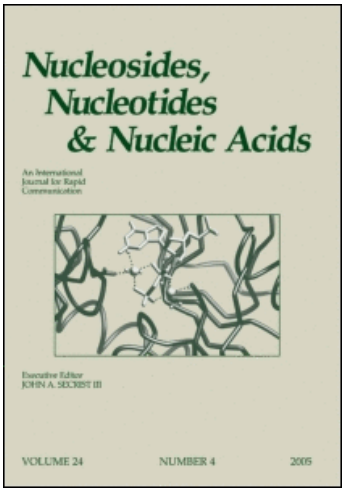

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TAUTOMERISM, PROTONATION, AND IONIZATION OF FORMYCIN IN AQUEOUS SOLUTION BY THE pH DEPENDENCE OF ^{13}C CHEMICAL SHIFTS AND ^{13}C-^{1}H COUPLING CONSTANTS§

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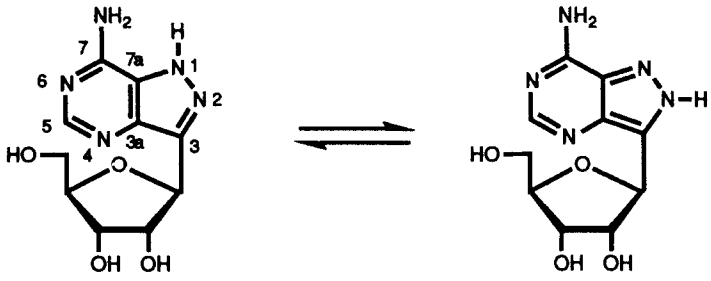
Abstract: *Analyses of the pH dependence of ^{13}C chemical shifts and ^{13}C-^{1}H coupling constants of formycin in aqueous solution revealed two pKa's, at 4.4 and 9.7, corresponding to a protonation at N_4 and an ionization at N_1. The N_4-protonation results in the transfer of a pyrazolo ring hydrogen from N_1 to N_2. At physiological pH, formycin was found to exist as a mixture of N_1H and N_2H tautomers, with the former being predominant (>94%).*

INTRODUCTION

Formycin is a naturally occurring C-nucleoside analog of adenosine.[1] The presence of a pyrazolo ring in formycin results in a N_1H-N_2H prototropic tautomerism (Figure 1) and the syn conformation about the glycosyl bond, both of which are thought to be important structural features for understanding its wide range of interesting pharmacological activities in various enzymatic systems.[2,3]

Consequently, the structure of formycin has been studied by a variety of experimental techniques.[4-15] Spectroscopic[4-7] and X-ray crystallographic[11] data, as well as NMR[8-12] and theoretical calculations,[16,17] have consistently suggested that the neutral form of formycin exists as a mixture of N_1H and N_2H tautomers, with the former being more abundant. However, this tautomeric equilibrium can be disturbed by protonation, further complicating the situation.[5-7] To date, experimental[2,7] and theoretical[3,17] evidence predicted protonation to

§This paper is dedicated to the memory of Dr. R. K. Robins.



N_1H Tautomer **N_2H Tautomer**

FIG. 1. Structure and the main tautomeric form of formycin

occur on one of the pyrimidine nitrogens, i.e., either the N_4 or N_6. Conflicting X-ray crystallographic results, however, have been reported for the specific site of protonation. Thus, while the hydrochloride salts of 3'-deoxyformycin[12] and 2',3'-dideoxyformycin,[13] and formycin 5'-monophosphate[18] were found to be protonated at N_4 with the pyrazolo ring hydrogen at N_1 (i.e., the N_4-protonated N_1H tautomer), Koyama et al.[15] have reported a protonation at N_6 for formycin hydrobromide with the migration of the pyrazolo ring hydrogen atom from N_1 to N_2 (i.e., the N_6-protonated N_2H tautomer). In aqueous solution, the latter form was detected by luminescence studies as the major tautomeric species, along with minor amounts of the N_4-protonated N_1H and N_2H tautomers.[5] A recent theoretical study, however, suggested the N_4-protonated N_2H tautomer as the most stable tautomeric form of formycin.[17]

Previous NMR studies regarding the tautomerism of formycin have been limited to non-aqueous media.[8-10] Earlier ^{13}C NMR studies[8,9] reported severe line broadenings of quaternary base carbons of formycin and their temperature dependence, which were taken as evidence for the presence of N_1H-N_2H prototropic tautomerism. In a detailed ^{13}C NMR study, Chenon et al.[10] have shown, using the temperature-dependent ^{13}C chemical shifts in DMSO and HMPT, that the population of the N_1H and N_2H tautomers exist in a ratio of ~85:15. Similar ^{13}C NMR structural studies of formycin in aqueous solution, however, have not been reported.

The biological importance of tautomerism in aqueous solution,[2] coupled with the excellent water solubility (~0.1 g/mL) of formycin, prompted us to

examine its tautomeric equilibria by pH-dependent ^{13}C NMR spectroscopy. In this communication, a series of ^{13}C NMR spectra of formycin have been recorded as a function of pH. The effect of pH on the ^{13}C shifts and ^{13}C-^{1}H coupling constants associated with the base carbons of formycin was analyzed in order to probe the sites of protonation and ionization, and to determine the relative N_1H-N_2H prototropic population at physiological conditions.

EXPERIMENTAL SECTION

Formycin was a generous gift from Dr. R. P. Panzica. All ^{1}H and ^{13}C NMR spectra were recorded at ambient temperature on a Bruker AM300 NMR spectrometer, operating at 300 and 75.5 MHz, respectively. The ^{1}H NMR chemical shifts in D_2O were reported in ppm vs DSS. For ^{13}C NMR measurements in DMSO-d_6, the highest intensity peak (at 39.5 ppm) was used as an internal reference. For pH-dependent ^{13}C NMR measurements, aqueous samples (~50 mg/mL) in 10 mm sample tubes were prepared in 2.0 mL of deionized H_2O, and the appropriate pH values were obtained by addition of dilute NaOH or HCl and measured with a pH meter. A coaxial capillary containing dioxane in D_2O was used as a deuterium lock and as an external reference at 66.5 ppm. ^{1}H-coupled ^{13}C NMR spectra were measured with gated decoupling with full NOE. Typical conditions were: flip angle, 60-80°; data size, 32K; spectral width, 20 KHz; and recycle time, 1.8s. For coupling constant measurements, the free induction decays were zero-filled to give a digital resolution of 0.61 Hz and processed with Lorentzian to Gaussian filtering using Bruker parameters of -1 Hz and 0.17.

RESULTS AND DISCUSSION

Assignments of ^{13}C Chemical Shifts. Unambiguous ^{13}C chemical shift assignments of the base carbons of formycin in D_2O have been made through ^{1}H-coupling and selective ^{1}H-decoupling experiments. The assigned ^{13}C chemical shift and coupling constant information of formycin in various media, including those in D_2O, are given in Table 1.

The carbon signal at 148.68 ppm was readily recognized as C_5 because of its large one-bond ^{13}C-^{1}H coupling ($^{1}J_{C5\text{-}H5}$ = 194.1 Hz). On the basis of its three-bond coupling with H_5 ($^{3}J_{C7\text{-}H5}$ = 9.8 Hz), the most deshielded doublet at 154.39 ppm was assigned to C_7. The doublet of doublets at 137.73 ppm must arise from C_{3a}, because it is coupled both with the H_5 ($^{3}J_{C3a\text{-}H5}$ = 10.4 Hz) and the anomeric sugar $H_{1'}$ ($^{3}J_{C3a\text{-}H1'}$ = 3.1 Hz) protons. The triplet at 140.57

TABLE 1. ^{13}C Chemical shifts and ^{13}C-^{1}H coupling constants of formycin in selected media.

	Chemical Shift (ppm)									
	C_3	C_{3a}	C_5	C_7	C_{7a}	$C_{1'}$	$C_{2'}$	$C_{3'}$	$C_{4'}$	$C_{5'}$
D_2O[a]	140.57	137.73	148.68	154.39	132.06	78.22	75.02	71.80	84.88	62.08
DMSO[b]	143.93[c]	138.68[c]	151.26	150.73[c]	122.22[c]	78.25[c]	74.88	72.19	85.77	62.43
H_2O (pH ~0.1)	137.05	127.70	146.18	153.66	125.30	76.95	75.51	71.24	84.94	61.46
H_2O (pH 7.5)	140.92	137.56	151.44	151.87	123.65	77.18	75.06	71.81	85.59	62.02
H_2O (pH ~14)	141.41	137.52	148.72	154.45	131.89	79.17	75.53	72.87	85.73	62.51

	Coupling Constant (Hz)		
	$^{3}J_{C7\text{-}H5}$	$^{1}J_{C5\text{-}H5}$	$^{3}J_{C3a\text{-}H5}$
D_2O[d]	9.8	194.1	10.4
DMSO[b]	ND[e]	199.2	ND[e]
H_2O (pH ~0.1)	10.4	212.4	7.9
H_2O (pH 7.5)	10.4	201.4	11.0
H_2O (pH ~14)	10.4	198.4	11.0

a Chemical shifts in ppm vs dioxane at 66.5 ppm.
b Chemical shifts in ppm vs DMSO at 39.5 ppm.
c Broad signal.
d Other pH-independent coupling constants; $^{3}J_{C3a\text{-}H1'}$ = 3.1 Hz, $^{3}J_{C3\text{-}H2'}$ = 1.8 Hz, $^{2}J_{C3\text{-}H1'}$ = 1.2 Hz.
e ND, not detected due to line broadening.

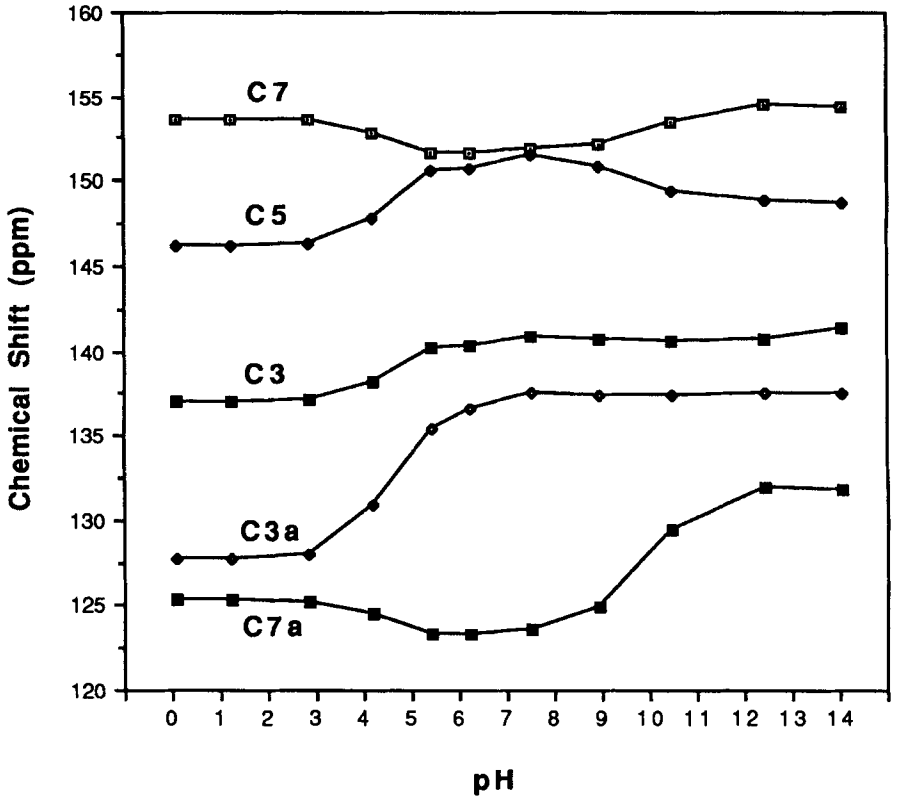


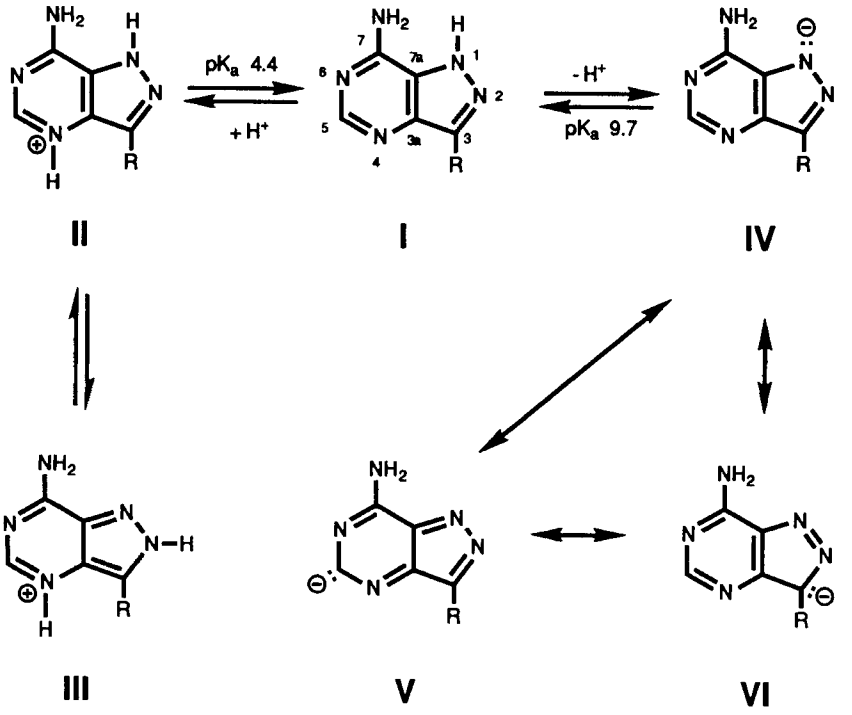
FIG. 2. The pH dependence of ^{13}C chemical shifts of the base carbons of formycin

ppm was assigned to C_3; this carbon coupled both with the sugar $H_{1'}$ ($^2J_{C3\text{-}H1'}$ = 1.2 Hz) and $H_{2'}$ ($^3J_{C3\text{-}H2'}$ = 1.8 Hz) protons. The remaining singlet resonance at 132.06 ppm was then delegated to C_{7a} by default.

The ribosyl sugar carbon signals were assigned by selective decoupling experiments and listed in Table 1. For this, ^1H NMR chemical shift assignments of formycin sugar protons in D_2O were made by COSY experiments, and its sequence was identical to that reported previously[19]; δ 8.03 (H_5), 5.36 ($H_{1'}$), 4.74 ($H_{2'}$), 4.46 ($H_{3'}$), 4.25 ($H_{4'}$), and 3.83, 4.03 ($H_{5'}$, $H_{5''}$). Although the sequence of ribose carbon resonances was the same as that of the N-nucleosides, $C_{1'}$ was shielded with respect to $C_{1'}$ in N-nucleosides. The $C_{2'}$ and $C_{3'}$ signals can also be conveniently identified on the basis of their characteristic pH-independent ^1H-coupling spectral patterns in aqueous

solution, i.e., the fine structure of $C_{2'}$ in the ^1H-coupled spectrum appeared as an apparent doublet, while that of $C_{3'}$ appeared as a broad apparent triplet.[20]

pH-Dependent ^{13}C NMR. In earlier ^{13}C NMR studies, Krugh[8] and Chenon et al.[9] have independently reported the extensive line-broadening of all the quaternary base (i.e., C_3, C_{3a}, C_7, and C_{7a}) and the anomeric sugar $C_{1'}$ carbons of formycin in DMSO. This was considered as evidence for the N_1H-N_2H prototropic tautomerism, which is possible in the pyrazolo ring moiety. In aqueous solution, however, such line-broadening was not observed. This allowed us to conduct unambiguous ^{13}C signal assignments and to perform detailed ^{13}C NMR studies in aqueous solution. As expected, the ^{13}C chemical shifts of base carbons were sensitive to protonation and the extent of ionization, while the sugar carbons exhibited minimal effects. In Figure 2, the ^{13}C chemical shift changes of the base carbons were monitored as a function of pH. The chemical shift and coupling constant information at three representative pH values is given in Table 1. Analysis of the pH-^{13}C shift titration curve yielded two pK_a values of 4.4 and 9.7, in close agreement with the literature values (4.4 and 9.6) determined previously by spectrophotometric methods.[4]



In the pH range from 7.5 to ~0.1, the C_{3a} and C_5 carbons of formycin experienced large shieldings, with the effect much greater for the former (+9.86 and +5.26 ppm, respectively, Table 1). Because these carbons are adjacent (α) to N_4, the results are consistent with N_4 as the principal site of protonation.[21] During the same period, the C_7 carbon was deshielded by 1.79 ppm, presumably due to the γ effect. Furthermore, the one-bond $^1J_{C5\text{-}H5}$ coupling was increased by 11.0 Hz, while the three-bond coupling $^3J_{C3a\text{-}H5}$ decreased (3.1 Hz); however, the $^3J_{C7\text{-}H5}$ value did not change. On the basis of the chemical shift[21] and coupling constant[22] information, it was concluded that N_4, and not N_6, of formycin is protonated (**II**).

In accord with expectation, smaller chemical shift changes were detected for C_3 and C_{7a}, both of which are two atoms away (i.e., β-position) from the protonated N_4 (+3.87 and -1.65 ppm, respectively). Surprisingly, the magnitude of β-shielding (+3.87 ppm) of C_3 is unusually large compared to that encountered with structurally similar compounds. For example, in adenosine and 8-oxoadenosine, both of which are known to protonate at N_1 (i.e., N_6 of formycin), approximately 0.5 ppm of chemical shift change was observed for the β-carbon C_5 (i.e., C_{7a} of formycin).[20] It should be emphasized that the C_3 carbon of formycin is located adjacent to N_2 and it is strongly influenced by the effect of the N_1H-N_2H tautomerism. For example, C_3 and C_{3a} were shielded (+8.8 and +4.2 ppm, respectively), while C_{7a} was deshielded (-7.7 ppm), going from N_1-methyl to N_2-methylformycin, which are model compounds for the N_1H and N_2H tautomers, respectively.[10] This illustrates how the chemical shifts of the pyrazolo ring carbons are affected by a combination of substituent and electronic effects and may explain the aforementioned unusual shieldings of C_3 and C_{3a} of formycin in acidic pH. Assuming a monoprotonation, therefore, the above data seem to support the view that the N_4-protonated formycin prefers the N_2H tautomeric form (i.e., **III**). This is in good agreement with the results of a recent theoretical study,[17] which predicted that N_4 is more basic than N_6 and that the N_1H tautomeric form is more stable (ca. 2 kcal/mole) than the N_2H tautomer for neutral formycin, while N_2H is the preferred tautomer for the protonated formycin.

The largest effects from pH 7.5 to ~14 were due to tautomeric changes accompanying removal of a proton from N_1 (**IV**).[21] The α-carbon C_{7a} was most deshielded (-8.24 ppm), followed by that (-2.58 ppm) of the β carbon C_7, while the C_3 and C_{3a} carbons were relatively unaffected (Table 1). Of particular interest is a unique shielding (+2.72 ppm) of the pyrimidine carbon C_5, which is

furthest from the ionization site. This is presumably due to the importance of the resonance form **V**, in which the anionic charge at N_1 after deprotonation was partially localized at C_5. A similar type of long-range polarization effect (+3.20 ppm) has been observed for the C_2 carbon (i.e., C_5 of formycin) of 8-oxo-adenosine in basic solution.[20] In the case of formycin, the anionic charge at N_1 can alternatively be localized at C_3, as indicated by the resonance structure **VI.** This species is unique for formycin and it must be emphasized that it can not occur in the isoelectronic N-nucleoside 8-oxoadenosine. The relative inertness of the chemical shift of C_3 indicated that the contribution of **VI** is insignificant.

Tautomerism. It is well known that carbons α and β to a protonated nitrogen atom are deshielded on formation of the corresponding anion, with the effect much greater for the former.[21] The chemical shifts of the C_3, C_{7a}, and C_{3a} carbons of formycin, therefore, are expected to be most influenced with the N_1H-N_2H tautomeric equilibrium by a combination of α- and β-effects.

Since the pK_a for deprotonation of formycin is 9.7, the base should exist mostly (>99%) in the unionized neutral form at physiological pH. As the pH of the medium increases, deprotonation either at N_1 or N_2 will take place. If we ignore the β-effect, it would be possible to evaluate the relative N_1H-N_2H tautomeric populations of formycin at physiological pH, through analysis of the pH dependence of the α-shifts of the C_{7a} and C_3 carbons. Figure 2 shows that the α-effect was much greater for C_{7a} (-8.24 ppm) than that of C_3 (-0.49 ppm), clearly indicating the predominance of the N_1H tautomer in neutral aqueous solution.

Semi-quantitative tautomeric evaluations of formycin can be made from the present ^{13}C data if the following basic assumptions are followed: first, the α-effects on C_{7a} and C_3 are solely due to the removal of a pyrazolo ring hydrogen on the N_1 or N_2 atom; second, the difference in ionization potential between the N_1H and N_2H tautomers is negligible. A total of 8.73 ppm deshielding could be accounted for the complete removal of a pyrazolo ring proton (a total α-effect). On the basis of this simple approximation, the contribution of the N_2H tautomer (-0.49 ppm) is calculated to be about 6% of the total. Therefore, the estimated population of the N_2H tautomer in aqueous solution is less than that (~15%) determined previously in DMSO.[10]

CONCLUSIONS

Analyses of the pH dependence of ^{13}C chemical shifts and ^{13}C-^1H coupling constants have provided detailed insights into the tautomeric behavior

of formycin in aqueous solution. The protonation of formycin at N_4 was accompanied with the migration of the pyrazolo ring hydrogen from N_1 to N_2, consistent with the prediction made by theoretical calculations.[17] The simplified quantitative treatment of the pH-dependent ^{13}C NMR chemical shifts revealed that formycin exists greater than 94% as the N_1H tautomer at physiological pH. The use of pH dependence of ^{13}C NMR chemical shifts allowed us to make a semi-quantitative determination of the relative tautomeric ratio of formycin in aqueous solution. This simple method may be of potential value since it eliminates the need for appropriately methylated analogues as models of fixed tautomeric forms, as they are often the source of undesirable steric and electronic effects.

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