NMR Quantification of Tautomeric Populations in Biogenic Purine Bases

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NMR Quantification of Tautomeric Populations in Biogenic Purine Bases

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Purine bases such as purine, adenine, hypoxanthine, and mercaptopurine are known to exist in several tautomeric forms. Characterization of their tautomeric equilibria is important not only for predicting the regioselectivity of their *N*-alkylation reactions, but also for gaining knowledge of the patterns with which these compounds of significant biological activity form hydrogen bonds with their biological targets. The tautomeric equilibria of purine and some purine derivatives in methanol and *N*,*N*-dimethylformamide solutions were investigated by low-temperature ^{1}H and ^{13}C NMR spectroscopy. The N(7)H and N(9)H tautomeric forms were quantified by integrating the individual ^{1}H NMR signals at low temperatures. The Gibbs free energy differences were calculated and the effects of substitution on the N(7)H/N(9)H ratio discussed. A previously published theoretically predicted mechanism of the tautomeric exchange is compared with our measurements in deuteriated solvents. The influence of concentration on the temperature of coalescence indicates that supramolecular clusters play a significant role in this proton transfer process.

Introduction

The characterization of purine bases and the highlighting of the significant role they play in living systems has attracted the attention of many scientific teams and has been an important stimulus for intensive investigations in this field. Purines are not only basic constituents of nucleic acids, but they also interact with enzymes and other proteins as components of cofactors and signal molecules. Adenosine 5′-triphosphate (ATP) controls the energy metabolism of every cell and nicotinamide adenine dinucleotide (NAD^+, NADH) and flavin adenine dinucleotide (FAD) are key coenzymes of the cellular citric acid cycle that is involved in cellular oxidation/reduction processes. Another molecule of biological relevance is acetyl-coenzyme A, which is of central importance for metabolism.[1] Naturally occurring purines have been excellently reviewed by Rosemeyer.[2] Purine derivatives bearing diverse types of substituents display a broad spectrum of biological activities, including an interferon inducing effect or more often an inhibitory effect against leukotrene A_4 hydrolase, sulfotransferase, phosphodiesterase, kinase, and other enzymes.[3] Furthermore, a number of purine derivatives currently find application in medicine and are used therapeutically as antiviral, anticancer, and antiasthmatic agents.[4]

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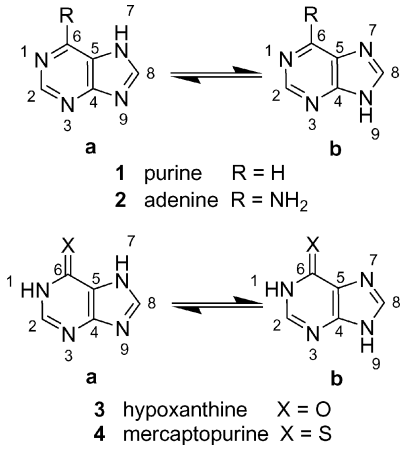
Although a broad range of biological activities of purine derivatives has been reported, all the facts dealing with their structure and their tautomeric equilibria have been neither well described nor completely understood. Purine (**1**), the fundamental representative of this group, is an aromatic compound consisting of five carbon and four nitrogen atoms. Due to the character of the purine skeleton, four prototropic tautomers of purine base can coexist. The structures of the particular tautomers are defined by the different positions of the hydrogen atom on the nitrogen atoms of the imidazole or pyrimidine ring. The tautomers are denoted as N(1)H, N(3)H, N(7)H, and N(9)H purine.[5] The structure and tautomeric equilibria of isolated nucleic acid bases and other purine derivatives have been extensively investigated by both theoretical and experimental approaches. Knowledge of their tautomeric equilibria is of great importance because this phenomenon affects the behavior of these compounds, from their chemical and spectral properties to their biological activities.[6]

According to quantum chemical calculations[7] and experimental IR, UV,[8] and NMR studies,[9] N(7)H and N(9)H represent the favored tautomers of purine derivatives (Scheme 1) as compared with the N(1)H and N(3)H forms. The relative populations of the individual tautomers are generally influenced by the substitution of the purine ring, the solvent, and the temperature. Biological effects are linked to the ability of the purine derivative to form supramolecular complexes with biological targets. Weak intermolecular interactions, especially hydrogen-bonding, play a crucial role in the formation of such complexes. The ability of purines to form hydrogen bonds and the regioselectivity

of the alkylation and glycosidation reactions are influenced by their tautomeric equilibria and by the distribution of electrons around the purine skeleton.[10] Moreover, several hypotheses claim that there could be a connection between purine tautomerism and the spontaneous mutation of DNA.[11]



Scheme 1. Schematic representation of the tautomeric equilibria for compounds **1**–**4**.

The aim of this study was to directly determine the ratio between the tautomeric forms of purine and its 6-substituted biogenetic analogues and to investigate the effect of the substitution and the solvent on the tautomeric populations. Although these biogenetic bases were investigated by ^{13}C NMR spectroscopy as early as 1975,[12] no direct experimental solution-state study of the tautomeric ratios of these compounds has been published. We recently reported a preliminary low-temperature ^{1}H NMR study of three representatives of 6-substituted purines.[9a] In this contribution we report the low-temperature NMR study of biologically important purine bases **1**–**4** in various solvents and compare the results obtained with the previously published findings.

Purine (**1**) represents the basic compound of this group, whereas adenine (**2**), hypoxanthine (**3**), and mercaptopurine (**4**) all bear substituents at the 6-position (Scheme 1). In addition to the N(7)H/N(9)H tautomeric equilibrium, the amino/imino (for **2**), hydroxy/keto (for **3**), and thiol/thione (for **4**) phenomena must also be considered. However, it has been known for a long time that the amino form of adenine,[12,13] the keto form of hypoxanthine,[14] and the thione form of mercaptopurine[12,15] are the predominant tautomeric forms that exist in solution.

Low-temperature NMR spectroscopy represents an important tool for investigating tautomeric equilibria. Applications of this method have been reviewed and used many times in investigations of tautomeric phenomena.[16] Recently it has been shown that isolated signals for the individual tautomers of substituted purines,[9] purine analogues,[17] and hydrogen-bonded clusters[10,18] can be detected at low temperatures. Typically, a rapid chemical exchange between the individual tautomeric forms occurs at laboratory temperatures, which results in NMR signals that are time-averaged and population-weighted. Cooling the system to lower the temperature slows down this chemical exchange process. This slower exchange process results in a spectrum in which the signals of the individual tautomers are resolved.

In this study, compounds **1**–**4** were investigated in [D_7]-DMF and [D_4]methanol, permitting the use of temperatures as low as roughly 210 and 180 K, respectively. In most cases, the solubilities of compounds **1**–**4** were sufficient for such NMR analyses, including the 2D ^{1}H-^{13}C chemical shift correlation.

Results and Discussion

The tautomeric equilibria in a group of purine bases (compounds **1**–**4**) were studied by using low-temperature NMR spectroscopy. *N*,*N*-Dimethylformamide (DMF) was used because of its good ability to dissolve this class of compounds and its sufficiently low freezing point (about 213 K), which can be further reduced, for example, by adding a few drops of [D_6]acetone. Methanol has somewhat poorer dissolving abilities than DMF, but it allows experimental temperatures as low as 175 K to be reached.

A typical temperature dependence of the ^{1}H NMR spectra for **1** in both [D_7]DMF and [D_4]methanol was observed. At laboratory temperature, one set of sharp time-averaged signals corresponding to the population-weighted contributions of the individual tautomers was recorded. When the system was cooled down, the signals broadened and at a sufficiently low temperature two sets of separated signals, belonging to the N(7)H (**a**) and N(9)H (**b**) tautomeric forms, were observed. An example of the temperature dependence of the ^{1}H NMR spectra for compound **1** (in [D_4]methanol) is shown in Figure 1.

The assignment of the ^{1}H resonances (Table 1) to the individual atoms of the two tautomers was accomplished on the basis of the ^{13}C NMR spectra and 2D ^{1}H-^{13}C shift correlation experiments and is discussed in the following paragraphs. The ^{13}C NMR spectra and 2D ^{1}H-^{13}C correlation experiments were also of central importance in assigning the particular sets of signals to the individual tautomers. It is known that the chemical shift of C-5 of the purine ring resonates in the range of approximately 110–140 ppm and that this chemical shift is significantly higher (8–12 ppm) for the N(9)H form than for the N(7)H form.[12,19] In contrast, the chemical shift of C-4 is typically located in the range of 145–165 ppm,[19] and the C-4 resonance of the N(9)H form is shielded (8–12 ppm) relative to that of the related N(7)H form. Similar tendencies have been observed for the chemical shifts of C-6 and C-8.[12,19] The ^{13}C NMR chemical shifts measured for compounds **1**–**4** are summarized in Table 2. Two sets of ^{13}C NMR signals were obtained for compounds **1**–**4** at low temperatures. However, due to the low solubility of **4** and the resultant insufficient concentration of **4b**, ^{13}C NMR signals of this, the minor component, were not observed in [D_4]methanol.

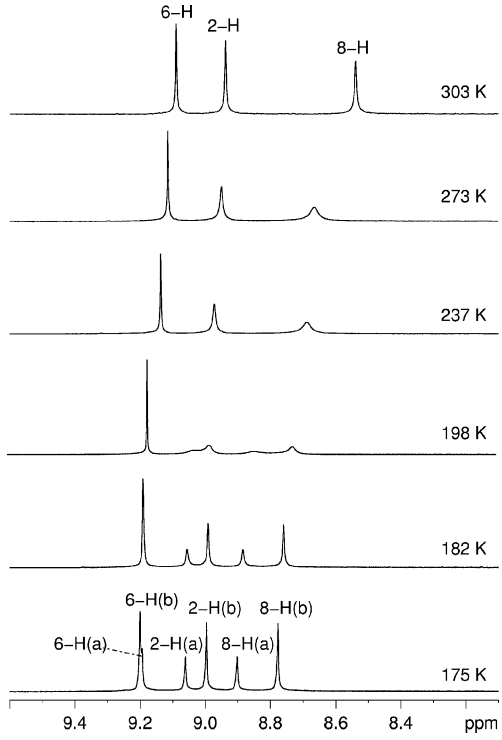


Figure 1. Temperature dependence of the ^{1}H NMR spectra for compound **1** in [D$_4$]methanol (17 mmol L^{-1}), showing the N(7)H (**a**) and N(9)H (**b**) tautomeric forms at low temperatures.

The assignment of structures was further confirmed by analysis of the ^{1}H-^{13}C coupling constants. It has been recognized,[19,20] that the value of $^3J_{H,C}$ for the HCNC fragment is greatly reduced by changing the =N– type of nitrogen to –NH–.[19] The values of the $^3J_{H8,C4}$ coupling constants measured for the major and minor tautomers **1b** and **1a** in [D$_4$]methanol are 5.3 and 12.6 Hz, respectively; the values of $^3J_{H8,C4}$ for the major and minor tautomers **2b** and **2a** are 5.4 and 12.1 Hz, respectively. Analogously, the values of the $^3J_{H8,C5}$ coupling constants for **1b** and **1a** in [D$_4$]methanol are 10.9 and 4.3 Hz, respectively, with $^3J_{H8,C5}$ = 10.5 for **2b** and $^3J_{H8,C5}$ = 4.6 Hz for **2a**. A representative portion of the ^{1}H-^{13}C GSQMBC spectrum of **1** used for extracting the ^{1}H-^{13}C coupling constants is shown in Figure 2. For hypoxanthine (**3**), the trends for the ^{1}H-^{13}C coupling constants $^3J_{H8,C5}$ and $^3J_{H8,C4}$ in the HCNC fragments are analogous to those discussed for **1** and **2**.

Compounds **1**, **3**, and **4** provided two sets of ^{1}H NMR signals (Table 1) at low temperatures in both [D$_7$]DMF and [D$_4$]methanol. In contrast, for compound **2**, two distinct sets of ^{1}H NMR signals were observed only in [D$_4$]methanol. No significant changes in the ^{1}H NMR patterns were observed on cooling the [D$_7$]DMF solution down to 213 K.

The populations of the N(7)H tautomeric forms (**a**) in compounds **1**–**4** presented in Table 3 were determined directly by integrating the ^{1}H NMR signals obtained at low temperatures. The relative concentrations of **a** and **b** were used to calculate the equilibrium constant $K_{7/9}$ = [N(7)H]/[N(9)H]. For example, the content of the N(7)H form in purine **1** was determined to be 33% in [D$_7$]DMF and 37% in [D$_4$]methanol at 206 K.

The Gibbs free energy difference ($\Delta G°$) between the tautomeric forms was calculated from the equilibrium constant $K_{7/9}$ by using Equation (1), where R is the gas constant and T is the absolute temperature. Again, by using **1a** as the example, the populations in [D$_7$]DMF (38%) and [D$_4$]methanol (41%) were subsequently calculated for a temperature of 303 K (Table 3). The signals obtained at several low temperatures were integrated and used to calculate the Gibbs free energy differences. The populations of **1a** were then calculated for the temperature 303 K. The differences in the calculated populations were comparable to the limit of the integration error (about 1%), and the entropic factor was therefore neglected.

$$\Delta G^0 = -RT\ln K_{7/9} \quad (1)$$

An analogous procedure was applied to investigate compounds **2**–**4**. The differences in free energy ($\Delta G°$) between the N(7)H and the N(9)H tautomeric forms are summarized in Table 3. According to our definition, the energy difference is positive when the N(9)H form is favored (as

Table 1. ^{1}H NMR chemical shifts for purine (**1**) and purine derivatives **2**–**4**.

	δ [ppm]					T [K], solvent
	2-H	6-H	8-H	7-H/9-H	1-H	
1a	9.16	9.42	9.14	14.37	–[a]	179, [D$_7$]DMF
1b	9.10	9.38	9.02	14.42	–[a]	179, [D$_7$]DMF
1a	9.06	9.18	8.91	–[b]	–[a]	175, [D$_4$]methanol
1b	8.99	9.19	8.78	–[b]	–[a]	175, [D$_4$]methanol
2a	8.26	–[a]	8.42	–[b]	–[a]	182, [D$_4$]methanol
2b	8.18	–[a]	8.29	–[b]	–[a]	182, [D$_4$]methanol
3a	8.39	–[a]	8.65	14.33	12.98	206, [D$_7$]DMF
3b	8.38	–[a]	8.42	13.93	12.90	206, [D$_7$]DMF
3a	8.23	–[a]	8.43	–[b]	–[b]	182, [D$_4$]methanol
3b	8.15	–[a]	8.18	–[b]	–[b]	182, [D$_4$]methanol
4a	8.69[c]	–[a]	8.94	14.57[c]	14.57[c]	199, [D$_7$]DMF
4b	8.64	–[a]	8.69 [c]	14.40	14.47	199, [D$_7$]DMF
4a	8.27	–[a]	8.54	–[b]	–[b]	187, [D$_4$]methanol
4b	8.21	–[a]	8.35	–[b]	–[b]	187, [D$_4$]methanol

[a] No proton at this position. [b] Not observed; the corresponding proton was replaced by deuterium from the solvent. [c] Overlapping signals.

Table 2. ^{13}C NMR shifts for purine (**1**) and purine derivatives **2**–**4**.

	δ [ppm]					T [K], solvent
	C-2	C-4	C-5	C-6	C-8	
1a	153.35[a]	161.30	125.77	143.06	148.69	198, [D_7]DMF
1b	153.35[a]	153.35[a]	135.51	148.69	146.77	198, [D_7]DMF
1a[c]	153.04	160.30	125.85	143.16	149.97	175, [D_4]methanol
1b[c]	153.26	153.42	133.65	147.86	147.41	175, [D_4]methanol
2a	_[d]	158.3[c]	111.41	153.65	144.69	213, [D_4]methanol
2b	153.65	150.55	119.35	157.18	141.16	213, [D_4]methanol
3a[e]	145.1	158.0	116.1	154.8	142.9	213, [D_7]DMF
3b[e]	146.3	149.9	124.5	157.7	139.4	213, [D_7]DMF
3a[c]	_[b]	157.2	116.5	156.0	_[b]	175, [D_4]methanol
3b[c]	_[b]	150.5	124.0	159.0	_[b]	175, [D_4]methanol
4a	145.90	153.13	128.21	170.93	146.81	204, [D_7]DMF
4b	146.22[f]	146.04	135.82	176.73	142.71[f]	204, [D_7]DMF
4a	145.80[g]	151.70	128.83	172.89	146.42[g]	187, [D_4]methanol
4b	_[d]	_[d]	_[d]	_[d]	_[d]	187, [D_4]methanol

[a] Overlapping signals. [b] Not detected. [c] Values determined from the ^1H-^{13}C GSQMBC spectra. [d] Not detected due to the low concentration of the minor form. [e] Values determined from the ^1H-^{13}C gs-HMBC spectra. [f] Signals could be interchanged. [g] Signals could be interchanged.

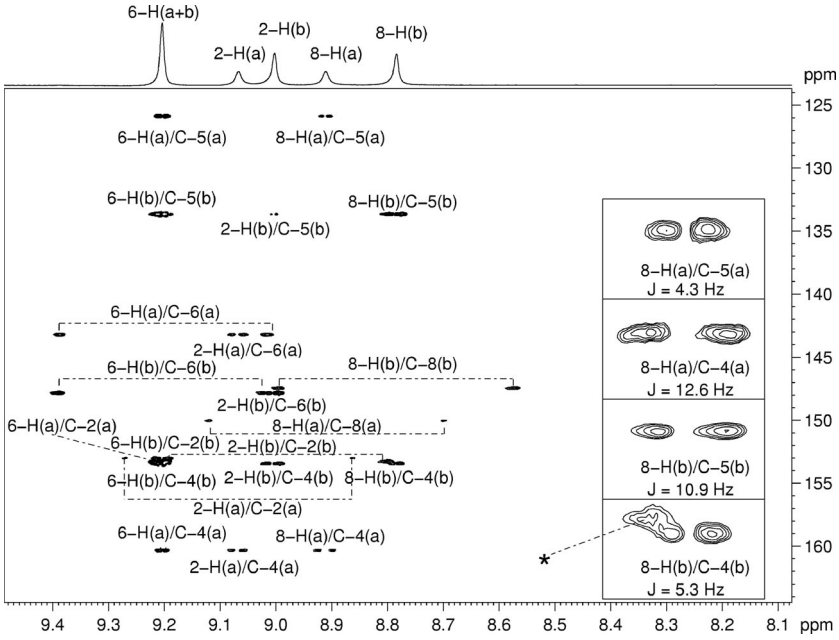


Figure 2. A portion of the ^1H-^{13}C GSQMBC spectrum of compound **1** measured in [D_4]methanol at 175 K with additional details of the 8-H/C-4 and 8-H/C-5 correlation signals for both the N(7)H (**a**) and the N(9)H (**b**) tautomeric forms. The asterisk indicates partial overlap of the 8-H(b)/C-4(b) with the 2-H(b)/C-2(b) correlation signal.

Table 3. Populations of the N(7)H tautomeric forms (**a**) of compounds **1**–**4** at low temperature and at 303 K, the energy difference between the N(7)H and the N(9)H tautomers ($\Delta G°$), and the populations of **a** as determined previously by analysis of the ^{13}C NMR chemical shift.[a]

	Solvent	Population of **a** [%] (temperature)	$\Delta G°$ [kJ mol^{-1}]	Population of **a** at303 K [%]	Population of **a** at 310 K in DMSO[a] [%]
1	[D_4]methanol	37 (206 K)	0.9	41	39–40
1	[D_7]DMF	33 (206 K)	1.2	38	39–40
2	[D_4]methanol	12 (213 K)	3.5	21	14–15
3	[D_4]methanol	54 (204 K)	–0.3	53	58
3	[D_7]DMF	52 (206 K)	–0.1	51	58
4	[D_4]methanol	92 (206 K)	–4.2	84	79
4	[D_7]DMF	90 (206 K)	–3.8	82	79

[a] See ref.[12].

for **1** and **2**) and the energy difference is negative if the N(7)H form predominates (compounds **3** and **4**). The absolute values of $\Delta G°$ were found in the range between 0.3 and 4.2 kJ mol^{-1}.

The populations of **1a** were determined to be 41 and 38% in [D_4]methanol and [D_7]DMF at 303 K, respectively. The 6-amino substituent (see adenine, **2**) reduces the percentage of the N(7)H form in [D_4]methanol to 21%. The presence of an oxygen or sulfur atom at the 6-position significantly influences the electron distribution, and the N(7)H forms are preferred for **3** and **4**. Whereas the N(7)H and N(9)H forms are almost equally populated for hypoxanthine (**3**), the N(7)H form of mercaptopurine (**4**) strongly predominates in both solvents (84 and 82% in [D_4]methanol and [D_7]DMF, respectively). Interaction of the hydrogen at N-7 with the lone pair of electrons of the oxygen atom in the C=O functionality (compound **3**) or with those at sulfur in the C=S fragment (compound **4**) is assumed to play a significant role in stabilizing the N(7)H form.

The individual tautomeric forms are stabilized differently in different environments. It is known that the N(7)H forms of the nucleic acid bases adenine and guanine are characterized by large dipole moments and should therefore be stable in polar media.[13] Thus, the value of the dipole moment, μ, for N(9)H adenine calculated by using B3LYP/6-31G* is 2.45 D and for N(7)H adenine it is 6.74 D. For comparison, a value of 4.09 D was calculated for the N(3)H form of adenine by using the same theoretical approach.[13a] The effect of the solvent on the percentage of the N(7)H form is apparent from Table 3. It is clear that the N(7)H forms are slightly more stable in [D_4]methanol than in [D_7]DMF. However, this relative stability is quite weak and the differences between the two solvents investigated are only in the order of 2–3% at a temperature of 303 K. The higher content of form **a** observed in the more polar methanol corresponds to the general assumption based on the dipole moments and on the results of previously published theoretical studies.[13a]

The tautomeric populations reported in this work agree relatively well with those determined previously by ^{13}C NMR spectroscopy utilizing *N*-substituted model compounds.[12] Despite a difference in the solvent applied, there is excellent agreement between the results obtained with purine (**1**; 38% for DMF, 41% for methanol, and 39–40% for DMSO[12]). However, the differences become larger for the other bases (3–7%). The largest difference observed between values for solvents of similar type (DMF vs. DMSO) approached 7% in the case of hypoxanthine (see Table 3).

Another issue connected with the phenomenon of tautomerism is the mechanism of the N(9)H/N(7)H exchange process, which has previously been widely investigated and reported.[21] The overwhelming majority of the reports describe the results of theoretical calculations performed at various levels of theory. Two recent studies[21] investigated theoretically the mechanisms for the N(9)H/N(7)H tautomeric process in a vacuum and in water solution. In the proposed solvent-mediated pathway,[21a] N(9)H purine is initially protonated at N-7 by a proton from the protic solvent (water). The intermediate subsequently loses a proton from C-8. In the following step, a proton is removed from N-9 and a proton from the solvent attaches to C-8, producing the N(7)H form. If the proton 8-H can exchange with a proton of the solvent or with one of the N(7)H or N(9)H groups, it should also exchange with a deuteron from deuterated solvent such as D_2O or [D_4]methanol. To prove or disprove the published mechanism involving 8-H exchange, all the compounds were measured in [D_4]methanol. Whereas protons N(7)H, N(9)H, and N(1)H were exchanged for deuterium and were invisible in the ^{1}H NMR spectra, the signal intensity of the proton 8-H remained unaltered. Similarly, the ^{1}H NMR signals of all the other C–H groups neither disappeared nor diminished in intensity due to deuterium replacement, as demonstrated for **1** in Figure 1. Different mechanisms for the tautomeric exchange must therefore be designed.

It may be speculated that the reaction mechanism could incorporate the formation of a purine anion by the removal of an N(7)H or N(9)H proton by the solvent, followed by a topological rearrangement and a proton jumping from the solvent to another position of the purine skeleton. In this case the solvent would be expected to play an important role with the sample concentration having no effect. Alternatively, the mechanism of the tautomeric exchange could include the formation of clusters of two or more purine molecules, enabling synchronous or asynchronous proton transfer. To evaluate the possible role of supramolecular clusters, the effect of sample concentration on the temperature of coalescence in the ^{1}H NMR spectra was investigated. Splitting of the ^{1}H NMR signals was generally observed for lower concentrations at higher temperatures as compared to the more concentrated samples. For example, the ^{1}H NMR spectra (T = 193 K) of four different samples of compound **1** at concentrations ranging between 0.003 and 0.344 mol L^{-1} in [D_4]methanol are shown in Figure 3.

Whereas at higher sample concentrations the ^{1}H NMR spectra indicate signal broadening, at lower concentrations they clearly show resolved signals, albeit still somewhat broadened. These results unequivocally demonstrate a change in the half-life of the individual tautomers induced by altering the sample concentration. It follows that either direct or solvent-mediated contacts between two or more purine molecules are crucial for the dynamics of the exchange process. It should be noted explicitly at this point that the sample concentration has no detectable effect on the equilibrium constant, as confirmed by determining the value of $K_{7/9}$ at various concentrations.

However, for characterizing the transition states and the reaction intermediates, the situation is somewhat complicated by the fact that various types of complexes[22] that show different levels of stability in various solvents can be born, as shown by recently published data for related structures.[23] According to theoretical calculations, hydrogen-bonded structures of nucleic acid bases are most stable in chloroform, T-shaped structures are preferred in dimethyl sulfoxide, and stacked structures predominate in methanol. Along the same line, the deshielding of the aromatic pro-

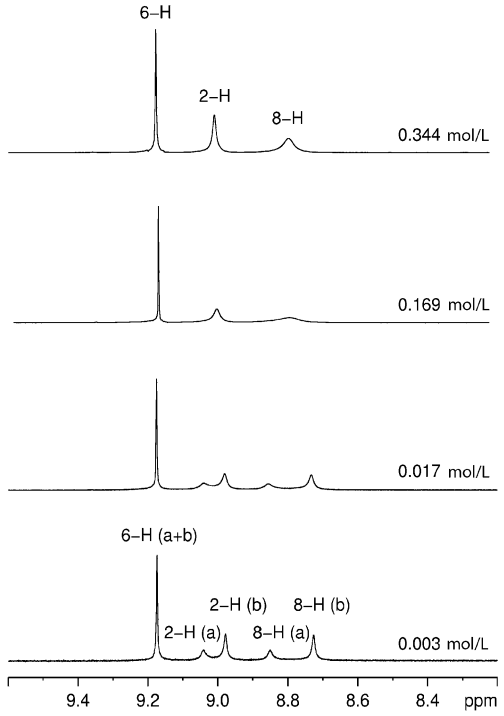


Figure 3. Portions of the ^{1}H NMR spectra for compound **1** at various concentrations in [D$_4$]methanol (T = 193 K), showing the N(7)H (**a**) and N(9)H (**b**) tautomeric forms.

tons observed upon cooling the methanol solutions employed in this study (see Figure 1) may indicate that both the stability and the population of stacked structures increase as the temperature is lowered. A higher population of stacked structures could induce additional deshielding of the aromatic protons caused by the magnetic properties of neighboring aromatic rings. Clearly, characterization of the topology of the supramolecular clusters presumably involved in these tautomeric exchange processes deserves further study.

Conclusions

Populations of the N(7)H and N(9)H tautomeric forms of purine, adenine, hypoxanthine, and mercaptopurine have been quantitatively evaluated by using low-temperature NMR spectroscopy. The populations were determined to be significantly affected by substitution at the 6-position. However, they were affected only very slightly by the nature of the solvent (methanol vs. dimethylformamide). The percentage of the N(7)H form was somewhat higher in methanol than in DMF. A previously published theoretical mechanism as the dominant reaction pathway for the tautomeric process[21] has been disproved by measurements made in [D$_4$]methanol. The role of the solvent was investigated in a series of experiments at various sample concentrations, and it was found that the temperature of coalescence rose in the case of less concentrated samples. This indicates that there is a relationship between the reaction mechanism and the self-association of purine molecules that results in the formation of supramolecular clusters. However, the topology and dynamic behavior of these clusters deserve further detailed investigation.

Experimental Section

General: Compounds **1**–**4** were obtained commercially. All 1D and 2D NMR spectra were recorded by using a Bruker Avance 500 spectrometer operating at frequencies of 500.13 (^{1}H) and 125.77 MHz (^{13}C). The NMR samples were prepared by dissolving purine bases **1**–**4** (2–5 mg) in [D$_4$]methanol or [D$_7$]DMF (0.55 mL). [D$_6$]Acetone (3 drops) was added to the DMF solutions to lower the freezing point of the solutions and to increase the quality of the spectra recorded at low temperatures. The NMR spectra were recorded in the range of 303–175 (methanol) or 303–210 K (DMF) with the exact values indicated in the text and in Tables 1 and 2. The thermocouple of the NMR probehead was calibrated by using a methanol sample.[24]

The ^{1}H and ^{13}C NMR chemical shifts (δ in ppm) are referenced to the signal of tetramethylsilane (TMS), which was used as an internal standard. A 5 mm QNP [^{13}C/^{19}F/^{31}P{^{1}H}] or a 5-mm multinuclear inverse BBI [^{1}H{BB}] probe with a self-shielded z-gradient coil was used to measure the ^{1}H, ^{13}C, and heteronuclear shift correlation spectra.

The following parameters were used in the recording of the NMR spectra. ^{1}H NMR spectra: pulse 90°, relaxation delay 5–10 s, number of scans 4–16, resolution <0.005 ppm per point. ^{13}C NMR spectra: pulse 45°, relaxation 3 s, number of scans 8192–16384, resolution ca. 0.02 ppm per point. The ^{1}H-^{13}C GSQMBC[25] and gs-HMBC[26] NMR experiments were adjusted for long-range couplings of 8–10 Hz. Computer processing was performed with Bruker TopSpin software. Indirect spin–spin coupling constants (reported in Hz) were determined with an accuracy of ±0.6 Hz for $^nJ_{H–C}$.

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