the electrode does not contain bulky substituents; (v) anthraquinones are adsorbed parallel to the surface and are not influenced by changes in concentration; (vi) the biphenyl moiety, initially adsorbed with both rings parallel to the surface, undergoes reorientation first to a structure in which both rings are bonded to the surface edgewise and then to a η^2 structure in which one ring is attached and the other is pendant; (vii) preferential adsorption of organic functional groups on Pt follows the order thiol > N heteroaromatic > diphenol ring ≈ quinonoid ring > benzene ring > alkene > amine > hydroxyl > ketone; (viii) in general, adsorbed molecules reorient to structures occupying smaller surface area.

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Registry No. **1**, 123-31-9; **2**, 106-51-4; **3**, 120-80-9; **4**, 95-71-6; **5**, 452-86-8; **6**, 1948-33-0; **7**, 98-29-3; **8**, 608-43-5; **9**, 615-90-7; **10**, 527-18-4; **11**, 771-63-1; **12**, 608-80-0; **13**, 16867-04-2; **14**, 123-33-1; **15**, 5721-21-1; **16**, 1126-61-0; **17**, 81255-51-8; **18**, 51-61-6; **19**, 2889-61-4; **20**, 81753-11-9; **21**, 1079-21-6; **22**, 4371-32-8; **23**, 571-60-8; **24**, 2066-93-5; **25**, 117-14-6; **26**, 84-50-4; Pt, 7440-06-4.

A Nitrogen-15 Nuclear Magnetic Resonance Study of the Acid–Base and Tautomeric Equilibria of 4-Substituted Imidazoles and Its Relevance to the Catalytic Mechanism of α-Lytic Protease[1]

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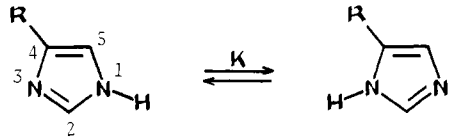
Contribution No. 6537 from the Laboratories of Chemistry, California Institute of Technology, Pasadena, California 91125. Received September 23, 1981

Abstract: The pH dependence of the ^{15}N NMR shifts of histamine, imidazole-4-propionic acid, imidazole-4-acetic acid, *trans*- and *cis*-urocanic acid, and *endo-cis*-3-(4-imidazoyl)bicyclo[2.2.1]hept-5-ene-2-carboxylic acid has been determined at the natural-abundance level of ^{15}N. The chemical-shift changes permit calculation of pK_a values for the acidic species present as well as reasonably accurate positions of the N1(H) ⇌ N3(H) tautomeric equilibria for those species having unprotonated imidazole rings. The ^{15}N shifts of *cis*-urocanic acid and *endo-cis*-3-(4-imidazoyl)bicyclo[2.2.1]hept-5-ene-2-carboxylic acid demonstrate that carboxylate–N3(H) hydrogen-bonding interactions can cause the N3(H) tautomers to be substantially more stable than the N1(H) tautomers. The unusual positions of these tautomeric equilibria are quite similar to that found for the histidine of the catalytic triad of α-lytic protease.

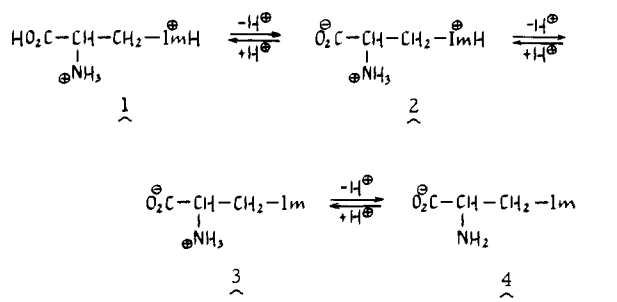
Imidazole rings are very important functional groups in many biological processes. In connection with the present research, the role of the histidine imidazole ring in the Asp-His-Ser catalytic triad of the serine protease is especially relevant.[3] For histidine, as with all 4-substituted imidazoles, the position of the N1(H)–N3(H) tautomeric equilibrium (eq 1) is potentially significant. In water solution, this equilibrium is established rapidly



(1)

on the NMR time scale, and even with ^{15}N NMR wherein the nitrogen resonances of the azine and N–H are expected to have chemical-shift differences of about 83 ppm,[4] the resonances are quite sharp[5] as expected for a mean lifetime before tautomeric proton shift of less than 10^{-4} s.

The equilibrium shown in eq 1 as a function of pH for histidine has been very carefully studied by Blomberg, Maurer, and Rüterjans[6] with the aid of ^{15}N NMR spectroscopy. The shift changes of the imidazole nitrogens of histidine are shown in Figure 1. Four levels of protonation (1–4) are covered in the pH range that was studied and, of these, it is evident from the shifts that a significant change in the equilibrium shown in eq 1 occurs in the change from **3** to **4**. The first ionization of histidine (**1** ⇌ **2**) has pK_a 1.8,[7] and on the change from **1** to **2** there appears to be no significant change in the ^{15}N shifts.[6] The second ionization (**2** ⇌ **3**) has pK_a 6.0[7] and deprotonation of the imidazole ring leads to a 53-ppm ^{15}N chemical-shift difference between N1 and N3



(1) Supported by the National Science Foundation and the National Institutes of Health.

(2) On leave from the Institute of Photographic Chemistry of the Academia Sinica, Peking, 1979–1981.

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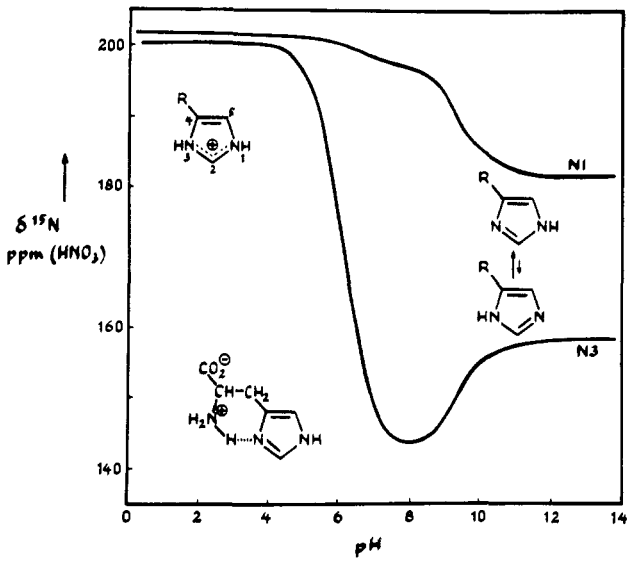
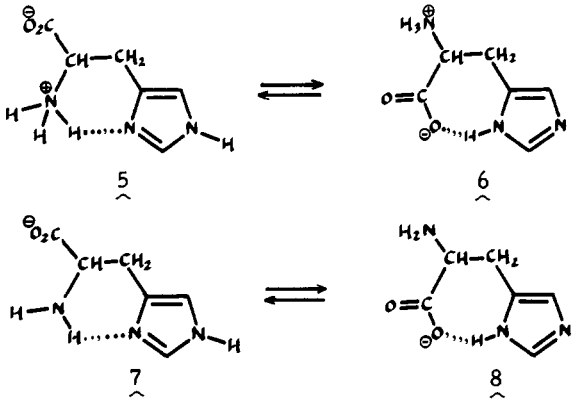


Figure 1. Histidine ^{15}N shift changes with pH data of Rüterjans and co-workers.[6]

at pH 7.8. Deprotonation of **3** (with pK_a 9.1[7]) finally results in a smaller N1–N3 shift difference of about 23 ppm,[6] which is still substantially larger than that for 4-methylimidazole of 8.4 ppm in water solution.[4] It is not known how much of the shift difference between the nitrogens of 4-methylimidazole arises from a differential methyl substituent effect on the resonances of N1 and N3 and how much arises from the equilibrium constant of eq 1 being different from unity. If we assume the equilibrium-constant effect dominates, then K of eq 1 for this imidazole is about 0.82. The same procedure suggests that K is about 0.22 for **3** and about 1.8 for **4**.[8] That these values are both larger than that for 4-methylimidazole and that for **3** is larger than that for **4** suggests different degrees of hydrogen bonding to N3 by the amino acid side-chain groups (**5**, **6**, **7**, and **8**).

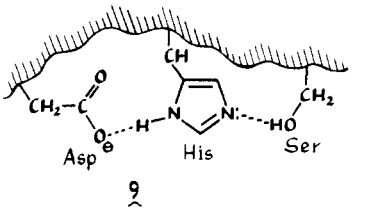


5 **6**

7 **8**

Although an a priori decision as to the relative importance of **5** vs. **6** or of **7** vs. **8** seems difficult, the evidence of the ^{15}N NMR shifts unequivocally suggests that **5** and **7** are more important because N3 is the downfield nitrogen.

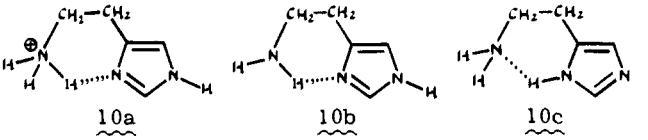
The position of the tautomeric equilibrium of eq 1 for histidine incorporated into peptide chains is clearly not going to be subject to the same influences as for free histidine because the carboxylic and amine groups are part of the peptide chain. It is interesting that, with the exception of the serine proteases,[3,9] azurin,[10] and ribonuclease A,[11] the N1(H) tautomers are reported[12] to dominate in the histidine-containing proteins so far studied. The exceptional serine proteases, where the N3(H) tautomer of the histidine at the active site clearly predominates, can be accounted for on the basis of hydrogen bonding involving N3(H) and the aspartate carboxylate group, as well as possible additional hydrogen bonding between N1 and the serine hydroxyl of the catalytic triad (Ser-His-Asp (**9**)).[3,9] However, the degree of the serine contribution



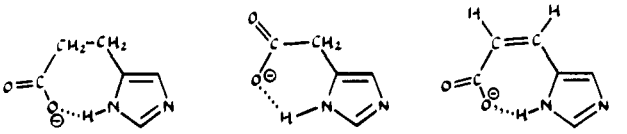
9

to stabilizing the N3(H) tautomer is rendered uncertain by X-ray studies of the catalytic triad of trypsin, which suggests that in the crystalline enzyme at pH 8 the serine hydroxyl forms a rather long (3.26 Å) and bent hydrogen bond to the histidine imidazole ring.[13]

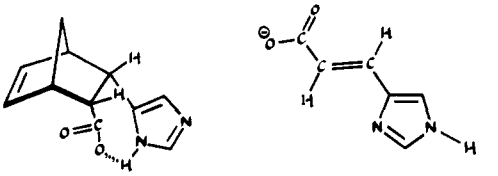
The present investigation is concerned with the possibility of perturbing the position of the N1(H) ⇌ N3(H) equilibria of the imidazole tautomers in aqueous solution through cyclic hydrogen bonding with suitable substituents located at C4. The substituent groups considered correspond to the hydrogen-bonded structures **10–14** of **10a** and **10b** would be expected (as for **5** and **7**) to favor the N1(H) tautomer while **10c–14** should favor the N3(H) tautomer. Urocanic acid **15** was also investigated as a control on the behavior of its cis isomer **13**.



10a **10b** **10c**



11 **12** **13**



14 **15**

Experimental Section

Histamine hydrochloride, the hydrochloride of imidazole-4-acetic acid, and urocanic acid were commercial products. Imidazole-4-propionic acid was prepared by reduction of urocanic acid with the aid of a procedure used for cinnamic acid.[14] Irradiation of a nitrogen-saturated aqueous solution of urocanic acid (mp 218–220 °C; $^3J_{H-H}$ = 16 Hz) in a quartz vessel for 6–20 h (depending on the diameter of the vessel) with a high-pressure 200-W mercury lamp[15–17] resulted in formation of a 60:40

(8) These values are in reasonable agreement with those reported by Alei and co-workers.[4b] Perhaps not surprisingly, we believe chemical shifts are likely to be more accurate than couplings for determining K values (see also later discussion). See Prado et al. (Prado, F. R.; Giessner-Puttre, C.; Pullman, B. *Org. Magn. Reson.* **1981**, *16*, 103–110) for theoretical speculations as to K, chemical shifts, and couplings in imidazoles and related ring systems.

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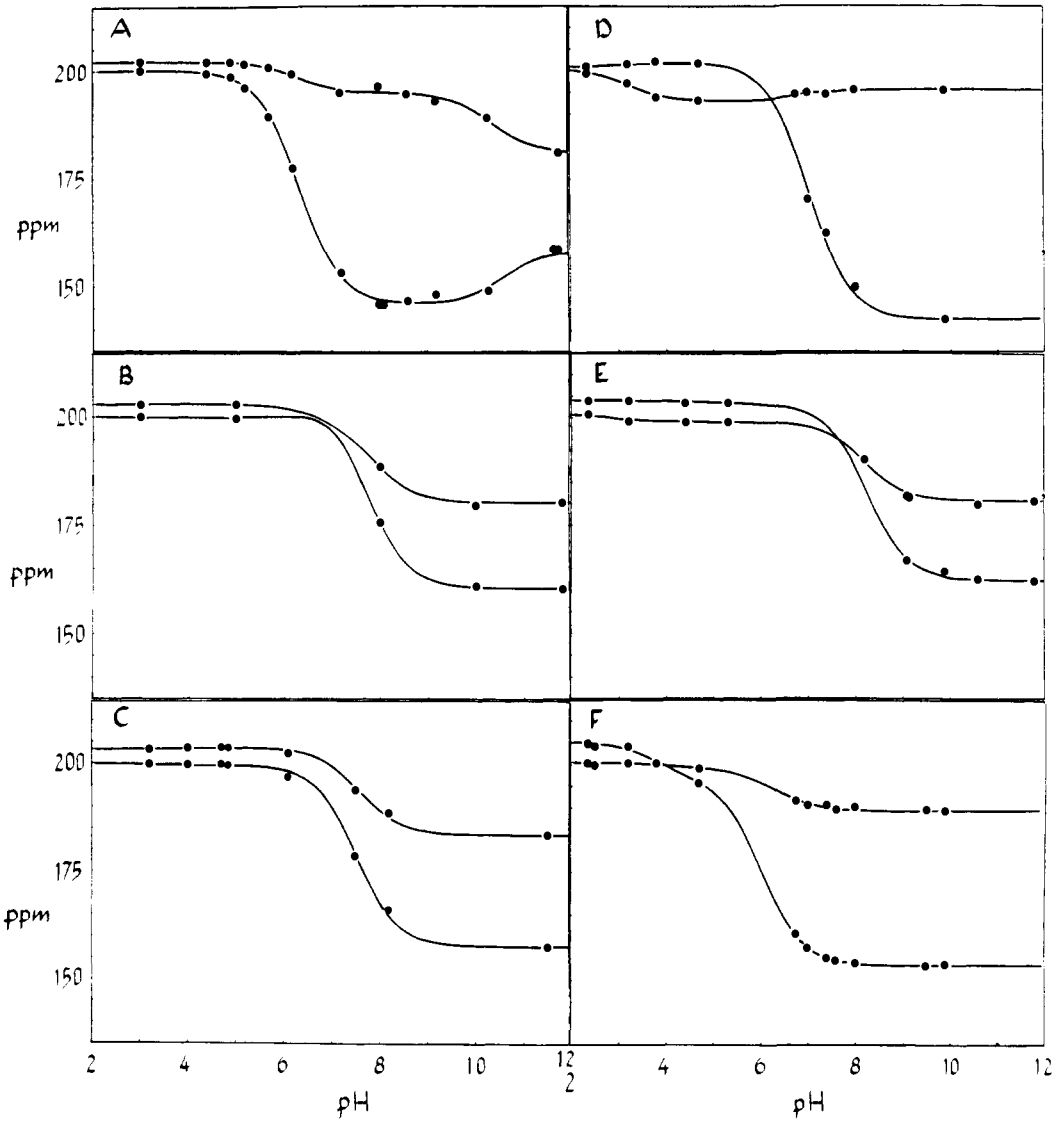

Figure 2. Changes in ^{15}N chemical shift with pH of imidazole nitrogens of the following: A, **10**; B, **11**; C, **12**; D, **13**; E, **14**; F, **15**. For all except D, N1 is the nitrogen with the most upfield shift at pH 12.

ratio of **13** to **15**. The pure cis acid was found to have mp 171–173 °C (lit.[17] mp 175–176 °C) and $^3J_{H-H}$ = 12 Hz.

endo-cis-3-(4-Imidazoyl)bicyclo[2.2.1]hept-5-ene-2-carboxylic acid (**14**) was prepared by Diels–Alder addition of **13** to 1,3-cyclopentadiene. A solution of 3 g (0.022 mol) of **13** in 22 mL of ethanoic acid and 8 mL of trichloromethane was mixed with 1.4 g (0.022 mol) of freshly distilled 1,3-cyclopentadiene and heated under reflux (60–65 °C) for 20 h. At this point, the ^1H NMR spectrum indicated that about 45% of the theoretical amount of **14** had been formed. The solvents were removed by distillation and the products separated on a dry silica column by elution with 95% ethanol. The melting point of **14** prepared in this way was 188–190 °C. The 500-MHz ^1H NMR spectrum of **14** was completely consistent with the assigned structure.

Proton-coupled and decoupled ^{15}N NMR spectra of **10**–**15** were taken with concentrations of 0.2–0.4 M in the Fourier transform mode with a Bruker WH-180 spectrometer at 18.25 MHz.[18] The reference external standard, a solution of 1 M $H^{15}NO_3$ in D_2O, also provided the deuterium field-frequency lock signal. The temperatures were close to 25 °C, except for **13** and **15** close to their isoelectric points (~pH 4.7) when the temperature was increased to 40 °C to improve the solubility. Assignments of the ^{15}N NMR resonances to N1 and N3 were made by comparisons with the reported values of the couplings between the nitrogens and H2 and H5.[4,6] Gated proton decoupling with NOE suppression and repetition rates of 20 s were helpful in obtaining satisfactory signal-to-noise ratios of the N1 resonances of **13** in mixtures with **15**. The pH of the samples were determined directly in the NMR tubes before and after each spectrum with a Radiometer pH meter and was adjusted by addition of small amounts of concentrated sodium hydroxide or hydrochloric acid solutions.

Results and Discussion

The simplest way to evaluate the importance (or nonimportance) of hydrogen bonding of the type exemplified by **10**–**15** is through measurement of the ^{15}N shifts of the imidazole nitrogens as a function of pH.[3,4] Information of ^{15}N shift–pH dependences for this analysis is shown graphically in Figure 2. The lines correlating the experimental points were obtained from nonlinear least-squares fits to the equilibria of eq 2 in accord with eq 3 where

$$\underset{16}{H\text{–}A\text{–}Im^{\oplus}H} \underset{K_1}{\overset{-H^{\oplus}}{\rightleftharpoons}} \underset{17a}{A\text{–}Im^{\oplus}H} \overset{K_3}{\rightleftharpoons} \underset{17b}{HA\text{–}Im} \underset{K_2}{\overset{-H^{\oplus}}{\rightleftharpoons}} \underset{18}{A\text{–}Im} \qquad (2)$$

$$S = (S_1[H^{\oplus}]^2 + S_2K_1[H^{\oplus}] + S_3K_1K_2)/([H^{\oplus}]^2 + K_1[H^{\oplus}] + K_1K_2) \qquad (3)$$

S = measured shift of a particular imidazole nitrogen; S_1 = shift of the particular nitrogen in species **16**; S_2 = average shift of the particular nitrogen for the species involved in the equilibrium **17a** $\rightleftharpoons$ **17b** with equilibrium constant K_3; S_3 = shift of the particular nitrogen in species **18**, and K_1 and K_2 are the acid ionization constants of **16** and **17**, respectively.

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(19) It is not unreasonable that the N3 shift might be somewhat abnormal because of the rather strong intermolecular interaction between the carboxylate group and N3(H) hydrogen as will be discussed later.

Table I. ^{15}N NMR Chemical Shifts and Acid Ionization Constants of Substituted Imidazoles

compd[a]	S_1,[b] ppm N1	S_1,[b] ppm N3	pK_{a_1}[c]	S_2,[b] ppm N1	S_2,[b] ppm N3	pK_{a_2}[c]	K[d]	S_3,[b] ppm N1	S_3,[b] ppm N3	K[d]
$Im(CH_2)_2NH_2$ (10)	202.2	181.0	6.3	194.7	146.0	10.6	0.26	181.0	158.4	0.60[f]
$Im(CH_2)_2CO_2H$ (11)	203.2	200.3	[g]	198.3	202.0	7.7		180.5	160.5	0.61
$ImCH_2CO_2H$ (12)	203.2	200.0	2.9[g]	203.6	199.4	7.5		183.4	157.5	0.53
ImCH=CHCO$_2$H (13) (cis)	200.8	200.5	3.3	202.0	192.6	7.0		142.4	195.5	5.2
$Im(C_7H_8)CO_2H$ (14) (endo-cis)	204.1	201.0	[g]	203.7	199.2	8.2		162.1	180.0	1.5
ImCH=CHCO$_2$H (15) (trans)	205.2	189.4	4.0	199.6	196.4	6.1		189.4	153.5	0.37

[a] Compounds are written in their conventional neutral forms as correspond to the species **10–15**. [b] Chemical shifts relative to an external standard of 1 M $H^{15}NO_3$ in D_2O, calculated from least-squares fits of shifts to eq 2. [c] Acid ionizations corresponding to K_1 and K_2 in eq 1, derived from least-squares fits to eq **2**. Separate values were obtained from the changes in shift of N1 and N3. In each case, the listed value corresponds to the larger change in shift resulting from loss of a proton. [d] Estimated tautomeric equilibrium constants for the equilibrium, N1(H) $\rightleftharpoons$ N3(H). [e] Value corresponds to equilibrium of **10a** with its respective N3(H) tautomer. [f] Value corresponds to the equilibrium between **10b** and **10c**. [g] Value inaccessible or very approximate because of small shift changes in the particular ionization.

In making the least-squares fits, the values of S_1 and S_3 were taken to be the observed shifts at low and high pH while K_1, K_2, and S_2 were calculated to give the best fit to the experimental points. The S_2 values and the pK_{a_n}'s corresponding to the K_n's obtained are summarized in Table I. With histamine, species **17b** (which corresponds to **5**) is assumed to be more stable than **17a** on the basis that imidazoles are weaker bases than aliphatic amines. With the various carboxylic acids **11–15**, the situation is reversed with **17a** expected to be more stable than **17b**.

Equilibrium constants K for the N1(H) $\rightleftharpoons$ N3(H) interconversion are also listed in Table I and these were estimated by using the 83-ppm difference in shifts between N1 and N3 of *N*-methylimidazole as a model shift.[3,4] The procedure cannot be verified as being highly accurate, but certainly it is more accurate than any other determination for solutions made heretofore.

The average of the N1–N3 nitrogen shifts of **10–15** is 170.38 ppm with a sample standard deviation of 0.83, which is about 1.9-ppm upfield of the 168.6-ppm N1–N3 average for *N*-methylimidazole. With the expected >80-ppm chemical-shift difference between the nitrogens for the individual N1(H) and N3(H) tautomers, even a 2–3-ppm uncertainty in the extreme shift values will allow quite reasonable estimates (±5–10%) of K near the midrange, but the estimates will become rather uncertain as K becomes either very large or very small. The values of K listed in Table I were calculated with use of 83 ppm as the shift spread and 170.5 ppm as the average shift. With these values, K calculated for each compound from the N1 shift agreed with K calculated from the N3 shift to within 11% except for **13** where the difference was 28.5%.[19] For all of the compounds, K corresponding to the N1 shift is the one given in the table because of the possibility of special perturbations of the shift of N3 by the substituent groups.

The situation with respect to the N1(H) $\rightleftharpoons$ N3(H) tautomeric equilibria for histamine **10** is very much like that discussed above for histidine (**3**),[6] in that K is large, the N1(H) tautomer being favored. That K for **10a** is virtually identical with that for **3** makes clear the competitive role of the histidine ammonium group in stabilizing the N1(H) tautomer **5** compared to the histidine carboxylate in stabilizing the N3(H) tautomer **6**. Furthermore, that K for neutral histamine is not significantly different from that for **4** indicates that **8** cannot be very important for histidine.

If we take $K \sim 0.82$ for 4-methylimidazole (see earlier discussion), it will be seen that **10b**, **10c**, **11**, and **12** exhibit a somewhat enhanced dominance of the N1(H) tautomer, despite the fact that hydrogen bonding as shown in **10c**, **11**, and **12** should in fact favor the N3(H) tautomer. However, **15** (with the trans configuration of the double bond preventing intramolecular hydrogen bonding) shows an even greater preference for N1(H). Perhaps **15**, in accord with the results of other NMR studies,[8] may have K closer to the "normal" value for 4-substituted imidazoles. If this is so, then slightly smaller K values may reflect some influence of **10c**, **11**, and **12** on the position of equilibrium. However, it is speculative to consider **15** to be "normal", because

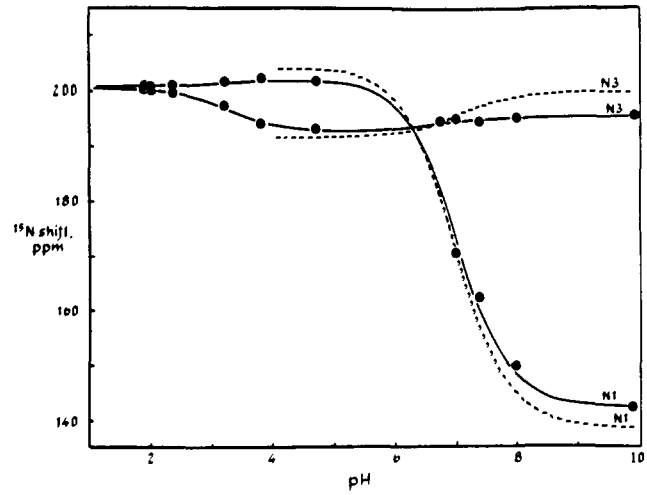


Figure 3. Changes in the ^{15}N chemical shifts of imidazole nitrogens of (—) *cis*-urocanic acid and (---) α-lytic protease[3] with pH.

there could well be a special electronic influence of the carboxyl group of **15** on the nitrogen shifts of the imidazole ring. Evidence that this may be the case comes from the fact that the N1 shift of the imidazolium ring moves downfield by 5.6 ppm while the N3 shift goes upfield by 7 ppm in ionization of the carboxyl group corresponding to K_1 (**16** $\rightleftharpoons$ **17a**). These changes are all the more striking because the identical ionization for the *cis*-urocanic acid results with the N1 and N3 resonances moving in opposite directions, a subject we shall take up shortly.

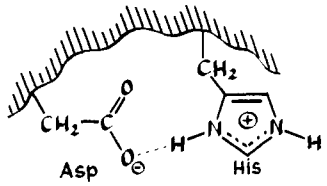
We were surprised to find that **10c**, **11**, or **12** are not important enough to perturb the N1(H) $\rightleftharpoons$ N3(H) equilibria to the N3(H) side. We conclude that, for these substances, either the bond angles are not correct for intramolecular hydrogen bonding (**12**) or that formation of an otherwise favorable hydrogen bond is accompanied by eclipsing of $-CH_2-CH_2-$ hydrogens in the side chain (**10c**, **11**) which is destabilizing enough, especially when the side-chain group is competing with water as the hydrogen bonder, to make the intramolecular hydrogen bond unimportant. To assess this hypothesis, we prepared and measured the ^{15}N shifts of *cis*-urocanic acid (**13**) and its bicyclic analogue **14**. For both of these compounds, the eclipsing problem is circumvented by building in stereochemical rigidity to increase the favorableness of intramolecular hydrogen binding as shown by **13** and **14**.

The values of K which correspond to **13** and **14** indicate that, indeed, providing the proper stereochemical relationships can result in favoring the N3(H) tautomer through carboxylate hydrogen bonding. It was unexpected that **13** corresponds to a substantially larger proportion of N3(H) than does **14**, because α,β-unsaturated carboxylic acids are substantially stronger acids (and their anions expected to be weaker hydrogen bonders) than saturated carboxylic acids. The most likely factor making **13** different from **14** is the

expected difference between the C–C–C bond angles in the connections between the carboxylate and imidazole groups.

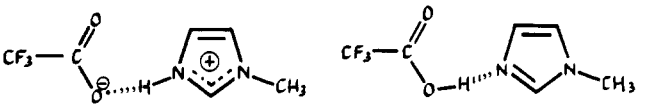
As can be seen in Figure 3, the changes in the ^{15}N shifts of *cis*-urocanic acid with pH are extraordinarily similar to those observed for the histidine of the catalytic triad of the serine protease α-lytic protease.[3] This parallelism provides confirmation of the conclusions drawn previously as to the nature of the species present at the Ser-His-Asp triad of the active site of α-lytic protease and especially the mode of interaction of the aspartic acid with the histidine. Nothing observed in the present research is contrary to the previous determination that His is a stronger base than the Asp carboxylate and that the "charge-relay" mechanism once postulated as the mode of action of serine proteases cannot be correct in its usual formulation.[3,20,21] This should hardly be taken to mean that the Asp carboxylate is unimportant—quite the contrary, it plays an important role in making *K* more favorable for attachment on the serine hydroxyl proton in the catalytic step. It will also stabilize the imidazolium cation when the Ser hydroxyl proton is transferred. In addition, the Asp carboxylate may help keep the imidazolium ring so oriented as to be effective in the protonation and cleavage steps necessary for breaking the peptide bond and formation of the serine ester from the "tetrahedral" intermediates.[3]

A special issue in the interpretation of the His ^{15}N shifts of α-lytic protease was the 12.6-ppm difference at pH 4.5 between the resonances of N1 and N3 with that of N3 being downfield (see Figure 3). This was interpreted as being a downfield perturbation of the N3 shift of the protonated histidine imidazole by the carboxylate anion of Asp (**19**). The assignment was made



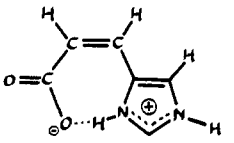
19

by analogy with an N1–N3 shift difference of 16 ppm observed for 2M each of *N*-methylimidazole and trifluoroethanoic acid in trichloromethane.[3,4] Infrared spectra and ^{15}N shifts indicated



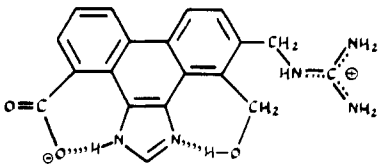
20 21

proton transfer and formation of a carboxylate imidazolium cation **20** rather than the alternative complex **21**.[4] The extrapolation from trichloromethane solution to aqueous solution was a long one and it is gratifying that *cis*-urocanic acid exhibits the same pattern of shifts at pH 4.7 with an N1–N3 shift difference of 9.7 ppm, which can be confidently ascribed to **22**, an analogue of **19**.



22

The similarity of ^{15}N shifts of **19** and **22** as well as of **9** and **13** suggests that it would be interesting to prepare substances such as **23** (with a guanidino or similar cationic group to enhance solubility) for testing as models for serine protease action. To



23

be sure, **23** lacks the "oxyanion hole" and other stabilizing groups for formation of the "tetrahedral intermediate" which characterize serine proteases, but it is possible that some modification of the cationic group shown as guanidinium in **23** could partly fulfill this purpose. An advantage of **23** over enzyme would be a substantially greater thermal stability.

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Effect of Isocyano Group Substitution in Simple Primary, Secondary, and Tertiary Carbanions

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Abstract: The stabilities, structures, and charge distributions of primary, secondary, and tertiary α-isocyano carbanions and their parent carbanions have been examined through ab initio (STO-3G) calculations. The data provide evidence that the isocyano group does not act as a delocalizing substituent but through an inductive effect, in agreement with the conclusions of Walborsky and Periasamy[4] obtained with a more complex isocyano carbanion.

The effect of substituents on carbanions is of interest for both synthetic and mechanistic reasons.[1] While a variety of substituent groups have been employed to enhance the stability of carbanions, the isocyanide group is one of the more effective for such purposes.

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