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Inactivation of Chymotrypsin by 5-Benzyl-6-chloro-2-pyrone: ^{13}C NMR and X-ray Diffraction Analyses of the Inactivator–Enzyme Complex†

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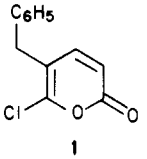
ABSTRACT: The inactivation of chymotrypsin by 5-benzyl-6-chloro-2-pyrone has been studied. Chloride analysis of the inactivated enzyme suggests that chlorine is no longer present in the complex. ^{13}C NMR spectroscopy of chymotrypsin inactivated with 5-benzyl-6-chloro-2-pyrone-*2,6*-$^{13}C_2$ shows the presence of two new resonances from the protein-bound inactivator. The chemical shift values of these resonances are consistent with an intact pyrone ring on the enzyme as well as the replacement of the C-6 chlorine by a different heteroatom. X-ray diffraction analysis at 1.5-Å resolution of the inactivator–enzyme complex demonstrates that the γ-oxygen of the active site serine residue (serine-195) is covalently attached to C-6 of the inactivator and that the pyrone ring is intact. The 5-benzyl group of the inactivator is bound to the enzyme in the hydrophobic specificity pocket. The conformational changes that occur in the protein as a result of complexation with the inactivator are discussed.

The inactivation of chymotrypsin and other serine proteases by substituted 6-chloro-2-pyrones has recently been reported (Westkaemper & Abeles, 1983). Inactivation of chymotrypsin by 5-benzyl-6-chloro-2-pyrone (**1**) and 3-benzyl-6-chloro-2-pyrone (**2**) occurs in a time-dependent manner.



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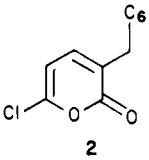

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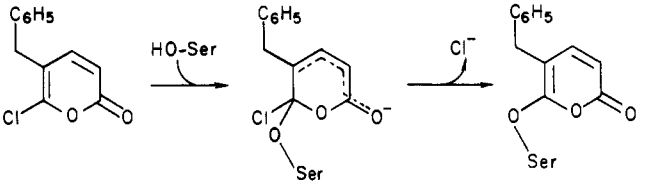
The benzyl group is important since the unsubstituted chloropyrones are not inactivators of chymotrypsin. The mechanism of inactivation depends on the position of the benzyl group. Scheme I shows a proposed mechanism for inactivation of chymotrypsin by **1**.

This mechanism was proposed based on the observation that the UV–vis spectrum of chymotrypsin inactivated with **1** shows a distinct absorbance at 320 nm (Westkaemper & Abeles, 1983). This long wavelength band is characteristic of an intact pyrone. It was suggested that **1** reacts with the active-site serine residue (serine-195) (Westkaemper & Abeles, 1983) although no direct evidence was presented in the earlier report.

Scheme I



In contrast, no near-visible band is seen in the spectrum of chymotrypsin inactivated with **2**, suggesting that the pyrone ring is no longer intact. Thus, the benzyl group adjacent to the carbonyl group in **2** directs the attack of the active-site serine onto the haloenol–lactone resulting in destruction of the pyrone ring.

This paper reports our structural characterization of the adduct formed between chymotrypsin and **1**. We have prepared **1** enriched with ^{13}C and have obtained the ^{13}C NMR spectrum of the inactivated enzyme complex. Crystalline chymotrypsin was inactivated with **1** and the structure of the complex determined by X-ray diffraction methods. These studies show that the pyrone ring remains intact in the complex with chymotrypsin. C-6 of **1** forms a covalent bond with the

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γ-oxygen of serine-195. The 5-benzyl group of **1** is bound in the hydrophobic specificity pocket on the enzyme.

Materials and Methods

γ-Chymotrypsin crystals were a gift from David R. Davies (NIH). The crystals were stored in 10 mM cacodylate buffer, pH 5.6, containing 75% saturated ammonium sulfate. α-Chymotrypsin (type II) and benzoyl-L-tyrosine ethyl ester (BTEE) were obtained from Sigma Chemical Co. The γ-form of the enzyme, rather than the α-form, was selected for the crystallographic studies since the packing in these crystals leaves the active site more accessible to inhibitors (Birktoft et al., 1970; Segal et al., 1971). α-Chymotrypsin was used for the NMR studies since it is commercially available in large quantities. Crystallographic studies of both enzyme forms show no major structural differences except at the protein surfaces (Segal et al., 1972). Barium carbonate-^{13}C (97–98% enriched) was from Mounds Facility, Monsanto. Sephadex G-25 gel filtration media was from Pharmacia Fine Chemicals.

General Methods. Chymotrypsin was assayed with BTEE (Hummel, 1959). Protein concentrations were determined spectrophotometrically by using $\epsilon_{280} = 5.1 \times 10^4$ M^{-1} cm^{-1} (Dixon & Neurath, 1957). All spectrophotometric measurements were made with either a Perkin-Elmer 559 or Lambda 3 UV–vis instrument with 1-cm quartz cells thermostated at 25 °C. ^{13}C NMR spectra at 100.6 MHz were obtained on a Bruker AM-400 spectrometer and at 22.6 MHz on a Bruker FT WH-90 spectrometer. All chemical shift values are reported relative to tetramethylsilane.

Chloride Analysis of Chloropyrone-Inactivated Chymotrypsin. Chloride analyses were carried out by adapting the colorimetric procedure of Zall et al. (1956). Solutions of mercuric thiocyanate and ferric perchlorate were prepared as described (Zall et al., 1956). All aqueous solutions were prepared with quartz-distilled water. A sample of inactivated enzyme for chloride analysis was prepared as follows: α-Chymotrypsin (10 mg) in 18 mL of 20 mM tris(hydroxymethyl)aminomethane (Tris)–$HClO_4$, pH 7.4, was inactivated by the addition of 1 mL of 5-benzyl-6-chloro-2-pyrone solution (10 mM in acetonitrile). After 10 min the sample was concentrated to 1–2 mL by ultrafiltration (Amicon PM10 membrane) at 4 °C and applied to a Sephadex G-25 column (1.8 × 30 cm) equilibrated with 20 mM Tris–$HClO_4$, pH 7.4. Protein-containing fractions were pooled and concentrated to 2 mL by ultrafiltration at 4 °C. The chymotrypsin concentration was 132 μM, and the enzyme was completely inactive. The enzyme solution was analyzed for chloride as follows: Enzyme solution (0.5 mL) was mixed with 30% trichloroacetic acid (0.5 mL). After the solution was chilled on ice for 10 min, the precipitated protein was removed by centrifugation. The supernatant fluid (0.5 mL) was mixed with $Hg(SCN)_2$ solution (0.15 mL) followed by ferric perchlorate solution (0.2 mL). The absorbance at 460 nm was immediately read. The amount of chloride was estimated from a standard curve prepared in an identical manner as above by using a standard KCl solution.

The following control experiments were carried out. A mixture of α-chymotrypsin and KCl was passed through a G-25 column (see above). The fractions were assayed for enzyme content (A_{280}) and chloride content (see above). Complete separation of protein from chloride was observed. When a solution containing chymotrypsin and a known amount of chloride was assayed for chloride content, greater than 90% of the chloride was always detected.

Synthesis of 5-Benzyl-6-chloro-2-pyrone-2,6-$^{13}C_2$. Sodium acetate-*1*-^{13}C was prepared from the reaction of methylmagnesium iodide and $^{13}CO_2$ (generated from 15.4 g of barium carbonate-^{13}C, 97–98% enriched) (LeMaster, 1980; Murray & Williams, 1958). The salt was fused, converted to the free acid by distillation from phosphoric acid, and brominated with Br_2 in trifluoroacetic anhydride containing a catalytic amount of PBr_3 (Roberts & Poulter, 1978). Bromoacetic-*1*-^{13}C acid was converted to cyanoacetic-*1*-^{13}C acid and then to diethyl malonate-*1*-^{13}C (Ott, 1981). Diethyl malonate-*1*-^{13}C (5.2 g) was converted in four steps to pure 3-benzyl-6-chloro-2-pyrone-*2,6*-$^{13}C_2$ (180 mg) and 5-benzyl-6-chloro-2-pyrone-*2,6*-$^{13}C_2$ (40 mg) (Westkaemper & Abeles, 1983). The chloropyrones were ca. 50% enriched with ^{13}C at C-2 and C-6. The carbon ^{13}C NMR spectrum of the enriched chloropyrone in $CDCl_3$ showed two enriched resonances at 146.9 and 160.1 ppm. These resonances were assigned to C-2 (160.1 ppm) and C-6 (146.9 ppm) as follows: The natural absorbance ^{13}C NMR of 5-benzyl-6-chloro-2-pyrone showed four quaternary carbon resonances at 114.8, 137.2, 146.9, and 160.1 ppm. On the basis of chemical shifts and ^1H-^{13}C coupling patterns, the resonances at 114.8 and 137.2 ppm were assigned to C-5 and the quaternary phenyl carbon, respectively. The natural absorbance ^{13}C NMR spectrum of 3-benzyl-2-pyrone, prepared by reduction of the chloropyrone with Zn dust (Westkaemper & Abeles, 1983), showed a quaternary resonance at 162.6 ppm, but the 146.9 ppm peak was replaced by a new nonquaternary carbon resonance at 149.5 ppm. Thus, the peak at 160.1 ppm must be from the carbonyl carbon (C-2) and the peak at 146.9 ppm from the haloenol carbon (C-6).

Preparation of an NMR Sample of Chloropyrone-Inactivated Chymotrypsin. α-Chymotrypsin (150 mg) was dissolved in 300 mL of 20 mM potassium phosphate, pH 7.0. A solution of 5-benzyl-6-chloro-2-pyrone-*2,6*-$^{13}C_2$ (9.9 mg in 0.58 mL of acetonitrile) was added in one portion and the solution stirred gently at room temperature. After 50 min an aliquot was withdrawn and assayed for catalytic activity. At this point the enzyme was 98% inactivated. The solution was concentrated to 3–4 mL by ultrafiltration (Amicon PM10 membrane) at 4 °C. The sample was chromatographed on a Sephadex G-25 column (1.8 × 30 cm) equilibrated with 20 mM potassium phosphate, pH 7.0, and the protein-containing fractions were pooled and concentrated to 2–3 mL by ultrafiltration at 4 °C. D_2O (1 mL) was added and the solution concentrated to a final volume of 2.0 mL. The final enzyme concentration was 1.33 mM, and the enzyme was 95% inactivated. The sample was kept on ice overnight prior to NMR analysis. Just prior to the NMR experiments, 1–2 μL of dioxane was added as an internal reference standard.

^{13}C NMR of Inactivated Chymotrypsin. ^{13}C NMR spectra at 100.6 MHz were obtained on a Bruker AM-400 spectrometer. Protein samples were placed in 10-mm tubes and maintained at 10 °C during data collection. To minimize sample heating and maintain the NOE, a two-level proton decoupling scheme was used. During the data acquisition period (90 ms) the decoupler power was 2 W. The power was reduced to 0.5 W during the relaxation period (2 s). A 35° flip angle was used. Satisfactory spectra were observed after 1000–1500 scans. The reported chemical shift values were measured from a dioxane internal standard (67.3 ppm).

X-ray Diffraction Analysis of Inactivated Chymotrypsin. Crystalline γ-chymotrypsin was inactivated with 5-benzyl-6-chloro-2-pyrone by soaking individual crystals in 1 mL of a solution containing 10 mM cacodylate buffer, pH 5.6, 75% saturated ammonium sulfate, 5% dioxane, and 0.7 mM inactivator for a minimum of 1 week at room temperature in the dark. Additional inactivator (50 μL of 13.6 mM inacti-

vator in dioxane) was added after 3 days. The final concentration of dioxane was 9% and that of inactivator 1.2 mM, assuming no hydrolysis. The enzyme was found to be totally inactive when the crystals were redissolved and assayed with BTEE for catalytic activity.

The native crystals belong to space group $P4_22_12$ with unit cell dimensions $a = b = 69.6$ Å, $c = 97.4$ Å, and $\alpha = \beta = \gamma = 90°$ (Segal et al., 1971). The inactivated crystals had unit cell dimensions $a = b = 69.3$ Å, $c = 96.9$ Å, and $\alpha = \beta = \gamma = 90°$, indicating no major changes in the structure of the unit cell.

Data to 1.5-Å resolution were collected on a Nicolet P3 diffractometer equipped with a modified LT 1 low-temperature device. Measurements were made at −1 °C on three crystals by using Wyckoff scans to keep radiation damage at less than 25% for each. The first crystal was measured from a resolution of infinity to 2.5 Å, the other two from 2.6 to 1.5 Å by using a longer counting time for each reflection. Agreement between reflections measured on more than one crystal was within 3–4% on F's. Data was reduced in the usual way (Ringe et al., 1983). A total of 28 000 observed reflections, with intensities greater than 2σ, were used to calculate a difference Fourier map by using phases and native amplitudes calculated from the Protein Data Bank coordinates for γ-chymotrypsin (Bernstein et al., 1977). The map showed the bound inhibitor and a number of changes in the positions of residues near the active site. A $2F$(obsd) − F(calcd) Fourier map displayed on a Vector General computer graphics system was used to build the inactivator structure into the observed electron density and reposition some of the necessary protein residues which had changed position in the inhibited enzyme. The structure of the complex was then refined by the restrained least-squares method (Hendrickson & Konners, 1979). Eighteen refinement cycles were used to give a final R factor of 21.4% for all data from 10 to 1.5 Å resolution (1751 non-hydrogen atoms). The restraints applied were (rms deviation from ideality) 0.025 Å on covalent bond distances, 0.04 Å on one to three bond angle distances and 0.02 Å on planar distances. The structure was repositioned twice by using the graphics system during the refinement in order to obtain the best fit.

Results and Discussion

Chloride Analysis of Chymotrypsin Inactivated with **1**. To determine if the inactivator–enzyme adduct contains chlorine, we treated the complex with acid and analyzed the mixture for chloride. No chloride was detected. Greater than 90% of the chloride could be detected when an equal molar amount of chloride was added to samples of inactivated enzyme. All of the chloride was detected when a sample of **1** was treated with acid. These results demonstrate that no acid-labile chlorine is present in the inactivator–enzyme adduct.

^{13}C NMR of Chymotrypsin Inactivated with **1**. ^{13}C NMR of chymotrypsin inactivated with ^{13}C-enriched **1** provides a direct method for determining if the pyrone ring is still intact in the complex with the enzyme. The ^{13}C NMR spectrum of chymotrypsin inactivated with 5-benzyl-6-chloro-2-pyrone-2,6-$^{13}C_2$ is shown in Figure 1. Two new peaks at 160.6 and 161.1 ppm appeared in the spectrum that are absent in the spectrum of the native enzyme. All other regions of the spectrum were identical with a spectrum of the native enzyme. The chemical shift values of C-2 and C-6 of the inactivator on the enzyme can be compared to the respective shift values of the inactivator alone. For the inactivator alone, C-2 resonates at 160.1 ppm and C-6 at 146.9 ppm. The chemical shift value of the carbonyl carbon (C-2) of 160.1 ppm is characteristic of the pyrone structure, occurring significantly upfield

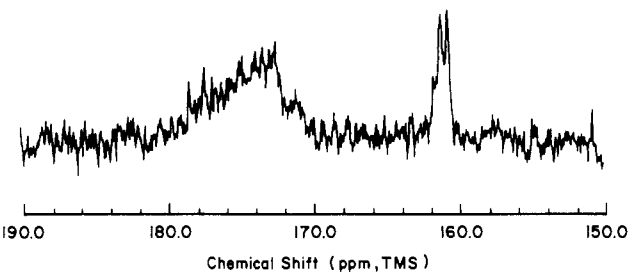

FIGURE 1: 100.6-MHz ^{13}C NMR of chymotrypsin inactivated with 5-benzyl-6-chloro-2-pyrone-2,6-$^{13}C_2$. The conditions for obtaining the spectrum are given under Materials and Methods. The two resonances at 160.6 and 161.1 ppm are from the ^{13}C-enriched inactivator. The broad envelope of resonances between 169 and 180 ppm are from the natural abundance ^{13}C of the protein carbonyl groups.

Table I: Atomic Coordinates (Å) and Isotopic B Factors (Å^2) for the Enzyme-Bound Inhibitor[a]

atom	X	Y	Z	B
serine-O_γ	37.04	77.41	87.21	19
O-1[b]	35.70	77.35	89.57	14
C-2[b]	35.87	77.50	91.06	16
C-3[b]	37.30	77.60	91.68	17
C-4[b]	38.54	77.57	90.75	17
C-5[b]	38.36	77.43	89.26	14
C-6[b]	36.93	77.31	88.65	18
carbonyl oxygen[b]	34.65	77.53	91.97	15
benzylic carbon[c]	39.51	77.37	88.21	18
C-1′[c]	40.89	78.00	88.49	27
C-2′[c]	41.68	78.45	87.24	25
C-3′[c]	43.06	79.09	87.39	23
C-4′[c]	43.65	79.29	88.78	26
C-5′[c]	42.87	78.84	90.03	27
C-6′[c]	41.48	78.20	89.89	28

[a] The coordinates correspond to the system previously defined (Bernstein et al., 1977). [b] Atoms of the pyrone ring. The numbering of the pyrone ring follows the conventional scheme. The ring oxygen atom is position 1. The remaining ring atoms are numbered consecutively in a counterclockwise manner around the ring. [c] Atoms of benzyl substituent. The carbon atoms are numbered consecutively around the ring beginning with the quaternary carbon as C-1′.

from the shift values of carboxylates and acyl carboxylate derivatives (ca. 167–185 ppm). The chemical shift value of the haloenol carbon (C-6) of 146.9 ppm has the expected value for an olefinic carbon with an oxygen substituent (Pretsch et al., 1976); the chlorine substituent creates only a small shift change with respect to hydrogen (only a 3.4 ppm shift was observed for C-6 when the chlorine was replaced by hydrogen).

The appearance of inactivator-associated ^{13}C resonances in the enzyme complex near 160 ppm demonstrates that the chemical shift of C-2 has not changed significantly following reaction of **1** with the enzyme, while the chemical shift of C-2 has moved downfield by ca. 14 ppm. The absence of a downfield resonance near ca. 170–180 ppm in the spectrum demonstrates that the pyrone ring remains intact. The downfield shift in the resonance of C-6 suggests that the chlorine has been exchanged for a different heteroatom. Both of the results are consistent with the mechanism of inactivation shown in Scheme I.

X-ray Analysis of Chymotrypsin Inactivated with **1**. In order to unambiguously identify the active-site residue of chymotrypsin that forms a covalent bond to **1**, we inactivated crystals of γ-chymotrypsin with **1** and obtained the structure of the bond inactivator at 1.5-Å resolution using X-ray diffraction methods.

The refined coordinates and B factors for the non-hydrogen atoms of the bound inactivator are listed in Table I. The inactivator is covalently bound to the γ-oxygen of serine-195 at C-6 of the pyrone in such a way that the 5-benzyl group

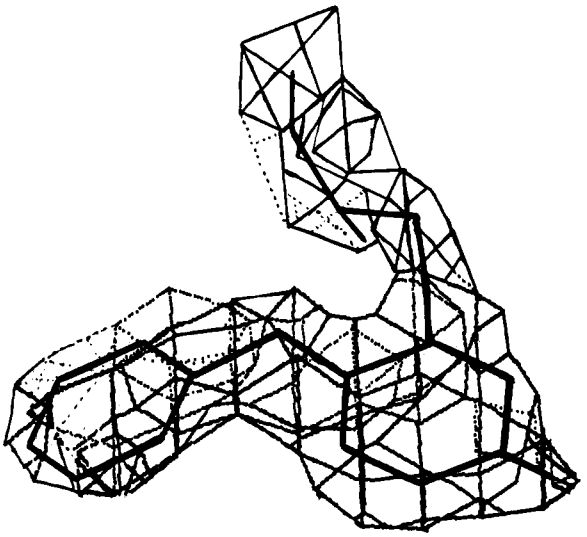

FIGURE 2: Model of the inhibitor fitted to the difference electron density map, $2F$(obsd) − F(calcd), at 1.5-Å resolution. Only the difference density corresponding to the bound inhibitor and serine-195 is shown. Changes in the protein structure as a result of complexation with the inactivator are discussed in the text and presented in Figure 3.

fits into the specificity pocket of the enzyme. The electron density of the pyrone ring is strong and clear enough to distinguish the carbonyl oxygen (Figure 2). The covalent attachment of the γ-oxygen of serine-195 to carbon 6 of the inhibitor is supported by the observation of a significant region of electron density between these two atoms (Figure 2). The attachment via a covalent bond is also supported by the ^{13}C NMR studies described above. The electron density of the phenyl ring (5-benzyl) is much weaker, although the shape and position are obvious. These observations are an indication that the phenyl ring has a greater mobility than the pyrone ring, i.e., is bound less tightly in the specificity pocket. This conclusion is supported by the average B factors observed for the pyrone ring (15 Å^2) and the phenyl ring (25 Å^2) (Table I). For comparison, the average B factor for the molecular complex as a whole is ca. 14 Å^2. A possible consequence of this observation is that the pocket is large enough to accommodate a larger side chain such as tryptophan, which would then be held more tightly in place.

The structure of the enzyme has undergone some changes as a consequence of the binding of the inactivator (Figure 3). The backbone and side chains of residues 194–196 have shifted position slightly so that the γ-oxygen of serine-195 moves 0.5 Å closer to the specificity site. The most striking changes have occurred at the specificity pocket formed by residues 212–214, 226–228, and 190–191 (Figure 3). Residues 189–191 and 213–216 have undergone a translational change in position, moving from 0.21 Å to as much as 1.5 Å inward toward the phenyl group. The motion brings most of these residues into van der Waals contact with the phenyl ring, enclosing it within the pocket. In addition, tyrosine-228, which normally lies to one side at the bottom of the site, has twisted around to close off the pocket (Figure 3) by a rotation of 19° about the C_α–C_β (χ_1) torsion angle and 20° about the C_β–C_γ (χ_2) torsion angle. In this new position, the tyrosine ring lies nearly perpendicular to the phenyl ring of the inactivator. The net movement undergone by the tyrosine ring involves only a side-chain rotation that causes a shift in position of about 0.8 Å. There appears to be a short contact (less than 3 Å) between one of the carbon atoms of the phenyl ring of the inactivator and the plane of the tyrosine phenol. This type of interaction has been observed in crystalline benzene and may be a form of structure stabilization (Cox et al., 1958).

Previous studies of inhibitors bound to chymotrypsin (Steitz et al., 1969; Segal et al., 1971; Matthews et al., 1967; Tulinsky et al., 1978; Henderson, 1970; Segal et al., 1972) have in general been done at lower resolution and have not involved full least-squares refinement. These studies have concentrated on the movements of the active-site residues (Asp, His, and Ser) and have not taken note of conformational changes in the binding pocket. Some of these studies, either because of direct binding of the inhibitor to histidine-57 or because of the involvement of histidine-57 with a group on the leaving group side of the inhibitor, show a change in the position of histidine. This is not observed here, nor is it expected, since the pyrone reacts with serine-195 and there is no group on the pyrone ring that can interact with histidine-57.

CONCLUSIONS

The results presented in this study characterize the complex formed between **1** and chymotrypsin. They show that attack of serine-195 onto C-6 of **1** with the loss of chloride has occurred to form an adduct in which the pyrone ring remains intact. Such a mechanism is also consistent with the known reactivity of the C-6 position of chloropyrones with nucleophiles such as hydroxide (Kagan et al., 1975; Westkaemper &

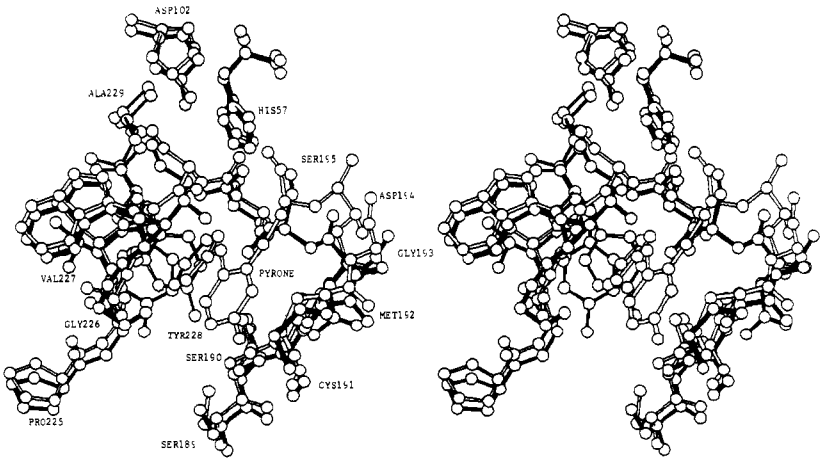

FIGURE 3: Stereoview of the active site of chymotrypsin with the bound inhibitor. The structure of the native enzyme (solid lines) is overlayed onto the structure of the enzyme–inhibitor complex (clear lines). The C-6 position of the inhibitor is covalently linked to the γ-oxygen of serine-195. Note the large change in the position of the side chain of tyrosine-228 that results from the interaction between the phenyl ring of the inhibitor and the phenolic ring of the tyrosine.

Abeles, 1983). The hydrophobic interaction of the 5-benzyl group of **1** with the specificity pocket of chymotrypsin positions the inactivator in such a way that the attack of serine-195 occurs onto the C-6 position of **1**. No enzyme-catalyzed esterolysis of the haloenol–lactone occurs. We consider it likely that the stability of the chymotrypsin–pyrone adduct is the result of the stable nature of the bond between the pyrone ring and the γ-oxygen of serine-195. This is in comparison to the relatively labile chlorine atom of the chloropyrone.

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Registry No. **1**, 85533-81-9; [2,6-^{13}C]-**1**, 93714-51-3; chymotrypsin, 9004-07-3.

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