A multinuclear NMR study of the restricted rotation in a bi-imidazole nucleoside †

Jukka Mäki,[a] Karel D. Klika,[b] Rainer Sjöholm[a] and Leif Kronberg*[a]

[a] *Department of Organic Chemistry, Åbo Akademi University, Biskopsgatan 8, FIN-20500 Turku/Åbo, Finland*
[b] *Department of Chemistry, University of Turku, Vatselankatu 2, FIN-20014 Turku, Finland*

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Ethenoadenosine, a common nucleoside adduct known to be formed by the action of several industrial and environmental genotoxic compounds on adenosine, undergoes ring-opening in aqueous NaOH solution with the consequent loss of one carbon atom (C5). The prototropic equilibrium and protonation of the resultant bi-imidazole nucleoside was studied by ^{1}H, ^{13}C, and ^{15}N NMR spectroscopy and it was found that in the neutral form an intramolecular H-bonding is in effect between N3″ of the outer imidazole ring and the N6 protons of the inner imidazole ring, giving rise to degenerate tautomeric forms. In the protonated form it is the pyridine-type nitrogen (N3″) of the outer imidazole ring where the proton is predominately located and the reduced intramolecular H-bonding results in essentially unrestricted rotation of the imidazole rings with respect to one another.

Introduction

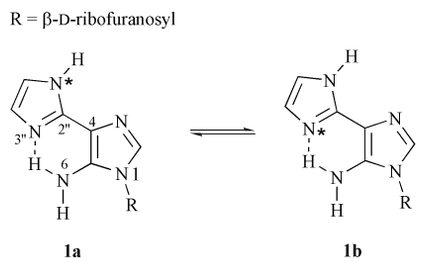
Many industrial and environmental genotoxic chemicals are known to produce exocyclic DNA adducts. Particular attention has been paid to the so-called ethenobases or etheno adducts[1] because they are poorly repaired and therefore prone to accumulate in DNA. Vinylic compounds, haloalkanes, halogenated aldehydes, and ketones are all known to form etheno adducts, either by direct reaction with nucleosides, nucleotides or DNA, or *via* the more reactive epoxide metabolites.[2] Mucochloric acid, a mutagenic drinking-water contaminant studied in our laboratory, is also known to produce etheno adducts.[3]

Ethenoadenosine undergoes ring-opening in basic aqueous solution and, as a consequence of the reaction, C5 in the ethenoadenosine moiety is eliminated by deformylation leading to the bi-imidazole nucleoside **1**.[4] It has been reported that in an oligonucleotide containing ethenoadenosine the analogous bi-imidazole lesion was generated when the oligonucleotide was stored under alkaline conditions.[5] The resultant bi-imidazole lesion was considered to be an even more harmful lesion than ethenoadenosine.[5]

Recently we have reported the characterisation of the bi-imidazole nucleoside (together with substituted structural analogues of **1**) by ^{1}H and ^{13}C NMR spectroscopy.[6] However, no distinction was made between the neutral and the protonated species of the compound. We now report on the prototropic tautomerism and protonation of the bi-imidazole nucleoside. The investigation relied primarily on ^{15}N NMR spectroscopy since nitrogen is a suitable nucleus to express the changes between different hybridisation states resulting from protonation or from prototropic tautomerism.

Results and discussion

The bi-imidazole nucleoside in its neutral form was found to be in slow exchange between two degenerate intramolecularly hydrogen-bonded forms, **1a** and **1b**, in DMSO solution (Scheme 1). The dynamic exchange process in effect is fully described as prototropic tautomerism (within the outer imidazole ring) concomitant with hindered rotation about the C4–C2″ bond adjoining the two imidazole rings as a result of intramolecular hydrogen bonding between the amino group (N6, as H donor) and the pyridine-type nitrogen (N3″, as H acceptor) of the outer imidazole ring.



Scheme 1 The bi-imidazole nucleoside in the neutral form showing the interconversion of the two degenerate forms **1a** and **1b** (the asterisk is used to depict the rotation of the outer imidazole ring about the C4–C2″ bond).

With two exceptions, the neutral form **1** displayed expected chemical shifts for all three nuclei, ^{1}H, ^{13}C, and ^{15}N, investigated (see Table 1). (The assignments relied heavily on ^{1}H{^{13}C} and ^{1}H{^{15}N} 2D heteronuclear multiple-bond coherence (HMBC) correlations and these are depicted in the supporting information.) The slow-exchange process, which is observed, is clearly indicated by the very broad carbon shifts at δ_C 127.29 and 114.27 for C4″ and C5″, respectively. (The exchange was confirmed by saturation-transfer experiments showing the saturation at one resonance upon irradiation of the other and *vice versa*.)‡ The existence of two interconverting forms is also mirrored in the nitrogen spectra by the presence of unique resonances for N1″ and N3″.§ This observation of slow exchange is remarkable as prototropic tautomerism in imidazoles is usually sufficiently fast that only averaged spectra,

† Electronic supplementary information (ESI) available: the ^{15}N HMBC spectra together with the ^{1}H{^{13}C} and ^{1}H{^{15}N} HMBC correlations. See http://www.rsc.org/suppdata/p1/b0/b010182f/

‡ *Ca.* 50-fold dilution of the sample had no effect on the linewidths of C4″ and C5″, indicating an intramolecular process to be responsible for the exchange-broadened lines.

§ Neither of these chemical shifts (N1″ and N3″) was observed directly and both were extracted from inverse-detected correlation spectra.

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Table 1 ^{1}H, ^{13}C, and ^{15}N chemical shifts (δ) and ^{1}H–^{1}H coupling constants of the neutral bi-imidazole nucleoside **1** and its protonated form **2**. Terms H1′–HO5′ and C1′–C5′ refer to protons and carbons in the ribosyl moieties. The proton chemical shifts and the coupling constants of the ribosyl units have been calculated with the PERCH program[11]

	Compound					
	1			**2**		
Atom	δ(ppm)	Multiplicity	$J_{H,H}$/Hz	δ(ppm)	multiplicity	$J_{H,H}$/Hz
H2	7.395	s		7.671	s	
H6	5.594 (2H)	br s		6.643 (2H)	br s	
H1″	11.829	br s		not observed		
H4″,H5″	6.884 (2H)	s		7.414 (2H)	s	
H1′	5.501	d	$J_{H2'}$ 6.2	5.584	d	$J_{H2'}$ 6.3
H2′	4.325	t	$J_{H1'}$ 6.2, $J_{H3'}$ 5.3	4.310	t	$J_{H1'}$ 6.3, $J_{H3'}$ 5.2
H3′	4.077	dd	$J_{H2'}$ 5.3, $J_{H4'}$ 3.5	4.090	dd	$J_{H2'}$ 5.2, $J_{H4'}$ 3.1
H4′	3.895	qt	$J_{H3'}$ 3.5, $J_{Ha5'}$ 3.2, $J_{Hb5'}$ 3.2	3.953	qt	$J_{H3'}$ 3.1, $J_{Ha5'}$ 3.1, $J_{Hb5'}$ 3.0
H^a5′	3.619	d(AB)d	$J_{H4'}$ 3.2, $J_{Hb5'}$ −11.9	3.624	d(AB)d	$J_{H4'}$ 3.1, $J_{Hb5'}$ −12.2
H^b5′	3.585	d(AB)d	$J_{H4'}$ 3.2, $J_{Ha5'}$ −11.9	3.606	d(AB)d	$J_{H4'}$ 3.0, $J_{Ha5'}$ −12.2
HO2′, HO3′, and HO5′ [a]	5.368, 5.180 (2H)			5.497, 5.368, 5.210		
C2	129.22			132.11		
C4	112.49			105.28		
C5	136.48			139.72		
C2″	144.59			140.48		
C4″	127.29			117.65		
C5″	114.27			117.65		
C1′	88.04			88.07		
C2′	73.17			73.17		
C3′	70.27			70.10		
C4′	85.31			85.64		
C5′	61.24			60.93		
N1	−211.3			−209.6		
N3	−132.3			−131.0		
N6	−338.1			−327.5		
N1″	≈−227			−220.2		
N3″	≈−141			−220.2		

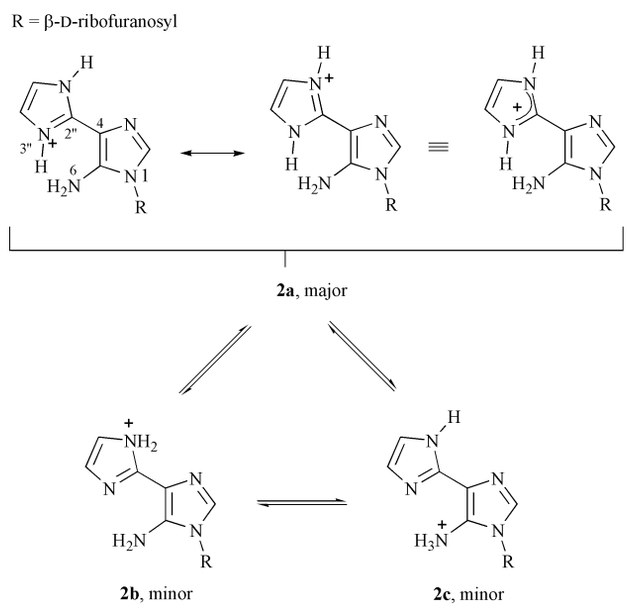
[a] No effort was made to assign the signals to specific hydroxy groups.

even in DMSO solution, are normally observed,[7] including the nitrogens which have considerably different chemical shifts.

The chemical shifts of note, though, are those of N6 (although this is only apparent in comparison with the protonated species, see below) and N3″. In comparison with 1-methylimidazole in DMSO solution[7] {δ(N1) −219.2, δ(N3) −119.1} and after taking into account a methyl-shift correction of −7.7 ppm, N1″ should have a chemical shift of −227 ppm, in accordance with what is in fact observed ($\delta_N \approx -227$). However, N3″ ($\delta_N \approx -141$) is significantly upfield from its expected resonance position by *ca.* 22 ppm. The chemical shift of this pyridine-type nitrogen is known to be particularly sensitive to protonation and moves dramatically upfield (in the order of several tens of ppm) with increasing degrees of protonation.[8] These chemical-shift data alone are compelling evidence for the proposed structures.

Upon protonation a new, faster exchange process is in effect and several other, rapidly interconverting tautomeric forms are possible (Scheme 2). The principal structure, though, in the dynamic-exchange system now consists of contributing resonance forms **2a** with essentially unrestricted rotation of the two imidazole rings with respect to one another and without the presence of, or at least otherwise markedly reduced, intramolecular hydrogen bonding.

The site of protonation is generally well indicated by the chemical-shift changes of the nitrogen bearing the proton, and the nitrogen chemical shifts played an essential role in assigning the tautomeric structures in this study. For aliphatic nitrogens a downfield shift of *ca.* 10 ppm is usual but may span the range from 16 ppm down to a few ppm or may even be negligible.[9] The pH dependency of the ^{15}N chemical-shift in imidazoles has been investigated in detail[8] and it is worth recounting the



Scheme 2 The bi-imidazole nucleoside in the protonated form showing some of the contributing resonance forms to the major form **2a**, and other contributing minor tautomeric forms to the dynamic equilibrium.

results here. (Note that the studies were performed in aqueous solution and the nitrogen chemical-shifts of imidazole are known to be very solvent sensitive.[10]) For 1-methylimidazole, which has NMR-distinct nitrogens, two resonances are observed at high pH, δ_N −133 and −213 for the pyridine-type and pyrrole-type nitrogens, respectively, with a chemical-shift

difference of 80 ppm and an average of δ_N −173. At low pH the pyridine-type nitrogen is protonated and both shifts are close to δ_N −204. For imidazole itself, at high pH a single, averaged resonance is observed at δ_N −174 and at low pH a single resonance is observed at δ_N −204.

Upon protonation of N3″, N1″ moves downfield from $\delta_N \approx -227$ to δ_N −220.2 (Table 1) and N3″ moves upfield by *ca.* 80 ppm to δ_N −220.2. These changes in chemical shift are similar to those observed for 1-methylimidazole.[8] Due to an increase in the exchange rate (dynamic effects for this process are no longer even observable in the ^{13}C spectra) and the reduction in chemical-shift difference, the signals of these two nitrogens, N1″ and N3″, effectively coalesce at 30 °C (based on integral values and $^{1}H\{^{15}N\}$ HMBC correlations). Dynamic effects are still evident though by the broad nature and shape of this peak. It is, however, the chemical-shift of N3″ (δ_N −220.2) which pointedly defines the site of protonation. In the neutral form **1**, N1″ and N3″ show a chemical-shift difference of approximately 86 ppm – akin to that observed for 1-methylimidazole[8] – and an average shift of δ_N −184. Protonation yields a new shift for the two nitrogens at δ_N −220.2, 36 ppm upfield from the average in comparison to a shift ≈31 ppm for 1-methylimidazole. Furthermore, it is the downfield shift of N6 by 10.5 ppm which is now of apparent note as this would normally allude to protonation, but in this case it is a reduction in intramolecular hydrogen bonding (as a H-donor) which accounts for the seemingly diagnostic downfield shift of N6. This downfield shift could also have a contribution from the deshielding effect caused by the protonation of the outer imidazole ring, see below. Although the multiplicities of the proton-bearing nitrogens generally could not be confirmed from fully coupled spectra or by distortionless enhancement/insensitive nuclei enhancement by polarisation-transfer experiments (DEPT/INEPT), integration of the proton spectra indicated that N6 was not protonated.¶

Finally, although the chemical shifts of the ^{1}H and ^{13}C nuclei of the imidazole rings at lower pH show that the aromatic integrity of the system remains intact, that it is significantly altered is indicated by the large chemical-shift changes of several of the nuclei. In particular the two methine protons of the outer ring (H4″ and H5″, +0.5 ppm), the quaternary carbon of the outer ring (C2″, −4.1 ppm), the carbon bearing the NH_2 group (C5, +3.2 ppm), the methine of the inner ring (C2, +2.9 ppm), and most dramatically, the quaternary carbon of the inner ring adjunct to the outer ring (C4, −7.2 ppm). The changes in chemical-shifts indicate a strong flow of electrons from the inner ring towards the outer ring upon protonation, displayed by the shielding effect of C4 and C2″, and deshielding of C2 and C5.

Conclusions

That protonation has occurred principally on the pyridine-type nitrogen (N3″) of the outer ring is the only postulate consistent with the large chemical-shift changes of this nucleus. Given the constancy of the nitrogen chemical shifts within the inner imidazole ring, these two nitrogens, N1 and N3, are effectively ruled out as sites of protonation. Although protonation at either N1″ or N6 (structures **2b** and **2c**, respectively) provides more viable alternatives, neither one accounts for the large chemical-shift changes of all the nuclei, only introspectively are they consistent with their own chemical-shift changes. Protonation at N6 would likely lead also to a slow exchange process, by intramolecular H-bonding, but even if fast, N1″ and N3″ should yield an average chemical-shift of δ_N −184, quite distinct to that actually observed (δ_N −220.2). Protonation at N1″ can mostly be excluded both on similar chemical-shift arguments and likely pK_b-values. Nevertheless, it is worth emphasising that the four protons H1″, H3″, and 2 × H6 form a labile system (together, of course, with the protons of the sugar OH groups and the residual water; this is evidenced by the proton spectra which clearly show exchange processes in effect and was confirmed by saturation-transfer experiments). Thus whilst the contributions of tautomers **2b** and **2c** cannot be completely ruled out, at best they represent only minor population counts.

In ethenoadenosine, the nitrogen atoms that are involved in the normal base-pairing and hydrogen bonding of DNA strands are blocked by the etheno bridge. However, the ring-opening reaction leading to the bi-imidazole nucleoside frees the nitrogen atoms (N1″, N3″ and N6) so again they are able to participate in intra- or interstrand H-bonding. At neutral pH significant amounts of both protonated and non-protonated bi-imidazole (**1** and **2**) are likely to be present. Although the H-bonding and protonation/deprotonation in water could differ markedly in comparison with DMSO solution, the results obtained in our study provide at least some possible insight into the exchange processes which are likely to take place in water. Whether the differences in mutagenicity between the two harmful lesions, ethenoadenosine and bi-imidazole, are simply due to different chemical structures and/or the altered H-bonding capability remains to be determined.

Experimental

General

NMR spectra were acquired at 30 °C in DMSO-d_6 solution (sample concentrations ≈ 2 mg/500 μl for ^{1}H, 100 mg/500 μl for ^{13}C and ^{15}N) on a JEOL Alpha 500 NMR spectrometer equipped with either a 5 mm normal-configuration tuneable probe or a 5 mm inverse *z*-axis field-gradient probe operating at 500.16 MHz for ^{1}H, 125.78 MHz for ^{13}C, and 50.69 MHz for ^{15}N. ^{1}H and ^{13}C spectra were referenced internally, δ 2.49 for ^{1}H (to DMSO-d_5) and δ 39.50 for ^{13}C (to DMSO-d_6); ^{15}N spectra were referenced externally to 90% nitromethane in CD_3NO_2 (0 ppm).

1D proton spectra were acquired with single-pulse excitation, 45° flip angle, pulse recycle time of 9.5 s and with spectral widths of 7 kHz consisting of 64 k data points (digital resolution 0.11 Hz/pt), zero-filled to 128 k prior to Fourier transformation. Saturation-transfer experiments were acquired using irradiation times of 5 s and with reduced resolution (0.22 Hz/pt).

1D carbon spectra were acquired with single-pulse excitation, 45° flip angle, pulse recycle time of 3.5 s and with spectral widths of 30 kHz consisting of 64 k data points (digital resolution 0.46 Hz/pt), zero-filled to 128 k and with 1 Hz exponential weighting applied prior to Fourier transformation. DEPT 135° spectra were acquired with similar spectral windows and with a pulse delay time of 3 s. Saturation-transfer experiments were acquired under similar conditions to the normal spectra and utilised irradiation times of 5 s. Field gradient 2D heteronuclear multiple quantum-filtered coherence (FG HMQC) and FG HMBC experiments were both acquired in magnitude mode with spectral widths and resolution appropriately optimised from the 1D spectra and processed with zero-filling (× 2, × 4), a π/(3–8)-shifted sinebell function, and exponential weighting (3–5 Hz, 5–25 Hz) applied in both dimensions prior to Fourier transformation. Both HMQC and HMBC spectra utilised a $^{1}J_{HC}$ coupling of 145 Hz, whilst the HMBC correlations were optimised for a long-range $^{n}J_{HC}$ coupling of 8 Hz.

1D nitrogen spectra with single-pulse excitation were acquired either with full coupling throughout or with inverse-gated decoupling; both experiments utilised a 60° flip angle, pulse recycle time of 9.3 s and with spectral widths of 25 kHz consisting of 32 k data points (digital resolution 0.76 Hz/pt),

¶ Comparison of the integrals of the H6 signals in **1** and **2** also supports the conclusion that N6 was not protonated.

zero-filled to 128 k and with 0.25 Hz exponential weighting applied prior to Fourier transformation. DEPT 45° and 135° and refocussed INEPT optimised on 2, 4, or 90 Hz spectra were acquired with similar spectral windows and with a pulse delay time of 3 s. FG HMQC and FG HMBC experiments were both acquired in magnitude mode with spectral widths and resolution appropriately optimised from the 1D spectra and processed with zero-filling (× 2, × 4), a π/4-shifted sinebell function, and exponential weighting (5–25 Hz, 25–125 Hz) applied in both dimensions prior to Fourier transformation. Both HMQC and HMBC spectra utilised a $^{1}J_{HN}$ coupling of 95 Hz, whilst the HMBC correlations were optimised for a long-range $^{n}J_{HN}$ coupling of 8 Hz.

5-Amino-4-(imidazol-2″-yl)-1-(β-D-ribofuranosyl)-1*H*-imidazole 1 and its protonated form 2

The structural characterisation of the bi-imidazole nucleoside has been described in detail previously.[6] In order to obtain both the neutral and the protonated forms of the bi-imidazole nucleoside, the method used for the preparation[6] was slightly modified. Ethenoadenosine (0.4470 g, 1.53 mmol) was heated for 10 hours at 70 °C in 100 ml of 0.05 M NaOH. After cooling to room temperature, the mixture was divided into two equal portions. One half of the mixture was concentrated to a volume of ≈10 ml by rotatory evaporation. Ethanol–diethyl ether (1 : 2 v/v) was added until the mixture became cloudy and it was stored in a refrigerator overnight. The product was collected by filtration, redissolved into a small amount of 0.01 M NaOH, precipitated by the addition of EtOH–Et_2O (1 : 2), collected by filtration, and dried in a vacuum desiccator over diphosphorus pentaoxide to yield **1** (0.1161 g, 53.8%) as a white powder.

The other half of the original reaction mixture was made slightly acidic (to pH 4) by the addition of dilute hydrochloric acid. The mixture was concentrated to a volume of ≈10 ml and the product was precipitated by the addition of EtOH–Et_2O (1 : 2) as described above. The product was redissolved into a small amount of water and the pH was adjusted to four by the addition of dil. HCl. The product was precipitated by the addition of EtOH–Et_2O (1 : 2), collected by filtration, and dried in a vacuum desiccator over diphosphorus pentaoxide to yield **2** (0.0984 g, 45.6%) as a white powder. ^{1}H, ^{13}C, and ^{15}N NMR for **1** and **2**, see Table 1; *m*/*z* (ESI) 282 (100%, MH^+), 150 (7%, MH^+ − ribosyl + H) [HRMS: Calc. for ($C_{11}H_{15}N_5O_4$ + H): *m*/*z*, 282.1202. Found: *m*/*z* 282.1202].[6]

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