portions of the ligand were used to prepare titrant ligand solutions. For experiments in the chloroform/methanol solvent, special attention was paid to sealing the calorimeter vessel against leaks; the head above the vessel was flushed with chloroform during the preequilibration period, which was longer than usual (approximately 1 h).

**Cation Transport Measurements.** The rates of transport of metal nitrate salts through stirred chloroform membranes containing the carriers listed in Table IV were determined by using a method described previously.[33] A 3-mL stirred chloroform membrane containing 1.0 mM ligand separated a 1.0 M source phase solution of metal nitrate (0.33 M in the case of $Ba(NO_3)_2$) and a distilled water receiving phase. The moles of cation transferred into the receiving phase in 24 h was determined by ion chromatography (Rb and Cs) or by atomic absorption spectroscopy (all other cations).

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**Supplementary Material Available:** Complete details on the syntheses and physical properties for compounds **3–15**, a table of $^1H$ NMR spectra of compounds **3–15** and the various alkylammonium complexes with compounds **2–21** (Table VI), and a table of temperature-dependent $^1H$ NMR spectral data and kinetic parameters for the complexation of primary alkylammonium salts with compounds **2–21** (Table VII) (18 pages). Ordering information is given on any current masthead page.

# Solvent Effects on the Deacylation of Acyl-chymotrypsins: A Critical Comment on the Charge-Relay Hypothesis

**Susan E. Hamilton and Burt Zerner***

*Contribution from the Department of Biochemistry, University of Queensland, St. Lucia, Australia 4067.* *Received July 25, 1980*

**Abstract:** The pH dependencies for deacylation of a series of acyl-chymotrypsins, prepared by using peptide-free enzyme, are reported. The $pK_a'$ values exhibit considerable variation, from 7.63 for α-benzamido-*trans*-cinnamoyl-chymotrypsin to 6.6 for 3,5-dinitrobenzoyl-chymotrypsin. It is proposed that the variation results from an interaction of variable strength between the carboxylate ion of Asp-102 and protonated His-57 and, further, that the kinetically determined values of the $pK_a'$ are attributable in all cases to the ionization of His-57. High values for the $pK_a'$ controlling deacylation are therefore indicative of a strong electrostatic interaction and low values reflect a relatively weak interaction. The effect of 20.4% (w/w) dioxane on the $pK_a'$ associated with the deacylation of a number of acyl-chymotrypsins is reported. The measured shifts in the value of the $pK_a'$ provide strong evidence for this description of the Asp–His pair in acyl-chymotrypsins. In the hydrolysis of the specific substrate *N*-acetyl-L-tryptophan ethyl ester, the ionizing group controlling deacylation behaves as a simple cationic acid. The results clearly indicate that a "charge relay" is not involved in the hydrolysis of specific substrates and argue against a kinetically important role for aspartic acid acting as a base in the mechanism of action of the serine proteinases.

A simple approach to the problem of characterizing catalytically important acidic groups in enzymes makes use of the differential effect of the dielectric constant of the solvent on the acidity of neutral and cationic acids. Simple neutral acids (RH) decrease in acidity and cationic acids ($RH^+$) increase slightly in acidity as the dielectric constant of the solvent is lowered; these effects are qualitatively accounted for on the basis of simple electrostatic models.[1-4] A decrease in the dielectric constant of the solvent thus affects the pH dependence for an enzyme-catalyzed reaction in a manner which depends on the charge type of the dissociating group or groups participating in the reaction.

Rabin's group first used the approach to characterize the ionizing groups involved in the mechanism of action of bovine pancreatic ribonuclease.[5] A refinement of their procedure resulted from the development by Hui Bon Hoa and Douzou[6] of a simple method for determining the hydrogen ion activity of buffered solvent–water systems. The buffers were used to determine directly the effect of solvent dielectric constant on the pH dependencies for several enzyme-catalyzed reactions.[7,8] Serine proteinases catalyze the hydrolysis of esters and amides via an acyl-enzyme intermediate (ES′, eq 1) in which the active-site serine residue

$$E + S \rightleftharpoons ES \xrightarrow{k_2} \underset{+\,P_1}{ES'} \xrightarrow{k_3} E + P_2 \qquad (1)$$

is acylated. The rate constants for acylation ($k_2$) and deacylation ($k_3$) exhibit similar pH dependencies, suggesting the participation of a single ionizable group, of $pK_a' \approx 7$, active in the free base form. Maurel's group determined the effect of a decrease in solvent dielectric constant on the pH dependence for the trypsin-catalyzed hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester[7] and for the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan ethyl ester.[8] A small decrease in $pK_a'$ was observed in the former case and negligible change in the latter, consistent in each case with the ionization of a cationic acid. There exists a considerable body of independent evidence which suggests that the group is a histidine residue (His-57 in α-chymotrypsin) and that it acts as a general base in the catalytic mechanism.

In 1969, Blow et al.[9] published a model of the active site of α-chymotrypsin that was based on an electron density map of the

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enzyme at 2-Å resolution and a revised amino acid sequence for residues 94–107. This revision was prompted by the observation by other groups that in bovine chymotrypsinogen B and trypsinogen residue 102 was aspartic acid, whereas the equivalent residue in α-chymotrypsin was determined by Hartley's group to be asparagine. In the revised sequence, residue 102 was redesignated aspartic acid. The residue was reported to be situated in a hydrophobic environment with its side-chain carboxylate group within hydrogen-bonding distance of the imidazole ring of His-57. Although the kinetically determined $pK_a'$ of ~7 was assigned by Blow to the Asp–His pair, Asp-102 was clearly designated the stronger base of the pair in the proposed reaction mechanism. Its postulated role in the deacylation step was that of a general base acting in tandem with His-57 to remove a proton from water. Hunkapiller et al.[10] subsequently provided experimental evidence to support a reversal of the expected $pK_a'$ values for Asp-102 and His-57. On the basis of $^{13}C$ NMR studies of α-lytic protease, a $pK_a'$ of ~7 was assigned to Asp-102, and the $pK_a'$ of His-57 was estimated to be lower than 4. A recent reassessment of these $pK_a'$ values by Roberts' group, using $^{15}N$ NMR, led to the opposite conclusion: the $pK_a'$ of His-57 is close to 7 and the $pK_a'$ of Asp-102 is considerably lower.[11]

Because the range of available techniques for titrating individual groups on enzymes is limited, and thus far has provided conflicting results for the serine proteinases, we have undertaken further to exploit the effect of the dielectric constant of the solvent on the pH dependence for α-chymotrypsin-catalyzed hydrolyses. The aim of these experiments was to comment critically on the acid–base properties of the Asp–His pair and on the strength of the interaction between the residues in a range of acyl-enzymes. Of central importance to the investigation was the previous observation by several groups that the $pK_a'$ controlling the deacylation of acyl-chymotrypsins varied considerably with change in the acyl group: from ~6.7 for 3,5-dinitrobenzoyl-chymotrypsin[12,13] to 7.64 for α-benzamido-*trans*-cinnamoyl-chymotrypsin.[14] Several explanations have been offered to account for the variation,[12,15,16] of which the most plausible was provided by de Jersey et al.,[16] who observed that the value of 7.64 reported by Brocklehurst and Williamson[14] for the $pK_a'$ controlling the deacylation of α-benzamido-*trans*-cinnamoyl-chymotrypsin might reflect catalysis of deacylation by peptides produced by autolysis of free enzyme. In support of the proposition, it was shown that removal of autolysis peptides from α-chymotrypsin by Sephadex G-25 chromatography decreased the pH-independent constant $\bar{k}$ for the deacylation of α-benzamido-*trans*-cinnamoyl-chymotrypsin by a factor of ~4. In the same paper, it was noted that the discrepancy between the $pK_a'$ for the deacylation of *N-trans*-cinnamoyl-chymotrypsin determined by Bernhard et al. (7.32)[12] and the value determined by Bender et al. (7.15)[17] might be attributable to peptide catalysis in the former case, since Bender and Zerner[18] had earlier observed that the value of 7.3 for the $pK_a'$ controlling the deacylation of *N-trans*-cinnamoyl-chymotrypsin determined at very high enzyme concentrations ($10^{-3}$ M) was significantly higher than the value (7.15) determined at enzyme concentrations of ~$4 \times 10^{-5}$ M.

While these and other reported discrepancies among values of the $pK_a'$ controlling deacylation in particular acyl-enzymes are almost certainly the result of peptide contamination, the present study demonstrates that there exists a genuine range of values of this constant. The value of the $pK_a'$ depends on the structure of the acyl group, and the effect of a decrease in the dielectric constant of the solvent on the pH dependencies for deacylation of several acyl-chymotrypsins has enabled us to comment on the source of this variation.

## Experimental Section

**General Methods.** Dioxane was refluxed with HCl, dried over KOH, and fractionally distilled from sodium under nitrogen; bp 101 °C (760 mmHg) (lit.[19] bp 101.32 °C). 2,6-Lutidine was purified by the procedure of Biddiscombe et al.;[20] bp 143.4 °C (760 mmHg) (lit.[20] bp 144.05 °C). *N*-Ethylmorpholine was fractionally distilled from KOH under nitrogen; bp 138–138.5 °C (760 mmHg) (lit.[21] bp 138–139 °C). Triethylamine was fractionally distilled under nitrogen; bp 89.2 °C (760 mmHg) (lit.[22] bp 89.35 °C). Diethylmalonic acid was recrystallized from benzene or chloroform/petroleum ether; mp 126–127 °C (lit.[23] mp 125 °C). Malonic acid was recrystallized from diethyl ether/petroleum ether; mp 135 °C (lit.[24] mp 135.6 °C). All other reagents were analytical grade and were used without purification. Buffers were prepared with deionized distilled water by titrating the appropriate buffer acid or base to the desired final pH and concentration with standardized NaOH or HCl and then adding solid sodium chloride to give the desired final ionic strength. Buffers containing dioxane were made up just before use. Densities for dioxane[25] of 1.0268 g cm$^{-3}$ and for water of 0.9971 g cm$^{-3}$ at 25 °C were used to calculate the weight percent of dioxane in buffer. Measurements of pH were made at 25 ± 0.1 °C with the use of a Radiometer pH meter 4c, standardized with aqueous buffers according to Bates.[26] The final pH in each assay was measured. pH* is defined as the pH meter reading at 25 ± 0.1 °C in the presence of 20.4% (w/w) dioxane, and $pK_a$* is the pH* at which $k_{obsd} = \bar{k}/2$ on the plot of $k_{obsd}$ vs. pH*.

**Preparation of Acylating Agents.** 4-*trans*-Benzylidene-2-phenyloxazolin-5-one (BPO) was prepared by the reaction of benzaldehyde with hippuric acid in the presence of acetic anhydride and recrystallized from benzene; mp 166–167 °C (lit.[27] mp 167 °C). *N-trans*-Cinnamoylimidazole was prepared according to the procedure of Schonbaum et al.;[28] mp 133–134 °C (lit.[28] mp 133.5–134 °C). β-(2-Furyl)acryloylimidazole was prepared by a similar method and recrystallized from hot cyclohexane; mp 111.5–112 °C (lit.[29] mp 113–114 °C). β-(3-Indolyl)acryloylimidazole was prepared from β-(3-indolyl)acrylic acid (Aldrich Chemical Co., mp 185 °C dec) by a mixed anhydride procedure and recrystallized from benzene; mp 193–193.5 °C dec (lit.[30] mp 199–200.5 °C). 3,5-Dinitrobenzoylimidazole was prepared by the reaction of imidazole (5 g, 73 mmol) and 3,5-dinitrobenzoyl chloride (10 g, 43 mmol) in 400 mL of freshly distilled chloroform. After 1 h at 25 °C in the dark, imidazolium chloride was removed by filtration, and the filtrate was washed rapidly with 0.1 M phosphate buffer, pH 7.0, and then with water at 4 °C. The filtrate was dried with sodium sulfate and filtered into 400 mL of petroleum ether (bp 65–68 °C) at −15 °C. The precipitated product was recrystallized rapidly from chloroform/petroleum ether; mp 158–159 °C dec. 4-Nitrobenzoylimidazole was prepared similarly by the reaction of imidazole (1.6 g, 23 mmol) and 4-nitrobenzoyl chloride (2.6 g, 14 mmol) in 50 mL of redistilled dioxane. The product was precipitated with petroleum ether at −15 °C and recrystallized from hot cyclohexane; mp 116–118.5 °C dec (lit.[31] mp 120–122.5 °C). 3,5-Dimethoxybenzoic anhydride was synthesized by reaction of imidazole (1.5 g, 22 mmol) and 3,5-dimethoxybenzoyl chloride (2.1 g, 10 mmol) in 110 mL of benzene at 70 °C for 10 min. Imidazolium chloride was removed by filtration, the reaction filtrate was evaporated to dryness at reduced pressure, and the product was recrystallized twice from hot cyclohexane; mp 134–135 °C (lit.[32] mp 134–135 °C). The identity of the product was verified by mass spectrometry. On alkaline hydrolysis, the compound

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yielded 98% of the theoretical yield of 3,5-dimethoxybenzoic acid.

**Preparation of Acyl-chymotrypsins.** α-Chymotrypsin was chromatographed to remove contaminating peptides.[33] Enzyme (150 mg of Worthington or Sigma thrice-recrystallized α-chymotrypsin) was dissolved in 1 mL of 0.1 M citrate buffer, pH 5.67, and chromatographed on a column of Amberlite CG-50 resin (2 × 20 cm) equilibrated with the same buffer, at a flow rate of 6 mL/h. Fractions from the peak and descending side of the profile of absorbance at 280 nm were pooled and used for preparation of the acyl-enzymes.

α-Benzamido-*trans*-cinnamoyl-chymotrypsin was prepared as follows. Acetonitrile was added dropwise to chromatographed α-chymotrypsin (~1 mg/mL) at pH 5.67 to give a final acetonitrile concentration of 10% (v/v). The enzyme was titrated with *p*-nitrophenyl acetate. A stoichiometric amount of BPO in acetonitrile was added to the enzyme over 5 min at 25 °C, and the pH of the acylated enzyme was adjusted to 3.5 with 2 M HCl. The acyl-enzyme was concentrated by ultrafiltration to 4 mL and then chromatographed on Sephadex G-25 (column dimensions 2 cm × 50 cm) equilibrated with 0.01 M NaCl, pH 3.5 (with HCl). The chromatographed acyl-enzyme was reconcentrated to ~1 mL by ultrafiltration. 3,5-Dimethoxybenzoyl-chymotrypsin was prepared similarly by addition of a fourfold molar excess of 3,5-dimethoxybenzoic anhydride to α-chymotrypsin. *N-trans*-Cinnamoyl-chymotrypsin, β-(2-furyl)-acryloyl-chymotrypsin, and β-(3-indolyl)acryloyl-chymotrypsin were prepared from the corresponding acylimidazoles in a similar way except that the pH of the enzyme was adjusted to 3.5 before acylation and the final acetonitrile concentration was 1% (v/v). 3,5-Dinitrobenzoyl-chymotrypsin and 4-nitrobenzoyl-chymotrypsin were prepared by addition of a slight molar excess of acylimidazole in acetonitrile to α-chymotrypsin at pH 5.67 (no acetonitrile added initially), followed by adjustment of the pH of the acyl-enzyme to 3.5 and chromatography on Sephadex G-25 as described above.

**Determination of Deacylation Rate Constants.** Deacylations were initiated by the addition of an aliquot (10–50 μL) of acyl-enzyme to buffer (1–3 mL) equilibrated at 25 ± 0.1 °C in the sample compartment of a Cary 17 spectrophotometer. Deacylation was monitored at the following wavelengths (acyl group, wavelength in nm): α-benzamido-*trans*-cinnamoyl, 310; *N-trans*-cinnamoyl, 310; β-(2-furyl)acryloyl, 320; β-(3-indolyl)acryloyl, 359; 4-nitrobenzoyl, 289; 3,5-dinitrobenzoyl, 250. The deacylation of 3,5-dimethoxybenzoyl-chymotrypsin was monitored by withdrawing aliquots (100 μL) from the deacylation assay and "quenching" the deacylation by adding each aliquot to 1 mL of 0.1 M acetate buffer, pH 4.0, at 4 °C. The free enzyme was assayed by addition of an aliquot (100 μL) of the quenched acyl-enzyme to 3 mL of 0.05 M phosphate buffer, pH 6.85, and 75 μL of 0.033 M Ac–Trp–OEt in acetonitrile. Initial rates were followed at 300 nm. First-order rate constants for deacylation ($k_{obsd}$) were determined by "infinity" or Guggenheim[34] plots of the data. Kinetic constants $k_{cat}$ and $K_m$ for the chymotrypsin-catalyzed hydrolysis of Ac–Trp–OEt were determined by adding an aliquot (50 μL) of chromatographed enzyme to 3 mL of a buffered solution of substrate equilibrated at 25 ± 0.1 °C in the sample compartment of a Cary 17 spectrophotometer and following the initial rate at 300 nm. Substrate concentrations were in the range $10^{-3}$–$3 \times 10^{-3}$ M in the presence of dioxane and $10^{-4}$–$5 \times 10^{-4}$ M in the absence of dioxane. All assays were performed in duplicate or triplicate, and $V$ was determined by linear least-squares analysis of a Lineweaver–Burk plot of the data. $\Delta\epsilon$ for the hydrolysis of Ac–Trp–OEt in buffer was determined to be 240 $M^{-1}$ $cm^{-1}$ in buffer by complete hydrolysis of the substrate, in good agreement with the previously reported value.[35] In the presence of 20.4% (w/w) dioxane, $\Delta\epsilon$ was 301 $M^{-1}$ $cm^{-1}$.

For all of the acyl-enzymes investigated, graphs of $1/k_{obsd}$ vs. $[H^+]$ were linear, consistent with the equation $k_{obsd} = \bar{k}/(1 + [H^+]/K_a')$. $[H^+]$ was routinely varied from $10^{-2}$ to 10 $K_a'$. The slope $(1/\bar{k}K_a')$ and the intercept $(1/\bar{k})$ were determined by linear least-squares analysis. The slope $(1/\bar{k}K_a')$, together with a value of $\bar{k}$ arrived at by curve fitting over the high pH range, yielded $K_a'$. The error in $K_a'$ was obtained from the standard error in the slope of the linear regression line, together with the estimated error in $\bar{k}$. The latter was obtained from the average deviation of experimental $k_{obsd}$ values from the calculated curve of best fit over the high pH range.

## Results

**Deacylation—pH Dependencies Using Peptide-Free α-Chymotrypsin.** When α-benzamido-*trans*-cinnamoyl-chymotrypsin was prepared from enzyme which had been chromatographed on Amberlite CG-50, no dependence of $k_{obsd}$ on acyl-enzyme concentration was observed at pH 7.75, over the range (2–4) × $10^{-5}$ M. Further, the measured value of $k_{obsd}$ ($0.031_3$ $s^{-1}$) was consistent with the value of 0.033 $s^{-1}$ previously determined at pH 7.88 by extrapolation of $k_{obsd}$ to zero enzyme concentration.[16] At pH values greater than 8.5, a small dependence of $k_{obsd}$ on acyl-enzyme concentration was observed with one preparation of the acyl-enzyme (Table I). In this case, $k_{obsd}$ was determined at pH values greater than 8.5 by extrapolation to zero acyl-enzyme concentration. The pH dependence for deacylation of α-benzamido-*trans*-cinnamoyl-chymotrypsin was also determined under steady-state conditions ($[S]_0 \gg [E]_0$). For the other acyl-enzymes investigated, no dependence of $k_{obsd}$ on acyl-enzyme concentration was observed. Values of $\bar{k}$ and $pK_a'$ obtained in the present work are given in Table I, together with some of the lowest values of $\bar{k}$ previously reported and the corresponding $pK_a'$ values.

Table I. Dependence of $k_{obsd}$ on pH for the Deacylation of Some Acyl-chymotrypsins

| | this study[a] | | lit. | |
|---|---|---|---|---|
| acyl group | $pK_a'$ | $10^2\bar{k}$, $s^{-1}$ | $pK_a'$ | $10^2\bar{k}$, $s^{-1}$ |
| α-benzamido-*trans*-cinnamoyl | 7.62[b]<br>7.65[d] | $4.5_0$[b]<br>$4.6_0$[d] | 7.64[c] | 15.4[c] |
| β-(3-indolyl)acryloyl | 7.63[e] | 0.17[e] | 7.68[f] | 0.19[f] |
| *N-trans*-cinnamoyl | 7.18[g] | 1.18[g] | 7.15[h] | 1.25[h] |
| β-(2-furyl)acryloyl | 7.15[g] | 0.24[g] | 7.26[f] | 0.24[f] |
| 3,5-dinitrobenzoyl | 6.60[e] | 5.85[e] | 6.70[f] | 7.6[f] |

[a] Determined in phosphate and barbital buffers; $\mu = 0.2$, 25 ± 0.1 °C. [b] Values of $k_{obsd}$ at pH 8.5 and above determined by extrapolation to zero acyl-enzyme concentration; in all assays, [acyl-enzyme] ≈ $10^{-6}$–$5 \times 10^{-6}$ M and [acetonitrile] = 11% (v/v). [c] Reference 14. [d] Steady-state data: $[E]_0 = 2.5 \times 10^{-7}$ M; $[S]_0 = 1.2 \times 10^{-6}$–$3.7 \times 10^{-6}$ M; [acetonitrile] = 11% (v/v). [e] 1% (v/v) acetonitrile. [f] Reference 12. [g] 0.1% (v/v) acetonitrile. [h] Reference 17.

**Deacylation—pH Dependencies in the Presence of Dioxane.** The effect of 20.4% (w/w) dioxane on the pH dependence of $k_{obsd}$ for the deacylation of some acyl-chymotrypsins is summarized in Table II. pH dependencies were determined separately in neutral-acid and cationic-acid buffers. There is agreement within experimental error among the values of $\bar{k}$, $pK_a'$, and $pK_a^*$ obtained in neutral acid buffers and the corresponding values determined in cationic acid buffers, indicating the absence of specific buffer effects.

The effect of 20.4% (w/w) dioxane on the values of the $pK_a'$ of a series of simple neutral, cationic, and zwitterionic acids was also estimated (Table III). Buffer solutions (0.1 M) were prepared from each acid, and the difference between the pH of the buffer in the absence of dioxane and the pH meter reading (pH*) in the presence of dioxane was determined. Inagami and Sturtevant[37] have previously shown that the differences measured in this way for formate and Tris buffers largely reflect the predictable effect of dioxane on the $pK_a'$ values of formic acid and $TrisH^+$, respectively, in the presence of concentrations of dioxane between 0 and 88% (w/w). Similarly, the magnitude of (pH* – pH) obtained in the present work is in each case qualitatively consistent with the effect predicted on the basis of simple electrostatics. Further, the values of (pH* – pH) are similar in magnitude to the $pK_a'$ shifts previously determined for acids of the same charge type. We have employed these values of (pH* – pH) as a reference for interpreting the results of Table II.

## Discussion

It was observed in a previous paper[16] that the deacylation of α-benzamido-*trans*-cinnamoyl-chymotrypsin was catalyzed by autolysis peptides and that the deacylation rate constant was

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Table II. Effect of Dioxane on the pH Dependence for Deacylation of Acyl-chymotrypsins[a]

| acyl group | buffer acid | $\bar{k}$, $s^{-1}$ (no dioxane)[b] | $\bar{k}$, $s^{-1}$ (20.4% w/w dioxane)[b] | $pK_a'$[c] (no dioxane) | $pK_a^*$[c] (20.4% w/w dioxane) | $pK_a^* - pK_a'$ |
|---|---|---|---|---|---|---|
| α-benzamido-*trans*-cinnamoyl | neutral | 0.061 | 0.075 | 7.64[d] | 7.90 | +0.26 ± 0.05 |
| | cationic | 0.062 | 0.073 | 7.67[d] | 7.87 | +0.20 ± 0.05 |
| 3,5-dimethoxybenzoyl | neutral | 0.00215[e] | | 7.27[f] | | |
| | cationic | 0.0023[e] | 0.00241[e] | 7.20[f] | 7.46[f] | +0.26 ± 0.08 |
| *N*-*trans*-cinnamoyl | neutral | 0.0126[g] | 0.0086[g] | 7.18[d] | 7.23 | +0.05 ± 0.05 |
| | cationic | 0.0125[g] | 0.0090[g] | 7.12[d] | 7.18[d] | +0.06 ± 0.04 |
| 4-nitrobenzoyl | neutral | 0.00052[h] | 0.00055 | 6.89[f,h] | 6.78[f] | −0.11 ± 0.08 |
| *N*-acetyl-L-tryptophanyl | neutral | 49[j] | 25 | 6.90[j] | 6.73 | −0.17 ± 0.06 |
| 3,5-dinitrobenzoyl | neutral | 0.060 | 0.095 | 6.60 | 6.56 | −0.04 ± 0.06 |
| | cationic | 0.057 | 0.094 | 6.60 | 6.64 | +0.04 ± 0.06 |

[a] 0.1 M buffers; $\mu = 0.229$; 25 ± 0.1 °C. Neutral-acid buffers were acetate, maleate, malonate, diethylmalonate; cationic acid buffers were 2,6-lutidine–HCl, *N*-ethylmorpholine–HCl, triethylamine–HCl. [b] Error ± 4% except where specified. [c] Error ±0.03 p*K* unit except where specified. [d] ±0.02. [e] ±9%. [f] ±0.04. [g] ±2%. [h] $pK_a' = 6.95$, $\bar{k} = 6 \times 10^{-4}$ $s^{-1}$.[13] [j] $pK_a' = 6.86$; $\bar{k} = 46.5$ $s^{-1}$; 0.8% (v/v) acetonitrile.[36]

Table III. Effect of Dioxane on Acidity of Neutral, Cationic, and Zwitterionic Acids

| acid | pH* − pH[a] | $pK_a'$ (20% w/w dioxane) − $pK_a'$ |
|---|---|---|
| diethylmalonic | +0.78 | |
| malonic | +0.81 | |
| maleic | +0.80 | |
| acetic | +0.43 | +0.536[b] |
| propionic | | +0.592[b] |
| triethylammonium | −0.23 | |
| *N*-ethylmorpholinium | −0.12 | |
| 2,6-lutidinium | −0.40 | |
| imidazolium | −0.18 | |
| anilinium | | −0.15[c] |
| *N*-methylanilinium | | −0.21[c] |
| glycine zwitterion | +0.05 | +0.129 ($pK_2'$, α-amino)[b] |

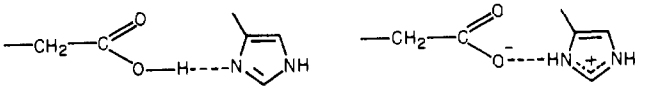
[a] 0.1 M buffers; $\mu = 0.229$; 25 ± 0.1 °C. pH* is the pH meter reading in the presence of 20.4% (w/w) dioxane. pH* − pH was determined in each case for a series of buffers covering a pH range from ($pK_a' - 0.4$) to ($pK_a' + 0.4$). No variation in pH* − pH was observed over this range. [b] Reference 3. [c] Reference 4.

therefore a very sensitive detector of such peptides.

The present study demonstrates the absence of a dependence of $k_{obsd}$ on [acyl-enzyme] at pH 7.75 after chromatography of α-chymotrypsin on Amberlite CG-50. Further, the value of $k_{obsd}$ ($0.031_3$ $s^{-1}$) was lower than the value obtained when unchromatographed enzyme ($0.048_7$ $s^{-1}$, [acyl-enzyme] = $4 \times 10^{-5}$ M) was used. These results, and their consistency with earlier measurements, demonstrate the effectiveness of the chromatography procedure for removing autolysis products from α-chymotrypsin. It was also observed that the procedure of Yapel et al.[38] for removing autolysis products by chromatography on Sephadex G-25 was much less effective than Amberlite CG-50 chromatography.

The value of 7.62 for the $pK_a'$ controlling the deacylation of α-benzamido-*trans*-cinnamoyl-chymotrypsin was confirmed by determining the pH dependence of $k_{cat}$, the steady-state rate constant for hydrolysis of BPO, at an enzyme concentration ~ 20-fold lower than was employed for the direct observation of deacylation. Good agreement between the $pK_a'$ and $\bar{k}$ values determined by the two methods was obtained (Table I). The value of $\bar{k}$ for the deacylation of 3,5-dinitrobenzoyl-chymotrypsin is significantly lower than the lowest value previously reported, suggesting that this acyl-enzyme is also susceptible to catalysis of deacylation by peptides. (Although only the lowest literature values of $\bar{k}$ are given in Tables I and II, a more extensive survey of published $\bar{k}$ values leads to a similar conclusion for *N*-*trans*-cinnamoyl-chymotrypsin and 4-nitrobenzoyl-chymotrypsin.)

It is clear, however, that the wide variation in the value of the $pK_a'$ controlling deacylation, from 6.60 for 3,5-dinitrobenzoyl-chymotrypsin to 7.62 for α-benzamido-*trans*-cinnamoyl-chymotrypsin, is not attributable to catalysis of deacylation by autolysis peptides. In all cases, the $k_{obsd}$–pH profile is a simple titration curve, consistent with the participation of a single ionizable group in the reaction. For the purpose of analyzing the effect of dioxane on the $pK_a'$, we shall consider several alternative descriptions of the group responsible for the ionization. The group may be (i) an aspartic acid residue, (ii) a histidine residue, or (iii) an interacting Asp–His pair. Two prototropic forms of (iii) may exist: **iiia** and **iiib**. The predominant form will depend on the relative



**iiia** **iiib**

acidities of aspartic acid and histidinium. In **iiia**, the interaction is relatively weak, and **iiia** is very similar to (i) in respect of solvent effects. In **iiib**, the interaction is relatively strong, and **iiib** will behave characteristically as a zwitterion in respect of solvent effects.

Two classes of acyl-enzyme are clearly distinguishable on the basis of the magnitude of the $pK_a'$ controlling deacylation and the effect of added dioxane on the $pK_a'$ (Table II). Those acyl-enzymes exhibiting relatively high values for the $pK_a'$ (α-benzamido-*trans*-cinnamoyl-, 3,5-dimethoxybenzoyl-) also exhibit an increase in this $pK_a'$ in the presence of 20.4% (w/w) dioxane, consistent with the effect of dioxane on the ionization of either a neutral acid or a cationic acid in a zwitterion (Table III). Those acyl-enzymes exhibiting relatively low values for the $pK_a'$ controlling deacylation (4-nitrobenzoyl-, *N*-acetyl-L-tryptophanyl-, and 3,5-dinitrobenzoyl-) exhibit either a decrease or no change in the $pK_a'$ in the presence of 20.4% (w/w) dioxane, consistent with the effect of dioxane on the ionization of a cationic acid. *N*-*trans*-Cinnamoyl-chymotrypsin is intermediate in behavior with respect to both the magnitude of the $pK_a'$ controlling deacylation and the effect of dioxane on the $pK_a'$.

These results may be understood in terms of the following propositions.

(1) In those acyl-enzymes exhibiting high values for the $pK_a'$ controlling deacylation, the Asp–His pair has substantial zwitterion character, and there exists an electrostatic interaction between the carboxylate anion of Asp-102 and the imidazolium cation of His-57, as in iiib. When the dielectric constant of the solvent is lowered, the $pK_a'$ controlling deacylation increases because the strength of the electrostatic interaction in the zwitterion increases (cf. glycine, Table III).

(2) In those acyl-enzymes exhibiting low values for the $pK_a'$ controlling deacylation (4-nitrobenzoyl-, *N*-acetyl-L-tryptophanyl-, 3,5-dinitrobenzoyl-), the electrostatic interaction between Asp-102 anion and His-57 cation is weak or nonexistent. The low $pK_a'$ values are a direct result of the decrease in electrostatic stabilization of the imidazolium ion. When the dielectric constant of the solvent is lowered, the $pK_a'$ controlling deacylation decreases, confirming that the ionizable group in these acyl-enzymes is a simple cationic acid whose acidity is substantially unaffected by any adjacent negative charge.

(38) Yapel, A.; Han, M.; Lumry, R.; Rosenberg, A.; Da, F. S. *J. Am. Chem. Soc.* **1966**, *88*, 2573–2584.

This description of the Asp–His pair requires the existence at the active site of α-chymotrypsin of an electrostatic interaction of *variable* strength between the carboxylate anion of Asp-102 and protonated His-57. That the active sites of the serine proteinases may indeed be flexible with respect to the relative positions of Asp-102 and His-57 is attested to by recently refined crystallographic data for the serine proteinase *Streptomyces griseus* protease A (SGPA). Although the extent of sequence homology between this bacterial enzyme and the pancreatic serine proteinases is small,[39] the geometries of the active-site residues Asp-102, His-57, Ser-195, and Ser-214 are almost identical in SGPA, β-trypsin, and α-chymotrypsin[39,40] (the numbering scheme used here is that of bovine chymotrypsinogen A[41]). The specific tetrapeptide aldehyde inhibitor Ac-L-Pro-L-Ala-L-Pro-L-Phe-H forms a stable covalent tetrahedral adduct with Ser-195 of SGPA, with a $K_i$ of $2 \times 10^{-6}$ M at pH 4.0.[42] X-ray crystallographic analysis of the complex crystallized at pH 4.1 shows that a major change in the position of the side chain of His-57 is associated with the binding of the inhibitor. Further, the hydrogen bond which has been proposed between $O^{\delta 1}$ of Asp-102 and $N^{\delta 1}$ of His-57 in the native enzyme is not able to be formed when His-57 occupies this altered position. Our results provide independent evidence which suggests the absence of a significant electrostatic interaction between Asp-102 and His-57 in an acyl-enzyme derived from a specific substrate (Ac–Trp–OEt) and in two relatively stable acyl-enzymes. Thus, the "charge-relay" as originally represented serves no useful catalytic purpose in any of these acyl-enzymes.

It is noteworthy that James' group has also reported that in SGPA, trypsin, α-chymotrypsin,[39,40] and α-lytic protease[43] the active-site aspartyl residue is situated in a polar environment. In earlier interpretations of crystallographic data for the serine proteinases,[9] the environment of the residue was described as hydrophobic, and it was on this basis that a $pK_a'$ of ~7 for the aspartic acid residue in α-lytic protease was rationalized by Hunkapiller et al.[10] In the present work we find that in several acyl-enzymes the ionizing group controlling deacylation has some zwitterion character. If the zwitterion is, as we have assumed, the (Asp-anion)–(His-57 cation) pair, then Asp-102 must have a lower $pK_a'$ than His-57 in these acyl-enzymes.

**Acknowledgment.** This work was supported in part by the Australian Research Grants Committee. The skilled technical assistance of Mr. Allen Tripp is gratefully acknowledged.

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# A Vinylsilane Route of (±)-Gymnomitrol

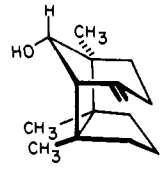
**Leo A. Paquette* and Yeun-Kwei Han**

*Contribution from the Evans Chemical Laboratories, The Ohio State University, Columbus, Ohio 43210.* *Received October 2, 1980*

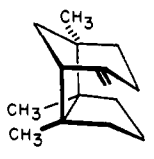
**Abstract:** Gymnomitrol, a sesquiterpenic alcohol of unusual structure, has been synthesized in an efficient and stereoselective manner. Selective ketalization of the bicyclo[3.3.0]octanedione (**4**), followed by Wolff–Kishner reduction and deketalization, afforded the $C_s$ symmetric ketone **5**. Methylenation with paraformaldehyde and *N*-methylanilinium trifluoroacetate gave the exocyclic methylene derivative (**6**) which entered into copper-catalyzed 1,4 addition with the Grignard reagent from (*E*)-2-(bromovinyl)trimethylsilane and subsequent in situ methylation to deliver **12** in 66% yield. Epoxidation and dilute acid hydrolysis of this intermediate furnished hydroxy ketone **15** directly. The coproducts **14a** and **14b** could also be converted to **15** which was oxidized to diketone **16**. The final stages of the synthesis involved regioselective addition of methyllithium to **16**, dehydration of the tertiary carbinol functionality, and ultimate lithium aluminum hydride reduction.

## Background

The liverwort *Gymnomitrion obtusum* (hindb) Pears has been shown by Connolly and co-workers[1] to be a rich source of sesquiterpenoids possessing the otherwise rare 4,8-methanoperhydroazulene carbocyclic framework.[2] The major metabolite, in alcohol called gymnomitrol, was assigned the interesting structure **1** on the basis of convincing spectroscopic and chemical evidence. Additional support for this formulation materialized later in the form of an *x*-ray crystal structure analysis of a derivative of two closely related hydrocarbons **2** and **3** with which it cooccurs.[3-5] The ring system of these molecules can also be regarded to be constructed of a diquinane[6] part structure which is further embellished by incorporation of one of the cyclopentane rings into a stereochemically well-defined functionalized bicyclo[3.2.1]octane network.



1



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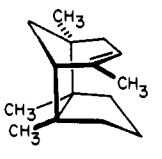

3

(1) Connolly, J. D.; Harding, A. E.; Thorton, I. M. S. *J. Chem. Soc., Chem. Commun.* **1970**, 1320. *J. Chem Soc., Perkin Trans. 1* **1974**, 2487.
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(3) Hydrocarbon **2** has been variously called gymnomitrene,[1] β-pompene,[4] and β-barbatene,[5] whereas **3** is known as isogymnomitrene,[1] α-pompene,[4] and α-barbatene.[5]
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