}H-^{15}N PFG-HMBC experimental data including four-bond correlations at natural abundance.

Antibiotics in the class of pyrimido[5,4-*e*]-1,2,4-triazine ring system such as toxoflavin (**1**),[1] fervenulin (**2**),[2] reumycin (**3**),[3] pyrizinostatin (**4**),[4] and structurally related nostocine A (**5**)[5] have received biological interest because of their high toxcities and broad-spectra of antibacterial activities together with chemical interest.[6] Those structures were mainly determined by X-ray crystallographic analyses and synthetic studies,[7] because their partial structures which consist of hydrogen and carbon atoms are divided by nitrogen atoms and could not be connected by ^{1}H and ^{13}C 2D NMR techniques unambiguously.



1 **2** R = Me, **3** R = H **4** **5**

The HMBC (Heteronuclear Multiple-Bond Correlation) spectroscopy[8] is widely applied to structure determination of organic molecules. Recently, pulsed field gradient (PFG) technique[9] has been applied to multiple dimensional NMR spectroscopy with a great success,[10] and the HMBC experiment was drastically improved by using PFG in selection of coherence transfer pathway and t_1 noise suppression.[11] Since our challenging attempts of PFG-HMBC experiments were applied to detect ^{1}H-^{15}N long-range correlations at natural abundance in 1993,[12] the successful applications of the ^{1}H-^{15}N PFG-HMBC experiments were reported.[13,14] Following the early efforts, a number of applications of the ^{1}H-^{15}N PFG-HMBC

experiments have been used for ^{15}N NMR study of several plant alkaloids[15-21] and fungal metabolites,[22-26] and antibiotics,[27-34] including structure detemination of new natural products[32-34] such as 11-isopropylcryptolepine,[19] fungerin,[22] agrocybenine,[23] brevicompanines A and B,[24] kaitocephalin,[25] pyralomicins,[27] 4-methylaeruginoic acid,[28] YM-75518,[30] mescengricin,[31] and ritterazine A.[35] ^{15}N NMR data obtained by ^{1}H-^{15}N PFG-HMBC experiments have been also applied to study of tautomerism in clitidine,[26] substituents effects in pyrazoles[36] and salicylaldoximes,[37] detemination of oxidation sites in *N*-oxides,[38] and characterization of palladium pyridinylazole catalysts.[39] In general, two- and three-bond correlations have been used for detection of ^{15}N resonance of non-protonated nitrogens in PFG-HMBC experiments. Detection of four-bond coupling correlations was reported in tubercidin cyclonucleoside,[12] 1,2,4-triazolo[1,5-*a*]pyrimidine,[13] strychnine[13,40] and other alkaloids,[17,18,21] in which correlated protons are located on the position of W-character relationship from the nitrogen atom or the *para* position on a 6-membered aromatic ring system. In some case, detection of ^{1}H-^{15}N four-bond correlations is difficult because of weak coupling and signal decay by T_2 relaxation, particularly in medium or large size molecules.[29] We now report the complete ^{15}N NMR assignments of toxoflavin (**1**), which has a nitrogen atom N8 without two- or three-bond coupling pathways and fervenulin (**2**) by using ^{1}H-^{15}N PFG-HMBC techniques.

Table 1. ^{1}H, ^{13}C, and ^{15}N NMR Data for Toxoflavin (1) and Fervenulin (2).*

No	(1)	(2)
H-3	8.82 s	9.84 s
Me-N1	4.16 s	-----
Me-N6	3.50 s	3.58 s
Me-N8	-----	3.92 s
C3	145.2	154.3
C4a	145.5	130.8
C5	158.5	159.3
C7	154.3	149.3
C8a	150.4	151.1
Me-N1	43.4	-----
Me-N6	29.2	29.5
Me-N8	-----	29.8
N1	187.0	365.2
	(^{2}J = ca. 3.0, ^{3}J = 6.2)	(^{3}J = ca. 2.0, ^{4}J < 1.5)
N2	304.5	374.7
	(^{2}J = 17.6, ^{3}J = 4.5)	(^{2}J = 13.7)
N4	296.1	273.8
	(^{2}J = 12.8)	(^{2}J = 13.2)
N6	142.3	136.1
	(^{2}J = ca. 2.0)	(^{2}J = ca. 2.0)
N8	172.8	96.5
	($^{4}J_{N,Me\text{-}N1}$ < 1.5, $^{4}J_{N,Me\text{-}N6}$ < 1.0)	(^{2}J = ca. 2.0)

*TMS at 0 ppm and $CDCl_3$ solvent at 77.0 ppm as internal standard for ^{1}H and ^{13}C chemical shifts, respectively. $^{15}NH_4NO_3$ in a DMSO-d_6 solution as external reference at 0 ppm for ^{15}N chemical shifts.[41]

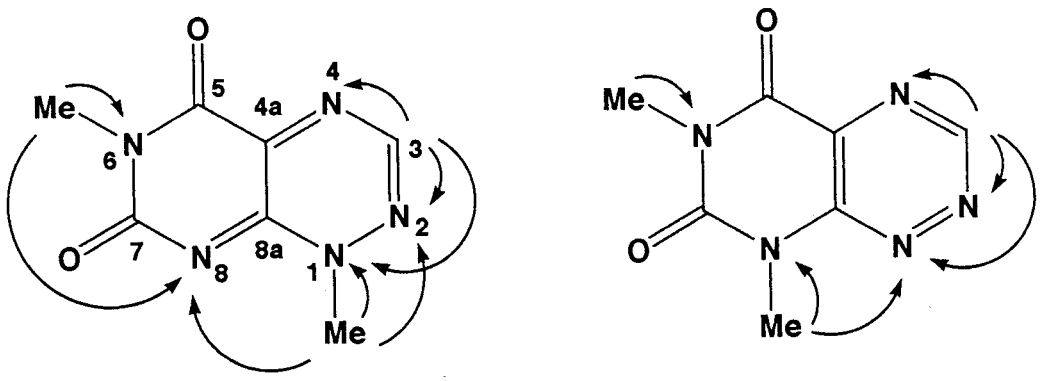


Figure 1. Observed ^{1}H-^{15}N Long-range Correlations in PFG-HMBC Spectra of Toxoflavin (1) and Fervenlin (2).

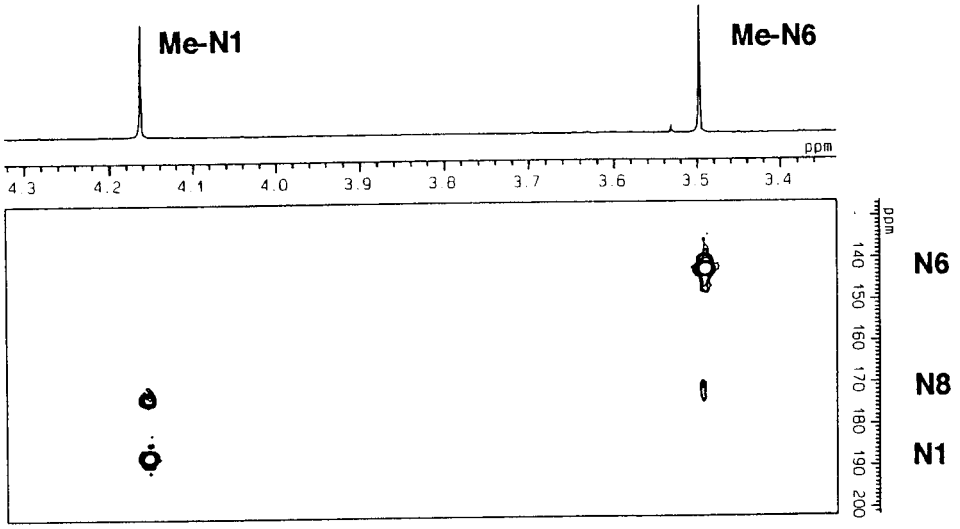


Figure 2. Expanded Region of ^{1}H-^{15}N PFG-HMBC Spectrum of Toxoflavin (1) using 300 ms duration time optimizing for 1.67Hz.

The ^{1}H NMR and ^{13}C NMR data of toxoflavin (**1**) and fervenulin (**2**) are summarised in Table 1. These assignments were confirmed by ^{1}H-^{13}C PFG-HMQC and PFG-HMBC experiments. In the ^{1}H-^{15}N PFG-HMBC spectrum of toxoflavin (**1**) recorded with the optimization of the long-range delay for 6.25 Hz (80 ms), all two and three-bond correlations were observed (Figure 1). Methyl protons at 4.16 ppm (N1-Me) were long-range coupled to N1 and N2 which resonated at 187.0 ppm and 304.5 ppm, respectively. Relatively high field chemical shift of N1 suggested that the methyl group was attached. This assignment was supported by the long-range correlations from H-3 and the coupling constant values of $^{3}J_{H3,N1}$ = 6.2 Hz and $^{2}J_{H3,N2}$ = 17.6 Hz. The later large coupling constant value is typical for two-bond coupling pathway in some azine systems.[42] The triazine proton H-3 had one more long-range coupling to N4 at 296.1 ppm in addition to N1 and N2. The two-bond coupling constant between H-3 and N4 was 12.8 Hz. Remaining methyl protons at 3.50 ppm were coupled with N6 at 142.3 ppm through two-bond coupling

pathway. One nitrogen N8, which have no two- and three-bond coupling pathway, remains to be assigned. In the ^{1}H-^{15}N PFG-HMBC spectra with the long-range delay of 150 ms (3.33 Hz) and 225 ms (2.22 Hz), Me-N1 protons were coupled to N8 through four-bond coupling pathway. The chemical shift of N8 was 172.8 ppm. In addition to this four-bond coupling pathway, the other four-bond correlation from Me-N6 protons to N8 was observed in the experiment optimized for 1.67Hz (300 ms) as shown in Figure 2.

In the case of fervenulin (**2**), H-3 proton has three long-range couplings to N1 at 365.2 ppm, N2 at 374.7 ppm, and N4 at 273.8 ppm which were observed in ^{1}H-^{15}N PFG-HMBC spectrum using 40 ms duration, and the observed long-range coupling constant values were $^{3}J_{H3,N1}$ = ca. 2.0 Hz, $^{2}J_{H3,N2}$ = 13.7 Hz, and $^{2}J_{H3,N4}$ = 13.2 Hz. Assignments of N1 and N2 from the chemical shift values were difficult, but relatively small coupling constant value of $^{3}J_{H3,N1}$ was useful for N1 assignment. Distinction between N2 and N4 was only difference of those chemical shifts. In the 1,2,4-triazine ring system, it is well known that resonance of N4 is appeared in higher field compared to N1 and N2, and shift difference between N2 and N4 is about 80 ppm in no substituted 1,2,4-triazine.[42,43] Methyl protons at 3.92 ppm (Me-N8) were coupled to N8 at 96.5 ppm and also correlated to N1 at 365.2 ppm through four-bond coupling pathway. This four-bond correlation was strongly supported the assignment of N1. Remainig N6 was easily assigned by two-bond correlation from methyl protons at 3.58 ppm (Me-N6) and observed at 136.1 ppm.

In the case of fervenulin, no four-bond correlation between protons of Me-N6 and N8, and between protons of Me-N8 and N6 was observed, by reason of low quantity of sample[44] or coupling constant value of almost 0 Hz. Practically in the ^{1}H-^{15}N PFG-HMBC experiments for caffeine as a model compound in large quantity, no four-bond correlation was observed from N-methyl protons.

In this study, four-bond coupling pathway between N-methyl protons and nitrogen at *peri* position in toxoflavin and fervenulin were effective to detect nitrogen nucleus possesing only four-bond coupling pathway, and very useful for complete assignments of ^{15}N resonances by the ^{1}H-^{15}N PFG-HMBC experiments.

EXPERIMENTAL

The 1D ^{1}H (at 600 MHz) spectra, and 2D ^{1}H-^{13}C PFG-HMQC and ^{1}H-X PFG-HMBC (X = ^{13}C and ^{15}N) spectra at 25°C were recorded with JEOL JNM-A600 spectrometer equipped with a JEOL 5 mm gradient inverse double resonance probe head. In 2D ^{1}H-^{15}N PFG-HMBC experiments, the matrix size was 2048 points in the F_2 (^{1}H) frequency domain and 128 or 256 points in F_1 (^{15}N) frequency domain. Various duration time for long-range coupling were used ranging from 40 ms (12.5 Hz) to 300 ms (1.67 Hz). Data were subsequently processed by zero-filling in both axes, and 30% shifted sine-bell window function was used prior to transformation. The number of scans was 96 and 512 for toxoflavin and fervenulin, respectively. The z-axis gradient ratio of 4.96: 4.96 : 1.00 was applied. The gradient pulses used for PFG-HMBC experiments were shaped to a sine-bell amplitude profile and lengths were 0.6 ms with pre and post 50 μs durations. The maximum gradient strength in use was 71.4G/cm. $^{n}J_{N,H}$ values were read from 2D spectra.

The concentration of toxoflavin and fervenulin was 3 mg/0.5 mL and 0.5 mg/0.14 mL in $CDCl_3$ solution, respectively. For fervenulin a symmetrical micro cell NMR tube (Shigemi) was used.

Toxoflavin and fervenulin were isolated from *Pseudomonas glumae* and those physico-chemical properties were identical with those reported values.[45,46]

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