iold-Samsøe, and O. Svendsen, *Eur. J. Pharmacol.*, 41, 153 (1977).
(14) U. H. Lindberg, S. O. Thorberg, A. L. Renyi, S. B. Ross, and S. O. Ögren, *Acta Pharm. Suec.*, **15** (no. 2) (1978).
(15) S. B. Ross, A. L. Renyi, and S. O. Ögren, *Eur. J. Pharmacol.*, **17**, 107 (1972).
(16) G. Ferrari, *Farmaco, Ed. Sci.*, **15**, 337 (1960).
(17) A. Vollmar and M. Dunn, *J. Org. Chem.*, **25**, 387 (1960).
(18) E. Schröder and K. Lübke in "The Peptides", Vol. I, Academic Press, New York and London, 1965, p 57.
(19) R. W. Roeske, *Chem. Ind. (London)*, 1121 (1959).
(20) E. Taschner, *Chem. Ber.*, **646**, 134 (1961).
(21) J. Rudinger and Z. Pravda, *Collect. Czech. Chem. Commun.*, **23**, 1947 (1958).
(22) A. Szent-Györgyi and I. Isenberg, *Proc. Natl. Acad. Sci. U.S.A.*, **46**, 1334 (1960).
(23) P. Millié, J. P. Malrieu, J. Benaim, J. Y. Lallemand, and M. Julia, *J. Med. Chem.*, 11, 207 (1968).
(24) P. H. Doukas in "Drug Design", Vol. V, E. J. Ariens, Ed., Academic Press, New York, N.Y., 1975, p 133.
(25) A. I. Salama, J. R. Insalaco, and R. A. Maxwell, *J. Pharmacol. Exp. Ther.*, **178**, 474 (1971).
(26) A. S. Horn in "Frontiers in Catecholamine Research", E. Usdin and S. H. Snyder, Ed., Pergamon Press, New York, N.Y., 1973, p 411.
(27) T. de Paulis, D. Kelder, S. B. Ross, and N. E. Stjernström, *Mol. Pharmacol.*, in press.
(28) B. K. Koe, *J. Pharmacol. Exp. Ther.*, **199**, 649 (1976).
(29) A. Szent-Györgyi, *Proc. Natl. Acad. Sci. U.S.A.*, **58**, 2012 (1967).
(30) U. H. Lindberg, S. B. Ross, S. O. Thorberg, S. O. Ögren, G. Malmros, and A. Wägner, *Tetrahedron Lett.*, in press.
(31) U. Thewalt and C. E. Bugg, *Acta Crystallogr., Sect. B*, **28**, 82 (1972).
(32) A. S. Horn and S. H. Snyder, *J. Pharmacol. Exp. Ther.*, **180**, 523 (1972).
(33) D. Carlström and R. Bergin, *Acta Crystallogr.*, **23**, 313 (1967).
(34) L. B. Kier, *J. Pharm. Pharmacol.*, **21**, 93 (1969).
(35) S. Abrahamsson, A. Carlsson, B. Carnmalm, and B. Dahlén, *Acta Chem. Scand., Ser. A*, **30**, 609 (1976).
(36) R. E. Counsell, P. Desai, T. D. Smith, P. S. Chan, P. A. Weinhold, V. B. Rethy, and D. Burke, *J. Med. Chem.*, **13**, 1040 (1970).
(37) J. Cason, K. L. Rinehart, Jr., and S. D. Thornton, Jr., *J. Org. Chem.*, **18**, 1594 (1953).

Inactivation of Trypsin-like Proteases by Sulfonylation. Variation of Positively Charged Group and Inhibitor Length

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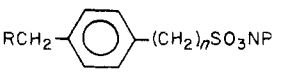
Attempts to achieve selective inactivation of serine proteases of closely related specificity (trypsin-like) by aryl sulfonylation have been extended. Nitrophenyl esters of benzenesulfonic acid and phenylmethanesulfonic acid containing various positively charged groups have been synthesized and examined as inactivators of trypsin, thrombin, plasmin, plasma kallikrein, and urokinase. Examples of selective inactivation by isothiouronium derivatives were found and attributed to differences among these enzymes in geometry and flexibility of the primary specificity sites.

Physiologically important proteases whose action involves a limited proteolysis of trypsin-like specificity often at unique sites in their normal substrate are found in coagulation, fibrinolysis, fertilization, and other processes[1]. Synthetic reagents capable of inactivating one of these enzymes without affecting the others may be of medicinal value. Affinity labeling offers a rational approach to enzyme inactivation based on the specificity of the target enzyme. However, proteases of similar specificity such as the trypsin-like enzymes cited which hydrolyze proteins at certain arginine or lysine residues have extended active centers which may encompass five or more amino acid residues[2,3] in their normal macromolecular substrate and may therefore achieve specificity by utilizing a large region of complementary interaction to position the substrate and promote productive cleavage. In view of this, the possibility of achieving selective inactivation with a small reagent occupying only the primary specificity site does not seem likely. Nevertheless, in earlier work we have shown that benzamidine and phenylguanidine are competitive inhibitors of trypsin[4] acting as structural analogues of the basic side chains of arginine and lysine and that carboxylate[5,6] and sulfonate[7,8] derivatives having these or other positively charged substituents may act as substrate analogues. Furthermore, among carboxylate derivatives the formation of acyl enzymes (intermediates of the normal hydrolytic pathway) of considerable stability in the case of plasmin[5,6] in contrast to thrombin indicated that selective inhibition was possible, involving a very limited region of the active centers.

Meanwhile the possibility has been explored[7,8] of using aryl sulfonate ester analogues instead of carboxylate in the expectation that selectivity of inhibition might be achieved in the acylation (sulfonylation) step rather than in the subsequent hydrolytic step since it was expected that desulfonylation would in all cases be negligible. In fact, *p*-nitrophenyl *p'*-amidinophenylmethanesulfonate was prepared and found to inactivate thrombin[8] but not trypsin, plasmin, or plasma kallikrein, although it complexed at the active center of all four proteases as shown by competition of the esterase action.

In the present work the effect of positively charged groups other than amidino and guanidino was examined on the sulfonylation of selected trypsin-like enzymes by *p*-nitrophenyl esters of the general structure



in which R is a positively charged group, $n = 0$ or 1, and NP is *p*- or *m*-nitrophenyl.

Chemistry. The *p*-nitrophenyl esters of *p*-bromomethylbenzenesulfonic acid and its next higher analogue, *p*-bromomethylphenylmethanesulfonic acid, were synthesized and reacted with dimethyl sulfide, thiourea, and its *N*-methyl derivatives, trimethylamine and pyridine, to

0022-2623/78/1821-0456$01.00/0

Table I. Nitrophenyl Esters of Substituted Benzenesulfonic Acids (BSA) and Phenylmethanesulfonic Acids (PMS)

No.	Ester isomer	Acid substituent	Mp, °C	Formula	Analyses	R'_n solvent[a] (days)	Crystn solvent[a]
1	Para	*p*-H_2N^+=C(NH_2)SCH_2BSA	192-194	$C_{14}H_{14}BrN_3O_5S_2$	C, H, N	a (1)	b
2	Para	*p*-H_2N^+=C($NHCH_3$)SCH_2BSA	169-170	$C_{15}H_{16}BrN_3O_5S_2$	C, H, N	a (2)	c
3	Para	*p*-CH_3N^+=C($NHCH_3$)SCH_2BSA	196-198	$C_{16}H_{18}BrN_3O_5S_2$	C, H, N	a (2)	c
4	Para	*p*-$(CH_3)_2S^+CH_2$BSA	101-103	$C_{15}H_{16}BrNO_5S_2$	C, H, N	a (1)	d
5	Para	*p*-$(CH_3)_3N^+CH_2$BSA	169-174	$C_{16}H_{19}BrN_2O_5S\cdot H_2O$	C, H, N	a (0.1)	e
6	Para	*p*-$C_5H_5N^+CH_2$BSA	192-194	$C_{18}H_{15}BrN_2O_5S$	C, H, N	a (0.1)	e
7	Para	*p*-H_2N^+=C(NH_2)SCH_2PMS	151-152	$C_{15}H_{16}BrN_3O_5S_2$	C, H, N	a (1)	b
8	Meta	*p*-H_2N^+=C(NH_2)SCH_2PMS	105-108	$C_{15}H_{16}BrN_3O_5S_2$	C, H, N	a (1)	f
9	Para	*p*-$(CH_3)_2S^+CH_2$PMS	97-100	$C_{15}H_{16}BrNO_5S_2$	C, H, N	f (1)	d
10	Para	*p*-$C_5H_5N^+CH_2$PMS	157-159	$C_{18}H_{15}BrN_2O_5S$	C, H, N	b (1)	b
11	Para	*p*-H_2N^+=C(NH_2)SCH_2PMS	83-85	$C_{15}H_{16}BrN_3O_5S\cdot H_2O$	C, H, N	g (1)	b
12	Para	*m*-$(CH_3)_2S^+CH_2$PMS	117-119	$C_{15}H_{16}BrNO_5S_2$	C, H, N	h (2)	d
13	Para	*m*-$(CH_3)_3N^+CH_2$PMS	177-178	$C_{17}H_{21}BrN_2O_5S$	C, H, N	c (0.2)	e
14	Para	*m*-$C_5H_5N^+CH_2$PMS	170-171	$C_{18}H_{15}BrN_2O_5S$	C, H, N	i (2)	b

[a] a, tetrahydrofuran; b, acetone; c, methanol; d, methanol-acetone; e, methanol-ethyl acetate; f, acetone-ethyl acetate; g, acetone-chloroform; h, chloroform; i, pyridine.

Table II. Rates of Inactivation of Trypsin-like Proteases by Aryl Sulfonates at pH 7.4, Enzyme Concentrations as in Figure 1[a]

	k_2, M^{-1} min^{-1}				
Ester	Plasma kallikrein	Plasmin	Thrombin	Trypsin	Urokinase
1	11	125	580	N.D.	92
2	0	0	0	0	N.D.
4	0	0	0	0	N.D.
5	0	50	147	N.D.	N.D.
6	N.D.	0	0	0	N.D.
7	4	7	0	1500	600
8	0	0	0	430	N.D.
9	0	0	0	0	N.D.
10	0	0	0	0	N.D.
11	0	100	260	23	N.D.
12	0	0	0	0	N.D.
13	0	0	0	0	0
14	0	0	0	0	0

[a] N.D. = not determined.

yield the desired esters with positively charged substituents. In some cases, the meta isomer of the alcohol or acid part of the structure was synthesized for comparisons as listed in Table I.

Results and Discussion

Of the positively charged groups used in the present study, the isothiouronium group provided the most interesting results. Compound **1**, *p*-nitrophenyl *p*-amidinothiomethylbenzenesulfonate, inactivated thrombin with a half-time of 22 min at an inhibitor concentration of 5.4×10^{-5} M, whereas a tenfold higher concentration required more than 2 h for a comparable effect on plasma kallikrein. Plasmin was susceptible to an intermediate degree. The results are presented as second-order rates in Table II. Methylation of a nitrogen atom in the isothiouronium group abolished activity.

The longer phenylmethylsulfonate ester, compound **7**, was not active on thrombin, plasmin, or plasma kallikrein although effective in inactivating trypsin itself. The ability of **7** to inactivate urokinase (Figure 1) provided an interesting example of selectivity of inhibition in view of the inertness of the reagent to three other physiologically important plasma proteases.

The loss of effectiveness against thrombin in going from the substituted benzenesulfonate **1** to the phenylmethanesulfonate **7** was not counteracted by altering the *p*-nitrophenyl ester to a *m*-nitrophenyl ester **8** as observed in an earlier case[8]. It appeared that the shape of the sulfonic acid part of the structure may not be suitable for promoting a covalent modification within the complex of **7** and **8** with a nonsusceptible protease. The formation of such complex was, however, verified. The phenylmethanesulfonate **7** was found to be a competitive inhibitor for plasma kallikrein, plasmin, thrombin, and trypsin with $K_i \times 10^{-4}$ = 3.2, 11.5, 9.3, 3.9, respectively, measured at pH 6. As in another example, in spite of comparable affinity for the active centers of four proteases, compound **7** inactivates only one enzyme of this group[7]. The geometric differences in the inhibitor-enzyme complex which limit covalent modification in this case to trypsin and urokinase are apparently less pronounced in the case of the isomer

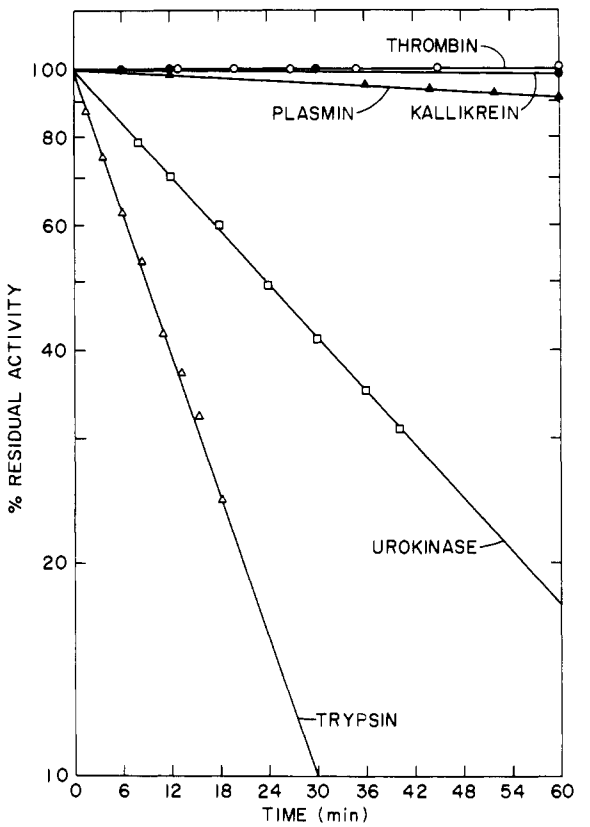


Figure 1. The action of **7** at 5×10^{-5} M on various trypin-related proteases at pH 7.4 in 0.1 M tris chloride, pH 7.4: enzyme concentration $\times 10^7$ M for bovine trypsin, 6.4; human plasmin, 4.8; human plasma kallikrein, 2.0; bovine thrombin, 16; and human urokinase, 51.

of the acid part of the structure, *p*-nitrophenyl *m*'-amidinothiomethylphenylmethanesulfonate (compound **11**), which inactivates three of the four proteases studied. Of particular interest are the antithrombin activity and lack of action on plasma kallikrein (Table II). It is of interest that the fundamental structure on which this reagent is based, *S*-benzylisothiouronium ion, has a relatively low affinity for trypsin with $K_i = 1.7 \times 10^{-3}$ M, and two orders of magnitude poorer than benzamidine[4]. Nevertheless, it provides the nucleus for a selective active-site-directed reagent, indicating again[7] that preoccupation with the binding phase of reagents[9] may represent misplaced emphasis as a step toward achieving selective irreversible inhibitors for serine proteinases.

Other positively charged groups such as the dimethylsulfonium (**4**, **9**, and **12**) and pyridinium (**6**, **10**, and **14**) provided sulfonate esters without ability to inactivate the proteases studied in contrast to the analogous carboxylate derivatives[6]. These studies are being extended to other trypsin-like proteases.

The primary specificity site of trypsin consists of a pocket on the protease surface containing the negatively charged β-carboxyl group of an aspartic acid residue which interacts with the positively charged substrate side chain[10,11]. This event helps complex the substrate at the active center and position it for catalysis. In trypsin-like enzymes for which sequence information is available, for example, in coagulation,[12] this specificity-providing feature (Asp-189) is conserved in a homologous relationship to the active center serine (Ser-195, conventional chymotrypsin numbering). The compounds under study are visualized as complexing in the normal way by charge interaction with the specificity site since competitive inhibition is observed, for example, with **7**. A subsequent transfer or sulfonylation of the active center serine depends on geometric suitability for this step which appears to be different among the proteases. Since the extended length of **7** from the positive charge to the sulfonate sulfur atom, 9.4 Å, is somewhat greater than the characteristic length of normal substrates (charge to carbonyl carbon), 7.8 Å, it is possible that the selectivity observed is due to differences in the extensibility of the primary specificity sites as well as to possible topographical differences. The existence of flexibility in the primary specificity site of trypsin, plasmin, and thrombin may be deduced from the normal substrate behavior[5] of esters of *p*-guanidinobenzoic acid whose length is 6.6 Å as well as by observations, in the case of trypsin, with guanidino acids of varied length[13].

Experimental Section

Inhibitor Evaluation. The sources of the proteases studied were the same as described earlier[8] except for urokinase, a purified sample which was kindly provided by Dr. Grant Barlow of Abbott Laboratories. The determination of K_i for competitive inhibition with Z-Lys-nitrophenyl ester followed published procedures[7]. For the detection of affinity labeling, protease and inhibitor were incubated at room temperature in 0.1 M tris chloride, pH 7.4, and aliquots were removed for assay with Z-Lys-nitrophenyl ester,[7,8] except in the case of urokinase for which the more sensitive substrate Z-Lys-thiobenzyl ester[14] was convenient. The concentration of proteases was established by titration with methylumbelliferyl *p*-guanidinobenzoate.[15,16] Incubations were not prolonged beyond 1 h in those cases in which a low or negligible loss of activity was detected.

Since the compounds under evaluation in the present study were not conveniently dissolved directly in buffer, stock solutions in DMF were used. However, the concentration of this solvent in a reaction mixture did not exceed 1% and had a negligible effect on enzyme stability.

When progressive enzyme inactivation occurred, the activity decay followed first-order kinetics, as in Figure 1, from which an apparent first-order rate constant was calculated from the half-time of inactivation, $k_1 = 0.693/t_{1/2}$(min) for a given inhibitor concentration. To permit comparison with data obtained at different reagent concentrations, the results are presented as a second-order rate obtained by dividing k_1 by the inhibitor concentration (Table II).

Synthesis of Reagents. Melting points were determined with a Fisher-John apparatus. Interatomic distances were estimated using Dreiding scale models obtained from Swissco Instruments.

***α*-Bromo-*p*-toluenesulfonyl Chloride.** *p*-Toluenesulfonyl chloride (38.0 g), *N*-bromosuccinimide (35.6 g), and benzoyl peroxide (2.4 g) were gently refluxed with stirring in CCl_4 (120 mL) for 2 h. The filtrate was concentrated to a syrup from which the product was extracted with 100-mL portions of boiling heptane. After removal of the solvent, the residue was recrystallized from hexane: yield 32 g; mp 67–72 °C.

***p*-Nitrophenyl *α*-Bromo-*p*-toluenesulfonate.** The sulfonyl chloride (10.8 g) and the sodium salt of *p*-nitrophenol (7.9 g) were stirred in acetone (60 mL) for 5 h. After removal of the solvent the residue in methylene dichloride was washed with 0.1 N NaOH and water to remove unreacted nitrophenol. The residue from the organic layer was crystallized from ethyl acetate providing 11.0 g, mp 111–114 °C. Anal. ($C_{13}H_{10}BrNO_5S$) C, H, N.

***p*-Nitrophenyl *p*'-Methylphenylmethanesulfonate.** *α*-Chloro-*p*-xylene (100 g) was stirred for 24 h at 105–110 °C with a solution of anhydrous sodium sulfite (94.5 g) in 300 mL of water. Saturated aqueous sodium chloride (150 mL) was added and the mixture cooled to room temperature. The sodium salt of the sulfonic acid was filtered with suction and washed with aqueous sodium chloride (2 × 50 mL) and drained thoroughly. After washing with acetone and ether, the product, 109 g, was dried at 100 °C overnight before conversion to the sulfonyl chloride.

The sodium sulfonate (21 g) was heated with phosphorus pentachloride (21 g) and phosphorus oxychloride (20 mL) for 3 h at 120 °C protected from moisture. The mixture was filtered and the filtrate combined with a carbon tetrachloride washing of the precipitate. Volatile material was removed by bringing the liquid fraction to 45 °C at 12 mmHg. The residue, without purification, was dissolved in dry acetone (100 mL) and stirred at room temperature with additions of sodium *p*-nitrophenolate until the yellow color persisted. Following removal of the solvent under reduced pressure, the residue was taken up in benzene–ethyl acetate (1:1) for extraction with 5% aqueous sodium carbonate and water. The residue from the organic layer was crystallized from ethyl acetate and hexane to provide 3.71 g of ester, mp 119–121 °C. Anal. ($C_{14}H_{13}NO_5S$) C, H, N. *p*-Nitrophenyl *m*'-methylphenylmethanesulfonate was similarly prepared, mp 113–114 °C. Anal. ($C_{14}H_{13}NO_5S$) C, H, N.

***p*-Nitrophenyl *p*'-Bromomethylphenylmethanesulfonate.** The foregoing ester (1.54 g) in carbon tetrachloride (50 mL) was refluxed with vaccum-dried *N*-bromosuccinimide (890 mg) and benzoyl peroxide (100 mg) until all of the brominating agent was consumed (starch–potassium iodide test) or about 75 min. After removal of the solvent, the residue was taken up in $CHCl_3$ (150 mL) and washed with 5% aqueous $NaHCO_3$, 1% HBr, and finally with water. The dried organic layer was concentrated to a small volume and the product crystallized by the addition of CCl_4 to yield 1.4 g, mp 119–122 °C. Anal. ($C_{14}H_{12}BrNO_5S$) C, H, N.

***m*-Nitrophenyl *p*'-Bromomethylphenylmethanesulfonate.** The *p*-methylphenylmethanesulfonate sodium salt (5.25 g) was stirred with 100 g of PCl_5 for 10 min at 150 °C protected from moisture. The reaction mixture was cooled, filtered through sintered glass, and washed with dry CCl_4, and the filtrate and washings were evaporated at 45 °C. The residue was dissolved in 40 mL of dry acetone and treated at room temperature with portions of sodium *m*-nitrophenolate until the yellow color persisted. The acetone was removed under reduced pressure, and the residue was dissolved in 100 mL of benzene–ethyl acetate, extracted with 5% $NaCO_3$ and water, and then dried over anhydrous magnesium sulfate. The residual oil after evaporation of the solvent was taken up in ethyl acetate and hexane was added for crystallization, yielding 334 mg of *m*-nitrophenyl *p*'-methylphenylmethanesulfonate. Recrystallization gave 222 mg, mp 79–80 °C. Anal. ($C_{14}H_{13}NO_5S$) C, H, N.

The bromomethyl derivative was prepared as described above, mp 93–94 °C. Anal. ($C_{14}H_{12}BrNO_5S$) C, H, N.

***p*-Nitrophenyl *m*'-Bromomethylphenylmethanesulfonate.** α-Chloro-*m*-xylene was converted to sodium *m*-methylphenylmethanesulfonate as described above for the para isomer; 46 g was treated with Dowex-50-H^+ to obtain the free acid which was dried overnight in high vacuum. The residue was stirred with $POCl_3$ (46 mL) at 105–110 °C, protected from moisture for 2.75 h. The filtrate and CCl_4 washings of the insoluble material were combined and concentrated with cautious warming at about 15 mm of pressure until an external temperature of 40 °C was reached. The residue was taken up in *n*-heptane (200 mL) and then crystallized at −20 °C to provide 14.1 g of product. In some runs the residue was used directly or distilled under reduced pressure.

The sulfonyl chloride (12.9 g) in dry CCl_4 (40 mL) was stirred while refluxing with *N*-bromosuccinimide (12.3 g) and benzoyl peroxide (0.5 g) for 3 h. The cooled reaction mixture was filtered and the filtrate plus $CHCl_3$ washings of the precipitate was concentrated. The residue without further purification was stirred with sodium *p*-nitrophenolate (14 g) overnight in acetone (80 mL). After removal of the acetone under reduced pressure, the residue was partitioned in $CHCl_3$ (200 mL) and water (100 mL). After washing with 5% sodium bicarbonate, water, 0.1 N HCl, and again with water, the organic layer was dried and concentrated to a small volume to induce crystallization; 14.6 g of the *p*-nitrophenyl ester of *m*-bromomethylphenylmethanesulfonate was obtained, mp 99–102 °C (ethyl acetate and hexane). Anal. ($C_{14}H_{12}BrNO_5S$) C, H, N.

Formation of Final Products. The bromomethylbenzenesulfonate or phenylmethanesulfonate esters were combined on a millimole scale at room temperature for varying periods with the appropriate nucleophile as described in Table I. In the case of trimethylamine, the 25% methanolic solution was used (w/w) with the bromo compound in the minimum amount of added solvent required for homogeneous solution. Yields varied from 50 to 90%.

Acknowledgment. The authors are grateful for the able technical contributions of John Ruscica. Research was carried out at Brookhaven National Laboratory under the auspices of the U.S. Energy Research and Development Administration with support from U.S. Public Health Service Grant 17849 from the National Institute of General Medical Sciences.

References and Notes

(1) E. Reich, D. Rifkin, and E. Shaw, Ed., "Proteases and Biological Control", Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1975, pp 455–465.
(2) D. Blow in "Proteinase Inhibitors", H. Fritz, H. Tschesche, L. J. Greene, and E. Truscheit, Ed., Springer-Verlag, New York, N.Y., 1974, pp 473–483.
(3) J. W. Nispen, T. C. Hageman, and H. A. Scheraga, *Arch. Biochem. Biophys.*, **182**, 227 (1977).
(4) M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, **240**, 1579 (1965).
(5) T. Chase and E. Shaw, *Biochemistry*, **8**, 2212 (1969).
(6) G. Glover, C.-C. Wang, and E. Shaw, *J. Med. Chem.*, **16**, 262 (1973).
(7) S.-C. Wong and E. Shaw, *Arch. Biochem. Biophys.*, **161**, 536 (1974).
(8) S.-C. Wong and E. Shaw, *Arch. Biochem. Biophys.*, **176**, 113 (1976).
(9) M. Yoshimoto and C. Hansch, *J. Med. Chem.*, **19**, 71 (1976).
(10) M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, **242**, 5782 (1967).
(11) M. Krieger, L. M. Kay, and R. M. Stroud, *J. Mol. Biol.*, **83**, 209 (1974).
(12) E. W. Davie, K. Fujikawa, M. E. Legaz, and H. Kato in ref 1, pp 65–78.
(13) F. Schwegler, *Experientia*, **32**, 1380 (1976).
(14) G. D. J. Green and E. Shaw, unpublished observations.
(15) G. W. Jameson, D. V. Roberts, R. W. Adams, W. S. A. Kyle, and D. T. Elmore, *Biochem. J.*, **131**, 107 (1973).
(16) P. Coleman, G. Latham, and E. Shaw, *Methods Enzymol.*, **45**, 12 (1976).