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The Determination of the Ionization Constants of C-nucleosides

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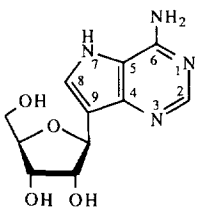
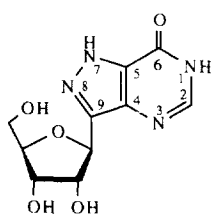
Abstract: We here report for the first time the ionization constant of 9-deazaadenosine (1) (pK_a 6.0) as well as the third ionization constant for Formycin B (2) (pK_a of 1.3) in addition to its two known pK_as of 8.8 and 10.4.

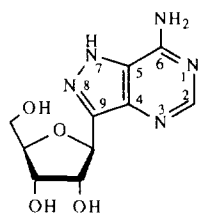
The C-nucleosides are found ubiquitously in various tRNAs[1], and their structural characteristic are distinguished by carbon-carbon bond linking the ribofuranosyl moiety to a heterocyclic base at the anomeric center in contradistinction to N-nucleosides (adenosine, guanosine, cytidine, uridine, ribothymidine and their 2'-deoxy counterparts), where carbon to nitrogen bond link the aglycone to the sugar. Many of these C-nucleosides are antibiotics and exhibit anticancer and/or antiviral activity[2]. The primary structure of a C-nucleoside is made of a purine or a pyrimidine aglycone which is covalently bonded from C9 of purine or C5 of pyrimidine to C1' of a D-ribopentofuranose in a β-configuration. While the aglycone moieties of the N-nucleosides are directly involved in carrying the genetic information and its propagation in the replication machinery by Watson-Crick or Hoogsteen hydrogen bonded base-pairing, very little is known on the physicochemical properties or the stereochemical role of C-nucleosides in biology in general, except for the fact that their presence in certain tRNAs is absolutely vital to the biochemical function[1].



(1)



(2)



(3)

A perusal of literature shows that the pK_as of C-nucleosides so far known are for formycin A (**3**) (pK_a 4.4 corresponding to protonation at N3, and 9.6 for deprotonation at N7)[3], formycin B (**2**) (pK_a 8.8 corresponding to deprotonation at N1, and 10.4 for deprotonation at N7)[4], pseudoisocytidine (pK_a 3.7

corresponding to protonation at N1, and 9.0 corresponding to deprotonation at N3)[5] and pseudouridine (pK_a 9.1 corresponding to mixed deprotonation at N1 and N3)[6].

We here report for the first time the pK_a of 9-deazaadenosine (**1**) (pK_a 6.0, σ=0.1 corresponding to protonation at N3). We have also found for the first time that formycin B (**2**) has a third pK_a of 1.3 corresponding to protonation at N3 in addition to the known pK_as of 8.8 and 10.4 for the deprotonation at N1 and N7, respectively[4].

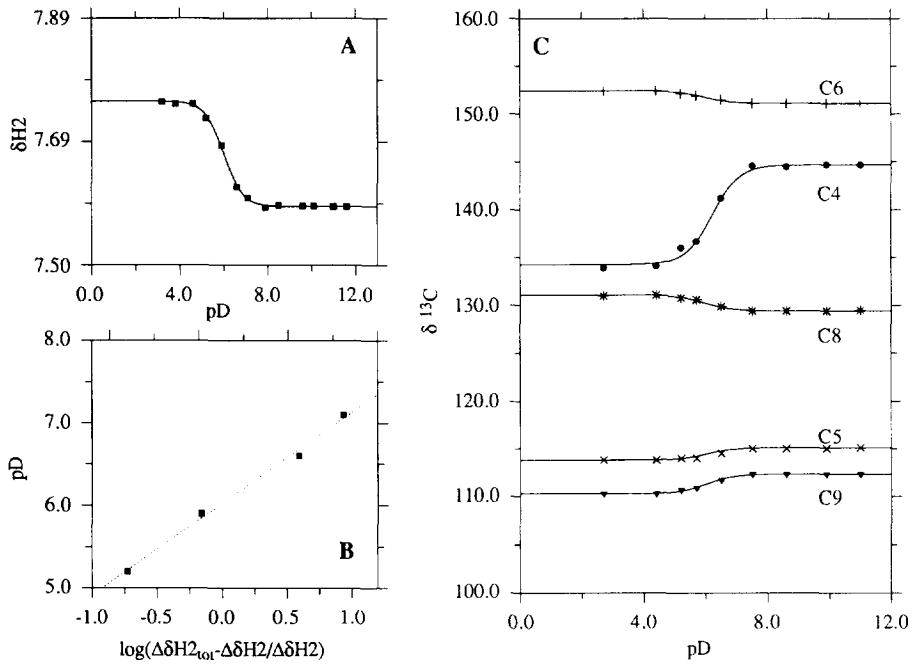


Fig. 1: Panel A is the evolution of proton chemical shift (H2) as a function of pD at 298K for 9-deazaadenosine (**1**), whereas Panel B is the Hill Plot derived from the titration curve, shown in the panel A, giving the pK_a at the intercept (see ref. 7 for details of the procedure). Panel C is the evolution of ^{13}C chemical shifts of aromatic carbons of 9-deazaadenosine (**1**) as a function of pD at 298K.

We have used pD-dependent aromatic proton (H2) chemical shift measurements for 9-deazaadenosine (Fig. 1A) and formycin B (**2**) (Fig. 2A) at 298K, and subsequently Hill plots[7] were used to extract their pK_as (Fig. 1B and 2B)). For formycin B (**2**), Monte Carlo fitting[8] has been used to obtain the ionization constant from the inflection point of the titration curves at the basic pH. The proton chemical shift change alone could not pinpoint the site of protonation or deprotonation in 9-deazaadenosine (**1**) and formycin B (**2**) because the probable number of sites for protonation or deprotonation in them were more than the number of aromatic protons available as markers for monitoring the evolution of the titration, and hence in those cases the measurements of pD-dependent ^{13}C chemical shift (heteronuclear HMBC experiment[9] with gradients) were used to determine the site of protonation or deprotonation (Fig. 1C and 2C). The pK_as obtained from the inflection points of these pD-dependent ^{13}C chemical shift studies are found to be the same as from the pD-dependent aromatic proton chemical shift measurements.

9-Dezaadenosine (**1**) potentially can be protonated either at N1 or N3. The fact that it is indeed protonated at N3 comes from the ^{13}C chemical shift titration profile (Fig. 1C), which shows that C4 is deshielded by 10.5 ppm compared to C6, which is only shielded by 1.3 ppm owing to the γ-effect. We could not have obtained the latter result if 9-deazaadenosine had the protonation site at N1. This is also fully consistent with the trend of the ^{13}C chemical shift change found for formycin A (**3**) where the N3 is the site of protonation, which was also evidenced by the largest deshielding of C4 (9.86 ppm) upon protonation[3].

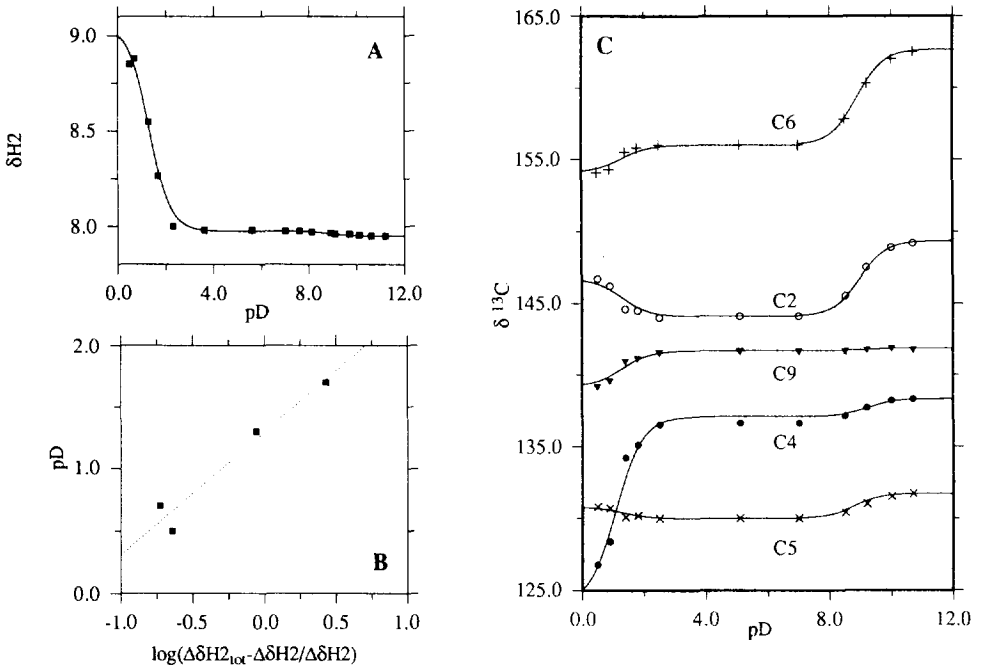


Fig. 2: Panel A is the evolution of proton chemical (H2) shift as a function of pD at 298K for formycin B (**2**), whereas Panel B is the Hill Plot derived from the titration curve, shown in the panel A, giving the pK_a at the intercept (see ref. 7 for details of the procedure). Panel C is the evolution of ^{13}C chemical shifts of aromatic carbons of for formycin B (**2**) as a function of pD at 298K.

Earlier, the ionization constant corresponding to deprotonation of formycin B (**2**) at N1 and N7 has been elucidated spectrophotometrically to be 8.8 and 10.4, respectively[4]. Our present proton chemical shift titration experiments (Fig. 2A) have however shown only two pK_as, one corresponding to deprotonation at N1, which is the same as the reported value (8.8), and the second pK_a of 1.3 (σ=0.1) corresponds to the protonation at N3; both of these sites have also been confirmed by ^{13}C chemical shift titration profile (Fig. 2C). This shows that C4 is shifted downfield by 13 ppm for protonation at N3 in the acidic medium in a very similar manner as those of 9-deazaadenosine (**1**) and formycin A (**3**), whereas the deprotonation at N1 at the alkaline pH is evident from the deshielding of C6 and C2 by 6.7 and 5.3 ppm, respectively. In our ^{1}H or ^{13}C chemical shift titration experiment, the chemical shift comes to a plateau after pH 10 (Figs 2A and 2C), hence the present study can not confirm the pK_a of 10.4 obtained by spectrophotometric titration[4].

As a minor supplement to this work, we have confirmed the ionization constants of pseudoisocytidine (pK_a 3.6, σ=0.2 corresponding to protonation at N1, and 9.0 corresponding to deprotonation at N3)[5], pseudouridine (pK_a 9.1, σ=0.3 corresponding to mixed deprotonation at N1 and N3)[6] and 1-methylpseudouridine (pK_a 9.7, σ=0.1 corresponding to deprotonation at N3), using the pD-dependent evolution of the proton chemical shifts at 298K.

Further work is in progress to determine the site of metal ion binding to the aglycone of C-nucleosides, and if the site of binding is determined by the hardness or softness of the metal ion or/and by the pK_a of the protonation or the deprotonation site of the C-aglycone. Work is also in progress to understand how the change of aromatic character of the C-aglycone, depending upon the protonation $\rightleftarrows$ deprotonation equilibrium or by the ligand (peptide or metal ion) complexation, drives the North [N (C3'-*endo*-C2'-*exo*)] $\rightleftarrows$ South [S (C2'-*endo*-C3'-*exo*)] pseudorotational equilibrium.

Experimentals

The NMR spectra were measured at 500 MHz in D_2O (99.9%) (4.5 mM for **1** and 10.0 mM for **2**) at 298K.The pK_a values for **1** and **2** were determined through Hill plots (ref. 7 for details). The ^{13}C chemical shifts were measured by Heteronuclear Multibond Correlation (HMBC) Experiment (ref. 9) at 125.76 MHz with Z-gradients with the delay of a multibond ^{13}C filter of 100ms, relaxation delay of 4s and datapoints of 512 in F1 and 2K in F2 dimensions, F1 dimension was subsequently zerofilled to 2K.

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