with three 10-ml portions of 0.5 *N* NaOH, once with 10 ml of brine, and then dried ($MgSO_4$). Spin evaporation *in vacuo* left a syrup which showed no uv change from pH 1 to 13.

This syrup was treated with 3.5 mmol of NaOEt (formed as described above) in 25 ml of absolute EtOH and refluxed with stirring for 4 hr. The cooled solution was acidified to pH 5 with AcOH, then spin evaporated *in vacuo*. The residue was triturated with several milliliters of *i*-PrOH, cooled, the product collected and then washed with *i*-PrOH; yield, 0.680 g (78%), mp 146–149°. Recrystallization from *i*-PrOH–EtOH gave clones of white pins, mp 153–154°. *Anal.* ($C_{14}H_{16}N_2O_3$) C, H, N.

Irreversible Enzyme Inhibitors. CLXXII.[1,2] Proteolytic Enzymes. XVI.[3] Covalent Bonding of the Sulfonyl Fluoride Group to Serine Outside the Active Site of α-Chymotrypsin by *exo*-Type Active-Site Directed Irreversible Inhibitors

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Three active-site directed irreversible inhibitors with a terminal SO_2F group (**1**–**3**) of α-chymotrypsin have been shown to covalently link a serine OH group by two independent methods. The sulfonate group on α-chymotrypsin in each case was displaced by mercaptoethylamine and resultant *S*-aminoethyl-L-cysteine identified after hydrolysis; the second method converted each sulfonate of serine into pyruvic acid which was identified enzymatically and by conversion into its 2,4-dinitrophenylhydrazone. Reaction of **2** with α-chymotrypsin was unequivocally shown not to occur on the active-site serine-195, but a serine outside the site tentatively identified as serine-223; these results establish the hypothesis that properly designed active-site directed irreversible inhibitors can covalently link an amino acid residue outside the active site by the *exo* mechanism.

Active-site directed irreversible inhibitors of enzymes operate by two steps.[5] A reversible complex is first formed between the enzyme and inhibitor; if a proper leaving group on the inhibitor is juxtaposed to a nucleophilic group on the enzyme, then a rapid and selective neighboring group reaction occurs with formation of a covalent bond that inactivates the enzyme.[5] There are two classes of active-site directed irreversible inhibtors, the *endo* type that forms a covalent bond inside the active-site and the *exo* type that forms a covalent bond outside the active-site.[5]

The *endo* type of irreversible inhibitor is of interest in protein structure studies for "labeling" amino acids inside the active-site; a now classical example is 1-chloro-4-phenyl-3-tosylamido-2-butanone (TPCK)[6] which specifically forms a covalent bond with histidine-57 in the active site of α-chymotrypsin.[7]

The *exo* type of irreversible inhibitor has a considerably wider utility in drug design than the *endo* type.[8] The best leaving group yet found[9] for the *exo* type of covalent bond is the F of the SO_2F moiety; such a moiety has the electrophilic capacity to react rapidly with a serine OH of an enzyme,[10] but direct chemical proof of covalent bond formation with a serine outside the active site had yet to be achieved. The SO_2F moiety properly positioned on an appropriate reversible inhibitor could inactivate a variety of enzymes, presumably by the *exo* type of active-site directed irreversible inhibition. Examples are dihydrofolic reductase,[9] xanthine oxidase,[11] guanine deaminase,[12] trypsin,[13] α-chymotrypsin,[14–16] the C′1a component of complement,[17] and cytosine nucleoside deaminase.[18] With dihydrofolic reductase, SO_2F-type inhibitors have been found that can inactivate an L1210 mouse leukemia enzyme with no appreciable inactivation of the enzyme in normal liver, spleen, and intestine of the mouse.[19]

Three successive questions can be asked about an active-site directed irreversible enzyme inhibitor of the SO_2F type that presumably operates by the *exo* mechanism. (1) Does the SO_2F form a covalent bond with a serine? (2) If the enzyme has a serine in the active-site, as in the case of α-chymotrypsin, has the covalent bond formed with the active-site serine (*endo*) or has the covalent bond been formed with a serine outside the active site (*exo*)? (3) If the covalently

(1) This work was supported in part by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper in this series see B. R. Baker, and J. L. Kelley, *J. Med. Chem.*, **13**, 461 (1970).

(3) For the previous paper on proteolytic enzymes see B. R. Baker and M. Cory, *ibid.*, **12**, 1053 (1969).

(4) Visiting research professor from the Department of Biology, Atomic Energy Commission, France, 1968–1969.

(5) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," John Wiley & Sons, New York, N. Y., 1967, pp 17–21.

(6) G. Schoellmann and E. Shaw, *Biochemistry*, **2**, 252 (1963).

(7) E. B. Ong, E. Shaw, and G. Schoellmann, *J. Biol. Chem.*, **240**, 694 (1965).

(8) B. R. Baker, *Biochem. Pharmacol.*, **11**, 1155 (1962).

(9) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967), paper CV of this series.

(10) For discussion of the chemistry of the SO_2F group, see ref 9.

(11) (a) B. R. Baker and W. F. Wood, *J. Med. Chem.*, **11**, 650 (1968), paper CXXIII of this series; (b) B. R. Baker and J. A. Kozma, *ibid.*, **11**, 652 (1968), paper CXXIV of this series; (c) B. R. Baker and J. A. Kozma, *ibid.*, **11**, 656 (1968), paper CXXV of this series; (d) B. R. Baker and W. F Wood, *ibid.*, **12**, 211 (1969), paper CXLVI of this series.

(12) B. R. Baker and W. F. Wood, *ibid.*, **12**, 214 (1969), paper CXLVII of this series.

(13) (a) B. R. Baker and E. H. Erickson, *ibid.*, **11**, 245 (1968), paper CXV of this series; (b) B. R. Baker and E. H. Erickson, *ibid.*, **12**, 112 (1969), paper CXLIV of this series.

(14) B. R. Baker and J. A. Hurlbut, *ibid.*, **11**, 233 (1968), paper CXIII of this series.

(15) B. R. Baker and J. A. Hurlbut, *ibid.*, **12**, 118 (1969), paper CXLV of this series.

(16) B. R. Baker and J. A. Hurlbut, *ibid.*, **12**, 221 (1969), paper CL of this series.

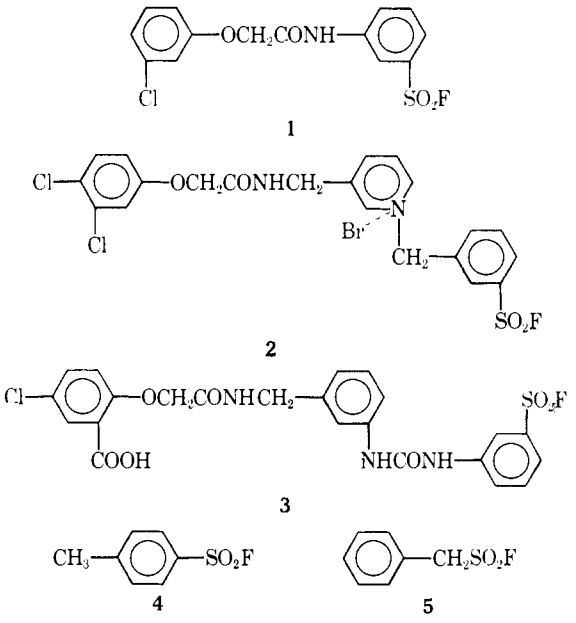
(17) B. R. Baker and J. A. Hurlbut, *ibid.*, **12**, 902 (1969), paper CLXI of this series.

(18) B. R. Baker and J. L. Kelley, *ibid.*, **12**, 1046 (1969), paper CLXIII of this series.

(19) (a) B. R. Baker, G. J. Lourens, R. B. Meyer, Jr., and N. M. J. Vermeulen, *ibid.*, **12**, 67 (1969), paper CXXXIII of this series; (b) for a recent review see B. R. Baker, *Accounts Chem. Res.*, **2**, 129 (1969).

bonded serine is outside the active site, which specific serine has been linked? Answers to the first two questions are of importance in drug design, but the third question is only of major importance for protein structure studies.

The irreversible inhibitors **1**,[14] **2**,[16] **3**,[15] **4**,[14,20] and **5**[20] were used to answer the first two questions; these answers with active-site directed irreversible (**1–5**) inhibitors give chemical proof of the *exo* mechanism and are the subjects of this paper.



Experimental Section

Reaction of Irreversible Inhibitors with α-Chymotrypsin.—To a solution of 25 mg (~1 μmol) of α-chymotrypsin in 4 ml of H_2O was added 7.5 ml of 0.05 *M* Tris buffer (pH 7.4). After addition of 1 ml of a 3 m*M* solution of inhibitor (**1**, **2**, or **3**) (3 μmoles) in DMSO, the solution was maintained at 37° for 1 hr, then cooled to 0°. Assay with *N*-glutaryl-L-phenyl *p*-nitroanilide (GPNA)[14,21] showed that <1% the enzyme remained; a control reaction with no inhibitor in the DMSO showed 93–97% of the enzyme activity remained.

The inactivated and control chymotrypsin solutions were exhaustively dialyzed against a slow flow of 1 m*M* HCl at 3° for about 18 hr, then freeze-dried in high vacuum. Assay again showed no activity in the inhibitor treated enzyme reaction, but >90% activity in the control solution.

Reaction of α-chymotrypsin with α-(**5**) or *p*-tolylsulfonyl fluoride (**4**) dissolved in *i*-PrOH was performed at pH 7 in the absence of buffer; the pH was maintained at pH 7 by addition of 0.1 *N* NaOH in a pH-Stat.[20] When no more alkali was consumed, the solution was dialyzed and freeze-dried as above. Less than 2% activity remained.

Displacement of the Sulfonate Group on α-Chymotrypsin by Mercaptoethylamine.—A solution of 4.8 g of urea and 2.27 g. of mercaptoethylamine·HCl in 8 ml of H_2O was adjusted to pH 8.0 with 6 *N* NaOH. The inactivated enzyme or the enzyme control in 2 ml of H_2O was added. The pH was then readjusted to pH 8.0 and the solution maintained at 40° for 6 hr. The solution was adjusted to pH 3 with 6 *N* HCl, then exhaustively dialyzed against a slow flow of 1 m*M* HCl at 3° for about 18 hr. The solution was freeze-dried. This is a modification of the literature[22] procedure.

Acid Hydrolysis of Modified α-Chymotrypsins.[22,23]—A solution of 5–10 mg of modified or unmodified α-chymotrypsin in 3 ml of 6 *N* HCl was frozen, then evacuated to 50 μ to remove air. The hydrolysis bulb was allowed to thaw, then refrozen, reevacuated, and sealed. The bulb was kept at 110° for 25 hr, then evaporated to residue *in vacuo*. The evaporation was repeated 3 times after addition of 5 ml of H_2O, then dissolved in H_2O for amino acid analysis.

Amino Acid Analysis of *S*-Aminoethyl-L-cysteine. The standard conditions for separation of amino acids on a Spinco 120 amino acid analyzer gave poor separation of *S*-aminoethyl-L-cysteine[24] from L-lysine. However, 0.35 *N* citrate buffer (pH 5.28) at 55° in a 50-cm column gave good separation;[22] at a flow rate of 68 ml/hr, the *S*-aminoethyl-L-cysteine appeared 19 ml after lysine and 38 ml before histidine. The yields of *S*-aminoethyl-L-cysteine are recorded as mol 14 mol of lysine or 2 mol of histidine present in α-chymotrypsin by comparison of peak areas of the three amino acids.

Conversion of Modified α-Chymotrypsins into Anhydrochymotrypsins and Pyruvic Acids.[25,26]—A solution of the 25 mg of modified α-chymotrypsin in 4.5 ml of H_2O was cooled to 0°, then treated with 0.5 ml of 1 *N* NaOH previously cooled to 0°. After 4 hr at 0°, the solution of anhydrochymotrypsin was treated with 2.5 ml of 9 *N* HCl, then heated 75 min at 100° to hydrolyze the protein. The solution was neutralized to pH 7.1 with 6 *N* NaOH, then made up to exactly 10 ml with H_2O and assayed for pyruvic acid from serine and α-ketobutyrate from threonine.

Enzymatic Assay of Pyruvate and α-Ketobutyrate.—In a 3-ml cuvet was placed 1.00 ml of solution containing 1–5 μg of pyruvate or α-ketobutyrate, 1.80 ml of 0.1 *M* phosphate buffer (pH 7.5), and 0.10 ml of NADH (5 mg/ml). The reaction was started by addition of 0.10 ml of lactic dehydrogenase (0.2 mg/ml) and the OD change noted. Pyruvate (1–5 μg) was reduced in 30 sec and the OD change was 0.029/μg; α-ketobutyrate was reduced in 3 min with an OD change of 0.027/μg. A solution containing both pyruvate and α-ketobutyrate could be determined at 1–2 μg/ml with an error of <10%; the OD change in 30 sec represented mainly pyruvate and the further OD change in 5 min represented α-ketobutyrate.

Spectrophotometric Determination of Pyruvate 2,4-Dinitrophenylhydrazone.—To 8.00 ml of solution from the hydrolysis of anhydrochymotrypsin was added 2 ml of 0.2% 2,4-dinitrophenylhydrazine in 2 *N* HCl. After 20 min the mixture was extracted with three 5-ml portions of Et_2O. The combined Et_2O solutions were back-extracted with 3.00 ml of 10% Na_2CO_3. The Na_2CO_3 solution was diluted fivefold, then the O. D. read at 380 mμ. A calibration curve for 8 ml of solution containing 0–10 μg/ml of α-ketobutyrate and pyruvate was made.

The presence of either the 2,4-dinitrophenylhydrazone of pyruvate or α-ketobutyrate was determined by tlc on Brinkmann cellulose MN with 7:1:2 *n*-BuOH–EtOH–0.5 *N* NH_4OH.[27,28] An aliquot of the undiluted Na_2CO_3 solution of dinitrophenylhydrazones was acidified and extracted with Et_2O, then concentrated and applied to the plate. The pyruvate 2,4-dinitrophenylhydrazone moved as two orange spots of *syn* and *anti* forms; the α-ketobutyrate derivative moved more rapidly.

Cleavage of Protein Chain at Amino Side of *S*-Aminoethylcysteine. Generation of a New Carboxyl Terminus.—In three simultaneous runs, α-chymotrypsin was inactivated with **2** and **5**, then the sulfonate displaced with mercaptoethylamine; an unmodified α-chymotrypsin control was run where the inhibitor was omitted. The protein sample was dissolved in 5 ml of 0.1 *N* HCl, cooled to 0°, and treated with a solution of 47 mg of BrCN[29] in 5 ml of 0.1 *N* HCl previously cooled to 0°. After 5 days at 0° in a stoppered flask, the mixture was treated with 20 ml of H_2O and spin-evaporated to dryness *in vacuo*. This treatment converts methionine and *S*-aminoethyl-L-cysteine into *S*-cyano sulfonium derivatives.

The residual protein was dissolved in 5 ml of 6 *N* HCl and allowed to stand 16 hr at room temperature. The mixture was dialyzed against H_2O for 2 hr, then lyophilized. The residue was

(20) D. E. Fahrney and A. M. Gold, *J. Amer. Chem. Soc.*, **85**, 997 (1963).
(21) B. E. Erlanger, F. Edel, and A. G. Cooper, *Arch. Biochem. Biophys.*, **115**, 206 (1966).
(22) A. M. Gold, *Biochemistry*, **4**, 897 (1965).
(23) A. M. Crestfield, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **238**, 622 (1963).
(24) (a) H. Lindley, *Austr. J. Chem.*, **12**, 296 (1959); (b) We wish to thank Professor J. P. Riehm for advice, discussion, and use of his amino acid analyzer.
(25) H. Weiner, W. N. White, D. G. Hoare, and D. E. Koshland, Jr., *J. Amer. Chem. Soc.*, **88**, 3851 (1966).
(26) A. Patchornik and M. Sokolovsky, *ibid.*, **86**, 1206 (1964).
(27) J. Cavallini, N. Frontali, and G. Toschi, *Nature*, **163**, 568 (1949).
(28) B. McArdle, *Biochem. J.*, **66**, 144 (1957).
(29) E. Gross and B. Witkop, *J. Amer. Chem. Soc.*, **83**, 1510 (1961).

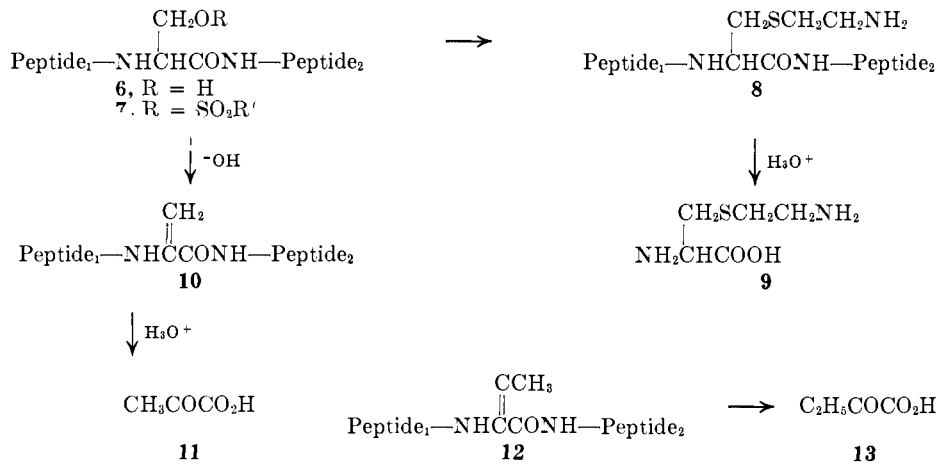
dissolved in 2 ml of H_2O and lyophilized with NaOH in the receiver; this operation was repeated once more. This treatment cleaves the protein chain at the carboxyl side of methionine and the amino side of *S*-aminoethyl-L-cysteine.

The dry residue was dissolved in 2 ml of H_2O, transferred to a hydrolysis bulb, then evaporated *in vacuo*, and dried over P_2O_5 *in vacuo* for 48 hr. The residue was dissolved in 0.50 ml of anhydrous N_2H_4, the bulb was sealed under vacuum, then placed in a 80° oven for 24 hr. The solution was evaporated *in vacuo*. The residue was dissolved in 2 ml of H_2O and transferred to a 3 × 0.9 cm column of Biorex-70 (H^+ form). The amino acid hydrazides were absorbed and the amino acids were eluted with 15 ml of H_2O.[30] The eluate was lyophilized, then the residue was dissolved in 2 ml of pH 2.2 citrate buffer and the ratio of amino acids determined on an amino acid analyzer. Hydrazinolysis converts all amide linkages to hydrazides leaving the carboxy terminal amino acid underivatized.[31]

Results and Discussion

α-Chymotrypsin was inactivated 97–100% when treated with any one of the inhibitors, **1–5**. Activity was not restored when the inactivated chymotrypsin was exhaustively dialyzed, thus showing that covalent bond formation had occurred; an α-chymotrypsin control under the same conditions still maintained most of its activity.

That covalent bond formation with **1–4** occurred with a serine residue was established by two methods. The first method was that of Gold[22] used for α-chymotrypsin inactivated with α-tolysulfonyl fluoride (**5**). The sulfonate esters (**7**) formed between **1** and **4** and α-chymotrypsin (**6**) were displaced to **8** with mercaptoethylamine and the *S*-aminoethyl-L-cysteine (**9**) resulting after hydrolysis was identified and quantified with an amino acid analyzer. Results are shown in Table I.



The yields of *S*-aminoethyl-L-cysteine were determined by peak areas compared to either the peak areas of 2 histidines or 14 lysines present in α-chymotrypsin;[32] the yields were 0.4–0.7 mol/mol of α-chymotrypsin for **1–4**, showing in each case a serine residue had been covalently linked as predicted.[9,14]

It was assumed by earlier workers that *p*-tolylsulfonyl fluoride (**5**)[33] and α-tolylsulfonyl fluoride (**4**)[20,22] attacked the active-site serine-195, based on analogy with diisopropyl phosphorofluoridate (DFP) and the fact that the enzyme was inactivated; although **5** was shown to attack a serine,[22] it was not proven to be serine-195. Later it was shown by X-ray studies that **4** did indeed form a covalent bond with serine-195.[34]

TABLE I

YIELD OF *S*-AMINOETHYL-L-CYSTEINE FROM MODIFIED α-CHYMOTRYPINS

Inhibitor	Mol/14 mol of Lys	Mol/2 mol of His
4	0.66	0.65
4	0.61	0.69
1	0.55	0.57
1	0.53	0.53
2	0.37	0.41
3	0.43	0.47

The second method for establishing that serine was attacked by **1–4** was conversion of the modified serine residue **7** into pyruvic acid (**11**) by the method of Koshland *et al.*;[25,26] this reaction proceeds *via* an anhydrochymotrypsin **10** formed by treatment of **7** with HO^-.

Pyruvic acid was determined enzymatically with lactic dehydrogenase. A standard curve was established by acid hydrolysis of 25 mg of unmodified chymotrypsin containing varying amounts of added pyruvic acid; the recovery of pyruvic acid was 80–86%. The results of analysis for pyruvic acid of the serine modified α-chymotrypsins are presented in Table II; no α-ketobutyrate (**13**) could be detected, which would be formed *via* **12** if **1–4** had attacked a threonine residue in α-chymotrypsin.

Unmodified α-chymotrypsin gave about 0.6 mol of pyruvic acid (Table II); thus the net yield of pyruvic acid from α-chymotrypsin modified by **1–4** was 0.4–0.8 mol/mol of α-chymotrypsin. Similarly, the spectrophotometric method used for the 2,4-dinitrophenylhydrazone of pyruvic acid showed a net yield of 0.4–0.8 mol/mol of α-chymotrypsin, although the method does not differentiate pyruvate from α-ketobutyrate; that the latter was absent (<10%) was shown by tlc.

α-Chymotrypsin modified with either α-tolylsulfonyl fluoride (**5**) or **2** were selected for further study to determine which serine of the 26 present in α-chymotrypsin was covalently linked. The reaction sequence

(30) P. De La Llosa, C. Tertrin, and M. Jutisz, *Experientia*, **20**, 204 (1964).

(31) H. Fraenkel-Conrat and C. M. Tsung, *Methods Enzymol.*, **11**, 151–155 (1967).

(32) B. S. Hartley, *Nature*, **201**, 1284 (1964); B. S. Hartley and D. L. Kauffman, *Biochem. J.* **101**, 229 (1966).

(33) D. H. Strumeyer, W. N. White, and D. E. Koshland, Jr., *Proc. Nat. Acad. Sci. U. S.*, **50**, 931 (1963).

(34) D. M. Blow, *Biochem. J.*, **112**, 261 (1969).

TABLE II

YIELDS OF PYRUVIC ACID FROM MODIFIED α-CHYMOTRYPSINS

Inhibitor	Mol of pyruvic acid/mol of α-chymotrypsin: Enzymatic	Spectrophotometric
4	1.17	1.09
1	1.36	1.35
2	1.02	0.98
3	1.37	1.35
None	0.57	0.62
None[a]	1.00	1.05
None[b]	0.53	0.98[c]

[a] Pyruvic acid (40 μg) added before acid hydrolysis. [b] α-Ketobutyric acid (40 μg) added before acid hydrolysis. [c] The spectrophotometric method does not distinguish between α-ketobutyrate and pyruvate.

TABLE III

C-TERMINAL ANALYSIS OF MODIFIED α-CHYMOTRYPSINS

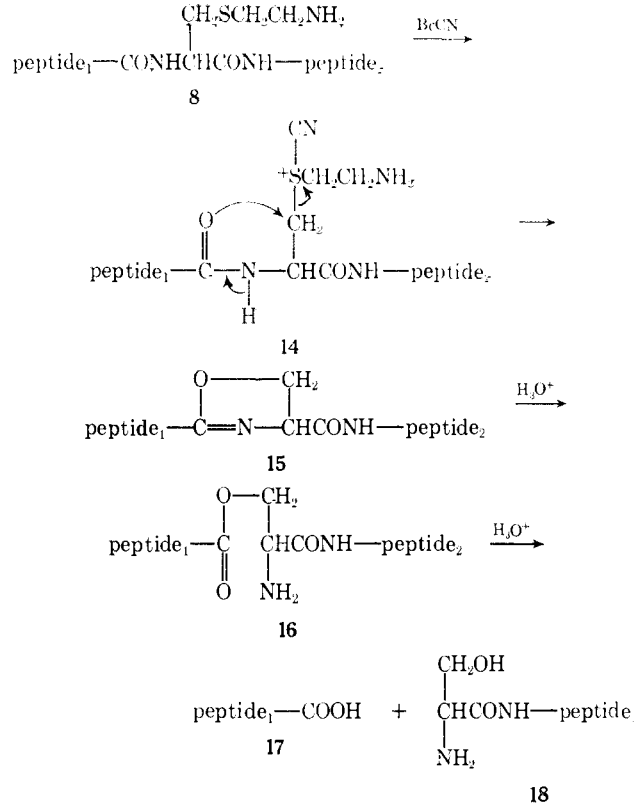
C-terminal amino acid	Modified α-chymotrypsin (CT)[a]: 5-CT	2-CT	CT	5-CT/CT	2-CT/CT
Leu[b]	1.00	1.00	1.00	1.00	1.00
Asp	0.165	0.106	0.068	2.43	1.56
Thr	0.036	0.047	0.033	1.09	1.42
Ser	0.107	0.095	0.073	1.47	1.30
Gly	0.130	0.140	0.082	1.58	1.82
Ala	0.101	0.092	0.079	1.28	1.16
Val	0.080	0.059	0.067	1.19	0.88

[a] CT = α-chymotrypsin. [b] Leu-13, the C-terminal end of the A-chain, is arbitrarily assigned the value of 1 mol/mol of CT and other amino acid peak areas are relative to Leu.

for selective cleavage of the peptide amino group of *S*-aminoethyl-L-cysteine (**8**) proceeded through **14–16** to generate a new carboxyl terminus (**17**). The 6 *N* HCl at 25° required for the steps **15–17** also gives some extraneous cleavage of the protein, particularly at glycine and glutamate residues; this "background noise" makes interpretation more difficult and results less exact. The results from the chromatograms coming off the amino acid analyzer could be quantitated by comparison with the peak area of Leu-13, the carboxyl terminus of the A chain; the peak area of Leu-13 was arbitrarily set at 1.00 and other C-terminal amino acids are relative to 1 mol of leucine/mol of α-chymotrypsin, as recorded in Table III.

The most easily understandable form for interpretation of the results is comparison of the ratios in the two right hand columns of Table III. It is clear that α-tolylsulfonyl fluoride (**5**) modified α-chymotrypsin generates Asp as a new carboxyl terminus in the sequence **14–17**; this result could arise if Ser-195 had been covalently linked by **5**, since Ser-195 is the only serine linked on its amino side to Asp.

The results with α-chymotrypsin modified by **2** clearly show the generation of much less carboxyl terminal aspartic acid than does α-chymotrypsin modified by α-tolylsulfonyl fluoride (**5**). Therefore it is clear that **2** has not reacted with the active-site Ser-195; this result confirms the hypothesis that *an active-site directed inhibitor such as* **2** *operates by the exo mechanism, that is,* **2** *forms a covalent linkage outside the active site.*



That **2** did not attack the active-site Ser-195 was unequivocally supported by the results in Table III. However, exactly which serine was attacked was more equivocal. Examination of the interaction of a model of **2** with a space-filling model of α-chymotrypsin[35] indicated that the SO_2F moiety could form a covalent linkage with one of the three serines between residues 217 and 223. Attack of Ser-217, Ser-218, or Ser-223 would generate a new carboxyl terminus at Gly-216, Ser 217, or Thr-222, respectively. Covalent linkage of **2** to Ser-218 can be eliminated since more new carboxyl terminus serine (217) is generated from α-chymotrypsin modified by α-tolylsulfonyl fluoride (**5**) than with **2**. In contrast, more threonine is generated by modification of **2** than with **5**; however, the difference is just outside experimental error, making the assignment of Ser-223 as the serine being linked by **2** only tentative. Although a glycine terminus (216) is higher with chymotrypsin modified by **2** than by **5**, the difference is probably within experimental error.

Although the concept[3] of the *exo* mechanism of active-site-directed irreversible inhibition has been unequivocally established by elimination of the active-site Ser-195 as the point of attack by **2**, new methods need to be developed to show unequivocally which serine has been covalently linked. Such methods should avoid the strong acid treatment that raises the "background noise" or else include a careful purification of the peptide fragments. Alternately, analysis of N-termini after selective cleavage at the dehydroalanine of anhydrochymotrypsin and unmasking of the amino-groups by alkaline hydrogen peroxide[29] would be a suitable method among others; such methods would also require a careful purification of the resultant peptides before end group analysis.

(35) We wish to thank Professor J. T. Gerig for use of his space-filling model of α-chymotrypsin.