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# Syn and Anti-Oriented Imidazole Carboxylates as Models for the Histidine-Aspartate Couple in Serine Proteases and Other Enzymes[1]

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**Abstract:** Several compounds containing an imidazole and a carboxylic acid group have been synthesized and studied as models for the His-Asp couple found in serine proteases and other enzymes. The ability of the carboxylic acid group to increase the basicity of the imidazole and its role in nucleophilic and general base catalysis by the imidazole is described.

Many aspects of catalysis by the serine proteases remain poorly understood.[2] Such is the case with the catalytic triad. Over two decades ago Blow reported that the "buried" carboxylate of Asp-102 was hydrogen bonded to His-57 in the active site of chymotrypsin.[3] This finding lead to his formulation of the charge relay mechanism in which the negatively charged carboxylate deprotonates Ser-195, through His-57, thereby generating its more nucleophilic alkoxide. While this proposal met with some skepticism, the proposal (Figure 1A) that the same process occurred in the transition state of attack of Ser-195 on the substrate carbonyl was more widely accepted.[4] In an alternative mechanism, a single proton is transferred from Ser-195 to His-57, while the carboxylate of Asp-102 serves to stabilize the charge of the incipient imidazolium ion (Figure 1B). The kinetic benefit of this stabilization might be expected to depend upon the increase in $pK_a$ of His-57 and the Brønsted coefficient for its general base catalyzed reaction. In addition to its potential role as a base or a charge stabilizing group, Asp-102 has been proposed to: 1) fix the tautomeric form of the imidazole and 2) anchor His-57 in the correct orientation.[5]

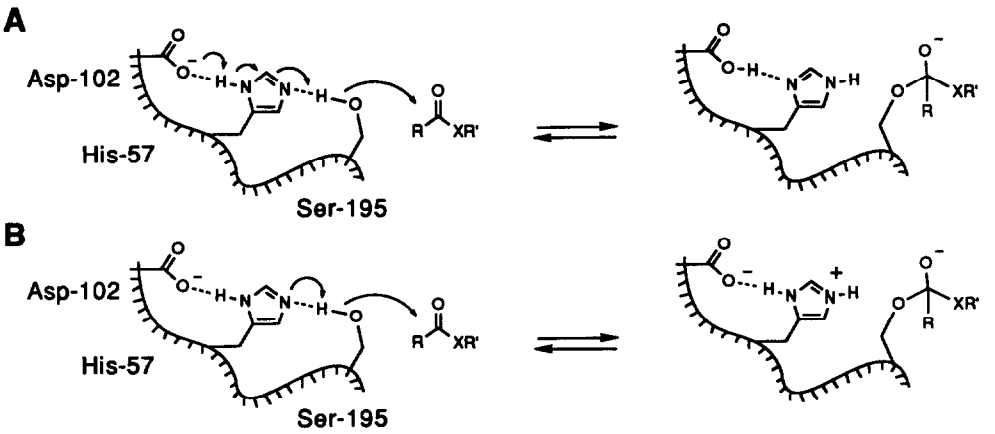



**Figure 1.** A. The charge relay mechanism in which Asp-102 acts as a general base in concert with His-57. B. Charge stabilization mechanism. Deacylation with water can occur by similar mechanisms.

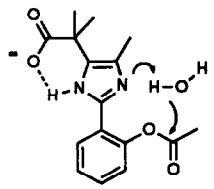
The importance of the buried Asp-102 is evidenced by the number of enzymes which contain an Asp-His-Ser triad. In fact, the bacterial and mammalian proteases present a textbook case of convergent evolution.[6] They have no sequence homology but contain identical catalytic triads. More recently, several other enzymes have been found to contain active site histidine residues which are hydrogen bonded to the carboxylate of aspartate or glutamate. This His-Asp couple, as it has become known, has been found in the active site of zinc containing enzymes,[7] glutathione reductase,[8] several lipases,[9] all serine proteases,[10] malate and lactate dehydrogenase,[11] DNAse I,[12] and possibly in RNase $T_1$.[13] Although the His-Asp couple functions differently in some of these enzymes, its prevalence suggests a considerable catalytic benefit is derived from the carboxylate.

In the serine proteases this benefit has been quantified by site-directed mutagenesis, a powerful technique for examining the role of individual amino acid residues in catalysis. Replacement of Asp-102 by asparagine (D102N) or alanine (D102A) results in mutant enzymes with a $10^4$ fold decrease in turnover number.[14] A careful study, however, showed that the large loss in activity was not due to Asp-102 alone since its replacement in trypsin causes a disruption in the normal hydrogen bonding pattern of the catalytic triad (vide infra).[15] Thus, these important studies document the importance of Asp-102, without distinguishing between mechanisms.

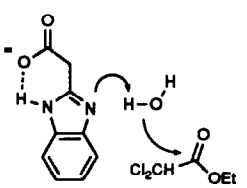
The most direct experimental evidence in support of charge relay comes from proton inventory studies, which have been reviewed by Venkatasubban and Schowen.[16] They conclude that the serine proteases studied thus far exhibit two proton transfer kinetics only when the correct combination of enzyme-substrate interactions are present near the scissile bond and at more distant subsites. The relationship between these interactions and the number of protons transferred has been further delineated by Stein and Strimpler.[17] These studies do not locate the sites of proton transfer and "non-specific" substrates are often processed nearly as efficiently with only a single proton transfer. In a related study, chymotrypsin methylated on His-57 exhibited multiple proton catalysis, although its mechanism is unknown.[18]

Evidence against the charge relay mechanism comes from NMR studies on α-lytic protease and trypsin where protonation of the catalytic triad occurred on histidine, not on aspartate.[5,19] Neutron diffraction studies of a complex between trypsin and a transition state analogue also show selective protonation of histidine.[20] These are by necessity ground state arguments whose relevance to the acylation transition state has been questioned.[2]

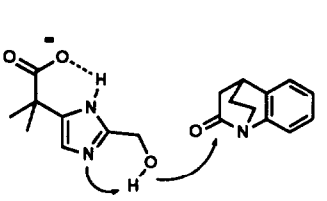
Organic models have great potential to shed light on this important mechanistic question. An extremely thorough kinetic study of the deacylation of compound **1** was reported by Rogers and Bruice in 1974.[21] The carboxylate in **1** was found to give only a 3-fold rate enhancement, which was taken as evidence against the



**1**



**2**



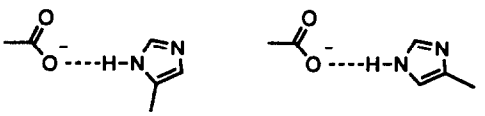
**3**

charge relay model. Bender studied the general base catalyzed hydrolysis of ethyl dichloroacetate by 2-benzimidazole acetic acid (**2**) and found an eightfold deviation in a Brønsted plot.[22,23] Although this deviation was used to support proton transfer to the carboxylate, the solvent isotope effect did not bear this out. The Brønsted

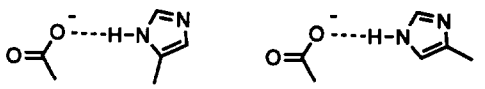
plot deviation might result from the choice of comparison compounds (vide infra). For example, the same analysis was used by Brown in the first demonstration of an intramolecular general base catalyzed O-acylation reaction with hydroxymethylimidazole compounds.[24] Although one of the compounds bore a pendant carboxylate (3), it fit well on a Brønsted plot and gave only a small additional rate enhancement attributed to charge stabilization.

Gandour has questioned the validity of these models on stereoelectronic grounds.[25] It was proposed that a *syn*- and an *anti*-oriented carboxylate may have basicities which differ by a factor of $10^3$-$10^4$, with the *syn* being more basic. Models **1-3** constrain the carboxylate to an *anti*-disposition, while the X-ray structures of serine proteases show that Asp-102 is *syn*-oriented to His-57. Of the enzymes that contain His-Asp couples there is a strong, though not absolute, preference for the N-3(H)-*syn* orientation (Chart I). The exceptions are phospholipase $A_2$ and human pancreatic lipase which contain His-Asp couples with N-3(H)-*anti* and N-1(H)-*anti*-orientations, respectively.[9b,c] Has the enzymic His-Asp couple evolved to prefer a *syn*-carboxylate as a result

**Chart I**



N-3(H)-syn N-1(H)-syn

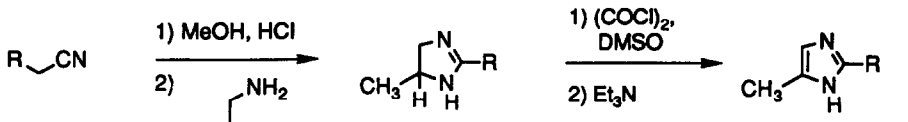


N-3(H)-anti N-1(H)-anti

of this proposed difference in carboxylate basicities? Do *syn*- and *anti*-carboxylates alter the basicity and catalytic properties of a proximate imidazole to different extents? In order to answer these questions we compare the structure, basicity, and catalytic properties of **4**, our previously described *syn*-oriented model of the His-Asp couple, with several *anti*-models of the His-Asp couple, and with the enzymic system.

## Results and Discussion.

**Compounds.** The compounds used in this study are shown in Chart II. His-Asp model **4** and amide **5**, a model for D102N trypsin, were obtained from the corresponding ester as described in the experimental section. Imidazoles **6** and **7** were synthesized by Swern oxidation of the corresponding imidazolines which in turn were



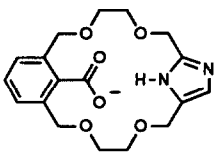
Eq. 1

**6**: R = $CH_2OCH_3$
**7**: R = $CH_2CH_2OCH_3$

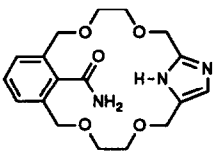
available from the reaction of 1,2-diaminopropane and the appropriate imino ether (equation 1).[26] The other compounds in Chart II were commercially available or readily synthesized from available materials (see experimental section).

**Structures and $pK_a$ Determinations.** One of the major issues addressed in this work is the importance of carboxylate orientation on the properties of a proximate imdazole ring. As pointed out by Gandour, these orientations fall into two general classes: *syn* and *anti*.[25] Of the compounds used as His-Asp models in this study, **10**, **13**, **16**, and **21** contain *anti*-oriented carboxylates while **4** has the *syn*-orientation that is found in the enzymic systems. Other important properties of the models include the distance between the carboxylate and the imidazole, the microenvironment (e.g. solvation) of the imidazole-carboxylate couple, and the geometry of the intramolecular hydrogen bond, assuming one can form.

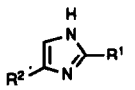
**Chart II**



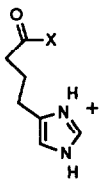
**4**



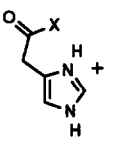
**5**



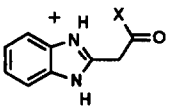
**6**: $R^1 = CH_2OCH_3$, $R^2 = CH_3$
**7**: $R^1 = CH_2CH_2OCH_3$, $R^2 = CH_3$
**8**: $R^1 = CH_2CH_3$, $R^2 = CH_3$



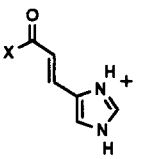
**9**: X = OMe
**10**: X = $O^-$



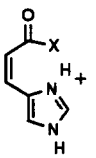
**11**: X = OMe
**12**: X = $NH_2$
**13**: X = $O^-$



**14**: X = OMe
**15**: X = $NH_2$
**16**: X = $O^-$



**17**: X = OMe
**18**: X = $O^-$



**19**: X = OMe
**20**: X = $NH_2$
**21**: X = $O^-$

The design of compound **4** was based on X-ray studies of two analogous crown ethers synthesized by Cram.[27] In the X-ray structures of both molecules a *syn*-transannular hydrogen bond was present between the carboxylic acid and the heteroatom occupying the position analogous to that of the internal imidazole nitrogen in **4**.[28] With **4**, standard methods for detecting an internal hydrogen bond were suggestive but not conclusive. Therefore, the structure of the imidazolium-carboxylate was examined computationally. The randomize function of PC MODEL[29] produced 21 conformations which were within 3 kcal $mol^{-1}$ of the lowest energy structure (Figure 2). Each of these low energy conformations contained a *syn*-oriented hydrogen bond, one of which was bifurcated.[30] The average $CO_2^-\cdots HN^+$ distance weighted for the different conformational populations was 2.54 Å, which is close to that found in the X-ray structures of enzymic systems (e.g. 2.7 Å in trypsin).[10] It is likely that a protonated His-57 is present in most of the X-ray structures of the enzymes.[31]

The first question we sought to answer is whether a *syn*-carboxylate can raise the $pK_a$ value of a proximate

imidazolium ion to a greater extent than can an *anti*-carboxylate. We chose an analysis in which comparisons are initially made between similar structures. Thus, the $pK_a$ of an imidazolium-carboxylate is compared to the same imidazolium ion lacking the proximate carboxylate. This $\Delta pK_a$ value is the increase in imidazolium ion basicity

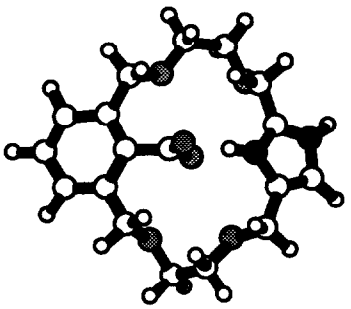


**Figure 2.** Low energy conformation of **4** containing a transannular hydrogen bond.

due to the carboxylate, and its magnitude is dependent on the strength of the $CO_2^-\cdots HN^+$ interaction. The interaction may occur through: 1) inductive effects and 2) through-space effects of hydrogen bonding and/or electrostatic stabilization. The strength of the through-space effect will depend upon: 1) the $CO_2^-\cdots HN^+$ distance, 2) solvation, and 3) carboxylate orientation. In order to determine the effects of orientation, comparisons must be made between systems with identical inductive effects, imidazole-carboxylate distances, and solvation.

The $pK_a$ values of compounds **4-5** and **9-21** and various $\Delta pK_a$ calculations are compiled in Table 1. As we have reported, the *syn*-systems (entries 9-10) have $\Delta pK_a$ values significantly higher than the *anti*-models (compare with entries 1-2). High *anti* $\Delta pK_a$ values (entries 4-8) can be obtained, but comparison with these $\Delta pK_a$ values is not valid because they have a large inductive component, in addition to the through space electrostatic/hydrogen bonding effect of interest.[24] This is seen in entry 11 where a remote carboxylate inductively increases the $pK_a$ of an imidazole by 0.91 units relative to an ester. The methylene group in **11-16** transmits the electronic effects to the same extent as the double bond in **18** (**21**), as evidenced by the Hammett ρ values for the dissociation of $XC_6H_4CH_2COOH$ ($\rho = 0.49$) and $XC_6H_4CH{=}CHCOOH$ ($\rho = 0.47$).[32]

**Table 1.** $pK_a$ Values of Imidazole-carboxylic Acids and Their Derivatives and $\Delta pK_a$ Values.

| Entry | Imidazole | pKa | Reference | pKa | ΔpKa |
|---|---|---|---|---|---|
| 1[a] | **10** | 7.62 | **9** | 7.3 | 0.3 |
| 2 | **21** | 6.78 ± 0.03 | **18** | 5.89 ± 0.03 | 0.89 |
| 3 | **21** | 6.78 ± 0.03 | **20** | 6.03 ± 0.02 | 0.75 |
| 4 | **21** | 6.78 ± 0.03 | **19** | 5.58 ± 0.03 | 1.20 |
| 5 | **13** | 7.46 ± 0.02 | **11** | 6.37 ± 0.03 | 1.09 |
| 6 | **13** | 7.46 ± 0.02 | **12** | 6.41 ± 0.02 | 1.05 |
| 7 | **16** | 6.28 ± 0.02 | **14** | 4.98 ± 0.02 | 1.30 |
| 8 | **16** | 6.28 ± 0.02 | **15** | 5.10 ± 0.01 | 1.18 |
| 9 | **4** | 7.05 ± 0.05 | **5** | 5.72 ± 0.02 | 1.33 |
| 10[b] | Trypsin | 6.8 | D102N Trypsin | 5.3 | 1.5 |
| 11 | **18** | 5.89 ± 0.03 | **17** | 4.98 ± 0.01 | 0.91 |

[a]Reference 33. [b]Reference15.

Which comparisons are the most appropriate? We believe entries 2, 9, and 10 are easily compared. The inductive effects are negligible in each, and the calculated $CO_2^-\cdots HN^+$ distances of 2.54 Å in **4** and 2.62 Å in **21**[29] are close to the value of 2.7 Å found in trypsin. Differences in solvation are difficult to quantify and could overwhelm the more subtle question of orientation. The carboxylate-imidazolium interaction in **4** occurs within a lipophilic crown ether, whereas that in the urocanic system is more exposed to solvent. In the serine proteases Asp-102 is found in both hydrophobic and highly polar environments.[34,35] Thus, with the proviso that solvation may play a role, it can be concluded that a *syn*-carboxylate can raise the $pK_a$ of a proximate imidazolium ion by ca. 0.4-0.6 $pK_a$ units over that of an *anti*-carboxylate.[36] This small increase does not invalidate the Gandour proposal because it is unknown to what degree a 3- or 4-fold increase in the $pK_a$ of the carboxylic acid would alter the basicity of the imidazole. Whatever the difference in basicity of *syn*- and *anti*-oriented carboxylates, the effect on the imidazole basicity is small, and it is questionable whether enzymes would have evolved in response to such a small energy difference.

**Kinetic Studies.** The substrates used for kinetic studies were chosen to have convenient rates and to be easily monitored by UV-visible spectroscopy. Since the enzymes which use His-Asp couples catalyze a wide range of transformations, the reaction examined is less important than its mechanism. We examined one reaction proceeding by a nucleophilic mechanism and a second reaction proceeding by a general base mechanism (Figure 3). Acyl transfer to imidazole (nucleophilic catalysis) was examined using 4-nitrophenylacetate (pNPA) as substrate.[37] Williams had shown that 4-nitrophenyl diphenylphosphinate underwent general acid catalyzed hydrolysis by imidazole;[38] however, with our 2,4(5)-substituted imidazoles this reaction failed to show good first order kinetics. A search of related substrates lead to 2-nitrophenyl diphenylphosphinate (**21**).

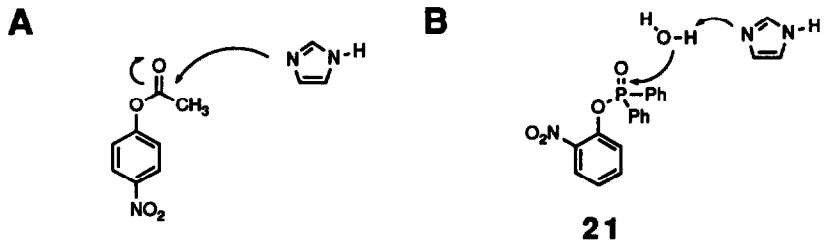



**Figure 3.** A. Nucleophilic catalyzed hydrolysis of p-nitrophenyl acetate. B. General base catalyzed hydrolysis of 2-nitrophenyl diphenylphosphinate by imidazole.

The kinetic role of the carboxylate in *syn*-His-Asp model **4** and in *anti*-models **13**, **16**, and **21** was determined through comparison with simple imidazole compounds using the Brønsted relationship. This relationship requires that all of the compounds in a series have similar steric constraints, but a wide range of $pK_a$ values. In the case of **4**, the comparison was made to the corresponding amide **5** and compounds **6-8**, the latter compounds prepared with a new imidazole synthesis (vide supra).[26] The kinetic results for all the compounds are compiled in Table 2. As seen in Figures 4 and 5, linear Brønsted plots can be constructed with *syn*-His-Asp model **4**, the *anti*-imidazole-carboxylates, and the comparison compounds. If the carboxylates were playing a special catalytic role (e.g. general base) this would show up as a positive deviation in the Brønsted plot. Such a deviation is not seen. A general base role for the carboxylate in **4** is further ruled out by identical solvent isotope effects for **4** ($k_{H2O}/k_{D2O} = 1.25 \pm 0.08$) and **8** ($k_{H2O}/k_{D2O} = 1.2 \pm 0.1$) in the hydrolysis of 4-nitrophenyl-

acetate, and again by identical solvent isotope effects for **4** ($k_{H2O}/k_{D2O}$ = 2.08 ± 0.03) and **8** ($k_{H2O}/k_{D2O}$ = 1.9 ± 0.2) in the hydrolysis of **21**.

The fact that benzimidazole acetic acid (**16**) fits the Brønsted plot is particularly significant because Bender found an 8 fold deviation in the general base catalyzed hydrolysis of ethyl chloroacetate by **16** and took this as support for the charge relay mechanism.[22] The deviation in his system might be attributed to the choice of

**Table 2.** Equilibrium $pK_a$ Values of Imidazolium Compounds and Second Order Rate Constants for their Reaction with 4-Nitrophenylacetate and 2-Nitrophenyl diphenylphosphinate (**21**).[a]

| Entry | Imidazole | $pK_a$ | Solvent | 4-Nitrophenylacetate $k_2^{max} \times 10^3$ $M^{-1}s^{-1}$ | 2-Nitrophenyl diphenylphosphinate $k_2^{max} \times 10^3$ $M^{-1}s^{-1}$ |
|---|---|---|---|---|---|
| 1 | **4** | 7.05 ± 0.05 | $H_2O$ | 10.0 ± 0.4 | 3.74 ± 0.04 |
| 2 | **4** | 7.50 ± 0.08 | $D_2O$ | 8.0 ± 0.4 | 1.80 ± 0.02 |
| 3 | **5** | 5.72 ± 0.02 | $H_2O$ | 2.0 ± 0.1 | 1.79 ± 0.08 |
| 4 | **5** | 6.15 ± 0.06 | $D_2O$ | | |
| 5 | **6** | 7.03 ± 0.01 | $H_2O$ | 10.2 ± 0.6 | 2.6 ± 0.2 |
| 6 | **7** | 8.13 ± 0.02 | $H_2O$ | 21.4 ± 0.1 | 5.0 ± 0.3 |
| 7 | **8** | 8.50 ± 0.02 | $H_2O$ | 60.0 ± 1.0 | 5.6 ± 0.4 |
| 8 | **8** | 8.62 ± 0.04 | $D_2O$ | 52.0 ± 6.0 | 2.9 ± 0.1 |
| 9 | **19** | 5.58 ± 0.03 | $H_2O$ | 15 ± 6 | |
| 10 | **20** | 6.03 ± 0.02 | $H_2O$ | 43 ± 5 | 1.6 ± 0.7 |
| 11 | **16** | 6.28 ± 0.02 | $H_2O$ | | 1.5 ± 0.5 |
| 12 | **11** | 6.37 ± 0.03 | $H_2O$ | 86 ± 16 | |
| 13 | **12** | 6.41 ± 0.02 | $H_2O$ | 73 ± 9 | 3.0 ± 0.5 |
| 14 | **21** | 6.78 ± 0.03 | $H_2O$ | 188 ± 25 | 3.5 ± 1.1 |
| 15 | **13** | 7.46 ± 0.02 | $H_2O$ | 408 ± 81 | 7.5 ± 1.8 |

[a]The data in entries 1-8 are taken from reference 1. The other $pK_a$ values were determined by potentiometric titration at 25 °C, μ = 0.1 (KCl) and second order rate constants ($k_2$) at 25 °C, μ = 0.1 (KCl) were determined from plots of $k_{obsd}$ against imidazole concentration. Maximal second order rate constants were calculated from $k_2 = k_2^{max} K_a/([H^+] + K_a)$.

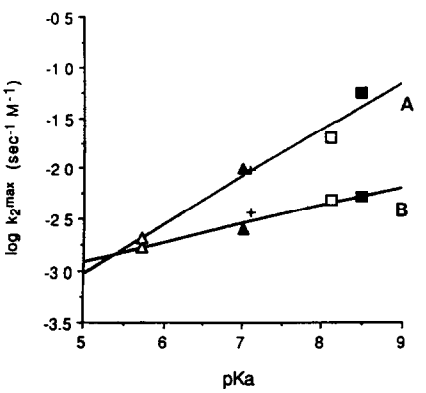



**Figure 4.** Brønsted plots for reaction of imidazole **4** (+), **5** (Δ), **6** (▲), **7** (□), **8** (■) with pNPA (A), β = 0.49, and **21** (B), β = 0.18.

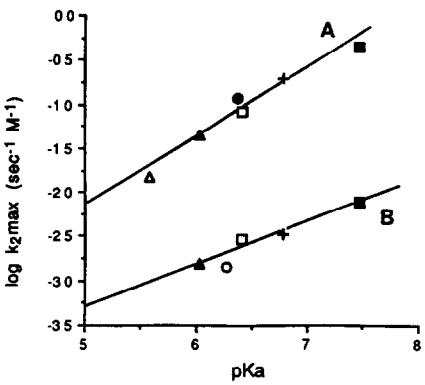



**Figure 5.** Brønsted plots for reaction of imidazole **11** (•), **12** (□), **13** (■), **16** (○), **19** (Δ), **20** (▲), **21** (+) with pNPA (A), β = 0.76 and **21** (B), β = 0.49.

comparison compounds, in particular the inclusion of acetate ion and N-methylimidazole. The results with **16** and the two other *anti*-models **13** and **21** are wholly consistent with the results of Bruice and Brown, that is, a

pendant *anti*-carboxylate provides a minimal benefit to imidazole catalyzed hydrolytic reactions.[21,24]

We conclude that no general base catalysis is operating in His-Asp models with either *syn*- or *anti*-oriented carboxylates. Indeed, the rate enhancement provided by the carboxylates in all His-Asp models is less than 10 fold, and limited to the increase in $pK_a$ of the imidazole as dictated by the Brønsted catalysis law. Why does the enzyme experience such a large rate deceleration when Asp-102 is mutated to Asn-102? Sprang has reported evidence that the amide of Asn-102 *donates* a hydrogen bond to His-57, reversing its tautomeric form so that it can no longer function as a base (Figure 4).[15] As such, the $10^4$ fold rate deceleration for the mutant enzyme (D102N) represents at least part of the contribution of the *entire* catalytic triad. While amides **12** and **20** could bear a similar reverse hydrogen bonding pattern, they are not constrained to as is trypsin. A negative deviation in the Brønsted plots is not seen for either of these compounds. Thus, the mutagenesis studies alone have confirmed the proposal that an essential role of Asp-102 is to provide the correct tautomeric form of His-57.[5]

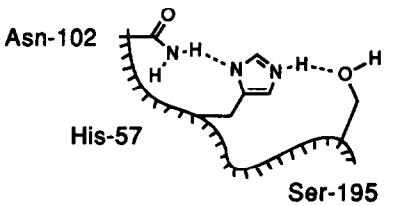



**Figure 4.** Reversed hydrogen bonding scheme in mutant (D102N) trypsin as proposed in Reference 15.

Our model studies are complementary to the mutagenesis work since they show that the *syn*-carboxylate provides only a small direct catalytic advantage in hydrolytic reactions. We cannot, however, rule out the possibility that the microenvironment of the His-Asp couple is altered in the enzyme-substrate complex such that the carboxylate provides a larger catalytic benefit.[39] Work with mixed aqueous-organic solvents should delineate the conditions under which this might occur.

**Experimental.**

**General.** Methanol was distilled from magnesium methoxide under nitrogen. Other solvents and reagents were of reagent-grade quality and used without further purification. All compounds used in this study had correct elemental analyses and had spectral data which were in accord with the assigned structure. Methyl 4-imidazoleacetate (**11**),[40] methyl benzimidazoleacetate (**14**),[41] benzimidazoleacetamide (**15**),[42] benzimidazole acetic acid (**16**),[42] methyl E-urocanate (**17**),[43] and methyl Z-urocanate (**19**)[44] were prepared by known methods. 2-Nitrophenyl diphenylphosphinate (**21**) was prepared according to the procedure of Williams,[44] mp 100-102 °C.

Analytical TLC was performed on 0.2-mm silica 60 coated plastic sheets (EM Science) with F-254 indicator. Melting points were measured on a Thomas-Hoover melting point apparatus and are uncorrected. Infrared (IR) spectra were obtained on either a Nicolet 7199C FT-IR or IBM FT-IR-32 spectrophotometer. $^1H$ NMR and $^{13}C$ NMR spectra were recorded on either a General Electric QE-300 (300 MHz, $^1H$; 75.5 MHz, $^{13}C$), a GN 500 (500 MHz, $^1H$; 125.8 MHz $^{13}C$), or a Varian XL-200 (200 MHz, $^1H$) and performed in chloroform-*d* unless otherwise stated. Chemical shifts are reported in parts per million (ppm) with TMS as an internal reference; coupling constants are reported in Hertz (Hz). Spectra in methanol-$d_4$ were referenced to the residual protio-

solvent peak (δ = 3.30). Mass spectra were obtained on Varian MAT CH-5 and 731 spectrometers. Elemental analyses were performed at the University of Illinois School of Chemical Sciences.

**3,6,13,16-Tetraoxa-9,23-diazatricyclo[16.3.1.1$^{8,11}$]-tricosa-1(22),8,10,18,20-pentaene-22-carboxylic acid (4).** To a stirred slurry of 624 mg (5.57 mmol) of potassium *tert*-butoxide in 15 mL of diethyl ether and 25 μL (1.39 mmol) of water at 0 °C was added a solution of 262 mg (0.697 mmol) of ester 4[26] in 15 mL of diethyl ether. The solution was allowed to warm to room temperature and stirred for 12 h. Ice water was added to the reaction until the mixture became homogeneous, the aqueous solution was extracted once with 30 mL of diethyl ether and once with 30 ml of methylene chloride. The aqueous solution was then adjusted to pH = 5.6 with 1 N HCl. Concentration of the reaction mixture under reduced pressure afforded a white powder. Chromatography (Sephadex LH-20, methanol) yielded the desired amino acid **4** as a white hygroscopic solid: mp 100-102 °C; IR ($CHCl_3$) 3600-3100, 2950, 2820, 1566, 1466, 1225, 1205, 1099 cm$^{-1}$; $^1$H NMR ($CD_3OD$) δ 7.37-7.21 (m, 3H, ArH), 6.88 (s, 1H, H-10), 4.66 (s, 2H, $CH_2$-2 or $CH_2$-17), 4.63 (s, 4H, $CH_2$-2 or $CH_2$-17), 4.54 (s, 2H, $CH_2$-7), 4.38 (s, 2H, $CH_2$-12), 3.67-3.73 (m, 8H, $OCH_2CH_2O$); $^{13}$C ($CD_3OD$) δ 173.59, 144.92, 139.10, 134.40, 130.62, 129.39, 129.09, 127.62, 118.24, 72.29, 71.90, 70.03, 69.98, 69.82, 69.36, 63.54, 62.77; mass spectrum (FAB), *m/z* 362 ($M^+$), exact mass calcd for $C_{18}H_{23}N_2O_6$ ($M^+$+1) *m/z* 363.1556, measured 363.1557. Anal. Calcd for $C_{18}H_{22}N_2O_6$: C, 59.64; H, 6.12; N, 7.73. Found: C, 59.22; H, 6.20; N, 7.42.

**3,6,13,16-Tetraoxa-9,23-diazotricyclo[16.3.1.1$^{8,11}$]-tricosa-1(22),8,10,18,20-pentaene-22-benzamide (5).** To a stirred solution of 209 mg (9.09 mmol) of sodium in dry ammonia at -50 °C was added 10 mg of ferric nitrate nonahydrate. The solution was stirred until the blue color turned to a brown-black. A solution of 250 mg (0.67 mmol) of the methyl ester of **4** in 10 mL of ether was added, the cold bath was removed and the solution was refluxed for 1 h. The ammonia was allowed to evaporate and the residue was taken up in 50 mL brine and extracted three times with 10% 2-propanol-chloroform. The organic layers were combined, dried over magnesium sulfate and concentrated under reduced pressure to give a yellow foam. Flash chromatography (8% methanol-$CH_2Cl_2$) yielded 100 mg (41%) of **5** as a white foam. Recrystallization from water gave colorless cubic crystals as a dihydrate: mp 187-189 °C ; IR ($CDCl_3$) 3477, 3439, 3155, 2904, 2873, 1662, 1603, 1586, 1465, 1381, 1356, 1279, 1243, 1169, 1095 cm$^{-1}$; $^1$H NMR δ 10.25 (br s, 1H, NH), 7.19-7.18 (m, 3H, ArH), 6.76 (s, 1H, H-10), 5.45 (br s, 1H, NH), 4.57 (s, 2H, $CH_2$-7), 4.52 (s, 2H, $CH_2$-2 or $CH_2$-17), 4.45 (s, 2H, $CH_2$-2 or $CH_2$-17), 4.25 (s, 2H, $CH_2$-12), 3.63-3.53 (m, 8H, $OCH_2CH_2O$); $^{13}$C NMR δ 170.89, 137.57, 135.72, 135.18, 130.57, 130.43, 128.97, 128.31, 72.17, 69.95, 69.79, 69.44, 69.30, 66.50; MS (FD, 15 ma), *m/z* 362 ($M^+$, 100). Anal. Calcd for $C_{18}H_{23}O_5N_3$•2 $H_2O$: C, 58.37; H, 6.53; N, 11.34. Found: C, 58.75; H, 6.24; N, 11.15.

**2-Methoxymethyl-4(5)-methyl-2-imidazoline.** Using the general procedure of Zimmerman,[26] 2.00 g (0.028 mol) of methoxyacetonitrile and 2.08 (0.028 mol) of 1,2-diaminopropane were reacted to give a crude product which was distilled by Kugelrohr to afford 2.40 g (67%) of product as a clear oil: bp 45-50 °C/ 0.3 mm; $^1$H NMR ($CD_3OD$) δ 4.00 (s, 3H, $CH_2$-2), 3.99-3.91 (m, 1H, H-4), 3.70 (dd, 1H, H-5), 3.35 (s, 3H, $OCH_3$), 3.16 (dd, 1H, H-5), 1.16 (d, 3H, $CH_3$-4); $^{13}$C NMR ($CD_3OD$) δ 165.56, 67.91, 58.14, 56.52, 20.97. Anal. Calcd for $C_6H_{12}N_2O$: C, 56.23; H,9.44; N, 21.86. Found: C, 55.77; H, 9.47; N, 21.50.

**2-Methoxymethyl-4(5)-methylimidazole (6).** Using the general procedure of Zimmerman,[26] 1.47 g

(0.011 mol) of 2-methoxymethyl-4(5)-methyl-2-imidazoline was oxidized to afford a crude product which was distilled by Kugelrohr to afford 800 mg (55%) of **6**. Recrystallization from ether-pentane gave waxy, white crystals: bp 70-80 °C/0.03 mm; mp 45-45 °C; UV (Borate buffer, pH = 8.0) $\lambda_{max}$ = 230 ($\varepsilon$ = 281); IR ($CCl_4$) 3468, 3109, 2988, 2824, 1549, 1448, 1252, 1190 $cm^{-1}$; $^1H$ NMR $\delta$ 6.71 (s, 1H, H-5), 4.49 (s, 2H, $CH_2$-2), 3.37 (s, 3H, $OCH_3$), 2.23 (s, 3H, $CH_3$-4); $^{13}C$ $\delta$ 144.14, 132.22, 118.17, 67.31, 58.05, 11.69; mass spectrum (EI, 70 eV), *m/z* (relative intensity) 127 ($M^+$+1, 3), 126 ($M^+$, 35), 96 (100), 95 (77), 45 (35). Anal. Calcd for $C_6H_{10}N_2O$: C, 57.12; H, 7.99; N, 22.21. Found: C, 57.13; H, 7.98; N, 22.21.

**2-[2-(Methoxy)ethyl]-4(5)-methyl-2-imidazoline.** Using the general procedure of Zimmerman,[26] 5.74 g (67 mmol) of 3-methoxypropionitrile and 5.0 g (67 mmol) of 1,2-diaminopropane were reacted to give a crude product which was distilled by Kugelrohr to afford 5.56 (58%) of **7** as a clear oil: bp 65-70 °C/0.02 mm; $^1H$ NMR $\delta$ 4.62 (br s, 1H, NH), 3.90 (m, 1H, H-4), 3.66 (dd, 1H, H-5, $J_{4,5}$ = 10.4), 3.56 (t, 2H, $CH_2OCH_3$, J = 6.29), 3.32 (s, 3H, $OCH_3$), 3.11 (dd, H-5, $J_{4,5}$ = 11.35), 2.48 (t, 2H, $CH_2$-2, J = 6.05), 1.15 (d, 3H, $CH_3$-4, J = 5.78); $^{13}C$ NMR $\delta$ 165.20, 69.50, 58.55, 56.63, 56.10, 29.58, 21.62; IR (CDCl3) 3854, 3630-3200, 2968, 2930, 2864, 1842, 1734, 1653, 1605, 1483, 1454, 1375, 1344, 1253, 1113, 1018 $cm^{-1}$.

**2-[2-(Methoxy)ethyl]-4(5)-methylimidazole (7).** Using the general procedure of Zimmerman,[26] 3.0 g (0.21 mmol) of 2-[2-(methoxy)ethyl]-4(5)-methyl-2-imidazoline was oxidized to give a crude product which was distilled by Kugelrohr to afford 1.5 g (51%) of **7** as a clear colorless oil: bp 100-110 °C/0.2 mm; $^1H$ NMR $\delta$ 9.50 (br s, 1H, NH), 3.67 (t, 2H, $CH_2OCH_3$, J = 5.93), 3.38 (s, 3H, $OCH_3$), 2.95 (t, 2H, $CH_2$-2, J = 6.0), 2.20 (s, 3H, $CH_3$-4); $^{13}C$ NMR $\delta$ 145.68, 132.01, 116.88, 70.78, 58.63, 28.81, 11.91; IR ($CDCl_3$) 3433, 3152, 2928, 2897, 2833, 2743, 1593, 1576, 1441, 1385, 1321, 1267, 1221, 1190, 1111, 1064 $cm^{-1}$; MS (EI, 70 eV), *m/z* (relative intensity) 140 (M+, 20), 125 (23), 110 (45), 109 (20), 96 (17), 95 (100). Anal. Calcd for $C_7H_{12}N_2O$: C, 59.98; H, 8.63; N, 19.98. Found: C, 59.87, 8.61, N, 19.92.

**4-Imidazoleacetamide (12).** Ammonia was passed for 25 minutes through a stirred solution of 0.59 g (4.2 mmol) of methyl 4-imidazoleacetate in 25 mL of dry methanol under nitrogen and in an ice bath. The solution was stirred at room temperature overnight and the solvent was removed at reduced pressure, leaving an off-white solid. Recrystallization from acetonitrile containing a small amount of methanol yielded 0.39 g (74%) of **12** as small white crystals: mp 145-147 °C; $^1H$ NMR ($CD_3OD$) $\delta$ 7.60 (s, 1 H, H-2), 6.95 (s, 1 H, H-5), 3.49 (s, 2 H, $CH_2$); $^{13}C$ NMR $\delta$ 175.96, 136.42, 133.16, 118.25, 35.02; MS (EI, 70 eV), *m/z* (relative intensity) 125 ($M^+$, 22), 82 (100), 81 (76), 54 (35); exact mass calcd for $C_5H_7N_3O$ *m/z* 125.05891, measured 125.05917. Anal. Calcd for $C_5H_7N_3O$: C, 47.99; H, 5.64; N, 33.58. Found: C, 47.95; H, 5.59; N, 33.49.

***E*-Urocanamide.** A stirred mixture of 1.98 g (14.3 mmol) of urocanic acid and 60 mL of thionyl chloride under a drying tube was heated to reflux for 3 hours. The thionyl chloride was removed under reduced pressure to afford a light orange powder which was gradually added to 70 mL of conc. ammonium hydroxide stirring in an ice bath. The reddish-brown solution was heated on a hot plate until only a small amount of ammonia was given off, and the remaining solvent was removed under reduced pressure. Preadsorption to silica gel (methanol) and flash chromatography (50 mm OD column, 1:4 methanol-dichloromethane) yielded 1.11 g (56%) of *E*-urocanamide as a yellow solid: mp 188-189 °C; $^1H$ NMR $\delta$ 7.75 (s, 1 H, H-2), 7.46 (d, $J_{6,7}$ = 15.5, 1 H, H-6), 7.34 (s, 1 H, H-5), 6.52 (d, $J_{6,7}$ = 15.5, 1 H, H-7); $^{13}C$ NMR $\delta$ 171.3, 138.5, 136.0, 133.3, 122.9, 118.7; MS (EI, 70 eV) *m/z* (relative intensity) 137 ($M^+$, 51), 136 (M-1, 34), 121 (29), 93 (100); exact mass calcd for $C_6H_7N_3O$ *m/z* 137.05891, measured 137.05839.

**Z-Urocanamide (20).** A solution of 3.0 g (22 mmol) of *E*-urocanamide in dry methanol under nitrogen was irradiated with a 450W medium pressure mercury-vapor lamp for 12 hours. TLC showed a 1:1 mixture of *E*- and *Z*-isomers. Preadsorption to silica gel (methanol) and flash chromatography (50 mm OD column, 1:4 methanol-dichloromethane) yielded 1.34 g (45%) of the *Z* isomer as a yellow solid. An analytical sample was obtained by dissolving this material in 2-propanol and filtering through a Millipore filter, removing the solvent under reduced pressure, dissolving in water, and refiltering. Evaporation afforded a residue which was recrystallized from water to yield **20** as a pale-yellow solid: mp 148-149 °C; $^1$H NMR δ 7.76 (s, 1 H, H-2), 7.25 (br s, 1 H, H-5), 6.75 (d, $J_{6,7}$ = 12.7, 1 H, H-6), 5.77 (br d, $J_{6,7}$ = 12.7, 1 H, H-7); $^{13}$C NMR δ 171.3, 137.8, 133.1, 130.6, 129.1, 116.8; MS (70 eV, EI) *m/z* (relative intensity) 138 (M+1, 6), 137 ($M^+$, 86), 136 (M-1, 97), 120 (100); exact mass calcd for $C_6H_7N_3O$ *m/z* 137.05891, measured 137.05870. Anal. Calcd for $C_6H_7N_3O$: C, 52.55; H, 5.14; N, 30.64. Found: C, 52.56; H, 5.15; N, 30.63.

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