Determination of the Preferred Tautomeric form of 4-Nitrohistidine

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Spectrophotometric p*K*a determinations of methyl derivatives of $N\alpha$-acetyl-4-nitrohistidine methyl ester **1** have been used to determine the position of the tautomeric equilibrium of **1**. The N_1-H tautomer is the predominant form with an equilibrium constant K_T of 48.

The conclusion is supported qualitatively by the study of ^{1}H-nmr and ^{13}C-nmr chemical shifts of the imidazole ring atoms and their changes from neutral to acidic media.

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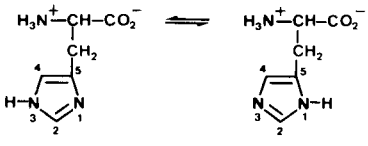
Imidazole rings play an important role in many biological processes. The amino acid histidine is present in the active center of several enzymes and is probably involved in the recognition of peptide-hormones at the receptor level [2]. In spite of the great effort made in the study of tautomerism in imidazole derivatives (see Elguero *et al.* [3] for an exhaustive review) nothing was known about the imidazole tautomerism in histidine until 1973 when Reynolds demonstrated, using ^{13}C-nmr, that the tautomeric equilibrium for this amino acid was clearly shifted towards the N_3-H form [4,5]. The same procedure has been applied to histidine-containing peptides as



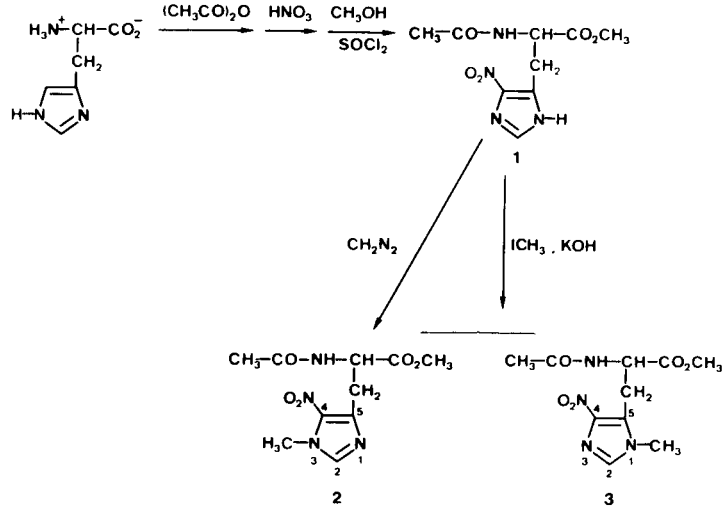
Scheme 1

bacitracin [5], thyroliberin [6], horse cyanoferrimyoglobin [7] and *P. aeruginosa* azurin [8]. In all the cases, with the exception of *P. aeruginosa* azurin, histidine is predominantly in the N_3-H tautomeric state. More recently, long range ^{13}C,H nuclear spin-spin coupling constants and ^{15}N-nmr chemical shifts have also been used in the study of imidazole tautomerism in L-histidine, histamine, and related compounds [9,10].

Tautomerism is probably a key factor for a good understanding of the differences in biological activity of analogues of histidine-containing peptide-hormones [6,11,12]. For some hormones it can be assumed that only one imidazole ring tautomer is the biologically active form. *N*-Methylated analogues can be viewed as frozen forms of the two tautomeric possibilities. As a consequence their activities depend on whether they correspond to the active or the inactive tautomer [6]. Synthesis of hormone analogues containing 4-nitrohistidine instead of native histidine could provide a useful tool for the diagnostic of these situations as the nitro group is expected to reverse the position of the tautomeric equilibrium of histidine imidazole ring [13]. In the present study we have proved that the N_1-H tautomer is the more stable form of 4-nitrohistidine in neutral water solution.

Results and Discussion.

The amino acid L-histidine can be nearly quantitatively $N\alpha$-acetylated by treatment during 3 minutes at 100° with acetic anhydride in slight excess. In these conditions the amino acid is partially racemized but this is irrelevant for the studies described in this paper [12]. $N\alpha$-Acetylhistidine can be transformed in $N\alpha$-acetyl-4-nitrohistidine methyl ester **1** following the procedure already described in the literature [14,15]. Treatment of compound **1** with diazomethane at room temperature affords crystalline N_3-methyl-$N\alpha$-acetyl-4-nitrohistidine methyl ester **2** in a 33% yield (Scheme 2). Alternatively, methylation of compound **1** with methyl iodide in methanolic potassium hydroxide gives a mixture of both N_3- and N_1-methyl derivatives **2** and **3** that can be resolved by silica gel column chromatography yielding 17% of **2** and 11% of **3**.



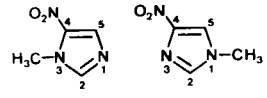
Scheme 2

The structural assignment of compounds **2** and **3** was possible after careful comparison of the ^{1}H-nmr and

^{13}C-nmr spectra with those of the model compounds 3-methyl-4-nitroimidazole **4** and 1-methyl-4-nitroimidazole **5** (see Table 1). Both N-CH_3 and C_2-H protons from compound **2** exhibit ^{1}H chemical shifts slightly more downfield than those from compound **3**. This behavior correlates with that of the parent nitroimidazole derivatives. The chemical shifts of the NH-hydrogen in the conjugate acids of *N*-substituted nitroimidazoles have been previously reported to give information about the position of the nitro group [16]. More recently McKillop has pointed out that ^{13}C-nmr is a good method for the structural assignment of the substitution pattern in nitroimidazoles [17]. The ^{13}C chemical shifts of the ring C_2 atom of compound **2** is 4-5 ppm more downfield than that of compound **3** as it is also the case for **4** with respect to **5** as well as for several other nitroimidazole derivatives studied by McKillop. ^{13}C Chemical shifts of the carbon bearing the nitro group also correlate well with the model compounds. On the contrary, the observed difference in the ^{13}C N-CH_3 chemical shifts cannot be used due to the chemical shift similarity between **4** and **5**.

Table 1

^{1}H-NMR and ^{13}C-NMR Chemical Shifts of Compounds **2**, **3** and *N*-Methyl-4-nitroimidazoles **4** and **5** [a]



4 [b] **5**

^{1}H-nmr

	N-CH_3	C_2-H		N-CH_3	C_2-H
4 [c]	4.05	7.64	**2**	3.93	7.99
5 [c]	3.90	7.54	**3**	3.71	7.79

^{13}C-nmr

	N-CH_3	C_2	C_4		N-CH_3	C_2	C_4
4	34.6	143.1	139.0	**2**	35.1	141.0	135.5
5	34.1	137.0	146.9	**3**	32.1	136.6	144.6

[a] All nmr spectra are recorded in d_6-DMSO except the ^{1}H-nmr spectra of compounds **4** and **5** which were dissolved in deuteriochloroform. [b] *N*-Methyl-4-nitroimidazoles are numbered in the same way as histidine derivatives for comparison purposes. [c] Data from G. B. Barlin and T. J. Batterham, *J. Chem. Soc.* (B), 516 (1967).

The p*K*a values of the protonated form of the imidazole ring in compounds **2** and **3** were determined spectrophotometrically from the observed uv absorbance changes of sulfuric acid solutions in the 310 nm region where the maximum absorbance of the neutral forms is found. The general Maroni-Calmon procedure [18] was followed for the calculations using the absorbance of the neutral forms as the only limit values. Results are shown in Table 2 and compared with the previously reported p*k*a of the imidazolium group of compound **1** [19].

Table 2

p*K*a Values of N^{α}-Acetyl-4-nitrohistidine Methyl Ester Derivatives

1	−0.26 ±0.06
2	0.88 ±0.04
3	−0.80 ±0.04

Acidity function measurements of frozen tautomeric derivatives provide one of the most fruitful procedures for studying tautomeric equilibria in heterocyclic compounds [3]. This procedure has been previously applied to determine the proportion of tautomers in several imidazole derivatives as histamine [20] and 4-nitroimidazole [13,21,22]. The tautomeric constant K_T for the equilibrium shown in Scheme 1 can be expressed as $K_T = f_1.K_{1\text{-}Me} / f_3.K_{3\text{-}Me}$ where $K_{1\text{-}Me}$ and $K_{3\text{-}Me}$ are the acidity functions of the protonated forms of **3** and **2** respectively and f_1 and f_3 are the ratio between acidity functions of each protonated tautomer of **1** and the corresponding methyl derivative. When the acidity function of the methyl derivatives cannot be determined experimentally the simplication $f_1 = f_3 = 1$ must be used. When, as in our case, both $K_{1\text{-}Me}$ and $K_{3\text{-}Me}$ values are available the weaker simplification $f_1 = f_3$ leads to the expression $K_T = K_{1\text{-}Me} / K_{3\text{-}Me}$ and from data of Table 2 a value of $K_T = 48 \pm 10$ is obtained. From the reported p*K*a value of protonated compound **1** (Table 2) and knowing that this acidity function (K_1) equals the sum of the acidity functions of each tautomer the expression $f = K_1 / K_{1\text{-}Me} + K_{3\text{-}Me}$ follows [3] and a value of $f = 0.28$, far from the unit, is obtained stressing the convenience of working with both methyl derivatives.

The value found for K_T is extremely low as compared with the reported $K_T = 500$ by Gallo [13] from basicity measurements of both methyl derivatives of 4-nitroimidazole. This difference could be accounted for in terms of the withdrawing electronic effect of the substituent in position 5 of the imidazole ring [23]. Charton [24] has derived empirical equations which correlate p*K*a values with Hammett coefficients in 4-substituted imidazoles. Assuming that the methyl effect is the same for both tautomers, the general equation to calculate K_T for monosubstituted imidazoles has the form $\log K_T = 3.2\sigma_m$. When this equation is applied to calculate the K_T of 4-nitroimidazole ($\sigma_m = 0.71$ for a nitro group) a value of $K_T = 186$ is obtained. The tripeptide thyroliberin (Glp-His-Pro-NH_2) can be taken as a suitable model for estimation of the Hammett coefficient of the substituent in position 5 of the imidazole ring of compound **1**. The p*K*a of

protonated thyroliberin and both N_1-methyl- and N_3-methyl-derivatives have been determined potentiometrically by Grant *et al.* [25]. Applying the Charton equations [24] a mean value of $\sigma_m = 0.10$ is obtained. From a qualitative point of view the electron withdrawing effect of the substituent agrees well with the experimentally found decrease of K_T in compound **1** with respect to 4-nitroimidazole. Quantitatively, if we assume that the effect of substituents is additive log K_T = 3.2 (0.71-0.10) = 1.95 and then K_T = 89.

Carbon-13 nuclear magnetic resonance is a very useful technique for the study of tautomerism in azole compounds [3,5,6,9,26]. A simple comparison of the values reported in Table 3 for the chemical shifts of C_2 and C_4 atoms of compound **1** with those of the model methyl derivatives **2** and **3** strongly suggests that the tautomeric equilibrium is shifted towards the N_1-H tautomer. The same is true when the chemical shifts of compound **6** are compared with those of the methyl derivatives **4** and **5**. However, such a direct comparison can give sometimes misleading conclusions by neglecting the effect of the methyl group in the ^{13}C-chemical shift. A safer conclusion can be obtained comparing the changes in chemical shift between the neutral and the protonated forms of the target molecule with those of the methyl derivatives. Such a comparison is shown in Scheme 3. In $N\alpha$-acetyl-4-nitrohistidine methyl ester **1** the observed variations in

Table 3

^{13}C-NMR Chemical Shifts of Compounds **1**,**2**,**3**,**4**,**5** and 4-nitroimidazole (**6**) in Neutral and Acidic Conditions

d_6-DMSO

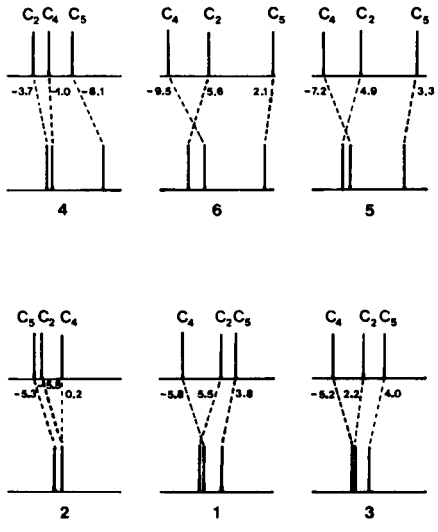
	C_2	C_4	C_5		C_2	C_4	C_5
6	136.8	147.6	120.2	**1**	133.9	144.1	130.0
4	143.1	139.0	132.7	**2**	141.0	135.5	142.8
5	137.0	146.9	122.5	**3**	136.6	144.6	131.0

d_6-DMSO-H_2SO_4

	C_2	C_4	C_5		C_2	C_4	C_5
6	142.4	138.1	122.3	**1**	139.4	138.3	133.8
4	139.4	138.0	124.6	**2**	135.5	135.7 [a]	137.5 [a]
5	141.9	139.7	125.8	**3**	138.8	139.4	135.0

[a] Unambiguous assignment between these two signals is not possible.

chemical shift during the protonation of the ring are very close to those observed in the N_1-methyl derivative **3** thus confirming the strong shift towards the N_1-H tautomer predicted from the basicity measurements discussed above. The behaviour of the parent compound **6** is also shown in Scheme 3 for comparison purposes. Although no quantitative information about K_T can be obtained from



Scheme 3

^{13}C-nmr measurements when equilibria are strongly shifted towards one tautomer, as it is in our case, data from Scheme 3 could be useful in the future to study the tautomeric equilibrium of other 5-substituted-4-nitroimidazoles where neither *N*-methyl derivative are available.

EXPERIMENTAL

Ultraviolet spectra have been recorded in a Perkin-Elmer 124 spectrophotometer. Proton nmr spectra have been obtained in a Perkin-Elmer R-12A and all chemical shifts have been referenced to TMS. ^{13}C-nmr spectra have been recorded either in a Varian CFT-20 or in a Brucker WH-90 apparatus. For tlc, Merck silica gel 60 plates (F-254, 0.2 mm) have been used.

$N\alpha$-Acetyl-4-nitrohistidine Methyl Ester (**1**).

i) A solution of 33 ml of acetic anhydride (0.342 mole) in 80 ml acetic acid was slowly added to a stirred solution of 50 g of L-histidine (0.322 mole) in 187 ml acetic acid. The mixture was stirred for three minutes at 100°, and after the addition of water was evaporated to dryness. The solid residue was recrystallized from water, giving 59 g of $N\alpha$-acetylhistidine (93% yield) mp 162-165°, lit 169° [27].

ii) To a stirred solution of 27.0 g (0.137 mole) of $N\alpha$-acetylhistidine in 56 ml of concentrated sulfuric acid, 30 ml of fuming nitric acid were slowly added keeping the temperature of the reaction mixture between 40 and 45°. The solution was stirred for two hours at the same temperature, then it was poured onto 300 g of ice and solid sodium carbonate was added to adjust the *p*H to 0.4. The product precipitated, it was filtered immediately and then recrystallized from water to give 20.8 g of $N\alpha$-acetyl-4-nitrohistidine (63%) mp 237-238.5°, lit 236-237° [14].

iii) A solution of 1.36 ml of thionyl chloride (0.0185 mole) in 126 ml methanol was poured onto 9 g of $N\alpha$-acetyl-4-nitrohistidine (0.0371 mole). The resulting mixture was stirred at room temperature for 8 hours, after which time the solvent was removed *in vacuo* below 40°. Water (200 ml) was added to the residue and the *p*H of the solution was adjusted to 4.0 with solid sodium bicarbonate. The solid was crystallized and recrystallized from water to yield 5.8 g of **1** mp 202-205°, lit 202-205° [15].

N_3-Methyl-$N\alpha$-acetyl-4-nitrohistidine methyl ester (**2**) and N_1-methyl-$N\alpha$-acetyl-4-nitrohistidine methyl ester (**3**).

i) Methylation of **1** with Diazomethane.

To a solution of 0.20 g of **1** (0.78 mmole) in 20 ml of methanol was added 20 ml of anhydrous ether and then, dropwise, 40 ml of a solution of diazomethane (about 15 mmoles) in dry ether. The mixture was stirred for 16 hours at room temperature, after which time some drops of acetic acid were added to destroy the excess diazomethane and the solvent was removed *in vacuo*. Fractionated crystallization from ethanol afforded 70 mg of **2** mp 148-149° (33% yield).

ii) Methylation of **1** with Methyl Iodide.

To a solution of 1.68 g of potassium hydroxide (0.02 mole) in 100 ml methanol were added first 5.12 g of **1** (0.02 mole) and then 1.87 ml of methyl iodide (0.03 mole). The solution was gently refluxed for 37 hours, then was filtered and the filtrate evaporated to dryness. The solid residue was dissolved in the minimal amount of methanol and chloroform was added to precipitate inorganic salts. After filtration the solvent was again removed *in vacuo*. The reaction proceeded with a 95% yield and the isomers were obtained in a proportion of 40% of **2** and 60% of **3**. Separation of the reaction products **2** and **3**, which were also contaminated with some starting product, was accomplished by careful silica gel (45 g, Merck, 0.04-0.063 mm) column chromatography eluting with chloroform/methanol/ammonia 40/10/1 or 20/10/1 using small batches (*ca.* 0.6 g) of the mixture obtained after a rough purification of the whole crude in 100 g of silica using the first eluent. Alternatively, fractionated crystallization from methanol/chloroform or ethanol/water were used to separate **2** and **3** after the first purification. The global amount of pure products obtained were 0.9 g of **2** (17% yield) and 0.59 g of **3** (11% yield). Analytical samples were further recrystallized from ethanol and were characterized as follows: mp, **2** 149-149.5°; **3** 165.5-167°; tlc, solvent systems: A = chloroform/methanol/ammonia 20/10/1, B = chloroform/methanol/ammonia 40/10/1; **2**, Rf (A) = 0.71, Rf (B) = 0.64; **3**, Rf (A) = 0.61, Rf (B) = 0.51; ^{1}H-nmr (DMSO-d_6): **2** δ 8.33 (d, NH, 1H), 7.99 (s, C_2-H, 1H), 3.93 (s, N-CH_3, 3H), 3.63 (s, O-CH_3, 3H), 1.84 (s, CH_3-CO, 3H); **3** δ 8.39 (d, NH, 1H), 7.79 (s, C_2-H, 1H), 3.71 (s, N-CH_3, 3H), 3.66 (s, O-CH_3, 3H), 1.87 (s, CH_3-CO, 3H); ^{13}C-nmr (DMSO-d_6): chemical shifts not indicated in Table III are : **2** δ 22.1 (CH_3-CO), 30.7 (CβH_2), 50.3 (CαH), 50.7 (CO_2-CH_3), 169.1 and 171.6 (carbonyls); **3** δ 22.0 (CH_3-CO), 26.2 (CβH_2), 49.9 (CαH), 52.1 (CO_2-CH_3), 169.3 and 170.9 (carbonyls); ir (potassium bromide): **2** 3310 (NH), 1740 (COO), 1640 (CONH), 1555 and 1370 (NO_2); **3** 3320 (NH), 1750 (COO), 1670 (CONH), 1505 and 1375 (NO_2) cm^{-1}; uv (water): **2** λ max = 312 nm, ϵ = 7700; **3** λ max = 311 nm, ϵ = 7000.

Anal. Calcd. for $C_{10}H_{14}N_4O_5$: C, 44.4; H, 5.2; N, 20.7. Found: **2** C, 44.9; H, 5.5; N, 20.6; **3** C, 44.4; H, 5.5; N, 20.6.

Determination of Acidity Functions.

A parent solution of product **2** or **3** (about 3.10^{-4} *M*) in water was prepared, as well as a series of standard solutions of sulfuric acid of different concentrations. For each concentration of acid, the ultraviolet spectrophotometer was adjusted with a solution prepared by mixing 5 ml of water and 10 ml of the acid solution, and then, the spectrum of an aliquot of 5 ml of the parent solution diluted with 10 ml of the corresponding acidic solution was recorded. The actual acid concentration of each solution was determined by addition of a known excess of sodium carbonate followed by back titration with hydrochloric acid.

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