Biochimica et Biophysica Acta, 165 (1980) 158—166

BBA 69087

KINETIC PROPERTIES OF TRIPEPTIDE LYSYL CHLOROMETHYL KETONE AND LYSYL *p*-NITROANILIDE DERIVATIVES TOWARDS TRYPSIN-LIKE SERINE PROTEINASES

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(Received March 13th, 1980)

Key words: Serine proteinase; Synthetic substrate; Synthetic inhibitor; Kinetic constant

Summary

The steady-state kinetic parameters of the tripeptides D-Val-Leu-Lys-, Ala-Phe-Lys-, and <Glu-Phe-Lys- in which the free carboxyl group was substituted with *p*-nitroaniline (substrate) or chloromethane (inhibitor), towards the serine proteinases plasmin (EC 3.4.21.7), thrombin (EC 3.4.21.5), urokinase, factor Xa, and trypsin (EC 3.4.21.4) were investigated. The *p*-nitroanilide derivatives were found to be very good substrates for plasmin, 2.5—40-times less efficient towards trypsin and very poor (100—10 000-times less efficient) substrates for thrombin, factor Xa and urokinase. The chloromethyl ketone derivatives were comparably efficient inhibitors of plasmin and trypsin and in general very poor (100—10 000-times weaker) inhibitors of thrombin, factor Xa and urokinase. D-Val-Leu-Lys-pNA however was a very poor substrate but D-Val-Leu-Lys-CH_2Cl a very efficient inhibitor for thrombin.

The variability in susceptibility of the substrates towards the enzymes was due to differences in their Michaelis constant, in their deacylation rate constant or both. The variable efficiency of the inhibitors was mostly due to differences in their dissociation constant and much less to differences in their alkylation rate constant. Only a poor correlation ($r = 0.25$) was found between the efficiency of the *p*-nitroanilides as substrate and their homologous chloromethyl ketones as inhibitor. The most notable discrepancy was observed with the D-Val-Leu-Lys derivatives towards thrombin.

Abbreviations: D-Val-Leu-Lys-, D-valyl-L-leucyl-L-lysyl-; Ala-Phe-Lys-, L-alanyl-L-phenylalanyl-L-lysyl-; <Glu-Phe-Lys-, L-pyroglutamyl-L-phenylalanyl-L-lysyl-; D-Val-Phe-Lys-, D-valyl-L-phenylalanyl-L-lysyl-; pNA, *p*-nitroanilide; -CH_2Cl, chloromethyl; Bz-Phe-Val-Arg-pNA, α-*N*-benzoyl-L-phenylalanyl-L-valyl-L-arginyl-*p*-nitroanilide; Bz-Ile-Glu-Gly-Arg-pNA, α-N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginyl-*p*-nitroanilide; <Glu-Gly-Arg-pNA, L-pyroglutamyl-glycyl-L-arginyl-*p*-nitroanilide; Boc, tertiary butyloxycarbonyl; Z, benzyloxycarbonyl.

Introduction

Trypsin-like serine proteinases such as thrombin (EC 3.4.21.5), plasmin (EC 3.4.21.7), factor Xa, urokinase, kallikrein (EC 3.4.21.8), etc., play an important role in many biological systems such as blood coagulation, fibrinolysis, kinin formation, complement activation, etc. These enzymes have the following factors in common, they hydrolyze peptide, ester or amide bonds in which a basic amino acid contributes the carbonyl group [1]. This specificity is due to a complementarity between the negatively charged aspartic acid side-chain in the primary binding site of the enzyme, called S_1 in the nomenclature of Schechter and Berger [2], and the positively charged side-chain of the basic amino acid preceding the scissile bond in the substrate, called P_1 [2]. With respect to the primary specificity (P_1), several enzymes such as thrombin, factor Xa, urokinase, etc., preferentially hydrolyze arginyl bonds in physiological substrates, others, such as plasmin, prefer lysyl bonds, whereas trypsin hydrolyzes both types equally well [3]. Additional selectivity is endowed not only by complementarity between amino acids beyond the basic P_1 residue on the amino-terminal side of the scissile bond in the substrate (positions P_2, P_3, etc.) and secondary binding sites in the enzyme (S_2, S_3, etc.) but also by complementarity between amino acids on the carboxy-terminal side of the scissile bond in the substrate (P_1', P_2', etc.) and binding sites in the enzyme (S_1', S_2', etc.) [2].

In view of the important physiopathological role of these enzymes, extensive research has been performed on the development of specific substrates as well as inhibitors of trypsin-like serine proteinases. Based on the assumption that the specificity may to a large extent be imposed by the sequence of the three or four amino acids preceding the scissile bond (P_1, P_2, P_3 and P_4) tri- or tetrapeptide derivatives have been most thoroughly investigated. In general, two approaches have been used for the design of these tripeptide derivatives, being either imitation of the sequences preceding scissile bonds in natural substrates or a more-or-less systematic kinetic investigation of several tripeptide sequences. The peptide research group at Kabi [4] has screened a large number of *p*-nitroanilide derivatives of tripeptides with lysine or arginine in the P_1 position. In general these studies have confirmed that thrombin, factor Xa and urokinase have a marked preference for arginine and plasmin for lysine. In several instances, but not always, the best substrates were tripeptide-*p*-nitroanilides which resembled or which were identical to the sequence preceding the scissile bond in physiological substrates.

Shaw et al. [5,6] have investigated chloromethyl ketone derivatives as inhibitors of serine proteinases and have again observed that thrombin is most sensitive to arginine derivatives tailored from sequence preceding the scissile bond in physiological substrates. In a recent study however, D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl ketone, a tripeptide sequence which does not occur in physiological thrombin substrates, was found to be an extremely efficient inhibitor [7].

A comparison of the kinetic parameters of *p*-nitroanilide and chloromethyl ketone derivatives of the same tripeptides might yield new information on the role of these functional groups for the selectivity of both types of derivative and might help to take advantage of the results obtained with substrates for the

design of inhibitors and vice versa. We have initiated such a systematic, comparative study primarily with the aim of obtaining more specific inhibitors which could be used in vivo. Already with the first series of lysyl derivatives some quite unexpected findings have been obtained which are reported in the present study.

Materials and Methods

Enzymes

Human plasmin was prepared by extensive activation (more than 95%) of purified human plasminogen [8] with streptokinase (0.02 mol/mol) in 0.1 M phosphate buffer, pH 7.3, containing 25% glycerol for 18 h at 4°C. Human α-thrombin [9] was a gift from Dr. J.W. Fenton, II (Division of Laboratories and Research, New York State Department of Health, Albany, NY). Bovine trypsin (twice crystallized, type I) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine factor Xa was a gift from Kabi AB, Stockholm, Sweden (courtesy of Mr. P. Friberger) and human urokinase was a gift from Abbott Laboratories, North Chicago, IL, U.S.A. (courtesy of Dr. Sewell). The active concentration of the enzymes was determined by active site titration with *p*-nitrophenyl-*p'*-guanidinobenzoate [10].

Substrates

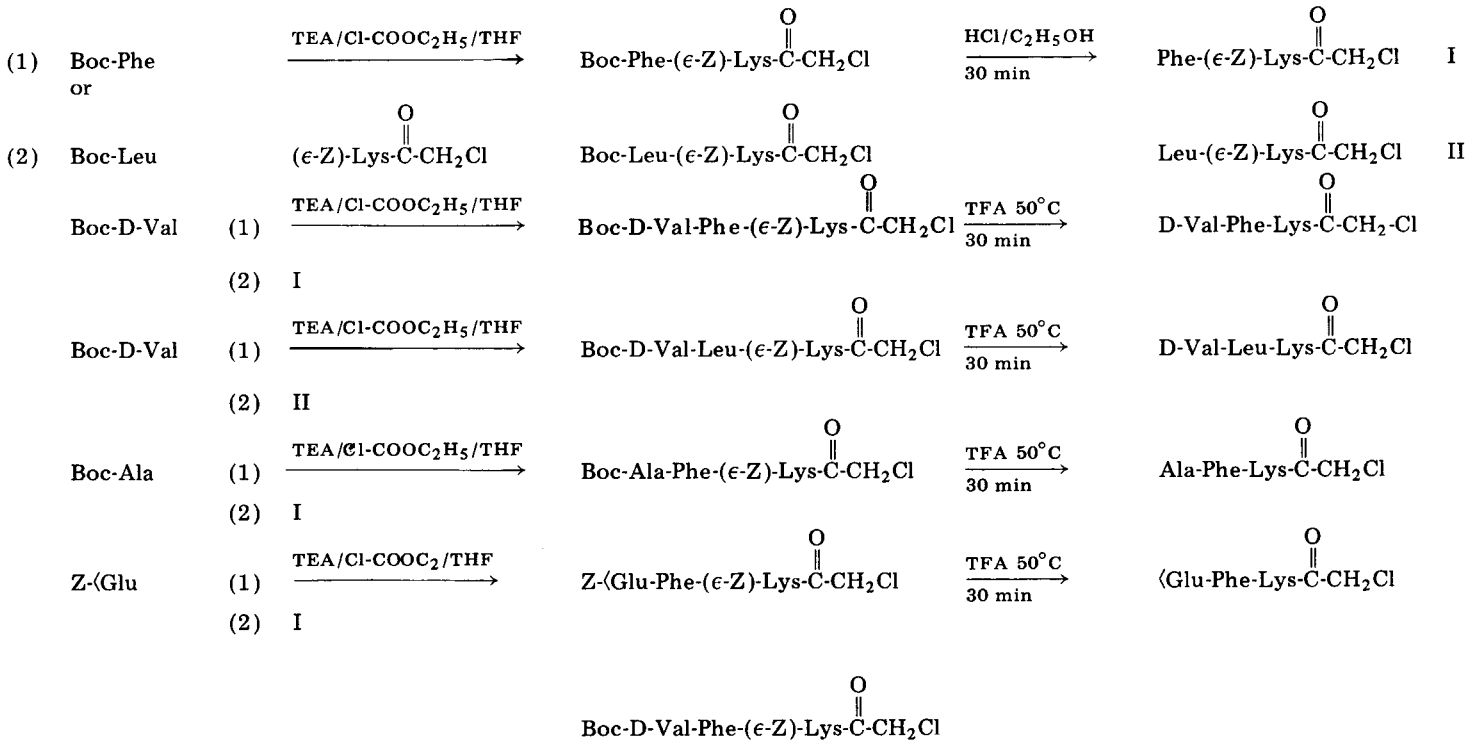
In the inhibition experiments, residual enzyme activities were measured using the specific tripeptide *p*-nitroanilide derivatives developed and generously donated by Kabi AB, Mölndal, Sweden (courtesy of Dr. G. Claeson). Thrombin and trypsin were measured with Bz-Phe-Val-Arg-pNA (S-2160) at a final concentration of 0.2 mM, plasmin with D-Val-Leu-Lys-pNA (S-2251) at a final concentration of 0.3 mM, urokinase with <Glu-Gly-Arg-pNA (S-2444) at a final concentration of 0.15 mM and factor Xa with Bz-Ile-Glu-Gly-Arg-pNA (S-2222) at a final concentration of 0.06 mM. The buffer solutions used for these measurements were those proposed by the manufacturer.

Inhibitors

N-α-Tosyl-L-lysine-chloromethyl ketone (TLCK) was purchased from Merck (Darmstadt, F.R.G.).

The chloromethyl ketone derivatives of D-Val-Leu-Lys-, Ala-Phe-Lys-, D-Val-Phe-Lys- and <Glu-Phe-Lys- were synthesized and evaluated at the Peptide Synthesis Unit of Union Chimique Belge (UCB, Brussels, Belgium) as described below. ε-Z-lysing chloromethyl ketone was prepared according to the procedure previously described [5]. The building up of the tripeptides was performed using the mixed anhydride procedure as shown in Scheme I.

The protected amino acids were dissolved in tetrahydrofuran (0.4 M) and cooled to −10°C. An equimolar amount of triethylamine and an equimolar amount of ethyl chloroformate were then added. The formation of the mixed anhydrides was allowed to proceed for 9 min at −10°C. The neutralized ε-Z-lysine chloromethyl ketone (0.4 M in tetrahydrofuran − half the amount of the protected amino acid) was then added and allowed to react for 20 min at 0°C and 2 h at 20°C. The solution was filtered and evaporated under reduced pres-



Scheme I. Scheme for the building up of the chloromethyl ketone tripeptides. TEA, triethylamine; THF, tetrahydrofuran; TFA, trifluoroacetic acid.

sure. The remaining material was dissolved in ethyl acetate and extracted with cold 10% citric acid, 5% $NaHCO_3$ and saturated NaCl in water. The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The protected dipeptides were then purified to homogeneity by several crystallizations from dichloromethane/petroleum ether. The purity of the products was assessed by thin-layer chromatography (silica gel developed with dichloromethane/acetone (8 : 2, v/v)). Staining was performed with ninhydrin, ninhydrin after HCl treatment and chlorine/toluidine reagents. Cleavage of the *t*-butyloxycarbonyl group was effected by a 30 min treatment in 5 M HCl/ethanol (1 g peptide/5 ml). After evaporation, the remaining material was dissolved in ethanol, precipitated with diethyl ether, dried over KOH under vacuum and used in the next coupling step without further purification.

The synthesis of the tripeptides was performed using the same conditions as described for the dipeptides. The purification was made in the same crystallization solvent except for the Z-<Glu-Phe-Lys-(Z)-$\overset{\overset{O}{\|}}{C}$-$CH_2Cl$ which was crystallized from acetonitrile/diethyl ether. The protected tripeptides were dissolved in trifluoroacetic acid (1—1.5 g/20 ml) and heated at 50°C for 30 min. The solution was evaporated to dryness under reduced pressure. The remaining oil was treated for 2 min in 5 M HCl/ethanol. After evaporation and drying over KOH, the deprotected tripeptides were purified by crystallization from acetonitrile. The purity of the final products was more than 95% as assessed by thin-layer chromatography (silica gel eluted with the solvent system : ethyl acetate/*n*-butanol/acetic acid/water (2 : 1 : 1 : 1) and stained with ninhydrin and chlorine/toluidine). During the synthesis, the structure of the intermediates and the final products was confirmed by NMR spectroscopy at 60 MHz. The presence of the $\overset{\overset{O}{\|}}{C}$-$CH_2Cl$ group was checked by its characteristic resonance as a sharp singlet at δ: 4.2—4.5.

Kinetic analysis

The kinetic parameters of the reaction between the enzymes (E) and the *p*-nitroanilide substrates (S) were determined using the classical Michaelis-Menten analysis in which the reaction is represented by

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

The Michaelis constant (K_m) and the maximal reaction rate (V) were determined from a Lineweaver-Burk plot:

$$\frac{1}{v} = \frac{K_m}{V} \cdot \frac{1}{[S]} + \frac{1}{V}$$

and k_2 by dividing V by the total enzyme concentration. The value k_2/K_m was calculated and used as an efficiency index of the substrate.

The kinetic parameters of the reaction between the enzymes (E) and the chloromethyl ketone inhibitors (I) were determined as described by Kitz and

Wilson [11]. The inactivation reaction was described by

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI'$$

in which the dissociation constant (K_i) and the rate constant for the alkylation step (k_2) are obtained from a plot of the inverse of the apparent pseudo-first-order rate constant (k_{app}) vs. the inverse of the concentration of the inhibitor (I), using the equation:

$$\frac{1}{k_{app}} = \frac{K_i}{k_2} \cdot \frac{1}{[I]} + \frac{1}{k_2}$$

The value of k_2/K_i was calculated and used as an efficiency index or specificity constant of the inhibitor.

All measurements of enzyme activities were carried out at 25°C in a volume of 1 ml in a cuvette with 1 cm path length. The change in absorbance at 410 nm was automatically recorded with a Pye Unicam SP 1800 double-beam spectrophotometer with a recorder scale of 100 mA. Incubation of the enzymes with the chloromethyl ketones was carried out under pseudo-first-order conditions (at least 5-fold higher concentration of inhibitor) in 0.1 M phosphate buffer, pH 7.3, at 25°C. Residual enzyme activities were then measured after 10-fold dilution and the kinetic constants were derived as described above. The K_m and V values of the reactions between the enzymes and the *p*-nitroanilides were calculated from measurements at 25°C in 0.1 M phosphate buffer, pH 7.3. Enzyme and substrate concentrations were adjusted to obtain a sufficiently large ΔmA at 410 nm.

Results and Discussion

The kinetic constants of the reactions between the inhibitors (D-Val-Leu-Lys-CH_2Cl, Ala-Phe-Lys-CH_2Cl, D-Val-Phe-Lys-CH_2Cl and <Glu-Phe-Lys-CH_2Cl) and the enzymes (human plasmin, human thrombin, human urokinase, bovine factor Xa and bovine trypsin) are summarized in Table I. All reactions could adequately be described by the scheme:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI'$$

and plots of the inverse of the pseudo-first-order rate constant (k_{app}) vs. the inverse of the inhibitor concentration (I) yielded straight lines as exemplified for D-Val-Leu-Lys-CH_2Cl in Fig. 1. Linear regression analysis yielded correlation coefficients of more than 0.97. The efficiency index k_2/K_i of these inhibitors ranged from 0.17 $M^{-1} \cdot s^{-1}$ for urokinase and Ala-Phe-Lys-CH_2Cl to 25 000 $M^{-1} \cdot s^{-1}$ for human plasmin and D-Val-Phe-Lys-CH_2Cl. These lysyl chloromethyl ketone derivatives appear to be good inhibitors for plasmin and trypsin and poor inhibitors for thrombin, factor Xa and urokinase with the exception of D-Val-Leu-Lys-CH_2Cl which is a very efficient inhibitor of thrombin (k_2/K_i =

TABLE I

KINETIC CONSTANTS OF THE INHIBITION OF SERINE PROTEINASES BY TRIPEPTIDE LYSYL CHLOROMETHYL KETONES

TLCK, *N*-α-tosyl-L-lysine-chloromethyl ketone

	Human plasmin	Human thrombin	Bovine factor Xa	Human urokinase	Bovine trypsin
D-Val-Leu-Lys-CH_2Cl					
k_2 (s^{-1}) X 10^2	6.5	6.6	4	2.9	1.5
k_i (M) X 10^6	54	2.6	1 300	33 000	7.8
k_2/K_i ($M^{-1} \cdot s^{-1}$)	1 200	23 000	30	0.85	2 000
Ala-Phe-Lys-CH_2Cl					
k_2 (s^{-1}) X 10^2	1.7	1.8	1.7	2.9	1.3
K_i(M) X 10^6	13	77	1 300	170 000	3.8
k_2/K_i ($M^{-1} \cdot s^{-1}$)	1 300	240	13	0.17	3 400
<Glu-Phe-Lys-CH_2Cl					
k_2 (s^{-1}) X 10^2	1.8	0.77	0.14	0.15	0.8
K_i(M) X 10^6	2.0	330	100	2 900	1.3
k_2/K_i ($M^{-1} \cdot s^{-1}$)	9 000	23	14.0	0.52	6 000
D-Val-Phe-Lys-CH_2Cl					
k_2 (s^{-1}) X 10^2	1.5	1.5	7.4	2.0	1.3
K_i(M) X 10^6	0.63	29	630	67 000	1.2
k_2/K_i ($M^{-1} \cdot s^{-1}$)	25 000	540	110	0.3	11 000
TLCK					
k_2/K_i ($M^{-1} \cdot s^{-1}$)	0.15				0.7

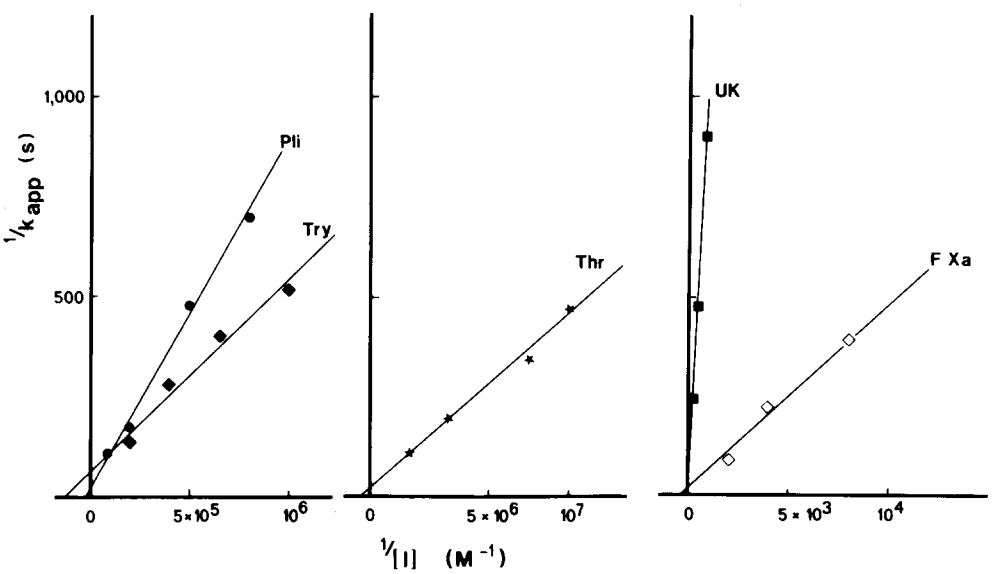


Fig. 1. Inactivation of human plasmin (Pli), human thrombin (Thr), bovine factor Xa (FXa), human urokinase (UK) and bovine trypsin (Try) by D-Val-Leu-Lys-CH_2Cl. Graphical representation of $1/k_{app}$ vs. 1/[I] according to Kitz and Wilson [11].

23 000 $M^{-1} \cdot s^{-1}$). The differences in efficiency index appear to be mainly due to differences in the dissociation constants (K_i) and much less to differences in alkylation rate constants (k_2). The tripeptide chloromethyl ketones are 10^4 times more efficient as inhibitors of plasmin than lysyl-chloromethyl ketone.

The kinetic constants of the reactions between the substrates (D-Val-Leu-Lys-pNA, Ala-Phe-Lys-pNA and <Glu-Phe-Lys-pNA) and the enzymes are summarized in Table II. All reactions could adequately be described by the model

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

and plots of $1/v$ vs. $1/[S]$ yielded straight lines, as exemplified for D-Val-Leu-Lys-pNA in Fig. 2. Linear regression analysis resulted in correlation coefficients of more than 0.98. The catalytic efficiency index k_2/K_m of these substrates ranged from 3.6 $M^{-1} \cdot s^{-1}$ for factor Xa and D-Val-Leu-Lys-pNA to 410 000 $M^{-1} \cdot s^{-1}$ for plasmin and <Glu-Phe-Lys-pNA. These lysyl *p*-nitroanilide derivatives are good substrates for plasmin and trypsin but very poor substrates for thrombin, factor Xa and urokinase. The differences in catalytic efficiency of these substrates appear to be due to differences both in their deacylation (k_2) rate constants and in affinity (K_m).

Linear regression analysis of the efficiency index of the *p*-nitroanilide derivatives of these tripeptides as substrates (k_2/K_m) vs. their chloromethyl ketone derivatives as inhibitors (k_2/K_i) gave a correlation coefficient of 0.25 for all pooled data obtained with the five enzymes and the three tripeptides. For the individual enzymes the correlation coefficients were 0.04 for urokinase, 0.51 for trypsin, 0.54 for factor Xa, 0.63 for plasmin and 0.98 for thrombin. For each tripeptide the correlation coefficients were 0.30 for D-Val-Leu-Lys-, 0.14 for Ala-Phe-Lys- and 0.83 for <Glu-Phe-Lys-.

It thus appears that in general tripeptide lysyl *p*-nitroanilides are good sub-

TABLE II

KINETIC CONSTANTS FOR THE HYDROLYSIS OF TRIPEPTIDE LYSYL p-NITROANILIDES BY SERINE PROTEINASES

	Human plasmin	Human thrombin	Bovine factor Xa	Human urokinase	Bovine trypsin
D-Val-Leu-Lys-pNA					
$k_2(s^{-1})$	12	0.67	0.0064	0.63	7.0
$K_m(M) \times 10^4$	2.4	25	18	83	4.0
$k_2/K_m(M^{-1} \cdot s^{-1})$	49 000	270	3.6	76	18 000
Ala-Phe-Lys-pNA					
$k_2(s^{-1})$	15	0.015	0.14	0.028	4.7
$K_m(M) \times 10^4$	1.7	10	130	4.5	20
$k_2/K_m(M^{-1} \cdot s^{-1})$	89 000	15	11	62	2 300
<Glu-Phe-Lys-pNA					
$k_2(s^{-1})$	24	0.07	0.72	0.51	5.4
$K_m(M) \times 10^4$	0.29	11	100	10	2.0
$k_2/K_m(M^{-1} \cdot s^{-1})$	410 000	62	72	510	27 000

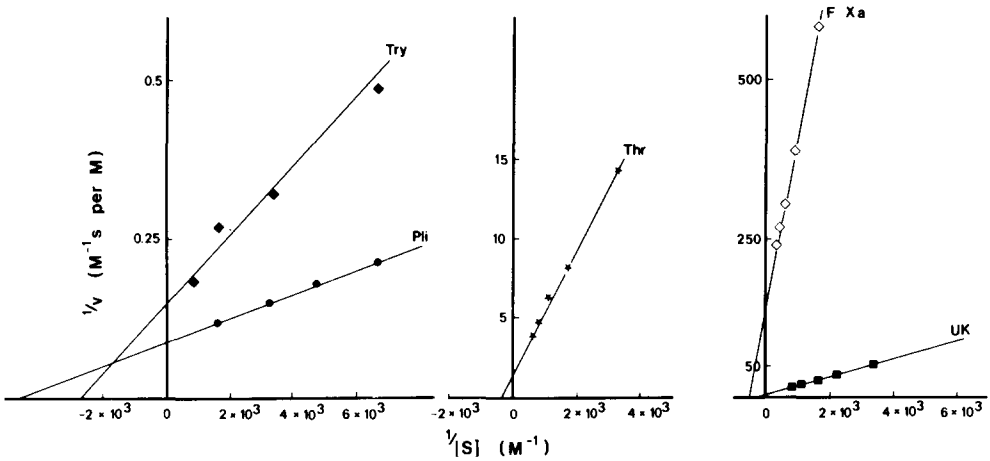


Fig. 2. Lineweaver-Burk plot of the reaction between human plasmin (Pli), human thrombin (Thr), bovine factor Xa (FXa), human urokinase (UK) and bovine trypsin (Try) and the chromogenic substrate D-Val-Leu-Lys-pNA.

strates and lysyl chloromethyl ketones good inhibitors for plasmin and trypsin and that these derivatives are poorly reactive towards thrombin, factor Xa and urokinase. There is however only a poor quantitative correlation between the efficiency of these *p*-nitroanilides and chloromethyl ketones, the most remarkable discrepancy being observed with the D-Val-Leu-Lys- derivatives towards thrombin. It is thus concluded that results obtained with *p*-nitroanilides cannot be extrapolated to chloromethyl ketones and vice versa. Furthermore sequences derived from natural substrates do not necessarily constitute the best models for designing peptide substrates or inhibitors for serine proteinases.

It may be anticipated that by changing the nature and configuration of the amino acids in the P_1, P_2 and P_3 positions even better inhibitors might be obtained than those reported in the present study. The safest way to find the best sequence (but unfortunately the most laborious one) seems to be a systematic synthesis and screening of a large number of derivatives.

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