# Kinetic Study on the Mechanism of Inhibition of Trypsin and Trypsin-like Enzymes by *p*-Guanidinobenzoate Ester

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**It was found that [4-(2-succinimidoethylthio)phenyl 4-guanidinobenzoate]methanesulfonate (E-3123) inhibits trypsin, thrombin and kallikrein, and its inhibitory activity is most potent toward trypsin. The interactions of these enzymes with E-3123 were studied mainly by using stopped-flow spectrophotometry. E-3123 behaved as a quasi-substrate of the enzymes and the inhibitory property was due to the efficient production of the stable acyl-enzyme. The acylation process with trypsin was exceedingly effective, and the resulting acyl-enzyme was the most stable among the three enzymes tested. This observation affords a rational basis for explaining the action of E-3123, which is a transient inhibitor most active toward trypsin.**

**Keywords** trypsin; thrombin; kallikrein; enzyme kinetics; synthetic inhibitor; acyl-enzyme

A number of proteases which have trypsin-like specificity play major roles in a variety of physiological processes. For example, thrombin and plasmin are key enzymes in the blood coagulation and fibrinolysis systems. Trypsin itself is also known to be involved in the pathogenesis of various diseases. Acute pancreatitis is believed to result from intraparenchymal activation of digestive enzymes within the pancreas followed by digestion of the gland, and trypsin is suggested to be the major cause of the pathogenesis.[1,2] From this view point, aprotinin,[3] a trypsin inhibitor extracted from bovine lungs, and various synthetic protease inhibitors have been proposed as therapeutic agents for acute pancreatitis.[4,5] In our search for new synthetic inhibitors of trypsin as potential anti-pancreatitis drugs, we have found that [4-(2-succinimidoethylthio)phenyl 4-guanidinobenzoate] methanesulfonate (E-3123) exhibits strong inhibitory activity toward trypsin. As reported in the previous paper,[6] E-3123 has been shown to possess a suppressing effect on the development of experimental acute pancreatitis. In the work described here, the kinetic characteristics of E-3123 in its interaction with trypsin and trypsin-like enzymes were analyzed with a view to elucidating the mechanistic basis of the enzyme inhibition and the anti-pancreatitis action.

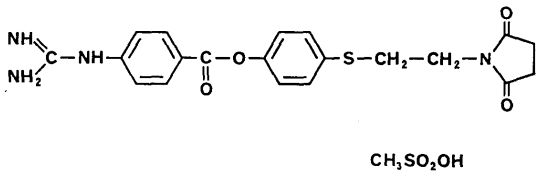


Fig. 1. Chemical Structure of E-3123

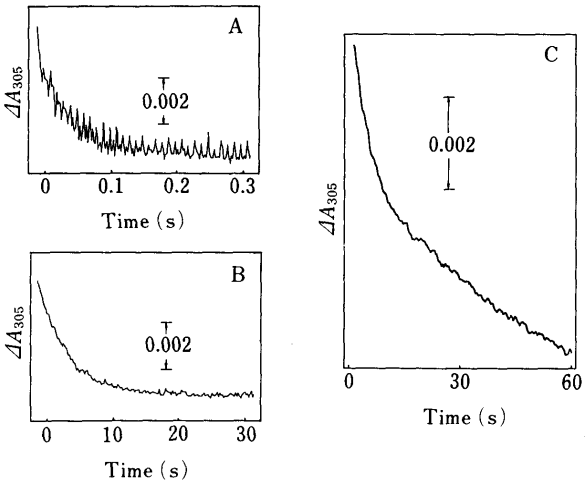


Fig. 2. Time Course of Trypsin-, Thrombin- and Kallikrein-Catalyzed Hydrolyses of E-3123 at pH 8.0, and at 25 °C

The linear part of the curve represents the deacylation stage. A: [trypsin] = $2.5 \times 10^{-6}$ M, [E-3123] = $3.98 \times 10^{-5}$ M. B: [thrombin] = $2.3 \times 10^{-6}$ M, [E-3123] = $3.98 \times 10^{-5}$ M. C: [kallikrein] = $2.5 \times 10^{-6}$ M, [E-3123] = $1.99 \times 10^{-5}$ M.

Hydrolysis of E-3123 was followed by monitoring the absorbance change at 305 nm due to the release of 4-(2-succinimidoethylthio)phenol (SEPT). The enzyme-catalyzed reaction was determined at pH 8.0 under the condition $[S]_0 \gg [E]$. As shown in Fig. 2, trypsin, thrombin and kallikrein caused a very rapid absorbance change which was monitored by a stopped-flow spectrophotometer. The rapid process was followed by a slow process. The result indicates that the catalysis proceeds in three steps—Michaelis complex formation, rapid acylation and subsequent slow deacylation. The reaction of E-3123 with the enzymes can be explained by the mechanism shown in Fig. 3. The acyl-enzyme accumulated during the course of the reaction can no longer exhibit catalytic activity. Consequently, E-3123 acts as a transient inhibitor and the duration of its action is governed by $k_3$.

The kinetic parameters for the reactions are compared in Table I. The $k_3$ value for trypsin was determined by measuring the reactivation rate of the isolated acyl-enzyme, which is exceptionally stable. E-3123 exhibits strong binding affinity to each of the three enzymes, and the $K_s$ values are in the range of $10^{-4}$—$10^{-6}$ M. The efficiency of inhibition by way of acyl-enzyme production will be reflected in the kinetic parameters $k_2$ and $k_3$ as well as $K_s$: a large $k_2$ (efficient production of inactive acyl-enzyme) and a small $k_3$ (stability of resulting acyl-enzyme) favor inhibition. The most efficient acylation process was observed in the reaction with trypsin. The process is so quick that the accumulation of acyl-trypsin is completed within 0.1 second. The $k_2$ value for trypsin is two orders of magnitude larger than those of other enzymes. Structural differences of the active centers within trypsin-like enzymes could be reflected in these acylation rate constants. Proximity of the enzyme catalytic residue to the ester part of E-3123 might

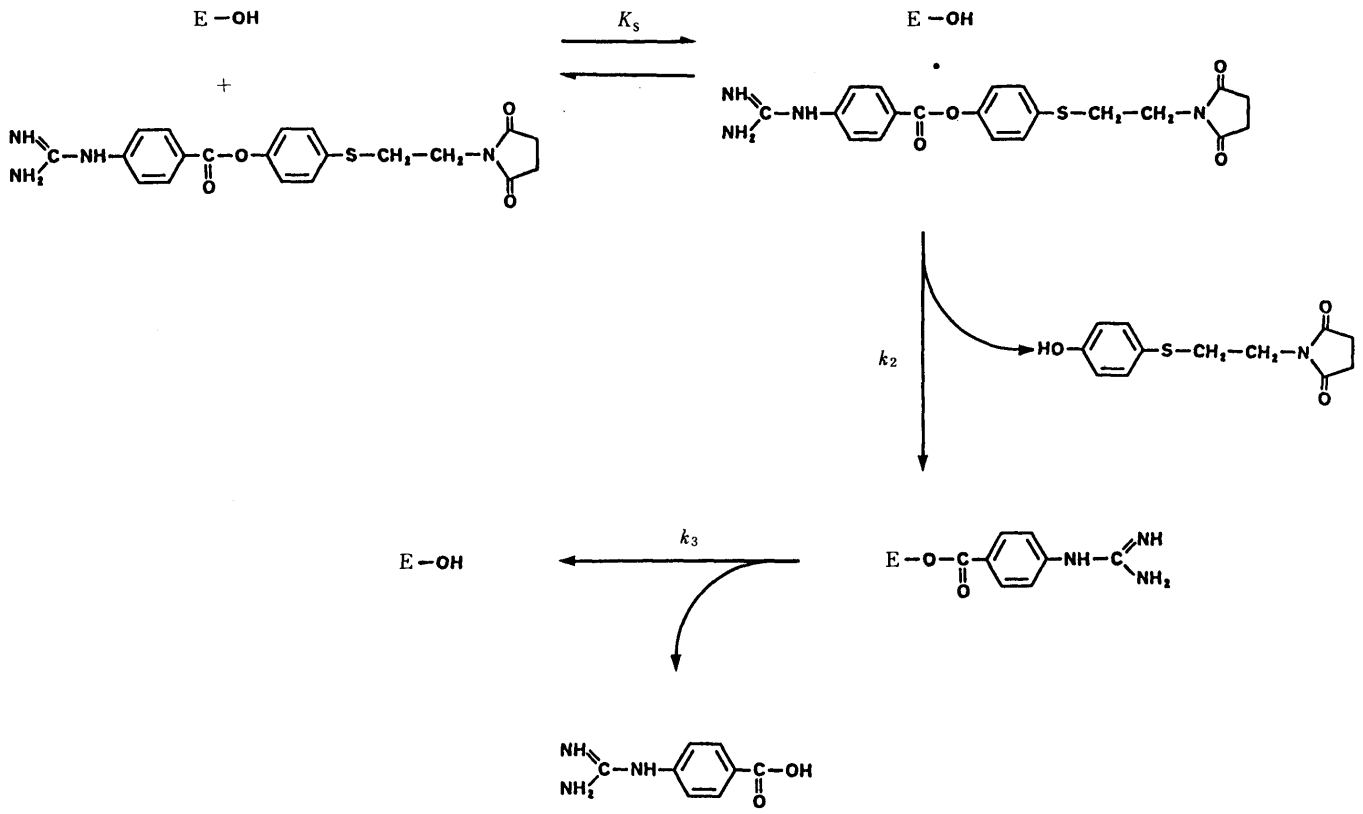


Fig. 3. Reaction Sequence of Trypsin and Trypsin-like Enzymes with E-3123
$K_s$, dissociation constant; $k_2$, acylation rate constant; $k_3$, deacylation rate constant.

be most favorably attained in the ES complex derived from trypsin. In contrast to the facile acylation process, deacylation is less favored in each case. A very small rate, $k_3 = 4.9 \times 10^{-5}\,s^{-1}$, was observed with trypsin and this value is one and three orders of magnitude smaller than those of thrombin and kallikrein, respectively. The apparent Michaelis constant ($K_m$app) was calculated from these parameters as described in the footnote to Table I. E-3123 shows the greatest inhibitory potency toward trypsin, with $K_m$app: $1.8 \times 10^{-10}$ M.

For hydrolytic enzymes, compounds which afford stable acyl-enzyme intermediates are expected to be inhibitors. This approach, transient inhibition, depends on the kinetic properties of compounds, *i.e.*, facile production of the stable acyl-enzyme is required. *p*-nitrophenyl-*p'*-guanidinobenzoate is widely used as a versatile titration reagent for trypsin and trypsin-like enzymes, since it affords stable *p*-guanidinobenzoyl-trypsin.[7-9] On the basis of the reported characteristics of the *p*-guanidinobenzoyl group, design of the inhibitor, E-3123, has been successfully carried out.

In the design of inhibitors, especially for drug use, the selectivity to a target enzyme among several enzymes of similar specificity is important. Inhibitors acting through the acyl-enzyme production mechanism are advantageous in this respect, since the differentiation of enzymes can be attained by each of three distinct kinetic parameters, *i.e.*, $K_s$, $k_2$, $k_3$.[10] This is in contrast to simple competitive inhibitors in which the inhibitory potency or selectivity is determined by the single parameter $K_i$. It is deduced from the values in Table I that the selective inhibition of trypsin by E-3123 in the presence of trypsin-like enzymes is feasible. At the concentration of $10^{-9}$ M, E-3123 completely inhibits trypsin activity without having a major effect on thrombin and kallikrein. The selectivity is also reflected in the difference in the duration of inhibition. It can be calculated from the $k_3$ values that the half-life time of acyl-trypsin (one turn-over) is 3.9 h, whereas that of kallikrein is only 1.1 min.

TABLE I. Comparison of Kinetic Parameters for Bovine Trypsin-, Human Thrombin- and Porcine Kallikrein-Catalyzed Hydrolyses of E-3123 at pH 8.0, and at 25 °C

| Enzymes | $K_s$ (M) | $k_2$ ($s^{-1}$) | $k_3$ ($s^{-1}$) | $K_m$ app (M)[a] |
|---|---|---|---|---|
| Trypsin | $2.0 \times 10^{-4}$ | 55 | $4.9 \times 10^{-5}$ | $1.8 \times 10^{-10}$ |
| Thrombin | $5.3 \times 10^{-5}$ | 0.34 | $4.3 \times 10^{-4}$ | $6.7 \times 10^{-8}$ |
| Kallikrein | $8.0 \times 10^{-6}$ | 0.15 | $1.1 \times 10^{-2}$ | $5.5 \times 10^{-7}$ |

*a*) Apparent Michaelis constant. $K_m$ app values were obtained by employing the equation: $K_m\,\text{app} = K_s[k_3/(k_2 + k_3)]$.

## Experimental

**Synthesis of E-3123** Succinimide (49.5 g, 0.50 mol) and 1,2-dibromoethane (187.9 g, 1.0 mol) were heated with $K_2CO_3$ (138 g, 1.0 mol) in 750 ml of 2-butanone under reflux for 9.5 h. Precipitated KBr was filtered off, and the residue obtained by evaporation of the solvent was dissolved in 2 l of chloroform and washed with water. The solvent was evaporated off, and the resulting residue was distilled *in vacuo*, bp 127 °C/2.5 mmHg, to give a colorless solid. The obtained solid was recrystallized from ethyl acetate and *n*-hexane to give 67.37 g of colorless needles of 1-bromo-2-succinimidoethane, mp 57.7 °C (65%). *Anal.* Calcd for $C_6H_8BrNO_2$: C, 34.98; H, 3.91; N, 6.80. Found: C, 35.03; H, 3.81; N, 6.56. 1-Bromo-2-succinimidoethane (175.92 g, 0.85 mol) and 4-hydroxythiophenol (107.6 g, 0.85 mol) were heated with $K_2CO_3$ (141.42 g, 1.0 mol) in 860 ml of ethanol

under reflux for 4 h. The resulting KBr was filtered off, and the residue obtained by evaporation of ethanol was dissolved in 1 l of water. The pH of the solution was adjusted to 1.0 by addition of hydrochloric acid, and the solution was extracted with ethyl acetate. The extract was washed with water, and the residue obtained by evaporation of the solvent was recrystallized from ethyl acetate to give 42.67 g of colorless prisms of 4-(2-succinimidoethylthio)phenol, mp 120.2 °C (20%). *Anal.* Calcd for $C_{12}H_{13}NO_3S$: C, 57.35; H, 5.21; N, 5.57. Found: C, 57.49; H, 5.20; N, 5.48. 4-(2-Succinimidoethylthio)phenol (32.04 g, 0.127 mol) and 4-guanidinobenzoyl chloride hydrochloride (32.8 g, 0.14 mol) were stirred in 260 ml of pyridine under cooling with ice for 16.5 h. Five liters of saturated aqueous solution of $NaHCO_3$ was added to the solution under cooling with ice, and the resulting precipitate was washed with acetone. This product and methanesulfonic acid (12.0 g, 0.125 mol) were heated in 910 ml of methanol to obtain a clear solution. The residue obtained by evaporation of the solvent was recrystallized from methanol to give 37.6 g of [4-(2-succinimidoethylthio)phenyl 4-guanidinobenzoate]methanesulfonate (E-3123) as colorless prisms, mp 201.6 °C (58%). *Anal.* Calcd for $C_{21}H_{24}N_4O_7S_2$: C, 49.60; H, 4.76; N, 11.02. Found: C, 49.58; H, 4.85; N, 10.76.

**Enzymes** Bovine trypsin was purchased from Sigma Chemicals Co. (Lot. T-8253). Enzyme concentration was measured spectrophotometrically at 280 nm ($E_{1\%,1\,cm}=15.5$).[11] Its molarity was determined to be 71% by titration with *p*-nitrophenyl-*p'*-guanidinobenzoate (NPGB)[7] taking the molecular weight of the enzyme to be 23300.[12] Human thrombin was prepared from Cohn fraction III (generously provided by Dr. B. H. Landis of Armour Pharmaceutical Co.) by the method of Fenton *et al.*,[13] modified by the use of Taipan venom (Australian Reptile Park, Gosfold, NSW, Australia). The obtained prothrombin was activated following the reported procedure.[14] Enzyme concentration was measured spectrophotometrically at 280 nm ($E_{1\%,1\,cm}=17.3$).[15] The molarity of the preparation was determined by NPGB titration to be 87%, taking its molecular weight as 37000.[15] Porcine kallikrein was purchased from Sigma Chemicals Co. (Lot. K-3627). Esterolytic activity of kallikrein toward *N*-α-benzoyl-l-arginine ethyl ester (BAEE) was determined to be 8.0 unit/mg (Lowry) at 25 °C and pH 8.0 in 0.1 M Tris–5 mM ethylenediaminetetraacetic acid (EDTA) by a spectrophotometric method at 254 nm.[16] One unit of esterolytic activity is defined as that amount of enzyme required to hydrolyze 1 mol of substrate per min (final substrate concentration, approximately 1 mM). Its molarity was determined by titration with NPGB.

**Determination of $k_2$ and $K_s$** Spectrometric measurements for the slow reaction were made on a Hitachi 330 spectrophotometer and those for the rapid reaction were performed on a Union Giken RA-401 stopped-flow spectrophotometer, taking $\Delta\varepsilon=1900$ at 305 nm.

The determination of $k_2$ and $K_s$ for trypsin- and thrombin-catalyzed hydrolyses of E-3123 was carried out in 0.1 M Tris buffer, pH 8.0 containing 0.02 M $CaCl_2$ and 2% dimethylsulfoxide at 25 °C (calcium ion was added to prevent autolysis). Under the condition of excess E-3123, the release of SEPT obeyed first-order kinetics with respect to enzyme concentration. In this case, the rate equation is given by: $k_{2obsd}=(k_2+k_3)[S]_0/(K_s+[S]_0)$, and the values of $k_2$ and $K_s$ were determined from plots of $1/k_{2obsd}$ against $1/[S]_0$. The concentration of E-3123 used in the analysis was 7.96—$1.39\times10^{-5}$ M, and the concentrations of trypsin and thrombin were $2.5\times10^{-6}$ M and $2.3\times10^{-6}$ M, respectively.

The determination of $k_2$ and $K_s$ for kallikrein was carried out in 0.1 M Tris buffer, pH 8.0 containing 5 mM EDTA and 2% dimethylsulfoxide at 25 °C (EDTA was added to avoid the effect of calcium ion). Concentrations of E-3123 and kallikrein were 7.96—$1.39\times10^{-5}$ M and $2.5\times10^{-6}$ M, respectively.

**Determination of $k_3$** The determination of $k_3$ for trypsin was carried out as follows. Trypsin, 5 mg, was dissolved in 1 ml of 0.1 M Tris–0.02 M $CaCl_2$ (pH 8.0). To this solution, 0.2 ml of an aqueous solution of E-3123 (0.02 M) was added and the mixture was kept at 25 °C for 15 min. The pH was adjusted to 2.0 by addition of 1 M HCl and the resultant solution was gel-filtered (Sephadex G-25 using pH 2.0 HCl as the eluant), and 5 ml of the eluate was collected after the void volume. The pH of the eluate was adjusted to 8.0 by addition of 1 M Tris–0.2 M $CaCl_2$. The solution was incubated at 25 °C, and reactivation of acyl-trypsin resulting from deacylation was monitored by measuring spectrophotometrically the residual activities of aliquots toward *N*-α-benzoyl-l-arginine-*p*-nitroanilide (l-BAPNA)[15] in 0.1 M Tris–0.02 M $CaCl_2$ (pH 8.0). The residual activity was assayed every 30 min after the reactivation reaction was started, and the assays were finished within 1 min to minimize the effect of the further progress of the deacylation during the assay. The value of $k_3$ was calculated from the slope of semilog plots. The substrate concentration was $2.0\times10^{-3}$ M and the acyl-trypsin concentration was $1.39\times10^{-7}$ M.

The overall catalytic constants, $k_{cat}$, for thrombin and kallikrein were obtained from the absorbancy change at 305 nm under the condition that the enzyme concentration was much smaller than the E-3123 concentration. The deacylation rate constant, $k_3$, was obtained from the equation: $k_{cat}=k_2k_3/(k_2+k_3)$.

In the case of thrombin catalysis, determination of $k_{cat}$ was carried out in 0.1 M Tris–0.02 M $CaCl_2$ (pH 8.0) at 25 °C. E-3123 concentration was $2.0\times10^{-4}$ M and the thrombin concentration was $8.5\times10^{-6}$ M. For kallikrein catalysis, determination of $k_{cat}$ was carried out in 0.1 M Tris–5 mM EDTA (pH 8.0) at 25 °C. E-3123 concentration was $1.0\times10^{-4}$ M and the kallikrein concentration was $8.0\times10^{-7}$ M.

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