Proton-coupled ^{15}N NMR spectra of neutral and protonated ethenoadenosine and ethenocytidine*

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ABSTRACT

The ^{15}N chemical shifts and ^{15}N , ^1H spin coupling constants were determined in the title compounds using the INEPT pulse sequence and assigned with the aid of selective proton decoupling. The $\delta /^{15}\text{N}/$ and $J/\text{N,H}/$ values are discussed in terms of involvement of the imidazole ring created by ethenobridging in the electronic structure of the whole molecule. Both spectral parameters indicate that the diligant nitrogen in this ring is the primary site of protonation in these modified nucleosides.

It is concluded that ^{15}N NMR of nucleoside bases can be largely a complementary method to ^1H and ^{13}C NMR studies and, in addition, can serve as a direct probe for studies of nitrogen environment in oligomeric fragments of nucleic acids even at moderately strong magnetic fields due to the higher spectral dispersion compared with ^1H and ^{13}C NMR spectra.

1. INTRODUCTION

The application¹⁻⁴ of multiple pulse sequences such as INEPT⁵⁻⁷ in FT NMR spectroscopy effectively removes the drawbacks in obtaining ^{15}N NMR spectra. Because of low natural abundance of the ^{15}N nucleus and its low sensitivity and negative gyromagnetic ratio, only a few studies of nucleosides and nucleotides were published so far⁸⁻¹⁵, despite the vital role played by the nitrogen atoms in biological activity of these compounds. Concurrently ^1H and ^{13}C NMR spectroscopy have proven powerful methods in studies of structure and conformation of nucleosides and nucleotides¹⁶. On the other hand, ^{15}N spectroscopy, characterized by high chemical shift dispersion and sensitivity to intra- and intermolecular environment^{8,9,17} of the nitrogen nucleus, can serve as a direct probe to study these phenomena in heteroaromatic bases. This can be a valuable tool in our approach to chemical synthesis of the oligo-

meric fragments of tRNA and studies of the tRNA structure - function relationship¹⁸.

In the present work, we report complementary results from ¹⁵N NMR spectroscopy to the recent investigations of a comparative structural analysis of parent and etheno-modified cytidine and its hydrochloride in the crystal by means of X-ray analysis¹⁹, IR spectroscopy²⁰, and by ¹H and ¹³C NMR in solution²¹. Furthermore, similar ¹⁵N NMR results for ethenoadenosine and its hydrochloride are presented.

2. EXPERIMENTAL

The ¹⁵N NMR spectra were measured at 20.28 MHz on a Varian XL-200 NMR spectrometer using a broadband variable temperature probe. Chemical shifts were determined against external CH₃¹⁵NO₂ contained in a coaxial capillary. The pulse sequence INEPT with or without refocusing pulses was used to acquire fully ¹H-coupled spectra. Typical parameters were: 50 ms delay time τ , a spectral width of 4000 Hz, 1 s acquisition time, and a pulse sequence delay of 1 s. Routinely 6-9 h were required to obtain sufficient S/N ratios for the spectra of 0.4 M solutions in DMSO-d₆. ¹H frequencies for selective decoupling were determined in the same solutions and decoupling power γH_2 was typically 20 Hz to avoid irradiation of other closely lying proton resonances.

3. RESULTS AND DISCUSSION

3.1. Assignments of chemical shifts and coupling constants

Due to significant changes introduced in purine and pyrimidine bases by ethenobridging also ¹⁵N chemical shifts change dramatically and, hence, should be assigned using unambiguous arguments. These primarily stem from proton-coupled spectra. In Table 1 N,H spin coupling constants are presented for the title compounds 2 and 3 and for adenosine /1/. In Table 2 chemical shifts for modified nucleosides in neutral and protonated forms are given.

The assignments of chemical shifts are based on the magnitude of resolvable coupling observed, signal disappearance upon re-

Figure 1:

In Figure 1, typical proton-coupled ^{15}N INEPT spectra are illustrated which were used for spectral assignments. ^{15}N NMR INEPT spectra of ethenoadenosine /0.4 M in DMSO-d_6 / referenced against $\text{CH}_3^{15}\text{NO}_2$ in an external capillary.

- a/ Refocused spectrum of ethenoadenosine hydrochloride /2a/ irradiated at the H-11 frequency in the proton spectrum. Experimental conditions: 4000 Hz spectral width, 50 ms delay time, 0.8 s pulse sequence delay time, 1 s acquisition time, 13240 transients. The inset spectra show expansions for the N-1 and N-9 resonances. The N-1 resonance /upper inset/ spectrum shows the nonrefocused signal taken from the fully coupled spectrum, separation of outer lines 16.4 Hz. The lower inset shows the expanded refocused signal at -182.0 ppm, separation of the outer lines 11.7 Hz. For the N-9 resonance the inset illustrates a refocused fully coupled signal and the nonrefocused signal after irradiation of the H-C/1'/ frequency, in upper and lower traces respectively.
- b/ Nonrefocused fully coupled spectrum of ethenoadenosine /2/ after neutralization of the hydrochloride in DMSO-d_6 . Experimental conditions as above /23,370 transients/. The inset shows expansion of the N-1 signal, separation of outer lines 16.2 Hz, and of the N-9 signal.

Table 1. ^{15}N , H spin coupling constants in nucleosides (abs. values, ± 0.2 Hz).

	^1J			^2J			^3J	
	a)	b)	c)	a)	b)	c)	c)	
 1	90.3	88.2	90.1	15.8	14.0	12.4	(N1-H2)	3.2 (N1-NH ₂)
				17.1	15.5	14.8	(N3-H2)	
				11.0	10.5	11.8	(N7-H8)	
						8.4	(N9-H8)	
 2 ^d				7.0	(7.7)	(N1-H2)	5.3 (4.3)	(N1-H10)
				3.9	(3.9)	(N1-H11)	(1.6)	(N9-H2')
				13.5	(13.0)	(N3-H2)		
				10.9	(7.7)	(N6-H10)		
				12.5	(12.7)	(N7-H8)		
				6.9	(7.7)	(N9-H8)		
						(1.6)	(N9-H1')	
 3 ^e				6.2	(6.0)	(N1-H6)	4.5 ^f (4.5) ^g	(N3-H5)
				3.3	(3.3)	(N3-H8)	3.3 ^f (3.3)	(N3-H7)
				12.9	(8.7)	(N4-H7)		

a) Values taken from Ref. 14, 1 M solution in DMSO.

b) Values for 3'-MP taken from Ref. 12, 0.08 M solution in water, pH=7.

c) Present work, 0.4 M solution in DMSO-d₆.

d) For experimental conditions see notes d) and e) in Table 2. Values in parentheses refer to protonated ethenoadenosine (2a).

e) For experimental conditions see notes d) and e) in Table 2. Values in parentheses refer to ethenocytidine hydrochloride (3a).

f) Assignments may be interchanged.

g) Assignment confirmed by selective decoupling of H-5.

Table 2. ^{15}N chemical shifts^{a)} of ethenoadenosine (2) and ethenocytidine (3) and the corresponding protonation shifts.

	N-1	N-3	N-6	N-7	N-9
Ethenoadenosine ^{b)} (2)	-178.5	-152.2	-150.1	-139.1	-207.3
Protonation shift ^{c)}	-3.5	+4.6	-40.4	-0.8	+2.0
	N-3		N-4		N-1
Ethenocytidine ^{d)} (3)	-190.8		-164.0		-232.0
Protonation shift ^{e)}	-0.9		-41.8		+5.9

a) δ (ppm) with respect to external CH_3NO_2 in a capillary.b) 0.4 M solution of ethenoadenosine hydrochloride in DMSO-d₆ after neutralization by an equivalent amount of powdered K_2CO_3 and filtering.c) 0.4 M solution of the hydrochloride in DMSO-d₆.d) 0.3 M solution of ethenocytidine hydrochloride in DMSO-d₆ after neutralization by an equivalent amount of powdered K_2CO_3 and filtering.e) 0.3 M solution of the hydrochloride in DMSO-d₆.

removal of the N,H coupling used in building up the INEPT signal for a given nitrogen, and chemical shift comparison with the parent nucleoside. Since coupling constants through three bonds can be observed, signals are easily assigned by their characteristic coupling pattern. While ethenocytidine poses no problems the ambiguity arises in the case of N-3 and N-7 atoms in ethenoadenosine which are coupled to only one proton with much the same coupling constants, see Table 1 and Fig. 1. The similarity of coupling constants does not allow for unequivocal assignments on this basis to either pyrimidine or imidazole rings^{14,22}. Hence, the assignment of N-7 to the signal at highest frequency is substantiated by comparison with the ¹⁵N NMR spectrum of parent adenosine 1 where this nitrogen, unambiguously assigned by deuteration of C/8/-H, absorbs at nearly the same frequency¹⁴.

Assignments of coupling constants are confirmed in ambiguous cases /N-1 in ethenoadenosine and N-3 in ethenocytidine/ by selective decoupling of one of the three protons involved in coupling. The fully coupled spectrum /compare upper inset for the N-1 signal in Fig. 1a/ gives a complicated pattern for which the sum of the involved couplings can be evaluated from the separation of the outer lines. It is worth noting that the shape of the signal, the signal-to-noise ratio obtained, and relative line intensities depend drastically upon the assumed J values introduced into the multipulse INEPT sequence program and, hence, a good S/N ratio is required to detect all lines of an unknown signal pattern.

In the case of ethenoadenosine irradiation one of the resonance frequencies of the protons involved in coupling simplifies the N-1 signal pattern to a pair of doublets /refocused and nonrefocused, compare lower inset in Fig. 1a/ so that one coupling constant can be evaluated and the sum of the remaining two.

Using this technique it was possible to assign three coupling constants, visible in the fine structure of the N-1 signal of ethenoadenosine hydrochloride, by selective irradiation of the H-10 and H-11 proton frequencies /Table 1/. Irradiation of H-10 simultaneously gives an unambiguous assignment of the N-6

resonance by observing its reduced intensity due to removal of $^2J_{N-6,H-10}$ coupling responsible for enhancement of the INEPT signal in this case. In ethenoadenosine this experiment allows for distinction between closely lying signals of the N-3 and N-6 atoms.

In ethenocytidine, either neutral or protonated, the signal of N-3 is a nonrefocused doublet $/J = 4.5 \text{ Hz/}$ of triplets $/J = 3.3 \text{ Hz/}$. This simplifies the assignments since irradiation of H-5 in the hydrochloride allows an assignment of the larger coupling to this proton /Table 1/.

3.2. Chemical shifts

The current rationale for an interpretation of ^{15}N chemical shifts in heteroaromatic compounds^{8,9,23,24} is based on a general consensus that the dominant role in nitrogen screening is played by the paramagnetic term expressed by equation:

$$\sigma_j^p \sim -/\Delta E_{av} \sqrt{1} \langle r^{-3} \rangle_{2p} \sum_i Q_{ij}$$

where the $\sum_i Q_{ij}$ term represents the distribution of the 2p electrons in the ring system, ΔE_{av} an average excitation energy and $\langle r^{-3} \rangle_{2p}$ the expectation value of the reciprocal cube of the 2p orbital radius.

The interpretation of terms in the above equation justifies^{8,9,23,24,25} the linear relationship found by Witanowski et al.²⁴ between nitrogen chemical shift and π -electron density q_N^π at the nitrogen atom in heterocyclic compounds which shows a decrease of σ^p with increasing π -electron density²⁵. In this respect it is useful to discuss the nitrogen shifts in heterocyclic bases in terms of two distinct types of nitrogen valence states referred to as "pyridine like" /or diligant/ and "pyrrole like" /or triligant/ nitrogen atoms²⁴. The latter nucleus provides two electrons to the delocalized π -orbital system whereas the former only one. For this reason, q_N^π values from MO calculations are higher for the "pyrrole like" nucleus, and its resonance is displaced to much lower frequencies than that of the "pyridine like" nucleus. In the latter, the lone pair is not involved in the π -orbital system and is, therefore, accessible for complexation such as protonation. The protona-

tion of "pyridine like" nuclei can formally be considered as a change to the "pyrrole like" valence state, i.e. the nucleus becomes triligant. Such a change causes an increase in π -electron density at the protonated atom and consequently a shift to lower frequencies. On the other hand, the possibility of delocalization of an increased π -electron density may partially counterbalance low-frequency shifts induced by protonation.

1-Methylimidazole 4 contains both types of nitrogen valence states discussed above and, therefore, its chemical shifts^{24,26-28} as shown in the Scheme can serve as reference values for the nucleosides studied:

N/1/	-217.7 /H ₂ O/ ⁸	-210.3 /H ₂ O/
	-219.2 /DMSO/ ²⁸	-
	-221.7 /CDCl ₃ / ²⁸	-
N/3/	-134.7 /H ₂ O/ ⁸	-209.8 /H ₂ O/
	-119.1 /DMSO/ ²⁸	-
	-124.1 /CDCl ₃ / ²⁸	-

Scheme

Noteworthy is a fact that the average chemical shift for 1-methyl imidazole in water agrees with that found in imidazole /-177 ppm⁸/ undergoing tautomerization. The protonation of N-3 shifts this characteristic value of the system to ca. -210 ppm indicating that both nitrogens adopt nearly equally "pyrrole like" valence states. Interestingly, formal protonation shifts in water are 7.4 and -75.1 ppm for N-1 and N-3, respectively /Scheme above/ whereas the protonation shift is negative for N-1 and much smaller for N-3 in imidazoles undergoing tautomerization^{25,27,28}.

The possibility of simultaneous modification of purine and pyrimidine bases in oligomeric nucleic acids poses the problem of a differentiation between ethenocytidine and ethenoadenosine

moieties. This can be judged from two regions in the ^{15}N NMR spectrum. In the spectral region of the glycosidic nitrogens this atom absorbs at distinctly lower frequencies in ethenocytidine than in ethenoadenosine ~ 24 ppm/. Even more specific changes can be figured out by comparison of the chemical shifts of the two nitrogens in the imidazole rings created in both nucleosides. In ethenocytidine both of them absorb at much lower frequency than corresponding atoms in ethenoadenosine /Table 2 and Fig. 1/ and fall into the spectral range free from other resonances, i.e. -160 ppm to -190 ppm.

The individual ^{15}N chemical shifts in both bases can also be discussed in terms of a comparison of the imidazole rings topologically similar. Thus in the imidazole ring present in purine, identified by the N-7 and N-9 atoms, the chemical shifts are very close to those in the isolated model imidazole 4 given in the Scheme above. On the other hand, in the imidazole ring formed by ethenobridging /i.e. in 2/, the spread in chemical shifts between the two nitrogen atoms is only half of that in the purine imidazole moiety, and distinct deshielding of N-1 vs. the formally similar N-9 is observed. These observations may indicate closer similarity of the valence states of these nitrogens than found in isolated imidazole and stronger conjugation of this ring with the purine moiety than in purine itself. A similar situation is found in ethenocytidine /3/ where the ^{15}N chemical shifts in the imidazole ring are spread as much as in the corresponding imidazole ring of ethenoadenosine, the resonances, however, being shifted by $12-14$ ppm to lower frequency.

Protonation of both nucleosides results in similar spectral effects despite the structural differences between both systems. Thus, diligent nitrogens, N-6 in 2 and N-4 in 3, are shifted to lower frequency by about -41 ppm whereas their tri-ligant partners in the imidazole ring show only small low-frequency effects of -3.5 and -0.9 ppm in 2 and 3, respectively. The topologically related N-3 and N-1 atoms show similar deshielding effects of $+4.6$ and $+5.9$ ppm in 2 and 3. The average resonance positions in imidazolium units are shifted to higher frequency compared with the 1-methylimidazole model 4a,

by 24 and 11 ppm in 2 and 3, respectively.

These results permit a rationalization regarding the site of protonation and the π -electron distribution in the protonated molecule. The primary site of protonation in both cases is the diligent nitrogen in the imidazole ring formed by ethenobridging. However, the protonation shift in DMSO /-41 ppm/ is in both cases only about half of that found in the model 1-methylimidazole in H_2O . The proton is in fast exchange as confirmed by broadening of the N-6 signal /Fig. 1a/ and absence of $^1J/N,H/$ for this nitrogen in the INEPT spectrum. Although these informations could, in principle, indicate intermolecular proton exchange or intramolecular tautomerization of a proton, they also support the idea of charge transfer¹⁹⁻²¹ from the imidazolium moiety to the pyrimidine rings. This can be judged from a high-frequency shift of the average imidazolium nitrogen resonances, and a participation of N-3 in 2 and N-1 in 3 in stabilizing the imidazolium cations is also evident from their high-frequency shifts upon protonation of the respective nitrogen atoms N-6 and N-4.

3.3. Coupling constants

N,H Coupling constants for compounds 2 and 3 and for the parent adenosine /1/ are collected in Table 1. A comparison between parent adenosine and the modified nucleosides reveals interesting trends. In adenosine, nitrogen spin coupling to the H-2 proton in the pyrimidine ring is larger than to the H-8 proton in an imidazole ring^{23,28}. In ethenoadenosine /2/ the coupling of N-1 with H-2 is reduced by about 40% apparently due to a change in hybridization of the nitrogen atom. Interaction of N-7 and N-9 with H-8 in the imidazole part are of the same order of magnitude as in adenosine. N,H spin interactions in the imidazole ring formed by etheno-bridging are quite different as compared with other imidazole rings. Thus, N-1 in ethenoadenosine and N-3 in ethenocytidine, formally related to N-9, reveal only weak coupling of 3.9 and 3.3 Hz to H-11 and H-8 in ethenoadenosine and ethenocytidine, respectively, as compared with 8.4 and 6.9 Hz for N-9 in the purine moieties of 1 and 2. Geminal coupling of the structurally related N-6 and N-4 atoms reveal much stronger coupling through formal single

bonds /10.9 and 12.9 Hz, respectively/. These interactions, of the same order of magnitude as found for corresponding two-bond N,H interactions, e.g. in thiazole /-10.6 Hz/^{28,29}, may indicate enhanced double-bond character of the N-4,C-7 /and N-6, C-10/ linkages due to conjugation of three double bonds as suggested recently¹⁹⁻²¹. This idea is further supported by the 30% drop in magnitude of these couplings upon protonation of the diligent nitrogen atom resulting in an inhibition of conjugation and effective isolation of the carbon-carbon double bond in the imidazole moiety. Since the above phenomenon is the most significant change revealed upon protonation of the two modified nucleosides it can be of diagnostic value in recognizing the site of protonation. The observations discussed above confirm conclusions deduced from chemical shift interpretation regarding the differences between both imidazole rings, their distinctly different involvement in the electronic structure of the whole molecule, the site of protonation and proton exchange.

In summary, the reported ¹⁵N NMR spectra of modified nucleosides allow the conclusion that this technique gives results complementary to the observations deduced from ¹H or ¹³C NMR spectra as described recently²¹. In addition, direct insight into the structural environment of nitrogen nuclei in nucleoside bases may be obtained. A distinctly larger spectral dispersion, even at moderately strong magnetic fields, in the region of diligent and triligant nitrogen atoms as compared with the ¹³C spectral range of double bonds, as well as large protonation shifts seem to indicate useful applications in studies of oligomeric fragments of nucleic acids. Further experiments in this direction are in progress.

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