REACTION OF SOME SULFONYL HALIDES WITH TRYPSIN

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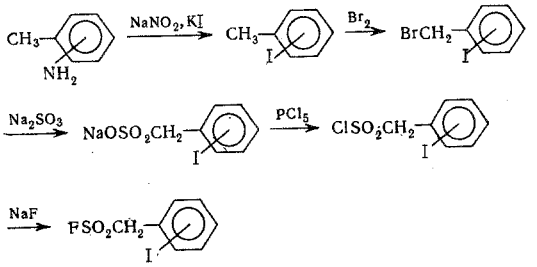
The catalytic activity of trypsin is blocked both reversibly and irreversibly by a number of compounds, including certain proteins [1] and acylating and alkylating agents [2-4]. Low-molecular irreversible inhibitors, which contain a heavy atom, are of special interest. The crystalline compound of a protein with such an inhibitor can be used in x-ray structure analysis [5]. An effective but nonspecific low-molecular inhibitor of trypsin is benzylsulfonyl fluoride (BSF) [6], which, in the value of the inhibition reaction rate constant, is close to diisopropyl fluophosphate [2] and 1-chloro-3L-tosylamido-7-amino-2-heptanone [3]. We obtained some BSF derivatives that contained the iodine atom in the *o*-, m-, and p-positions of the ring, and studied the inactivation of trypsin with these compounds.

The inhibitors were synthesized by the scheme:



The inhibition of trypsin was studied in tris-buffer solution at pH 7.2 in 30% acetonitrile. The reaction course was checked by the change in the activity of the trypsin, which was measured as described in [7], using benzoyl-L-arginine ethyl ester (BAEE) as the substrate, and an SF-4 spectrophotometer, equipped with an automatic recording attachment, to record the optical density with time. The hydrolysis rate of the BSF under these conditions was measured on a TTT-1 titrator, and the pH was maintained automatically at 7.2 by the addition of 0.047 N NaOH solution. This made it possible to compare the effect of the position of the substituent in the ring on the hydrolysis rate of these compounds and their reaction with trypsin. The corresponding kinetic curves are shown in Figs. 1 and 2. The values of the pseudofirst order rate constants for the hydrolysis (k_h) and inhibition (k_i) reactions were calculated from the initial sections of the kinetic curves. The second order constants were obtained by dividing by the concentration of the reagent present in excess (Table 1). The accuracy of measuring the constants was ±10%.

The insertion of iodine into the aromatic ring of BSF increase the hydrolysis rate, in which connection the following order of change in the reaction rate is observed: *o*-I–BSF, m-I–BSF > p-I–BSF > BSF.

TABLE 1

Compound	Hydrolysis		Inhibition	
	$k_I^h \cdot 10^{-4}$, sec^{-1}	$k_{II}^h \cdot 10^{-2}$, $M^{-1}\cdot$sec^{-1}	$k_I^i \cdot 10^{-4}$, sec^{-1}	k_{II}^i, $M^{-1}\cdot$sec^{-1}
	0,95	1,71	6,5	1,6
o-I — BSF	3,5	6,31	4,1	1,0
m-I — BSF	3,5	6,31	12,5	3,1
p-I — BSF	2,7	4,86	12,3	3,0

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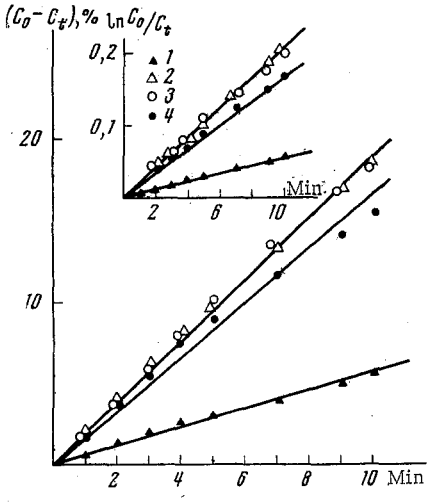

Fig. 1. Hydrolysis of BSF and isomeric I–BSF: 1) BSF; 2) *o*-I–BSF; 3) m-I–BSF; 4) p-I–BSF. pH 7.2; 25°C; 0.01 M $CaCl_2$; 30% acetonitrile; $C_0 = 4 \cdot 10^{-4}$ mole /liter; C_t is the concentration at time t.

The hydrolysis and alcoholysis of aromatic and aliphatic sulfonyl halides in aqueous–organic solvents is an S_N2 reaction of bimolecular nucleophilic substitution at the S atom* [8, 9]. It is natural that in the S_N2 mechanism electron-donor substituents favor a decrease in the reaction rate, while electron-acceptor substituents favor an increase. In our case an acceleration when hydrogen is replaced by iodine is most probably associated with the electron-acceptor properties of iodine due to the induction effect. Although iodine in the *o*- and p-positions also exhibits a mesomeric effect in the opposite direction, still in the given structure the reaction center is separated from the aromatic ring by a methylene group, as a result of which the influence of the mesomeric effect is weakened. †

In the reaction of BSF and the iodo-substituted BSF with trypsin, the same as in hydrolysis, the insertion of an iodine atom in the m- and p-position of the ring almost doubles the pseudomonomolecular reaction rate constant (k_I^i). This is in agreement with the hypothesis that the hydrolysis and inhibition have a common mechanism. The *o*-isomer is an exception: for it the first order rate constant not only does not increase, but it even decreases somewhat when compared with the unsubstituted BSF. The upsetting of the steric requirements of the active center of the enzyme toward the inhibitor is the most probable reason for this behavior of the *o*-isomer.

It is interesting to compare the hydrolysis rate of the inactivators with the rates of their reaction with trypsin. For the unsubstituted BSF, k_I^i is nearly seven times as great as k_I^h. The isomeric m- and p-I–BSF have k_I values that are respectively 3.6 and 4.3 times greater than the hydrolysis constant. As a result, faster rates are observed when reaction is with a stronger nucleophile (enzyme). This dependence of the rate on the strength of the nucleophilic reagent is characteristic for the S_N2 mechanism, whereas for S_N1 the rate should be determined primarily by the ionization rate of the substrate. It should be mentioned that in all of the cases the presence of iodine in the aromatic ring of the inhibitor hinders somewhat its reaction with the enzyme (the increase in k_I is smaller than could be expected when compared with the corresponding hydrolysis constants). This could be associated with an increase in the total size and a change in the geometry of the reagent when iodine is inserted.

Under our conditions (30% acetonitrile, 0.1 mole tris-buffer solution, 0.01 M $CaCl_2$), the rate constant k_{II} for the inactivation of trypsin by BSF is 2.8 times smaller than that determined in [6] in 10% aqueous propanol solution. This difference can be explained by the inhibiting effect of acetonitrile [10], as well as by the high concentration of the organic solvent, which upsets somewhat the native conformation of the proteolytic enzyme. However, the relatively high solubility of the iodinated BSF derivatives in aqueous acetonitrile makes it possible to create more favorable conditions for progress of the reaction by the pseudomonomolecular mechanism.

EXPERIMENTAL METHOD

The isomeric iodobenzyl bromides were obtained by the bromination of the iodotoluenes [11]: *o*-iodobenzyl bromide, mp 52° (from alcohol); see [12]; m-iodobenzyl bromide, mp 49° (from alcohol); see [13]; p-iodobenzyl bromide, mp 75–78° (from alcohol); see [11]. The sodium salts of the iodo-substituted benzylsulfonic acids were obtained in 70% yield [14].

Preparation of p-Iodobenzylsulfonyl Chloride. To 3.17 g (0.01 M) of the Na salt of *o*-I–BSF was added 2.45 g (0.017 M) of PCl_5 in 20 ml of $POCl_3$, the mixture was stirred at 120° for 11 h, the solution was decanted, the precipitate was extracted several times with benzene, the benzene extract was combined with the supernatant liquor, and the whole was distilled to dryness. The crystalline residue was dissolved in

*It should be mentioned that the composition of the solvent is important for both the reaction rate and the mechanism: an increase in the amount of the organic solvent favors realizing the S_N2 mechanism.
†Due to the fact that the influence of iodine on the reaction center (sulfur atom) can be transmitted through the methylene group only by the induction mechanism.

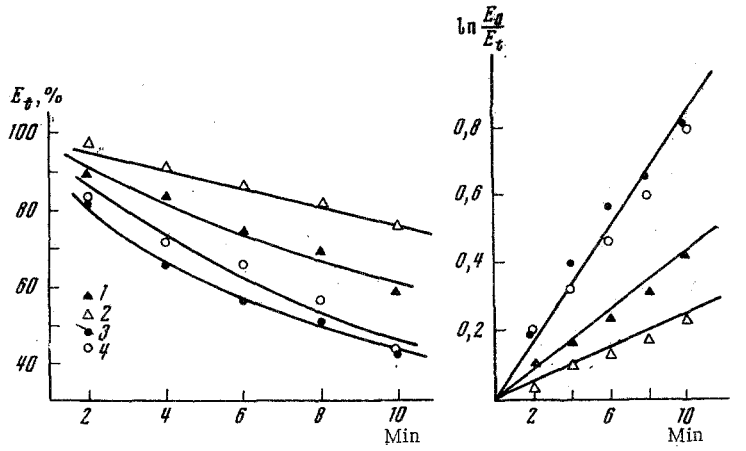

Fig. 2. Inhibition of trypsin by BSF and isomeric I–BSF: 1) BSF; 2) *o*-I–BSF; 3) m-I–BSF; 4) p-I–BSF. pH 7.2; 25°; 0.1 M tris-buffer solution; 0.01 M CaCl; 30% acetonitrile. Concentration of trypsin $2 \cdot 10^{-6}$, and of inhibitor $4 \cdot 10^{-4}$ mole/liter; E_t is the activity of trypsin at time t.

benzene and washed with ice water. We obtained 1.7 g (50%) of a product with mp 130-132° (from benzene). Found: C 26.73; H 1.98; S 10.16%. $C_7H_6FClO_2S$. Calculated: C 26.55; H 1.91; S 10.13%. *o*-Iodobenzylsulfonyl chloride (mp 78-81°) and m-iodobenzylsulfonyl chloride (mp 81-84°) were obtained in a similar manner.

The isomeric iodobenzylsulfonyl fluorides were obtained as described in [15]. To 3.0 g (0.01 M) of m-iodobenzylsulfonyl chloride in 10 ml of acetonitrile was added 2.1 g (0.05 M) of NaF in 6 ml of acetonitrile, the mixture was stirred at 85-90° for 3 h, the solution was decanted, the precipitate was extracted several times with hot benzene, and the combined solutions were evaporated. We obtained 1.8 g (64%) of product, mp 89-92° (from benzene). Found: C 28.00; H 1.77%. $C_7H_6FIO_2S$. Calculated: C 28.01; H 2.02%. *o*-Iodobenzylsulfonyl fluoride, mp 78-82°, was obtained in 60% yield, while p-iodobenzylsulfonyl fluoride, mp 180°, was obtained in 70% yield. Found: C 28.00; H 2.05; F 6.43%. $C_7H_6FIO_2S$. Calculated: C 28.02; H 2.02; F 6.33%.

The trypsin used in the study was obtained from the Leningrad Meat Combine, with an average activity of 10,000 units when determined by the Schwert–Takenaka method. The activity was calculated using the equation

$$A = \frac{\Delta D/\Delta t}{C} \cdot 5000,$$

where $\Delta D/\Delta t$ is the change in the optical density at 253 nm in 1 min, and C is the enzyme concentration, mg/ml.

To prepare a $4 \cdot 10^{-6}$ M trypsin solution we dissolved 9.6 mg of the protein in 100 ml of 0.2 M tris-buffer solution, pH 7.2, which contained $CaCl_2$ at a concentration of 0.02 M. Either 11.6 mg of BSF or 20 mg ($0.66 \cdot 10^{-4}$ M) of the I–BSF was dissolved in 50 ml of acetonitrile. Immediately before experiment the inhibitor solution was diluted with distilled water to a concentration of 60% acetonitrile. The inhibition of the trypsin was run at 25° in a thermostatted vessel. To 10 ml of the trypsin solution was added 10 ml of the inhibitor solution in 60% acetonitrile, and the pH was brought to 7.2 on a Radiometer TTT-1 titrator using 1 N NaOH solution. Samples of 0.1 ml of the solution were removed at 2 min intervals in the time from 2 to 10 min, diluted 20-fold with distilled water, and 0.8 ml was used to determine the activity.

The hydrolysis rate of benzylsulfonyl fluoride and of the iodo-substituted isomers at pH 7.2 and 25° was determined on an automatic titrator of the TTT-1 type, with an automatic recording of the titration curves. For this 9.9 mg ($3.3 \cdot 10^{-7}$ M) of the inhibitor was dissolved in 25 ml of freshly distilled acetonitrile. To 8.4 ml of the acetonitrile solution was added 19.6 ml of 0.014 M $CdCl_2$ in water, and the pH was maintained with 0.047 N NaOH solution.

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CONCLUSIONS

1. We synthesized the *o*-, m-, and p-iodobenzylsulfonyl fluorides (I–BSF) and measured the hydrolysis rate constants of these compounds in 30% acetonitrile at pH 7.2. The insertion of iodine into the aromatic ring of BSF increases the hydrolysis rate constant in the order: BSF < p-I–BSF < *o*-, m-I–BSF.

2. The rate constants for the inhibition of trypsin by BSF and the isomeric I–BSF were measured. The insertion of iodine in the p- and m-position of BSF facilitates the hydrolysis and the inhibition of the enzyme.

3. The *o*-I–BSF has the smallest inhibition rate constant, which is probably associated with steric hindrance during its reaction with the enzyme.

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