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SYNTHESIS OF ACTIVE SITE-DIRECTED ORGANOMETALLIC IRREVERSIBLE PROTEASE INHIBITORS

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Summary

p-Antimonybenzenesulfonyl fluoride and *p*-mercurybenzenesulfonyl fluoride irreversibly inhibit chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), and chromosomal protease, and these inhibitors appear to be as active as phenylmethanesulfonyl fluoride. The pretreatment of the proteases interferes with the phosphorylation of the active-site serine by diisopropylfluorophosphate suggesting that the organometallic inhibitors may also interact with the active site serine.

The organometallic inhibitors may be used for localization of proteases in different parts of the cell by electron microscopy and *p*-mercurybenzenesulfonyl fluoride could also be used for isolation of proteases by sulfhydryl affinity chromatography.

Introduction

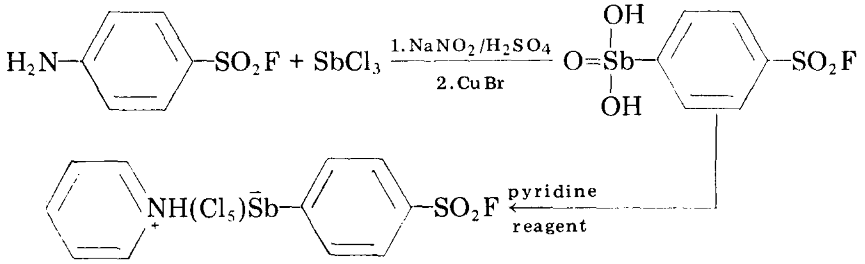
Cellular proteases appear to play an important role in various aspects of cellular events [1,2]. The role proteases play can be better defined by studying the distribution of proteases in different compartments of the cell. Various cytological agents have been developed for localization of proteases in the cell [3], and most of these rely on the detection of reaction products of proteases.

We report here the synthesis of metal-containing irreversible protease inhibitors which could be used for detection of serine proteases by electron microscopy and also for purification of proteases by affinity chromatography. The inhibitors are derivatives of benzenesulfonyl fluoride which contain either antimony or mercury at the *para* position of the benzene ring.

Materials and Methods

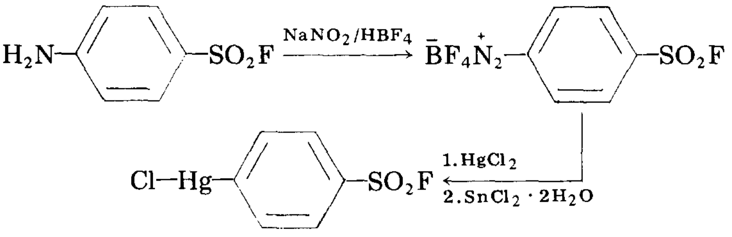
Preparation of metallobenzenesulfonyl fluorides

I. The synthesis of pyridinium p-(fluorosulfonyl)-phenylchloroantimonate (Sb-inhibitor). The general procedure of Doak and Steinman for the synthesis of arylstibonic acids by the Scheller reaction was followed [4], as shown in Scheme I. 12.5 g sulfanilyl fluoride was dissolved in 180 ml of 100% C_2H_5OH and cooled in an ice-bath. 7.2 g H_2SO_4 and 20 g $SbCl_3$ were added and stirred until solution was obtained. 5 g $NaNO_2$ in 10 ml H_2O was added to effect diazotization and the resulting suspension was stirred for 30 min. 1.4 g powdered CuBr was added and the reaction mixture was stirred for an additional 2 h while slowly reaching room temperature. N_2 evolution was evident at this time. Most C_2H_5OH was removed in vacuo and the dark residue was poured into ice/water stirring. The precipitate was filtered by suction, washed with water and pressed as dry as possible. This crude stibonic acid was stirred at 60° C with conc. HCl, during which time it became a dark oil. Addition of 50 ml HCl/pyridine (4 : 1, v/v), with cooling, resulted in a dark, gummy precipitate. The conc. HCl was decanted off, and the product was washed twice with conc. HCl, dissolved in 250 ml conc. HCl/ethanol (1 : 1, v/v) at 65—70° C, filtered and stored at 5° C. The resulting precipitate was filtered by suction, washed with conc. HCl, then with water and dried under vacuum over $CaCl_2$ to yield 6.1 g (16%) of a light-brown powder; mp. 140—143° C; $C_{11}H_{10}Cl_5FNO_2SSb$.



Scheme I. Synthesis of pyridinium *p*-(fluorosulfonyl)-phenylchloroantimonate

II. The synthesis of 4-(chloromercurio)-benzenesulfonyl fluoride (Hg-inhibitor). The general procedure of Dunker et al. [5] was followed as shown in Scheme II. 12.5 g sulfanilyl fluoride was suspended in 35 ml 48% tetrafluoro-



Scheme II. Synthesis of 4-(chloromercurio)-benzenesulfonyl fluoride boric acid with cooling. 5.5 g sodium nitrite in 11 ml H_2O was added in portions, with stirring, to effect diazotization. The diazonium tetrafluoroborate salt was collected on a filter and washed with water, C_2H_5OH and finally diethyl ether and air-dried to yield 12.7 g (65%) of the salt; mp. 130—131°C; $C_6H_4BF_5N_2O_2S$.

7.1 g diazonium tetrafluoroborate salt and 10.5 g $HgCl_2$ in 200 ml acetone was slowly added to a stirred suspension of 12.7 g $SnCl_2$ dihydrate and 7 g $HgCl_2$ in 50 ml acetone/20 ml H_2O. The mixture was then refluxed for 20 min, filtered by suction while hot and the volume of the filtrate was reduced in vacuo. Storing at 50°C overnight produced a precipitate which was collected and washed with cold diethyl ether to give 2.5 g (25%) of the mercury-containing compound; mp. 249—251°C; $C_6H_4ClFHgO_2S$.

Chromosomal protease

At the present time, we have difficulty in separating the chromatin-bound protease [6,7] from histones. The enzyme extract, therefore, contains histones, and we determined the degradation of histones by the chromatin-bound protease. The enzyme extract was prepared as follows: chromatin prepared from rat liver nuclei by the method of Stein et al. [8] was dissociated in 3 M NaCl, 10 mM Tris-HCl (pH 7.9) by stirring for 2 h at 4°C, and chromosomal proteins and DNA were separated by centrifugation for 12 h at 200 000 $\times g$ at 4°C. The supernatant was dialyzed against 10 mM Tris-HCl (pH 7.9) and used as the enzyme source. The concentration of protein was 0.5 mg/ml.

α-Chymotrypsin

A standard solution of α-chymotrypsin (Worthington Biochemicals) was prepared in 1 mM HCl at approx. 1 mg/ml (measured by the absorbance at 280 nm ($E_{1\,\mathrm{cm}}^{1\%} = 20.0$)) and diluted with 1 mM HCl immediately before use [9].

Trypsin

Approx. 10 mg/ml trypsin (Mann Research, Laboratories) in 0.01 M HCl at 0°C was dialyzed overnight at 4°C against 1 mM HCl. After removing of any traces of insoluble material by centrifugation, the precise concentration was established by measuring the absorbance at 280 nm after 1 : 10 dilution with 1 mM HCl ($E_{1\,\mathrm{cm}}^{1\%} = 15.4$). Stock solution was diluted with 1 mM HCl immediately before use [9].

Preparation of labeled histone

Arginine-rich histones (H2A, H3, and H4) were acetylated with [^{3}H]acetic anhydride (0.5 Ci/mmol) as described earlier [6] and excess [^{3}H]acetic anhydride was removed by chromatography on a Sephadex G-75 column. The labeled histones were extensively dialyzed against 5 M urea/10 mM Tris-HCl, (pH 7.9) to remove acid-soluble peptides and finally against deionized water. Histones were concentrated by lyophilization.

Assay of proteases

The activity of proteases was determined by following the release of acid-soluble peptides from radioactive histones as described earlier [6]. The effect of inhibitors on the proteases tested was determined at the initial 10% of the reactions. The details of reaction conditions are given in the Tables.

Results

Our main research interest has been studies on the biological role the chromatin-bound protease plays. Chromatin prepared from various tissues contains a common 25 000 dalton chymotrypsin-like protease [6,7]. The metal-containing inhibitors were synthesized to study localization of the protease in the nucleus by electron microscopy and for purification of the enzyme by affinity chromatography. Therefore, we used radioactive histones as substrate for the proteases we tested in this report.

The inhibitory activity of the metal-containing benzenesulfonyl fluorides was compared with that of phenylmethanesulfonyl fluoride which is a widely

TABLE I

INHIBITION OF PROTEASES BY METAL BENZENESULFONYL FLUORIDES

Reaction mixture contained ($5 \cdot 10^{-9}$ M α-chymotrypsin (or $1 \cdot 10^{-9}$ M trypsin, 0.25 mg/ml protein for chromosomal protease), $1 \cdot 10^{-6}$ M [^{3}H]histones (specific radioactivity $1.5 \cdot 10^{11}$ cpm/mmol), 40 mM Tris-HCl (pH 7.6), 20 mM sodium citrate. Inhibitors dissolved in dimethylsulfoxide were added to the reaction mixture containing 10% dimethylsulfoxide. Incubation time was for 20 min for α-chymotrypsin and trypsin and 3 h for chromosomal protease which gave about 10% acid-soluble peptides. At the end of reaction, an equal volume of cold 50% Cl_3CCOOH was added, and, after 15 min in ice, the acid-soluble material, after centrifugation for 10 min at 10 000 × *g*, was counted for radioactivity. PMSF, phenylmethanesulfonyl fluoride.

Protease	Inhibitor	Concn. (mM)	% Inhibition	$[I]_{0.5}$ (mM)
α-Chymotrypsin	PMSF	1.00	100	0.02
		0.10	83	
		0.01	26	
	Sb-inhibitor	1.00	100	0.01
		0.10	93	
		0.01	56	
	Hg-inhibitor	1.00	100	0.02
		0.10	76	
		0.01	40	
Trypsin	PMSF	1.00	94	0.20
		0.01	34	
		0.01	2	
	Sb-inhibitor	1.00	57	0.70
		0.10	19	
		0.01	0	
	Hg-inhibitor	1.00	96	0.05
		0.10	63	
		0.01	5	
Rat liver chromosomal protease	PMSF	1.00	92	0.04
		0.10	69	
		0.01	11	
	Sb-inhibitor	1.00	91	0.03
		0.10	66	
		0.01	34	
	Hg-inhibitor	1.00	71	0.07
		0.10	55	
		0.01	29	

used potent inhibitor of proteases. Phenylmethanesulfonyl fluoride interacts with the serine residue of active sites of many proteases (serine proteases) [10]. Both of the metal-containing compounds resemble phenylmethanesulfonyl fluoride in structure.

Sb-inhibitor inhibits chymotrypsin, trypsin, and the chromosomal protease as phenylmethanesulfonyl fluoride. The concentrations of Sb-inhibitor which is required for 50% inhibition of the enzymes are quite comparable with those of phenylmethanesulfonyl fluoride. Sb-inhibitor appears to be somewhat less effective against trypsin compared to phenylmethanesulfonyl fluoride (Table I). Sb-inhibitor tends to precipitate in the reaction mixture, and the addition of 10 mM sodium citrate prevents the precipitation. Hg-inhibitor also is a good inhibitor for the proteases tested, and the inhibitory activity of the compound is quite comparable with that of phenylmethanesulfonyl fluoride (Table I). The mercury group of Hg-inhibitor, however, can interact with sulfhydryl groups on the surface of any proteins, and this can limit the usefulness of the compound. Therefore, we tested the inhibitor activity of Hg-inhibitor in the presence of suflhydryl compounds such as mercaptoethanol and L-cysteine. Hg-inhibitor precipitates in the presence of mercaptoethanol but the compound remains soluble in the presence of L-cysteine presumably due to the charged groups of the amino acid even after the SH group of cysteine interacts with the mercury of Hg-inhibitor (Table II). Cysteine at the concentration of 0.1—5 mM does not affect the inhibitory activity of Hg-inhibitor against chymotrypsin, trypsin and the chromosomal protease. Therefore, Hg-inhibitor can specifically interact with proteases in the presence of excess cysteine.

We have also studied the possible site of interaction of Sb- and Hg-inhibitors on the proteases. Diisopropylfluorophosphate has been shown to phosphorylate the hydroxyl group of serine at the active site of serine proteases [11]. Therefore, if Sb- and Hg-inhibitors also interact with the active site serine

TABLE II

INHIBITION OF PROTEASE BY Hg-INHIBITOR IN THE PRESENCE OF L-CYSTEINE

Reaction condition was the same as that of Table I except that the reaction mixture contained 5% dimethylsulfoxide.

Protease	Concn. Hg-inhibitor (mM)	Concn. L-cysteine (mM)	% inhibition
α-Chymotrypsin	1.0	None	100
		0.1	97
		1.0	96
		10.0	91
Trypsin	1.0	None	95
		0.1	90
		1.0	90
		10.0	60
Rat liver chromosomal protease	1.0	None	73
		0.1	79
		1.0	77
		10.0	68

TABLE III

EFFECT OF PRETREATMENT OF PROTEASES BY Sb- AND Hg-INHIBITORS ON THE LABELING BY [^{3}H]DIISOPROPYLFLUOROPHOSPHATE

The proteases were incubated with the inhibitors for 1 h at 4°C in the reaction mixture described in Table I without histones and exhaustively dialysed against 40 mM Tris-HCl (pH 7.6), 20 mM sodium citrate, 10% dimethylsulfoxide. The dialyzed enzyme was tested for its activity using radioactive histones as substrate was described in Table I. A 0.5 ml aliquot of the dialyzed enzyme was incubated with 25 μCi [^{3}H]-diisopropylfluorophosphate (DFP) (3.4 Ci/mmol) for 1 h at room temperature. Excess of the label was removed by exhaustive dialysis against 1% sodium dodecyl sulfate, 0.01 M sodium phosphate, 0.1% 2-mercaptoethanol and the enzyme sample was counted for radioactivity. The results were expressed in terms of control which was not exposed to the metal-containing inhibitors.

Inhibitor (1 mM)	Enzyme	Enzymatic activity (% control)	[^{3}H]DFP uptake (% control)
Sb-inhibitor	α-Chymotrypsin	0	14
	Trypsin	0	14
	Rat liver chromosomal protease	16	15
Hg-inhibitor	α-Chymotrypsin	16	6
	Trypsin	11	11
	Rat liver chromosomal protease	7	13

pretreatment of the proteases with Sb- and Hg-inhibitor will prevent the binding of labeled diisopropylfluorophosphate to the proteases. Chymotrypsin, trypsin, and the chromosomal protease were treated with 1.0 mM of Sb- and Hg-inhibitor, and the inhibitors were removed from the enzymes by extensive dialysis. It was found that after the removal of the inhibitors the enzyme has lost its activity (Table III). This suggests that both Sb- and Hg-inhibitors are irreversible inhibitors of the proteases tested. As shown in Table III the proteases pretreated with Sb- and Hg-inhibitors take up only limited amount of [^{3}H]diisopropylfluorophosphate compared to the enzymes not exposed to the inhibitors. This experiment shows that Sb- and Hg-inhibitors interfere with the binding of [^{3}H]diisopropylfluorophosphate. This result suggests the possibility that Sb- and Hg-inhibitors may interact with the active site serine as phenylmethanesulfonyl fluoride [10]. Since the binding of histidine of the active site of trypsin by tosyllysine chloromethylketone also hinders the phosphorylation of the active site serine by diisopropylfluorophosphate [12] no definite conclusion can be drawn from the results shown in Table III.

Time course studies on the inhibition of chymotrypsin and chromosomal protease by these inhibitors also show that the metal-containing inhibitors are irreversible inhibitors as phenylmethanesulfonyl fluoride (Fig. 1). The antimony inhibitor, however, appears to require a high inhibitor/chymotrypsin ratio (at least 200 000) for complete inhibition of chymotrypsin (compare Fig. 1A and B). Both Sb- and Hg-inhibitor were found to be stable during the incubation period at pH 7.6. However, phenylmethanesulfonyl fluoride was rapidly inactivated under the same condition as in agreement with earlier reports [13].

Sb- and Hg-inhibitors do not inhibit non-protease enzymes such as RNAase and others.

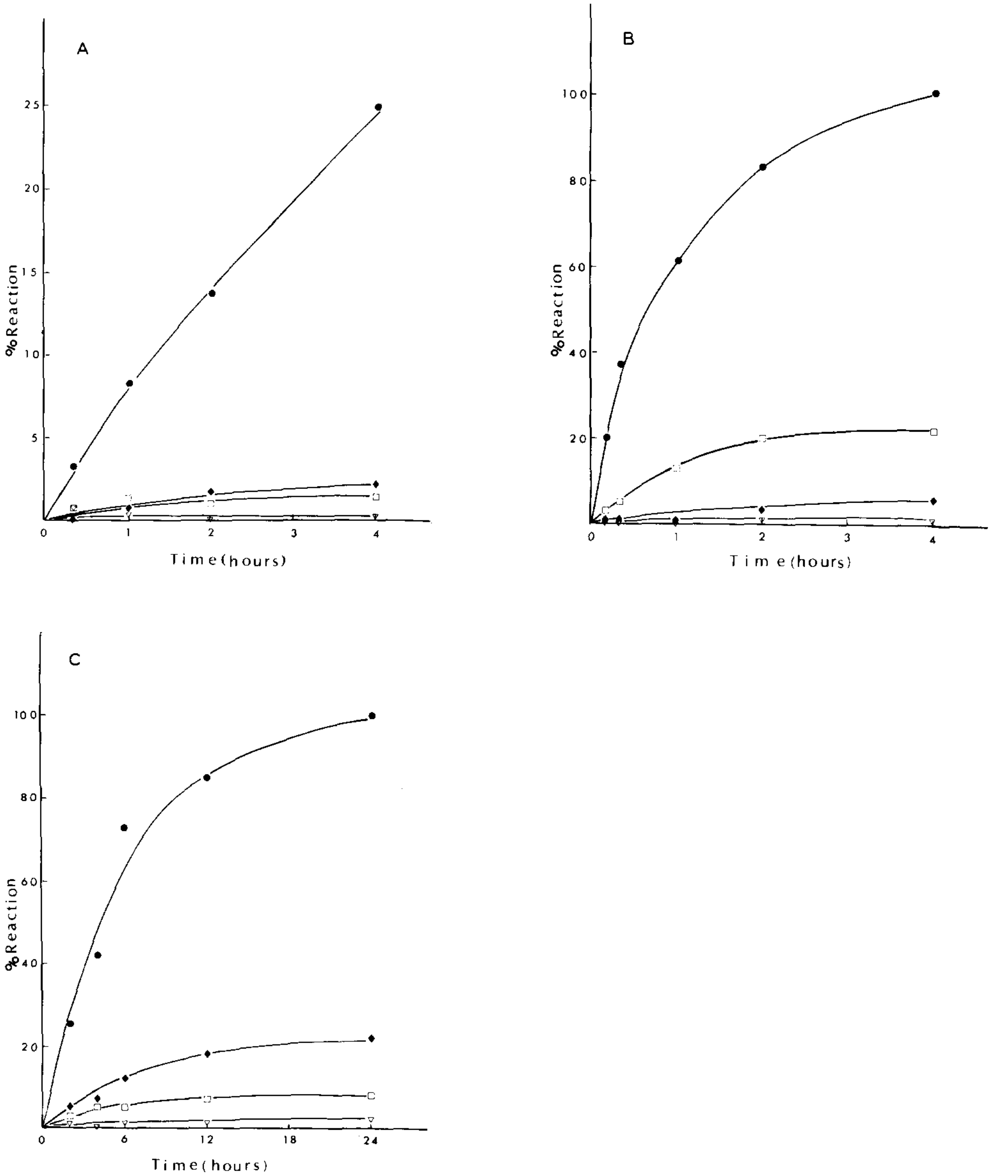


Fig. 1. Time course of inhibition of chymotrypsin and chromosomal protease by Sb- and Hg-inhibitors. The reaction conditions were essentially the same as described in Table I. Hg-inhibitor was incubated in the presence of 10 mM L-cysteine. (A). $1 \cdot 10^{-6}$ M [^{3}H]histones, $5 \cdot 10^{-9}$ M chymotrypsin; (B). $1 \cdot 10^{-6}$ M [^{3}H]histones, $5 \cdot 10^{-8}$ M chymotrypsin; (C). chromosomal protease, 0.25 mg/ml proteins. ●, no inhibitor; ▽, Hg-inhibitor; □, Sb-inhibitor; ◆, phenylmethanesulfonyl fluoride. Inhibitor concentration was 1 mM.

Discussion

The metal-containing inhibitors described in this report appear to be as effective as phenylmethanesulfonyl fluoride for inhibition of chymotrypsin, trypsin and the chromosomal protease. We have not attempted to determine various kinetic constants for the compound since we used radioactive histones as substrate, and histones obviously have multiple cleavage sites. Also the chromosomal protease preparation contained histones, and our aim was to compare the effectiveness of the inhibitors against several proteases using the same protein substrate.

Sb- and Hg-inhibitors appear to inhibit serine proteases irreversibly possibly by interacting with the active site serine. Sb- and Hg-inhibitors don't appear to interact with other non-protease proteins.

We are presently testing Sb-inhibitor as a cytological agent for localization of serine proteases in different parts of the cell by electron microscopy, Also Hg-inhibitor is being tested for purification of the chromosomal protease by sulfhydryl affinity chromatography. We are encouraged by the fact that the chymotrypsin treated with Hg-inhibitor binds to SH-affinity column, and the enzyme eluted from the column with 2-mercaptoethanol can be reactivated at pH 2 or pH 10 (Kim, Y.-J., unpublished data).

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