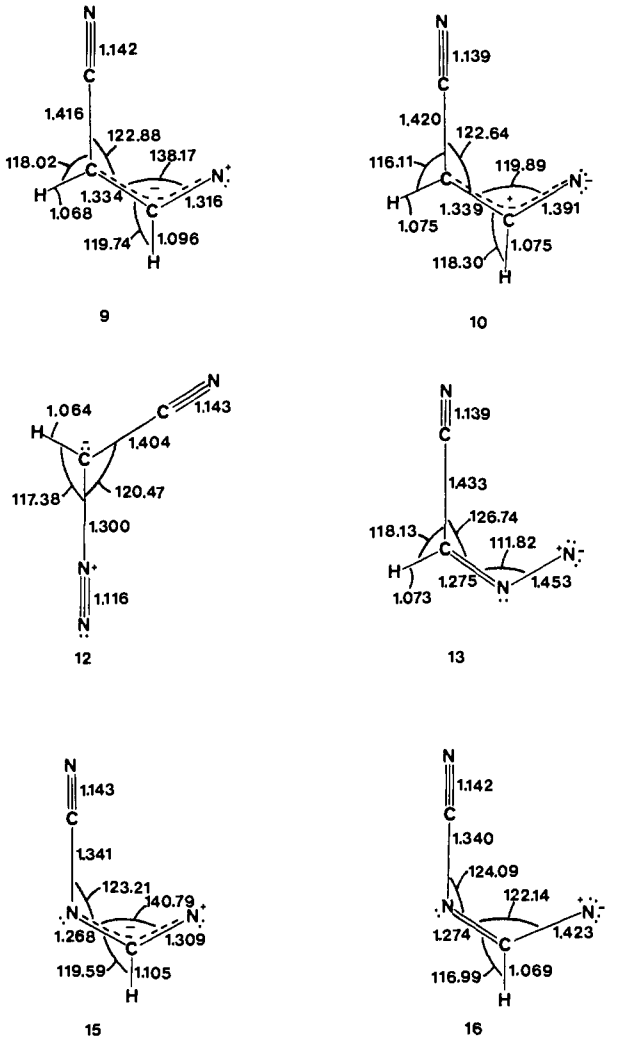


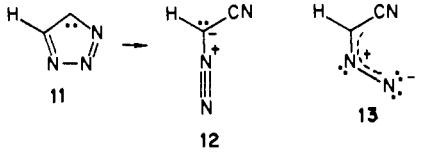
Figure 1.

Table I. 3-21G Optimized Energies of Open Carbenic Species

struct	E, au	struct	E, au
9	−222.240 89	**13**	−238.092 90
10	−222.230 61	**15**	−238.125 67
12	−238.213 94	**16**	−238.104 44

higher in energy than the six-π-electron structure **9** (see Table I).[8]

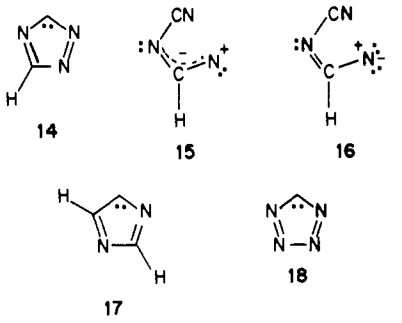
Calculations on the six-π-electron, singlet **11** also result in the spontaneous opening to the cyanodiazomethane structure **12**. The



11 12 13

alternative four-π-electron open structure **13** possesses a different geometry (Figure 1) and is considerably higher in energy (75.8 kcal per mol at the 3-21G basis level, see Table I).

Of the other possible azolylidene species, only the 2,3,5-triaza six-π-electron species **14** undergoes spontaneous ring opening to **15**. (As in the other cases, the four π-electron structure **16** is higher in energy). 2,4-Diazolyidene (**17**) does not spontaneously undergo ring opening, which is consistent with experimental observations.[9]



14 15 16 17 18

Interestingly, the 2,3,4,5-tetraza species **18** does not undergo spontaneous ring opening. Experimentally, **17** is observed to undergo fragmentation to generate a free carbon atom.[10,11]

In summary, the ring opening of an azolylidene occurs spontaneously from the six-π-electron carbene species and only when there are nitrogen atoms in the 2- and 3-positions.

An analysis of the forces indicate a repulsion between N2 and N3 and an attraction between C1 and N2. The repulsion between N2 and N3 appears to arise from a repulsive nonbonded pair interaction. These calculations support the mechanistic proposals put forth earlier and predict the ring opening of **14** not previously observed.

Acknowledgment. We thank the Computing Center of the University of Notre Dame for computer time and Dr. Daniel Chipman of the Radiation Laboratory of the University of Notre Dame for helpful discussions.

(8) Reaction coordinate calculations on the possible ring closure of **9** and **10** to **4** have not yet been attempted, and, thus, it is not known which electronic state closes more favorably to **4**.

(9) Kang, U. G.; Shechter, H. *J. Am. Chem. Soc.* **1978**, *100*, 651.

(10) Dyer, S. F.; Shevlin, P. B. *J. Am. Chem. Soc.* **1979**, *101*, 1303.

(11) Our present calculations do not indicate which electronic state of **14** undergoes this fragmentation reaction. All electronic states thus far calculated give rise to local minimum energy structures. Reaction coordinate calculations will be carried out in order to gain this information.

Stereospecific Reaction of 3-Methoxy-4-chloro-7-aminoisocoumarin with Crystalline Porcine Pancreatic Elastase

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There has been considerable interest in designing small molecules that will inhibit elastases because these proteolytic enzymes have been associated with pancreatitis,[1] emphysema[2] and arthritis.[3] Recently, a variety of nonpeptide inhibitors of elastases, and other serine proteases, have been investigated, including *N*-acylsaccharins,[4] isocoumarins,[5] benzoxazinones,[6,7] sulfonyl fluorides,[8] and 5-butyl-3*H*-1,3-oxazine-2,6-dione.[9] The mode of binding and mechanism of inhibition of these compounds have been previously

(1) Geokas, M. C.; Rinderknecht, H.; Swanson, V.; Haverback, B. J. *Lab. Invest.* **1968**, *19*, 235–239.

(2) Janoff, A. "Molecular Basis of Biological Degradative Processes"; Berlin, R. D., Herrman, H., Lepow, I. H., Tanzer, J. M., Eds.; Academic Press: New York, 1973; pp 225–260.

(3) Janoff, A. "Neutral Proteinases of Human Polymorphonuclear Leukocytes"; Havemann, K., Janoff, A., Eds.; Urban and Schwartzenberg: Munich, 1973; pp 390–417.

(4) Zimmerman, M.; Morman, H.; Mulvey, D.; Jones, H.; Frankshun, R.; Ashe, B. M. *J. Biol. Chem.* **1980**, *255*, 9848–9851.

(5) Harper, J. W.; Hemmi, K.; Powers, J. C. *J. Am. Chem. Soc.* **1983**, *105*, 6518–6520.

(6) Teshima, T.; Griffin, J. C.; Powers, J. C. *J. Biol. Chem.* **1982**, 257, 5085–5091.

(7) Hedstrom, L.; Moorman, A. R.; Dobbs, J.; Abeles, R. H. *Biochemistry* **1984**, *23*, 1753–1759.

(8) Yoshimura, T.; Barker, L. N.; Powers, J. C. *J. Biol. Chem.* **1982**, *257*, 5077–5084.

(9) Wiedmann, B.; Abeles, R. H. *Biochemistry* **1984**, *23*, 2373–2376.

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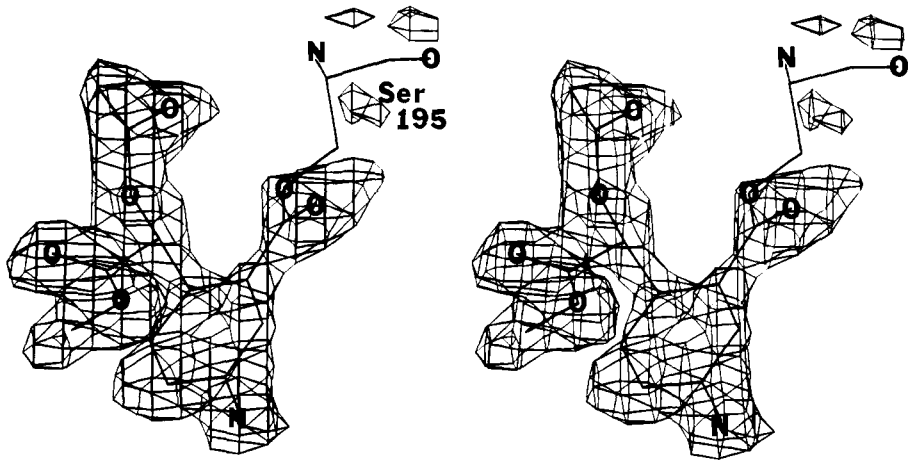

Figure 1. Stereopicture of electron density of the inhibitor in the active site of porcine pancreatic elastase. This difference ($F_o - F_c$) map was prepared by omitting the inhibitor–complex atoms from the structure factor (F_c) calculation. The contours are drawn at the 2 σ level and clearly indicate the fit of methoxy and acetate groups within the electron density envelope. The "doughnut" hole of density in the center of the benzene ring is a further indication of data quality.

surmised from kinetic data alone. In this paper, we report a high-resolution crystal structure of the mechanism-based, non-peptide inhibitor 3-methoxy-4-chloro-7-aminoisocoumarin (I)[10] with porcine pancreatic elastase (PPE).

Several large (0.3 × 0.3 × 0.7 mm) crystals were harvested at pH 5.0 from a 20 mg/mL solution of PPE in 0.1 M sodium acetate and 0.1 M sodium sulfate. They were soaked for 48 h in 1 mg/mL I with 0.1 M sodium acetate and 0.1 M sodium sulfate (pH 5.0) buffer. A crystal was mounted in a capillary in contact with a minimum of buffer and, at each end, an excess of solid inhibitor. Using the rotation method, a 1.8-Å resolution data set was measured in 10 days (70% completion: 42053 total and 15614 unique reflections). No correction was necessary for crystal decay. The agreement of symmetry-related reflections within a film ranges from 4.1% to 7.1%; the overall agreement of equivalent reflections from different films ranges from 6.0% to 9.1%, with a mean value of 7.1%. Photographic data evaluation is based upon the intensities, I, while the observed and calculated structure factors (F) are used to determine the agreement factor, R. The 1.65-Å resolution native structure of PPE[11] (R = 18% for 1937 atoms, including 117 atoms of hydration) was used as a starting point for a difference Fourier synthesis, which clearly indicated residual electron density in the active site region of the enzyme. After four cycles of refinement (EREF,[12] without the inhibitor included R = 21%), a model of the inhibitor was fit into the electron density; the agreement (R factor) of this model is 18%. Further refinement of the structure and assignment of additional bound HOH molecules is in progress. No unusual bond distances nor angles were required to bring the complex into electron density.

The electron density showed that an ester was formed between I and the active site Ser 195 residue, the ester being co-planar with the benzene ring of the inhibitor. This was the only covalent attachment of I to the enzyme (Figure 1). The amino group of the inhibitor is pointed toward solvent and is not hydrogen bonded to any residue of the enzyme. The chlorine atom, which would be expected to be the most electron-dense peak of the inhibitor, was absent. Other electron density for I has been determined to be a methyl ester group and an acetoxy group, the latter being situated in the primary specificity pocket of the enzyme. Because the density is well-defined for these two groups, the carbonyl oxygen atoms and methyl carbon atom were easily located: the acetoxy group fit in the density with a terminal "Y" shape and the methyl ester fit in density with a terminal "U" shape (Figure 1). The assignment of the acetoxy and methyl ester moieties is unambiguous and therefore only the R configuration is present at the benzylic carbon atom.

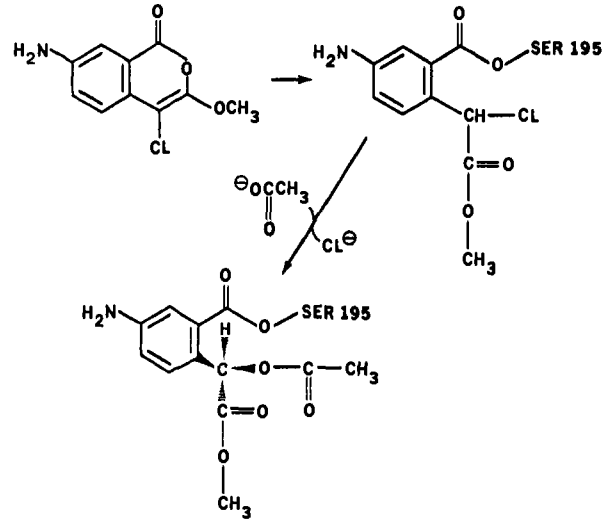

Figure 2. Schematic of reaction of 3-methoxy-4-chloro-aminoisocoumarin with porcine pancreatic elastase.

The proposed inhibition mechanism for I holds that the Ser 195 attack leads to cleavage of the heterocyclic ring and formation of an acyl p-quinonimine methide, which is capable of alkylating an active site nucleophile.[10] The most likely candidate for alkylation is the His 57 residue. Although in this crystal structure the only covalent bond between inhibitor and enzyme is the Ser 195 ester, this does not refute the proposed inhibition mechanism. The kinetic data were obtained at pH 7.5,[10] but the crystallographic experiment was performed at pH 5.0 since, at this pH, the crystals are much less soluble in buffer. At pH 5.0, the His 57 is protonated and could not have acted as a nucleophile. Rather, we conclude that an acetate group from the buffer acted as the nucleophile, resulting in the stereospecific synthesis of the R enantiomer.

Solution studies previously showed[10] that, at pH 7.5 with HEPES buffer, the inhibited enzyme is not reactivated with hydroxylamine. Subsequent to our structural determination, the reaction in solution was investigated at pH 5.0 in 0.1 M acetate buffer. At this pH the inhibited enzyme is reactivated by hydroxylamine,[13] supporting our structural analysis in the solid state and confirming that the reaction is dependent upon pH and buffer. Another possible explanation for the pH dependence of the reaction is that at pH 7.5, only the Ser 195 ester is formed but the inhibitor

(10) Harper, J. W.; Powers, J. C. *J. Am. Chem. Soc.* **1984**, *106*, 7618–7619.
(11) Meyer, E. F., Jr.; Cole, G. M., unpublished results.
(12) Jack, A.; Levitt, M. *Acta Crystallogr., Sect. A.*, **1978**, *A34*, 931–935.
(13) Powers, J. C.; Harper, J. W. *Biochemistry*, submitted.

adopts a conformation that makes the ester inaccessible to hydroxylamine.

We next plan to investigate whether a second active site nucleophile will make the postulated attack at pH 7.5 in the absence of a nucleophilic buffer. We have found that crystals grown at pH 5.0 (acetate buffer) can be converted to pH 7.5 (HEPES buffer) and then the inhibitor diffused into the crystals.

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Supplementary Material Available: X-ray data for native and complex crystals of PPE including tables of bond lengths and angles, nonbonded contacts, atomic coordinates, and temperature factors (12 pages). Ordering information is given on any current masthead page.

Mimics of Tryptophan Synthetase and of Biochemical Dehydroalanine Formation

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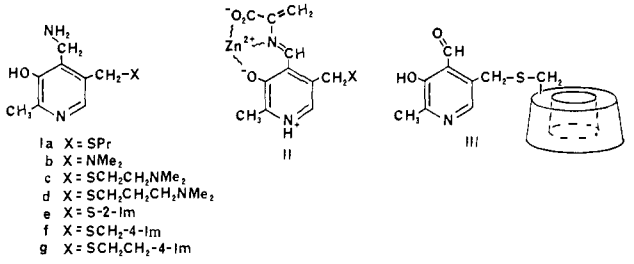
We have described compounds consisting of pyridoxamine coupled to the primary side of β-cyclodextrin,[2] the secondary side of β-cyclodextrin,[3] or a synthetic macrocyclic binding group.[4] These converted keto acids into amino acids with selectivity for substrates, such as indolepyruvic acid, that bound into the macrocyclic cavities while adopting the correct geometry for transamination. The enzyme tryptophan synthetase uses pyridoxal to dehydrate serine to a pyridoxal–dehydroalanine Schiff base (cf. structure II) while binding indole so as to promote carbon–carbon



bond formation.[5] To mimic this enzyme, we have prepared the pyridoxal–β-cyclodextrin conjugate III. Molecular models suggested that III should be able to imitate tryptophan synthetase, binding indole into the cyclodextrin cavity and holding it near a dehydroalanine group on the pyridoxal unit of III (see Scheme I). The product would be the doubly bound tryptophan that was involved in our earlier[5] transamination study.

Although III is formed in transaminations by the pyridoxamine–cyclodextrin conjugate we have described previously,[2] we

Scheme I

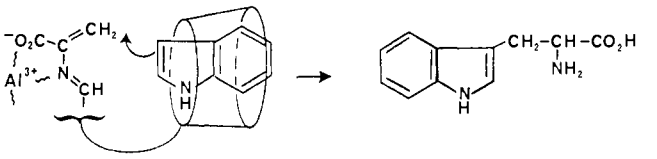


Table I. Rates of Dehydroalanine–Pyridoxal Imine Formation from Chloropyruvic Acid and of Ketimine to Aldimine Conversion with Pyruvic Acid by the Pyridoxamine Derivatives Ia–g

reagent	X	k_{elim}[a]	k_{rel}	k_{trans}[b]	k_{rel}
Ia	SPr	0.77	1.0	0.009	1.0
Ib	NMe_2	3.3	4.3	0.13	14
Ic	$S(CH_2)_2NMe_2$	9.2	12	0.23	26
Id	$S(CH_2)_3NMe_2$	2.6	3.4	0.33	37
Ie	SIm	6.4	8.3	0.05	5.6
If	SCH_2Im	5.3	6.9	0.11	12
Ig	$S(CH_2)_2Im$	2.8	3.6	0.68	76

[a] Pseudo-first-order rate constant for development of the chromophore at 414 nm, $\times 10^3$ s^{-1}, at 30.0 °C in methanol at pH 4.0. [b] Pseudo-first-order rate constant for conversion of the ketimine to the aldimine in transamination, $\times 10^3$ s^{-1}, at 30.0 °C in methanol at pH 4.0—data from ref 2.

preferred to prepare it in analytical purity[6] by conversion of pyridoxal to the dithioacetal with ethanedithiol, conversion of the CH_2OH group to CH_2SH (by Mitsunobu thioacetylation, then hydrolysis), displacement by the thiol on β-cyclodextrin-6-tosylate, and deprotection with $AgNO_3$. Pure III, from Sephadex SP-C50-120 cation exchange chromatography, showed the expected ^1H NMR and IR spectra. The UV spectrum at pH 10 had λ_{max} 398 nm; in H_2O, pyridoxal has λ_{max} 391 nm at this pH, shifting to 400 nm in 50% H_2O/THF solution. This spectroscopic indication that the pyridoxal group is partially bound in the cyclodextrin cavity of III is also supported by the strong CD of III at the pyridoxal chromophore. We have previously observed a related situtation in a thiamine–cyclodextrin conjugate.[7]

Metzler and Snell have reported[8] a ca. 1% yield of tryptophan from serine and indole with pyridoxal; we observe somewhat higher yields when *d,l*-β-chloroalanine is substituted for serine. We find that III indeed produces 3–5 times more tryptophan (still only a few percent) when it (at 21 mM) is incubated with 10 mM indole, 51 mM β-chloroalanine, and 2.5 mM $Al_2(SO_4)_3$ for 30 min at 100 °C and pH 5.2, compared with the same reaction in which III is replaced by pyridoxal. As expected, this kinetic advantage disappears at higher (40–80 mM) concentrations of indole, because of saturation of the binding group. Furthermore, the tryptophan produced with 10 mM indole has ca. 10% excess of the L enantiomer; under milder conditions conversion of indolepyruvic acid to tryptophan by the pyridoxamine derivative related to III had produced a 33% excess of the L isomer.[3] In these different syntheses the new asymmetric center is introduced by an identical step, so the difference in optical yield must reflect the more vigorous conditions in our present study.

The magnitude of the cooperative effect was undoubtedly diminished by alternative modes of binding of indole to III and by the internal binding detected in III itself. However, that such a cooperative effect is observed at all is good evidence for the dual binding of reagents to III, as a mimic of enzymes such as tryptophan synthetase that promote reaction of two bound substrates.

We have also described[9,10] mimics of transaminase enzymes in which basic groups attached to pyridoxamine by flexible chains acted to transfer protons from the CH_2 group of pyridoxamine

(1) Support of this work by the NIH, and by American Cancer Society Postdoctoral Fellowships to Wayne Weiner and Jeffrey Winkler and an NIH postdoctoral fellowship to Anthony Czarnik, is gratefully acknowledged.
(2) Breslow, R.; Hammond, M.; Lauer, M. *J. Am. Chem. Soc.* **1980**, *102*, 762–763.
(3) Breslow, R.; Czarnik, A. W. *J. Am. Chem. Soc.* **1983**, *105*, 1390.
(4) Winkler, J.; Coutouli-Argyropoulou, E.; Leppkes, R.; Breslow, R. *J. Am. Chem. Soc.* **1983**, *105*, 7198–7199.
(5) See: Walsh, C. "Enzymatic Reaction Mechanisms"; W. H. Freeman & Co.: San Francisco, 1979; pp 819–821.
(6) As the trihydrate of III. Anal. Calcd for $C_{50}H_{83}O_{39}NS$ (Found): C, 44.34 (44.07); H, 6.18 (6.40); N, 1.03 (1.53).
(7) Hilvert, D.; Breslow, R. *Bioorg. Chem.* **1984**, *12*, 206–220.
(8) Metzler, D.; Ikawa, M.; Snell, E. E. *J. Am. Chem. Soc.* **1954**, *76*, 648.
(9) Zimmerman, S. C.; Czarnik, A. W.; Breslow, R. *J. Am. Chem. Soc.* **1983**, *105*, 1694–1695.
(10) Zimmerman, S. C.; Breslow, R. *J. Am. Chem. Soc.* **1984**, *106*, 1490–1491.

0002-7863/85/1507-4093$01.50/0