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Acid–Base Properties of 4-Nitro-L-histidine and Related Compounds

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Acid–base properties of 4-nitro-L-histidine (**3**), N^{α}-acetyl-4-nitro-L-histidine (**2**), and N^{α}-acetyl-4-nitro-L-histidine methyl ester (**1**) are studied. Their pK_a^{II} values can be conveniently determined by ultraviolet spectroscopy. Potentiometric titration and ^{1}H-nuclear magnetic resonance (nmr) titration can also be used. Introduction of a nitro group strongly enhances the acidity of all the compounds. pK_a^1 of compound (**3**) has been also spectrophotometrically determined. Observed differences in acidity, in those cases where solvation does not play a major role, can be explained by assessing the influence of electrostatic charges on pK_a following Bjerrum's general theory with the aid of data from ^{1}H-nmr conformational analysis.

INTRODUCTION

4-Nitro-L-histidine (**3**) can be obtained by nitration of L-histidine (*1*). A strong decrease in the basicity of the imidazolic ring of this product can be expected as a result of the electron-withdrawing character of the nitro group. Replacement of 4-nitro-L-histidine for L-histidine in biologically interesting peptides could thus become a useful tool to evaluate the contribution of the acid–base properties of the side chain of histidine to the biological activity of natural peptides or proteins (*2*).

Usually, acidity constants have been determined by the classic potentiometric titration method. Since 1954, however, spectrophotometric methods have been used when the substance has convenient chromophoric properties (*3*). In the present paper, the spectrophotometric determination of the ionization constants of N^{α}-acetyl-4-nitro-L-histidine methyl ester (**1**), N^{α}-acetyl-4-nitro-L-histidine (**2**), and 4-nitro-L-histidine (**3**) is described. A conformational study by ^{1}H-nmr spectroscopy is used to discuss the results obtained.

From a biological viewpoint, compound (**1**) is the most interesting of the three, since its blocked amino and carboxyl functions reproduce to a better degree the situation of a nitrohistidine residue in a protein sequence.

EXPERIMENTAL

Ultraviolet and nmr (60 MHz) spectra were recorded in Perkin–Elmer 124 and Perkin–Elmer R-12 spectrometers. pH measurements were made with a Radiometer 51 pH meter equipped with a glass–calomel combined electrode.

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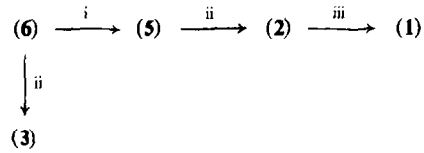
Syntheses

Compounds (**1**), (**2**), (**3**), and (**5**) were synthesized following described procedures with minor modifications (*1*, *11*). All the products were thoroughly purified by recrystallization prior to pK_a determinations and gave correct spectra and elementary analyses.



Reagents: i, Ac_2O; ii, HNO_3–H_2SO_4; iii, $SOCl_2$–MeOH

pK_a Determinations

Aliquots (5 ml) of 3.04 10^{-4} *M* (**1**), 2.56 10^{-4} *M* (**2**), and 2.05 10^{-4} *M* (**3**) parent solutions were taken and diluted with 10 ml each of different glycine–sodium hydroxide buffer solutions (*12*). Observed absorbances are listed in Tables 1 and 2 as well as the resulting pK_a^{II} values.

TABLE 1

ABSORBANCE OF BUFFERED SOLUTIONS OF (**1**) AND (**2**) AT 356 AND 357 nm RESPECTIVELY[a]

Compound (**1**)				Compound (**2**)			
pH	*A*	$\log \frac{A_{A^-} - A}{A - A_{AH}}$	pK_a^{II}	pH	*A*	$\log \frac{A_{A^-} - A}{A - A_{AH}}$	pK_a^{II}
5.88	0.115			5.65	0.115		
8.76	0.250	0.60	9.36	9.00	0.320	0.47	9.47
8.97	0.315	0.39	9.36	9.02	0.325	0.46	9.48
8.99	0.315	0.39	9.38	9.08	0.355	0.37	9.45
9.06	0.232	0.28	9.34	9.15	0.395	0.28	9.43
9.14	0.257	0.21	9.35	9.19	0.410	0.24	9.43
9.18	0.272	0.17	9.35	9.31	0.475	0.11	9.42
9.27	0.322	0.04	9.31	10.21	0.813	−0.81	9.40
9.30	0.332	0.02	9.32	11.50	0.924		
10.21	0.610	−0.91	9.30				
11.50	0.793						

[a] Measurements made at 28°C.

Aliquots (5 ml) of the parent solution of (**1**) described above were taken and diluted each one with 10 ml of sulfuric acid standard solutions (*13*). After recording the spectra, samples were titrated with sodium carbonate in order to ensure an exact H_0 value. Results are shown in Table 2.

A parallel pK_a^{II} determination of (**1**) was performed following standard procedures (*5*) by potentiometric titration of 100 ml of a mechanically stirred 0.01 *N* (**1**) solution with 1 *N* sodium hydroxide in nitrogen atmosphere.

H^1-nmr-pK_a^{II} determination of (**1**) was performed on aqueous solutions of the product at different pHs. Chemical shifts of the 2-imidazole proton were determined using

TABLE 2

DETERMINATION OF pK_a^{II} OF (3) AND pK_a^{I} OF (1) FOLLOWING MARONI-CALKMON PROCEDURE[a]

Compound (3)			Compound (1)		
H_0	A	$10^9(A_{AH} - A)/h_0$	H_0	A	$10^2(A_A - A)/h_0$
6.12	0.403		5.0	0.705	
8.18	0.340	41.3	0.28	0.608	18.6
9.02	0.288	11.0	0.07	0.573	15.6
9.03	0.285	11.1	−0.12	0.525	13.3
9.07	0.283	10.2	−0.25	0.490	11.8
9.16	0.280	8.5	−0.38	0.445	15.6
9.17	0.278	8.5	−0.53	0.418	8.3
9.36	0.236	6.1	−0.66	0.400	6.6
10.00	0.195	2.1	−0.91	0.353	4.3

[a] Absorbances measured at 304 nm for compound (1) and 310 nm for compound (2).

acetonitrile as internal reference. Since water proton did not interfere, nondeuterated water was used in order to get more accurate pH determinations, correction for the different D^+ and H^+ activities being not necessary. All measurements were performed at 36°C. Data treatment was carried out in a similar way to that proposed by Maroni and Calmon (*6*) for spectrophotometric determinations, i.e., pK_a^{II} value is obtained from the slope of the straight line:

$$\delta = \delta_{AH} + K_a^{II} \frac{\delta_{A^-} - \delta}{h_0}$$

deduced from:

$$pK_a^{II} = H_0 + \log \frac{\delta - \delta_{A^-}}{\delta_{AH} - \delta},$$

where

$$h_0 = \text{antilog } (-H_0).$$

TABLE 3

DETERMINATION OF pK_a^{II} OF (1) BY NMR-FOLLOWED TITRATION

pH	δ	$10^{-7}(\delta_{A^-} - \delta)/h_0$
3.65	5.78	-2.38×10^4
8.13	5.54	−4.04
8.68	5.52	−13.2
8.94	5.48	−20.3
9.03	5.43	−20.2
9.17	5.41	−24.6
9.26	5.41	−30.3
9.36	5.39	−33.1
9.40	5.37	−30.7
9.58	5.34	−38.0
10.17	5.29	−65.7

Conformational Analysis

Aminoacid H^1-nmr spectra were recorded in D_2O using NaOD and CF_3COOD to adjust the pH to the suitable value. α, β, and β' protons appeared as A_2X or ABX systems. In order to obtain the data of Table 6, the higher field proton was identified as β', i.e., the proton antiperiplanar to the methinic one in conformer (I). The assignment was made by analogy with data from malic acid (*14*). Recently, this assignment has been substantiated by analysis of nmr spectra of stereospecifically deuteriated derivatives of serine, phenylalanine, and other aminoacids (*15*). Coupling constants can be calculated from the following equations:

$$J_{\alpha\beta'} = (p_I + p_{III})J_g + p_{II}J_t,$$
$$J_{\alpha\beta} = (p_{II} + p_{III})J_g + p_I J_t,$$
$$1 = p_I + p_{II} + p_{III},$$

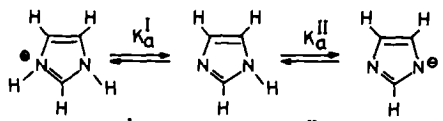
where J_t and J_g are the coupling constants between two protons in antiperiplanar and synclinal positions respectively [estimated values (*16*), J_t = 13.56 Hz and J_g = 2.60 Hz].

Electrostatic Effect Calculations

Bond angles and lengths were taken from crystallographic data (*17*). According to minimization energy calculations (*18*), the imidazolic ring was supposed to adopt $\chi^2 = \pm 90°$ relative positions (*19*). Calculations were made for both values and results were averaged. All compounds under study were considered to be derived only from the predominant tautomers of L-histidine (*20*) and nitroimidazole (*21*).

RESULTS

Two acid–base equilibria can be stated for imidazoles, giving rise to two ionization constants pK_a^I and pK_a^{II}.



The pK_a^{II} of compound (**1**) was determined spectrophotometrically according to Albert and Serjeant (*5*). Figure 1 shows uv spectra obtained from buffered solutions containing a fixed amount of (**1**) whose pHs ranged from 5.88 to 11.50. Let A_{A^-} and A_{AH} be the absorbances corresponding to the extreme pHs and A the absorbance of a solution at any intermediate pH. Combined application of Henderson–Hasselbalch equation and Beer's law results in:

$$pK_a^{II} = H_0 - \log \frac{A_{A^-} - A}{A - A_{AH}},$$

where H_0 is the Hammet acidity function which can be assimilated to pH for dilute aqueous solutions. pK_a^{II} then will be 9.34 ± 0.04 (Table 4). This value is in good agree-

ment with those obtained by potentiometric titration and H^1-nmr-followed titration, i.e., 9.32 ± 0.18 and 9.32 ± 0.05 respectively. It is also very close to the reported value for 4-nitroimidazole (**4**) (pK_a^{II} 9.30) (*4*).

pK_a^I has been determined from uv spectra of solutions of (**1**) in different concentrations of sulfuric acid whose H_0 values are tabulated. The absorption maximum corresponding to the neutral form remains constant at 310 nm, whereas the maximum of the protonated form moves hypsochromically as more negative values of H_0 are reached (275 nm at $H_0 = -0.43$; 260 nm at $H_0 = -5.80$). This fact suggests that not all the

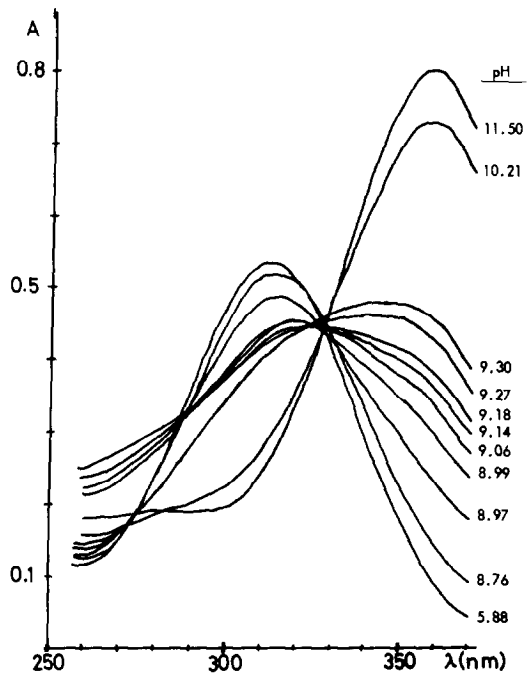


FIG. 1. Ultraviolet spectra of N^{α}-acetyl-4-nitro-L-histidine methyl ester (**1**) at different pH values.

titration points fulfill the Henderson–Hasselbalch equation. In view of this we have applied the Maroni–Calmon procedure (*6*) which uses an unique limiting value, in our case the absorbance of the neutral form. Results are shown in Fig. 2. Points corresponding to the two more acidic solutions do not follow ideal behavior and therefore they have not been considered. Straight slope leads to $pK_a^I = -0.26 \pm 0.06$.

pK_a^{II} of compound (**2**) has been determined as above. A value of 9.44 ± 0.04 is obtained. In contrast, determination by the same method of pK_a^{II} of (**3**) was not successful due to the absence of an isosbestic point in the solutions of highest basicity. This behavior is probably due to loss of a proton from the

$$-\overset{\oplus}{N}H_3 \text{ group}$$

in alkaline solution. Application of the Maroni–Calmon procedure using as unique limiting value the absorbance of the neutral form leads to $pK_a^{II} = 8.27 \pm 0.05$.

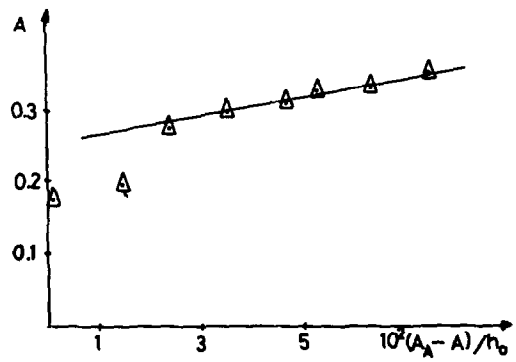


FIG. 2. Determination of the pK_a^I of N^α-acetyl-4-nitro-L-histidine methyl ester (1) following the Maroni–Calmon formalism.

DISCUSSION

The low value of pK_a^{II} of 4-nitro-L-histidine (3) compared with those of (1), (2), and 4-nitroimidazole (4) may seem surprising. Of these four compounds, (3) is the only one bearing a positive charge. At first sight it may seem reasonable to conclude that a positive charge has a greater effect than a negative one on the pK_a. Similar behavior can be observed in L-histidine (6), N^α-acetyl-L-histidine (5), and imidazole (7) (Table 4). Sachs *et al.* (7) have explained this effect on the basis of delocalization of negative charge on the carboxylate group.

In order to assess the influence of an electric charge on the pK_a of imidazolic ring,

TABLE 4

ACID–BASE CONSTANTS OF 4-NITRO-L-HISTIDINE AND RELATED COMPOUNDS

	1	2	3	4
pK_a^{II}:	9.34	9.44	8.27	9.30

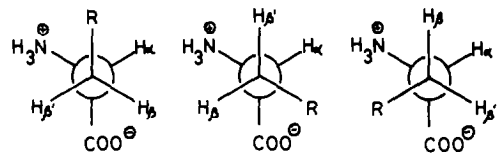
	5	6	7
pK_a^{I}:	7.08	6.00	7.08

we have used Bjerrum's electrostatic theory (*8*). The variation of pK_a caused by an electric charge is given by:

$$\Delta pK_a = \frac{1}{kT(\ln 10)} \frac{Ze^2}{\varepsilon_H R},$$

where k is the Boltzmann constant, T the absolute temperature, e the electron charge, Z the charge number, and R the distance from the charge to the proton to be ionized. Different methods for evaluating ε_H (effective dielectric constant) have been reported. We have used the one reported by Tanford (*9*), which consists of a plot of ε_H against R.

The value of R is evidently a function of the conformation of the molecule. We may assume that the molecule exists mainly as an equilibrium of the three staggered conformers I, II, and III. Application of Bjerrum's equation to each conformer leads to the



(I) (II) (III)

results shown in Table 5, ΔpK^- and ΔpK^+ being the electrostatic influence upon the pK_a of the negative and positive charge respectively. Conformer populations (p_I, p_{II}, p_{III}) can be obtained from the nmr spectra. Results obtained applying this method to compounds (**2**), (**3**), (**5**), and (**6**) at different pH values are shown in Table 6.

TABLE 5

ELECTROSTATIC INFLUENCE OF CHARGES UPON THE pK

	Conformer		
	(I)	(II)	(III)
ΔpK^-	+0.83	+2.83	+2.83
ΔpK^+	−2.28	−1.16	−2.28

TABLE 6

CONFORMER POPULATIONS OF COMPOUNDS(**2**), (**3**), (**5**), AND (**6**)

Compound	pH[a]	$J_{\alpha\beta}$	$J_{\alpha\beta'}$	p_I	p_{II}	p_{III}
(**2**)	5.6	9.6	4.8	0.64	0.20	0.16
(**2**)	11.6	10.5	4.3	0.72	0.15	0.13
(**3**)	10.6	10.9	3.5	0.72	0.08	0.17
(**5**)[b]	4	9.0	4.4	0.58	0.16	0.26
(**5**)	12	9.7	4.1	0.64	0.13	0.23
(**6**)[b]	4	6.7	6.7	0.37	0.37	0.26
(**6**)[b]	7.5	6.5	6.5	0.35	0.35	0.30
(**6**)	11	9.0	3.6	0.58	0.09	0.33

[a] Noncorrected pH meter readings.

[b] Ref. 10.

Results of Tables 5 and 6 can now be combined in order to account for experimental pK_as of the compounds under study. The influence of electric charges on imidazolic pK_a^I of L-histidine (**6**) is given by:

$$\Delta pK = \sum p_l^0(\Delta pK_l^+ + \Delta pK_l^-).$$

p_l^0 are the conformer populations present when pH = pK. By definition, as such pH protonated and nonprotonated forms are present in equal amounts in the solution, conformer populations can be easily obtained as the average values corresponding to pH 4 and 7.5 in Table 6. This leads to $\Delta pK = +0.24$. Similarly, for N^{α}-acetyl-L-histidine (**5**) $\Delta pK = \sum p_l^0 \Delta pK_l^-$ leads to $\Delta pK = +1.61$. From these results it can be stated that electric charge causes (**5**) to be more basic than (**6**), and, other factors being equal, the difference in pK_a values should be +1.34, which is not far from the experimental values of +1.08 (Table 4).

This kind of reasoning cannot be applied to imidazole (**7**), since in this case solvation effects may play a leading role; in effect, whereas protonation of (**7**) is clearly an ionogenic reaction, protonation of (**5**) or (**6**) are nearly isoionic reactions. Solvation must then provoke a strong increase in the basicity of (**7**) vs (**6**), as can be seen experimentally (Table 4).

The differences of pK_a^{II} of (**1**), (**2**), and (**3**) can be explained likewise. In this case, however, direct determination of the conformer populations of (**3**) has not been possible due to its low solubility in neutral or acidic water, so the same populations as for L-histidine (**6**) have been assumed. Calculations give $\Delta pK = +1.47$ and $\Delta pK = +0.24$ for compounds (**2**) and (**3**) respectively. These values show that, other factors being equal, electric charges cause (**2**) to be a weaker acid than (**3**), as is experimentally found, Moreover, the theoretical pK_a difference (+1.23) is in good agreement with the observed experimental value (+1.17; Table 4). Due to solvation effects pK_a^{II}s of (**4**) and (**1**) are not comparable with those of (**2**) and (**3**). Both compounds have no electric charges, and so very close values of pK_a^{II} can be expected for them. Indeed, the experimental pK_a^{II} difference is found to be only 0.04.

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