The Interaction of α -N-(*p*-Toluenesulphonyl)-*p*-guanidino-L-phenylalanine Methyl Ester with Thrombin and Trypsin

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The syntheses are described of *p*-guanidino-L-phenylalanine and some of its derivatives. α -N-(*p*-Toluenesulphonyl)-*p*-guanidino-L-phenylalanine methyl ester is an excellent substrate of bovine trypsin (EC 3.4.21.4) (K_m 57 μ M; k_{cat} 320 s⁻¹ at pH 7.4–8.0) and a very poor substrate of human thrombin (EC 3.4.21.5) (K_m 190 μ M, k_{cat} 0.2 s⁻¹) and bovine chymotrypsin (EC 3.4.21.1). The ester inhibits thrombin clotting activity. It also inhibits the amidase and esterase activities of human thrombin, this inhibition being of the mixed type. The inhibition constant, K_i , of the order of 1 μ M, increases with increasing inhibitor concentration. This suggests that the enzyme binds the inhibitor at multiple sites. The importance of the residue at the P₁ position [notation of Berger & Schechter (1970) *Philos. Trans. R. Soc. London Ser. B* **257**, 249–264] in determining the selectivity of a substrate or quasi-substrate among trypsin-like enzymes is borne out. *p*-Guanidino-L-phenylalanine may have a use in the synthesis of selective peptide inhibitors of thrombin.

It is widely recognized that the efficiency of action of a proteinase on its substrate depends on amino acid residues at both sides of the cleavable bond, which are complementary to subsites in the enzyme polypeptide chain (Berger & Schechter, 1970). The specificity of some proteinases has been explained on such a basis (Steitz *et al.*, 1969; Shotton & Watson, 1970; Hartsuck & Lipscomb, 1971).

Trypsin-like enzymes, such as thrombin (EC 3.4.21.5), plasmin (EC 3.4.21.7) and kallikrein (EC 3.4.21.8), possess a similar specificity for α -N-substituted arginine and lysine esters or amides, although their kinetic parameters with respect to these substrates may vary greatly. Some of these enzymes, however, are entirely different in their selectivity for protein substrates. The differentiation between these enzymes on the basis of modifications or substitutions at the P₁ and adjacent positions (notation of Berger & Schechter, 1970) has been the subject of

several investigations (Chase & Shaw, 1969; Glover *et al.*, 1973; Wong & Shaw, 1974, 1976). Such investigations are important for the mapping of enzyme active sites and may contribute to a better understanding of enzyme selectivity.

The present investigation deals with the differentiation between trypsin (EC 3.4.21.4) and thrombin by use of Tos-Gph-OMe. The syntheses of *p*-guanidino-L-phenylalanine, an analogue of arginine, and some of its derivatives are described. It is shown that Tos-Gph-OMe is an excellent substrate of bovine trypsin, but a very poor substrate and powerful inhibitor of human thrombin, and a very poor substrate of bovine chymotrypsin (EC 3.4.21.1). A preliminary report of the interaction of Tos-Gph-OMe with trypsin, thrombin and chymotrypsin has appeared (Klausner & Rigbi, 1975).

The synthesis of *p*-guanidino-DL-phenylalanine has been described by Elliott & Harington (1947), and that of α -N-(*p*-toluenesulphonyl)-*p*-guanidino-L-phenylalanine-*p*-toluenesulphonate by Jameson *et al.* (1973).

Experimental

1-Guanyl-3,5-dimethylpyrazole nitrate was the product of Aldrich Chemical Co., Milwaukee, WI, U.S.A. Trypsin and α -chymotrypsin (both three times crystallized and freeze-dried) were purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. The chymotrypsin was treated with

Abbreviations used: Tos-Gph-OMe, α -N-(*p*-toluenesulphonyl)-*p*-guanidino-L-phenylalanine methyl ester; Tos, *p*-toluenesulphonyl; Gph, *p*-guanidino-L-phenylalanine; Bz, benzoyl; Har, homoarginine; H₄furan, tetrahydrofuran; k_{cat} , catalytic rate constant; Nap, *p*-nitroanilide; Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one; Sar, sarcosine.

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Tos-Lys-CH₂Cl (Shaw, 1967) to inactivate traces of trypsin that might be present. Bovine thrombin was the product of Upjohn, Kalamazoo, MI, U.S.A. Bovine fibrinogen (Fraction 1, 90% clottable) was the product of Research Products Division, Miles Laboratories, Kankakee, IL, U.S.A. Bacto Ac-globulin and Prothrombin 2-Stage Reagent were obtained from Difco Laboratories, Detroit, MI, U.S.A. Factor II- and Factor VII-deficient plasma (B2-7D) and Russell's-Viper Venom in cephalin (RVC-L) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and Bz-L-Phe-L-Val-L-Arg-Nap,HCl was from AB KABI Diagnostica, Stockholm, Sweden. All other chemicals were of analytical or reagent grade. Deionized distilled water was used throughout.

Thin-layer chromatography

Silica-gel plates (Kieselgel 60F₂₅₄, Merck, Darmstadt, West Germany; 0.25 mm thick) were used with butan-1-ol/acetic acid/water (4:1:1, by vol.) as developing system. Chromatograms were viewed and photographed in short-wave u.v. light.

t-Butoxycarbonyl-*p*-guanidino-L-phenylalanine (I)

t-Butoxycarbonyl-*p*-nitro-L-phenylalanine (Schwyzer & Caviezel, 1971; 3.10 g, 10 mmol) was dissolved in methanol (80 ml) containing acetic acid (0.6 ml). The mixture was cooled to 0–5°C and hydrogenated at atmospheric pressure over 10% Pd/C (500 mg) for 3 h. The catalyst was removed by filtration, the solvent removed *in vacuo*, and the residue dried in high vacuum over P₂O₅ and NaOH for 16 h (yield: quantitative). The product was then dissolved in H₄ furan (20 ml). *NN*-Di-isopropylethylamine (3.1 ml; 19 mmol) was added, followed by 1-guanyl-3,5-dimethylpyrazole (Habeeb, 1959; 3 g, 15 mmol). The reaction mixture was refluxed for 12 h. On the following day the solvent was removed *in vacuo*, and methanol (40 ml) and then acetic acid (2.5 ml) were added. After 4–8 h at –18°C the crystalline product was collected on a filter, washed with several portions of cold methanol and dried in air to give 2.6 g yield (81%); m.p. 198–200°C (uncorr.), $[\alpha]_D^{25} + 28^\circ$ ($c = 1$ in water). *t*-Butoxycarbonyl-*p*-guanidino-L-phenylalanine (I) has R_F 0.52 on t.l.c. The material is Sakaguchi-positive (violet colour) (Acher & Crocker, 1952), u.v.-positive and ninhydrin-negative. On heating (Wolman & Klausner, 1966) the spot becomes ninhydrin-positive. (Found C, 54.6; H, 7.2; N, 16.6; C₁₅H₂₂O₄N₄·½H₂O requires C, 54.5; H, 7.0; N, 16.9%).

α -*N*-(*p*-Toluenesulphonyl)-*p*-guanidino-L-phenylalanine (Tos-Gph, II)

Compound (I) (3 mmol, 993 mg) was dissolved in 5M-HCl (6 ml). After 5 min, acetic acid (15 ml) was

added, and the reaction mixture freeze-dried. The residue was dissolved in 1M-NaOH (6 ml). H₄furan (10 ml) was added, followed by tosyl chloride (660 mg) in H₄furan (10 ml) in five portions over 1 h. NaOH (1M) was used to maintain the reaction mixture at pH 9.0–9.5. After a further 2 h the pH was adjusted to 5–6 with acetic acid, and the organic solvent was removed *in vacuo*. The product was kept overnight at 0°C, filtered, washed with water, and dried over P₂O₅ to give 470 mg, m.p. 275–276°C (uncorr.); R_F 0.51 on t.l.c. The filtrate was concentrated *in vacuo* to a small volume, the water decanted and methanol (about 1 ml) added. Crystallization occurred at once. Water (5 ml) was added and after a few hours the product was filtered, washed with water, and dried over P₂O₅ (yield 270 mg, m.p. 274–275°C). Total yield was 740 mg (63%). A sample for analysis was thoroughly triturated with methanol, water was added, and the material was filtered and dried in a high vacuum for 4 h at 100°C. α -*N*-(*p*-Toluenesulphonyl)-*p*-guanidino-L-phenylalanine (II) has m.p. 275–276°C (uncorr.), $[\alpha]_D^{25} + 2^\circ$ ($c = 1$, in 80% acetic acid), λ_{max} in 0.04M-Tris/HCl buffer, pH 8.0, 230 nm (ϵ 21 580 litre·mol^{–1}·cm^{–1}). (Found: C, 53.7; H, 5.5; N, 14.3; S, 8.3; C₁₇H₂₀O₄N₄S, ½CH₃OH requires C, 53.6; H, 5.7; N, 14.3; S, 8.2%).

α -*N*-(*p*-Toluenesulphonyl)-*p*-guanidino-L-phenylalanine methyl ester hydrochloride (Tos-Gph-OMe, III)

Compound (II) (1 mmol, 392 mg) was suspended in methanol (15 ml). HCl gas was passed through the solution for 1 h and the mixture was kept at room temperature (25°C) overnight. Next day it was concentrated *in vacuo* and dry ether (50 ml) was added. After 6 h in the cold the ether was decanted and the residue was dried *in vacuo* over P₂O₅ and NaOH. The material was purified by chromatography on a column (4 cm × 90 cm) of Sephadex LH-20 with methanol as eluent (flow rate 20 ml/h), u.v. monitoring at 280 nm; 5 ml fractions were collected. Fractions 135–148 contained the desired material (as judged by t.l.c.). These were combined, the solvent was evaporated, and the residue dried *in vacuo* over P₂O₅ to give 290 mg (yield 68%) of amorphous material. α -*N*-(*p*-Toluenesulphonyl)-*p*-guanidino-L-phenylalanine methyl ester hydrochloride (III) has $[\alpha]_D^{25} + 35^\circ$ ($c = 1$ in methanol), R_F 0.60 on t.l.c.; λ_{max} in 0.04M-Tris/HCl buffer, pH 8.0, 230 nm (ϵ 22 500 litre·mol^{–1}·cm^{–1}). (Found: C, 50.8; H, 5.9; N, 12.8; C₁₈H₂₂O₄N₄S·HCl requires C, 50.5; H, 5.5; N, 13.1%).

p-Guanidino-L-phenylalanine (Gph, IV)

Compound (I) (200 mg) was added to 2 ml of trifluoroacetic acid/acetic acid/anisole (7:3:1, by vol.). After 25 min the acids were removed *in vacuo*. Dry ether (50 ml) was added and the precipitate was

filtered, washed with dry ether, and dried *in vacuo* over P_2O_5 and NaOH. The entire quantity (200 mg) was dissolved in acetic acid (5 ml). Picric acid (415 mg) was added, and the product separated within 1 h. It was filtered, washed with acetic acid and dry ether, and dried *in vacuo* over P_2O_5 and NaOH (yield 120 mg; 41%). *p*-Guanidino-L-phenylalanine (IV) has m.p. 141–143°C [softens at 135°C (uncorr.)]; $[\alpha]_D^{25} + 6^\circ$ ($c = 0.5$ in 50% acetic acid), pK_3' (guanidino), 11.79 ± 0.04 (0.2 M-KCl, 27°C), λ_{max} in 0.04 M-Tris/HCl buffer, pH 7.32, 233 nm (ϵ 6000 ± 100 litre·mol $^{-1}$ ·cm $^{-1}$). (Found: C, 41.7; H, 4.2; N, 19.7; C $_{10}H_{14}O_2N_4$ ·C $_6H_3O_7N_3$ · $\frac{1}{2}$ H $_2$ O, the monopicrate hemiacetate hemihydrate, requires C, 41.7; H, 4.1; N, 19.8%.)

T.l.c. showed that Tos-Gph-OMe is rapidly and completely cleaved by trypsin (Plate 1a, tracks 1 and 2). This probably indicates that no racemization took place during the synthetic steps.

Thrombin clotting activity

Clotting assays were carried out in 12 mm × 64 mm tubes placed on a base over individual magnetic stirrers in a thermostatically controlled water bath (ICN Paris-Labo, Vincennes, France), at 35°C. A 0.5% solution of bovine fibrinogen (0.2 ml) in 0.01 M-Tris/HCl/0.15 M-NaCl, pH 7.4 (Stenn & Blout, 1972), was stirred by means of a 0.5 mm × 4 mm iron stirring bar. A test solution of human thrombin (0.20 ml, 3–8 NIH units/ml), preincubated with or without Tos-Gph-OMe or Tos-Arg-OMe as inhibitor, was added to the fibrinogen, and clotting times were measured as the time it took for the rotary movement of the bar to be arrested. Clotting times are given as the mean of three determinations within the range 12–60 s (S.E.M. $\pm 4.7\%$). Clotting activity is expressed in NIH units as read from a linear log-log plot of clotting time versus concentration, of U.S. Standard thrombin (lot no. 8–3; kindly supplied by Dr. E. B. Seligmann, Jr., Division of Biologic Standards, NIH, Bethesda, MD, U.S.A.).

Spectrophotometric measurement of Tos-Gph-OMe hydrolysis

Fig. 1 shows the difference spectrum of Tos-Gph-OMe versus its tryptic hydrolysate in the 1.00 absorbance range as measured in the Cary 14 recording spectrophotometer. Like the spectra of Tos-Gph and Tos-Gph-OMe, the difference spectrum has a peak at 230 nm. $\Delta\epsilon$ for the hydrolysis is calculated to be -908 litre·mol $^{-1}$ ·cm $^{-1}$. Owing to the high molar absorbance of Tos-Gph-OMe and its hydrolysis product, spectrophotometric measurements are not feasible at concentrations much higher than 0.1 mM (1 cm light-path). The calculation of $\Delta\epsilon$ on the basis

Fig. 1. Difference spectrum of Tos-Gph-OMe versus Tos-Gph

The difference spectrum of Tos-Gph-OMe (0.167 mM) versus its tryptic hydrolysate (2.7 μ M trypsin) in 0.04 M-Tris/HCl buffer, pH 8.0, was determined with a Cary 14 recording spectrophotometer, in the absorbance range of 1.00. Tandem cells with 0.437 cm light-path for each compartment were used. The sample cell contained Tos-Gph-OMe in the first compartment and trypsin in the second. Tos-Gph-OMe and trypsin were mixed in the first compartment of the reference cell, and time was allowed for complete hydrolysis. The second compartment contained buffer.

of the low absorbance difference shown is thus subject to inaccuracy.

$\Delta\epsilon$ was therefore calculated from the total decrease in A_{230} by following the tryptic hydrolysis of different concentrations of Tos-Gph-OMe to completion in the Gilford 2000 recording spectrophotometer in the 0.150 absorbance range, in 0.04 M-Tris/HCl buffer at pH 8.0 and 25°C. Initial Tos-Gph-OMe concentrations ranged from 0.028 to 0.05 mM. The average of 11 such determinations gave $\Delta\epsilon = -920$ (S.E.M. ± 20) litre·mol $^{-1}$ ·cm $^{-1}$. This value is used subsequently.

Spectrophotometric measurements of enzyme activity

The rate of hydrolysis of substrate was measured with a Gilford 2000 recording spectrophotometer and 1 cm light-path cuvettes of 3 ml capacity. Reactions were carried out at 25°C, except when stated otherwise. Hydrolysis of Tos-Arg-OMe and Tos-Gph-OMe took place in 0.04 M-Tris/HCl/0.1 M-KCl/0.01 M-CaCl $_2$ buffer, pH 7.4–8.0. Specific thrombin esterase activity was routinely assayed at pH 8.0 with Tos-Arg-OMe as substrate (0.8 mM). As Tos-Gph-OMe was found to be a very poor thrombin substrate, it was also tested as an inhibitor. $\Delta\epsilon$ for hydrolysis of Tos-Arg-OMe was $+715$ litre·mol $^{-1}$ ·cm $^{-1}$ at 246 nm [we found it to be the same at pH 7.4 and 8.1 (cf. Hummel, 1959)]. For the value of $\Delta\epsilon$ for Tos-Gph-OMe hydrolysis, see the preceding section.

As may be inferred from Fig. 1, it is possible to follow the hydrolysis of Tos-Arg-OMe at 246 nm (Hummel, 1959) without interference from Tos-Gph-OMe hydrolysis taking place simultaneously in the

same cuvette. Stock solutions of Tos-Gph-OMe and Tos-Arg-OMe were 0.1M in methanol and were diluted in buffer before use. The concentration of methanol did not exceed 0.25% by vol. in any enzyme reaction.

Hydrolysis by human thrombin of the amide substrate Bz-Phe-Val-Arg-Nap (Svendsen *et al.*, 1972) was carried out at pH 8.0 in 0.08M-Tris/0.08M-imidazole/0.08M-(NaCl+HCl)/0.008M-CaCl₂ buffer. Stock buffer solution was prepared by adding 0.1M-Tris/0.1M-imidazole/0.1M-NaCl/0.01M-CaCl₂, until pH 8.0 was reached, to 0.1M-Tris/0.1M-imidazole/0.1M-HCl/0.01M-CaCl₂ (approx. 0.5vol. of the former was required). Reaction mixtures (2.0ml) contained 1.6ml of stock buffer. Reaction was begun by addition of enzyme (0.1ml) and was monitored at 405nm ($\Delta\epsilon = +10510 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) (data sheet: S2160, A chromogenic substrate for serine proteases; AB KABI Peptide Research, Box 156, S-431 22 Mölndal, Sweden). Hydrolysis of this substrate by bovine thrombin was carried out under somewhat different conditions (Table 4).

Active-site titration

Trypsin and thrombin were titrated with *p*-nitrophenyl-*p*'-guanidinobenzoate (Chase & Shaw, 1967, 1969), and chymotrypsin was titrated with *p*-nitrophenylacetate (Bender *et al.*, 1966).

Prothrombin assay

Prothrombin was assayed by the two-stage method of Ware & Seegers (1949), or with Factor II (prothrombin)- and Factor VII-deficient plasma as described by Hjort *et al.* (1955).

Human thrombin

Freshly frozen human plasma, cryoprecipitate removed, either acid citrate/dextrose plasma or citrate/dextrose/phosphate plasma, was kindly supplied by Dr. Shulamith Bar-Shany, Magen David Adom Central Blood Bank, Tel-Aviv. Human prothrombin was prepared from the plasma as described for bovine prothrombin by Stenn & Blout (1972). It was activated in 25% (w/v) sodium citrate, pH 7.8, until a high clotting activity was reached, between 50 and 90% of the initial prothrombin activity (Lanchantin *et al.*, 1965). The solution was then dialysed against water, and chromatographed on a column (0.9cm×13cm) of Bio-Rex 70 (Lanchantin *et al.*, 1967). Thrombin emerged as a single peak between 0.18M- and 0.5M-NaCl, in 0.1M-sodium acetate, pH 7.0, with more or less uniform specific clotting and esterase activity across it. Pooled fractions contained about 1000 NIH units/ml. Specific activities

ranged between 1100 and 1700 NIH units/mg, except for one lot with considerably higher clotting activity, and 42–71 nmol of Tos-Arg-OMe hydrolysed/s per mg of enzyme. The columns were made of acrylic plastic (Pharmacia, Uppsala, Sweden) and fractions were collected in polyethylene tubes. Combined fractions were stored at –20°C. The stability of different lots varied. Experiments were performed with a lot as long as it retained full or almost full activity (down to 85%), and generally within 1 week of preparation.

Enzyme concentrations

To calculate molarity, a conversion factor of $1A_{280} \text{ unit} = 0.65 \text{ mg/ml}$ and a mol.wt. of 24000 was used for bovine trypsin (Keil, 1971). The same factor, based on the average tryptophan and tyrosine contents of bovine thrombin and a mol.wt. of 40000, were used for human and bovine thrombin (Magnusson, 1971). Molarities are expressed on the basis of active-site titration only where stated specifically. Percentage of active enzyme and, for thrombin, specific clotting activity, are indicated in each experiment.

Calculations and statistical methods

To interpret our results best, it was necessary to analyse them statistically. Straight lines in Lineweaver–Burk plots were drawn on the basis of weighted least-squares, with weights inversely proportional to $1/[S]$. Correlation coefficients and confidence intervals for the intercept α on the $1/v$ axis and the slope β , were calculated by ordinary linear-regression equations. From these, confidence intervals for the parameters $K_m (= \beta_0/\alpha_0)$, $K_{m(\text{app.})} (= \beta_i/\alpha_i)$ and $K_i [= \beta_0/[I]/(\beta_i - \beta_0)]$ were calculated, where the subscripts 0 and *i* refer to slopes or intercepts in the absence of inhibitor and presence of a fixed concentration of inhibitor respectively. The method used for the construction of the confidence intervals of these parameters was that for ratios by Roy & Potthoff (1958). Let *a* and *b* denote regression estimators of α and β respectively. For K_m , for example, the construction of the confidence interval is based on the fact that, from the usual regression assumptions, $(b - K_m a)/S_{b-K_m a}$ has a Student's *t* distribution, where $S_{b-K_m a}$ is an appropriate estimator of the standard deviation of $b - K_m a$ obtained from the regression. This estimator contains a quadratic expression K_m , which, on being solved, results in asymmetric confidence intervals about b/a . Confidence intervals are also asymmetric about $k_{\text{cat.}}$, as its calculation is based on the reciprocal of *a*. Confidence intervals for $K_{m(\text{app.})}$ and K_i are similarly calculated: 95% confidence intervals are used throughout and are given in parentheses immediately after the parameter values.

EXPLANATION OF PLATE I

Thin-layer chromatograms of Tos-Gph-OMe as a substrate of bovine trypsin, human thrombin and bovine chymotrypsin
 Tos-Gph-OMe (2.5 mM) was incubated with enzyme for the indicated time, at 25°C, in 0.04M-Tris/HCl/0.1 M-KCl/0.01 M-CaCl₂ buffer, pH 7.4. In each chromatogram, the Tos-Gph marker is at the extreme left and the Tos-Gph-OMe marker at the extreme right. Concentrations, based on active-site titrations were: trypsin or thrombin, 1 and 2, 0.086 μ M and 0.172 μ M respectively; chymotrypsin, 0.172 μ M. (a) 1, 2, trypsin, 5 min; (b) 1, 2, thrombin; 3, chymotrypsin, 4 h; (c) 1, 2, thrombin, 29 h; 3, chymotrypsin, 27 h; (d) 1, 2, thrombin; 3, chymotrypsin, 146 h. O and SF, origin and solvent front respectively. The Tos-Gph-OMe marker was incubated with buffer concurrently with Tos-Gph-OMe enzyme mixtures. Specific clotting activity of thrombin, 1450 NIH units/mg.

Results

Thin-layer chromatography

T.l.c. was used for a preliminary examination of Tos-Gph-OMe as a trypsin, thrombin and chymotrypsin substrate. Plate 1 shows that, given conditions in which trypsin-catalysed hydrolysis of Tos-Gph-OMe was complete in 5 min, thrombin-catalysed hydrolysis of the ester was only partly completed in 29 h. Chymotrypsin-catalysed hydrolysis of the ester was still slower. Hydrolysis catalysed by thrombin was complete in about 47 h (results not shown). Tos-Gph-OMe is therefore an excellent substrate for trypsin and a very poor one for thrombin and chymotrypsin. As shown below, it is a potent thrombin inhibitor.

Action of bovine trypsin on Tos-Gph-OMe

The action of trypsin on Tos-Gph-OMe follows Michaelis-Menten kinetics. The Lineweaver-Burk

Fig. 2. Lineweaver-Burk plot for the action of bovine trypsin on Tos-Gph-OMe

Trypsin (80% active) was present at a concentration of 2.05 nM. Points are results of separate determinations: $\odot$, two identical points. The buffer was 0.04 M-Tris/HCl/0.1 M-KCl/0.001 M-CaCl₂, pH 7.4, 25°C.

plot for pH 7.4 is shown in Fig. 2. Kinetic parameters for trypsin with Tos-Gph-OMe and, for comparison, the currently considered trypsin substrate of choice, Tos-Arg-OMe, are presented in Table 1. When the percentage of active enzyme is taken into account, together with the increased activity in the presence of Ca²⁺ ions (36% increase; Trowbridge *et al.*, 1963), it can be calculated that $k_{cat.}$ for trypsin with Tos-Gph-OMe is the same at pH 7.4 and 8.0. Though K_m for trypsin with Tos-Gph-OMe is roughly of the same order of magnitude as with Tos-Arg-OMe, within a similar concentration range Tos-Gph-OMe is hydrolysed about twice as fast. Higher values of $k_{cat.}$ often given in the literature for trypsin with Tos-Arg-OMe are obtained at higher substrate concentrations and are due to substrate activation (Trowbridge *et al.*, 1963).

Tos-Gph-OMe as a thrombin substrate

In spite of the extremely slow action of human thrombin on Tos-Gph-OMe, and the fact that the concentration of thrombin solutions as they emerged from the Bio-Rex 70 columns was of the order of 1 μ M only, an attempt was made to measure the kinetic parameters of thrombin with respect to Tos-Gph-OMe with some degree of accuracy. The highest concentration of thrombin obtained, 1.54 μ M, was used. Because of the extremely slow reaction rates, the variance of replicate measurement was very high. Nevertheless a correlation coefficient of 1/[S] with respect to 1/v of 0.67 was obtained. K_m was calculated to be 190 μ M, with a lower limit of 60 μ M, and $k_{cat.}$ was 0.2 s⁻¹ with a lower limit of 0.1 s⁻¹. The variance of replicate measurements was so high that the upper limit for either K_m or $k_{cat.}$ was infinity, which is absurd. The ratio $k_{cat.}/K_m$ characterizes Tos-Gph-OMe as an exceedingly poor substrate of human thrombin, with defined lower and upper confidence limits (Table 3). The $k_{cat.}$ values for bovine

Table 1. Kinetic parameters for the action of bovine trypsin on Tos-Gph-OMe and Tos-Arg-OMe

Trypsin solutions (40 μ M in 0.001 M-HCl) were diluted before use (1/200 or more) in 0.04 M-Tris/HCl/0.1 M-KCl/0.01 M-CaCl₂ at the stated pH. Trypsin (0.1 ml) and substrate (1.9 ml) in the same buffer solution were rapidly mixed in the cuvette, and the rate of change of A_{246} at 25°C, between 0.002 and 0.4/min, was measured.

Substrate	pH	Active enzyme (%)	$10^4 \times$ Substrate concentration range (M)	$k_{cat.}$ (s ⁻¹)	$10^5 \times K_m$ (M)	$10^{-4} \times k_{cat.}/K_m$ (s ⁻¹ ·M ⁻¹)	Reference
Tos-Gph-OMe	7.4	80	0.533–1.080	320 (316–324)	5.7 (5.5–5.9)	561.4 (547.5–567.6)	Fig. 2
	8.0*	54	0.14–0.95	161 (150–175)	11.8 (10.8–13.1)	136.4 (130.6–143.7)	Present work
Tos-Arg-OMe	8.0	~61	0.025–0.56	60	1.25	480.0	Trowbridge <i>et al.</i> (1963)†
	8.0	40	0.52–4.32	73 (72–75)	7.0 (6.4–7.7)	104.3 (100.1–110.9)	Present work

* Determinations carried out in the absence of CaCl₂.

† Determination carried out in a pH-stat in 0.2 M-KCl/0.05 M-CaCl₂, at 25°C and in the absence of buffer.

trypsin and human thrombin are consistent with the picture obtained from t.l.c.

Inhibition of human thrombin by Tos-Gph-OMe

As Tos-Gph-OMe is only very slowly hydrolysed by thrombin, its effect as an inhibitor was studied. Fig. 3 shows the inhibition of thrombin clotting activity by Tos-Gph-OMe, compared with that by the substrate Tos-Arg-OMe. Tos-Gph-OMe is the much stronger inhibitor. Preincubation of thrombin with Tos-Gph-OMe up to 10 min did not affect clotting time.

The order of addition of components and the time of addition of inhibitor have a pronounced effect on

Fig. 3. Inhibition of human thrombin clotting activity by Tos-Gph-OMe and Tos-Arg-OMe

Tos-Gph-OMe or Tos-Arg-OMe in dilute buffer and at 35°C (0.2 ml) was mixed to give the indicated ester concentrations, with 0.2 ml of thrombin (clotting specific activity 3900 NIH units/mg; 45% active), and the mixture was assayed immediately for clotting activity. Conditions in mixture: thrombin, 5.5 NIH units/ml, 35.2 nM; buffer, 0.008 M-Tris/HCl/0.02 M-KCl/0.002 M-CaCl₂, pH 7.4, 2% methanol (by vol.). Points are means of triplicate determinations (s.e.m. $\pm$ 4.7%). ▲, Tos-Arg-OMe; ●, Tos-Gph-OMe.

clotting time. When the addition procedure described in Fig. 3 is altered, and Tos-Gph-OMe or Tos-Arg-OMe is added first to fibrinogen, and thrombin next, clotting times are still longer (Table 2). Here again Tos-Gph-OMe exerts a stronger effect. Under these conditions one would expect fibrinogen to compete with inhibitor for enzyme more effectively, and clotting time to be shorter than when inhibitor is added to thrombin first. The observed effect is probably due to a direct action of the esters on fibrinogen. It was also found that when Tos-Gph-OMe is added to fibrinogen immediately after thrombin, the inhibition is less pronounced than when a mixture of Tos-Gph-OMe and thrombin is added. Evidently the initial cleavages by thrombin are over very rapidly, and subsequent inhibition of the enzyme does not affect clotting. If Tos-Gph-OMe is added just before the expected time of clot formation, no inhibition is observed.

The action of thrombin on the specific chromogenic amide substrate, Bz-Phe-Val-Arg-Nap, and its inhibition by Tos-Gph-OMe is shown in Fig. 4. The non-linear part of the graphs at high substrate concentrations (Fig. 4a) indicates substrate inhibition. The effect is much stronger than that reported for bovine thrombin with this substrate (Svendsen *et al.*, 1972), which we have confirmed (see also under 'Bovine thrombin'). Substrate inhibition indicates the presence of an additional substrate-binding site. Tos-Gph-OMe does not diminish, and even enhances, substrate inhibition. This suggests that the ester may bind to thrombin at yet another separate site.

In Fig. 4(b) substrate concentrations corresponding only to the linear part of the graphs of Fig. 4(a) were used. For each straight line at inhibitor concentrations of 8.9 and 17.8 μ M a pattern for mixed inhibition is obtained. However, in linear mixed inhibition all Lineweaver-Burk plots should meet at a single point on the left side of the $1/v$ axis and above the $1/[S]$ axis (Webb, 1963). It is not clear at present whether all three lines may be constrained to do so, within the

Table 2. Some effects of the addition procedure of Tos-Gph-OMe and Tos-Arg-OMe on the clotting activity of human thrombin. Thrombin (8 NIH units/ml) and ester (10 or 1 mM) were in 0.15 M-NaCl, pH 7.4. Ester was mixed with either thrombin or fibrinogen in the ratio 1:20 (v/v), then 210 μ l of the mixture was mixed with the remaining component (200 μ l) and clotting time was measured. In controls, 0.15 M-NaCl at pH 7.4 was added in the same way. Components in parentheses indicate mixtures.

Addition procedure	$10^5 \times$ Ester concn. (M) in clotting mixture	Clotting time (s)	Clotting activity (% of control)
Thrombin to fibrinogen (control)		13	100
(Thrombin+Tos-Gph-OMe) to fibrinogen	2.44	49	16.3
Thrombin to (fibrinogen+Tos-Gph-OMe)	2.44	59	12.5
(Thrombin+Tos-Gph-OMe) to fibrinogen	24.4	136	*
Thrombin to (fibrinogen+Tos-Gph-OMe)	24.4	210	*
(Thrombin+Tos-Arg-OMe) to fibrinogen	24.4	30	31.3
Thrombin to (fibrinogen+Tos-Arg-OMe)	24.4	55	13.8

* Clotting time outside 13–60 s range. Clotting activity considerably less than 12%.

Fig. 4. Lineweaver-Burk plots for the action of human thrombin on Bz-Phe-Val-Arg-Nap and its inhibition by Tos-Gph-OMe

(a) Thrombin, 45% active, clotting specific activity 3900 NIH units/mg, 3.52 nM, 0.55 NIH unit/ml: (1) No inhibitor; symbols ●, ○, ⊙, respectively represent single determination, two identical and three identical determinations; (2) □, Tos-Gph-OMe, 8.35 μM. (b) Thrombin, 44% active, clotting specific activity 1100 NIH units/mg, 18.1 nM, 0.8 NIH unit/ml: (1) ○, no inhibitor (for each concn., two identical points); (2) ▲, and (3) □, Tos-Gph-OMe 8.9 μM and 17.8 μM respectively (□, two identical points). Buffer was 0.08 M - Tris/0.08 M - imidazole/0.08 M - (NaCl+HCl)/0.008 M - CaCl₂, pH 8.0. Each determination is shown separately. Vertical bars indicate confidence limits for 1/v intercept of line (2); horizontal bars indicate confidence limits for the 1/[S] intercepts of lines as numbered. Dashed lines (2) are the lines of least squares constrained to pass through the intercept of line (1).

95% confidence limits of any given parameter. It should be mentioned that line 2 (Tos-Gph-OMe, 8.9 μM) could also be constrained to pass through the 1/v intercept of line 1 (no inhibitor), and inhibition would appear to be competitive. On the other hand, line 3 (Tos-Gph-OMe, 17.8 μM) could similarly be constrained to pass through the 1/[S] intercept of line 1, and inhibition would appear to be non-competitive. The confidence intervals for the two K_i values obtained from Fig. 4(b) do not overlap (Table 3 and Fig. 6), and K_i is higher at the higher inhibitor concentration. It may be that the concentration of Tos-Gph-OMe has some effect on the properties of the enzyme. Kinetic parameters obtained from the

Fig. 5. Lineweaver-Burk plots for the inhibition of human thrombin esterase activity by Tos-Gph-OMe

Substrate was Tos-Arg-OMe. Concentration of thrombin in the reaction mixtures was 0.122 μM. They contained 7 NIH units/ml. Thrombin specific clotting activity was 1450 NIH units/mg. (1) ●, No inhibitor; (2) ○, and (3) ▲, presence of 2.92 μM- and 8.76 μM-Tos-Gph-OMe respectively. Points are means of duplicate or triplicate measurements. Vertical bars indicate confidence limits of 1/v intercepts of lines as numbered. Buffer was 0.04 M-Tris/HCl/0.1 M-KCl/0.001 M-CaCl₂, pH 8.0, 25°C.

data of Figs. 4(a) and 4(b) are presented in Table 3. The difference between the two determinations of k_{cat} for the action of thrombin on the amide substrate Bz-Phe-Val-Arg-Nap, 2.9 and 14.9 s⁻¹, may be related to the specific clotting activity of the enzyme. Esterase activity, on the other hand, is not necessarily related to clotting activity (Lanchantin *et al.*, 1967; Glover *et al.*, 1973).

The inhibition of thrombin esterase activity by Tos-Gph-OMe at pH 8.0, with Tos-Arg-OMe as substrate, is shown in Fig. 5. No difference in inhibition was observed whether Tos-Gph-OMe was added to substrate and enzyme was added last, or whether Tos-Gph-OMe was preincubated with the enzyme and the mixture was added to substrate. Within their confidence limits, all three lines can be constrained to pass through a single intercept, and inhibition would appear to be competitive. Kinetic parameters for the action of human thrombin on Tos-Arg-OMe and its inhibition by Tos-Gph-OMe are presented in Table 3. K_i values at the two inhibitor concentrations hardly overlap (see also Fig. 6), and K_i is higher at the higher concentration. Similar experiments at higher and lower inhibitor concentrations (graphs not shown) gave a similar

Table 3. Kinetic parameters for the action of human thrombin on some substrates and for its inhibition by Tos-Gph-OMe

Thrombin from the peak emerging from the Bio-Rex 70 column was used. Hydrolysis of Tos-Arg-OMe and Tos-Gph-OMe was carried out in 0.04M-Tris/HCl/0.1M-KCl/0.01M-CaCl₂ buffer at the stated pH and 25°C; that of Bz-Phe-Val-Arg-Nap was in 0.08M-Tris/0.08M-imidazole/0.08M-(NaCl+HCl)/0.008M-CaCl₂ at pH 8.0 and 25°C. The reaction was followed with the Gilford 2000 recording spectrophotometer. Thrombin concentrations were adjusted to give a 0.002-0.04 absorbance change/min. For Tos-Arg-OMe, $\Delta\epsilon_{246} = +715$ (Hummel, 1959); for Tos-Gph-OMe, $\Delta\epsilon_{230} = -920$; for Bz-Phe-Val-Arg-Nap, $\Delta\epsilon_{405} = 10510$. Reactions were begun by adding 0.1 ml of thrombin to 1.9 ml of substrate in the absence or presence of inhibitor. See also the Experimental section.

Substrate	pH	Specific clotting activity (units/mg)	Active enzyme (%)	k_{cat} (s ⁻¹)	$10^5 \times K_m$ (*) or $10^5 \times K_{m(app.)}$ (†) (M)	$10^{-4} \times k_{cat}/K_m$ (s ⁻¹ ·M ⁻¹)	Tos-Gph-OMe as inhibitor		Reference
							$10^6 \times$ concn. (M)	$10^6 \times K_i$ (M)	
Tos-Arg-OMe	8.0	1450	Not determined	1.8 (1.7-1.9)	4.4 (3.5-5.6)*	4.09 (4.05-4.20)	0	—	Text
	8.0	1450	Not determined	—	10.2 (8.8-12.0)†	—	0.8	0.8 (0.6-1.2)	Text
	8.0	1450	Not determined	—	14.3 (12.1-17.4)†	—	1.6	1.3 (0.9-1.8)	Text
	8.0	1450	Not determined	2.6 (2.5-2.7)	11.1 (10.0-12.3)*	2.34 (2.22-2.50)	0	—	Fig. 5
	8.0	1450	Not determined	—	21.0 (17.3-26.1)†	—	2.92	4.5 (3.6-5.9)	Fig. 5
	8.0	1450	Not determined	—	31.8 (26.3-39.8)†	—	8.76	6.2 (5.5-7.0)	Fig. 5
	7.4	1350	32	3.2 (3.0-3.3)	8.2 (7.3-9.2)*	3.90 (3.62-4.13)	0	—	Text
	7.4	1350	32	—	45.7 (35.2-63.5)†	—	25.0	7.7 (7.1-8.4)	Text
Tos-Gph-OMe	8.0	1700	30	0.2 (lower limit 0.1)	19.0 (lower limit 6.0)	0.11 (0.08-0.16)	—	—	Text
Bz-Phe-Val-Arg-Nap	8.0	3900	45	14.9 (12.4-18.7)	10.7 (9.3-12.6)*	13.93 (12.54-15.49)	0	—	Fig. 4(a)
	8.0	3900	45	—	26.0 (16.8-53.8)†	—	8.35	3.9 (3.1-5.1)	Fig. 4(a)
	8.0	1100	44	2.9 (2.8-3.0)	5.5 (5.2-5.9)*	5.27 (5.11-5.40)	0	—	Fig. 4(b)
	8.0	1100	44	—	14.5 (8.0-38.1)†	—	8.9	3.8 (3.1-4.8)	Fig. 4(b)
	8.0	1100	44	—	6.4 (4.5-9.3)†	—	17.8	6.5 (5.5-7.9)	Fig. 4(b)

Fig. 6. Inhibition of human thrombin by Tos-Gph-OMe: dependence of K_i on inhibitor concentration

Each symbol represents determinations within one experiment. For Tos-Arg-OMe as substrate points $\blacksquare$ (Fig. 7) and ∇ (Lineweaver-Burk graph not shown), were obtained in 0.04M-Tris/HCl/0.1M-KCl/0.01M-CaCl₂ buffer, pH8.0; points $\bullet$ (Lineweaver-Burk graph not shown), were obtained in buffer of the same components and concentrations, but at pH7.4. For Bz-Phe-Val-Arg-Nap as substrate points $\circ$ (Fig. 4a and b) were obtained in 0.08M-Tris/0.08M-imidazole/0.08M-(NaCl+HCl)/0.008M-CaCl₂ buffer, pH8.0: 95% confidence intervals are shown. See also the Experimental section and Table 3.

inhibition pattern, with similar K_m and $k_{cat.}$ values, but over the entire range ($[I] = 0.8\text{--}50.0\mu\text{M}$), K_i increases with inhibitor concentration. It should be noted that within each experiment, for a given concentration of Tos-Gph-OMe and apart from cases of substrate inhibition, double-reciprocal plots are linear. The calculation of K_i is therefore valid. The dependence of K_i on Tos-Gph-OMe concentration, with Tos-Arg-OMe or Bz-Phe-Val-Arg-Nap as substrates, is shown in Fig. 6.

There is an apparent inconsistency between mixed-type inhibition of thrombin by Tos-Gph-OMe when Bz-Phe-Val-Arg-Nap is the substrate and the apparently competitive inhibition when Tos-Arg-OMe is the substrate. Closer examination of the inhibition of esterase activity shows that it too is of the mixed type. In competitive inhibition $K_{m(app.)}$ is a linear function of inhibitor concentration. In mixed-type inhibition $K_{m(app.)}$ is a hyperbolic function of inhibitor concentration (Cornish-Bowden, 1976). The hyperbolic dependence of $K_{m(app.)}$ on Tos-Gph-OMe concentration is shown in Fig. 7. The inhibition of thrombin esterase activity, as of amidase activity, is therefore of the mixed type.

Effect of Tos-Gph on human thrombin

Some difficulty was encountered in attempting to study the effect on thrombin of the hydrolysis product, Tos-Gph. This product is insoluble in water and organic solvents, but on enzymic hydrolysis of

Fig. 7. Action of human thrombin on Tos-Arg-OMe: Dependence of $K_{m(app.)}$ on inhibitor concentration

Symbols and conditions as in Fig. 6: 95% confidence intervals are shown. The curve originates from the point that is the average of three determinations of K_m (obtained in the absence of inhibitor).

Tos-Gph-OMe, solutions remain clear. On prolonged incubation of Tos-Gph-OMe with thrombin until hydrolysis is complete, some clotting activity is regained (approx. 30% in 50h, as compared with that of a thrombin control). It appears therefore that Tos-Gph inhibits thrombin, but less strongly than does Tos-Gph-OMe. The free amino acid, *p*-guanidino-L-phenylalanine, inhibits neither the esterase nor the clotting activity of thrombin.

Bovine thrombin

Tos-Gph-OMe and Tos-Arg-OMe inhibit the clotting activity of bovine thrombin in essentially the same way as they inhibit human thrombin (cf. Fig. 3). We did not continue the study of bovine thrombin inhibition by Tos-Gph-OMe any further. However, we determined K_m and the activity of bovine thrombin towards Tos-Arg-OMe and Bz-Phe-Val-Arg-Nap (Table 4) to facilitate comparison with our data for human thrombin and with the data of other workers for bovine and human thrombin. In general, our K_m values are of the same order of magnitude as those of other workers. However, activity data vary considerably (Curragh & Elmore, 1964; Sherry *et al.*, 1965; Kézdy *et al.*, 1965; Seegers *et al.*, 1968; Svendsen *et al.*, 1972). As already mentioned under 'Inhibition of human thrombin by Tos-Gph-OMe, substrate inhibition by the amide was much less pronounced than with the human enzyme.

When differences in experimental conditions are taken into account, it is seen that our kinetic parameters for both human and bovine thrombin with Tos-Arg-OMe and Bz-Phe-Val-Arg-Nap respectively (Tables 3 and 4) are not very different especially when differences in enzyme activity and experimental conditions are considered. For comparison, it may also be

Table 4. *Kinetic parameters for the action of bovine thrombin on Tos-Arg-OMe and Bz-Phe-Val-Arg-Nap*

Bovine thrombin (Upjohn) was dissolved in water to give the required clotting activity. Hydrolysis of Tos-Arg-OMe was carried out in 0.04M-Tris/HCl/0.1M NaCl buffer at pH8.4 and 25°C, and that of Bz-Phe-Val-Arg-Nap in 0.08M-Tris/0.08M-imidazole/0.07M-(NaCl+HCl) buffer at pH8.4 and 40°C. CaCl₂ was not included in the reaction mixtures. Reactions were begun and monitored as described in Table 3. Because of the low specific clotting activity of the thrombin preparation (14 NIH units/A₂₈₀ unit), enzyme concentration for the calculation of k_{cat} is based on active-site titration, and not on the conversion of A₂₈₀ into molarity.

Substrate	pH	Clotting activity (NIH units/ml)	k_{cat} , (s ⁻¹)	10 ⁵ × K_m (M)	10 ⁻⁴ × k_{cat}/K_m (s ⁻¹ · M ⁻¹)	10 ¹⁰ × Substrate hydrolysed (mol/s per NIH unit)
Tos-Arg-OMe	8.4	6	9.8 (8.6–11)	17.3 (13.5–22.6)	5.66 (5.16–6.35)	1.1
Bz-Phe-Val-Arg-Nap	8.4	0.4	56 (54–58)	15.6 (14.3–17.2)	35.90 (35.09–36.06)	3.9

calculated from the data in Figs. 6 and 7 that the rate of hydrolysis of these substrates by human thrombin is approx. 0.03 and 0.07–0.1 nmol/s per NIH unit respectively. In view of the fact that Tos-Gph-OMe inhibits bovine thrombin clotting activity, and in view of the similarity of the enzyme from bovine and human sources, it may be presumed that Tos-Gph-OMe would also strongly inhibit amidase and esterase activity of bovine thrombin.

Discussion

In the design of synthetic enzyme inhibitors, two requirements should be met: strong binding and poor or arrested catalysis, or none. For trypsin and trypsin-like enzymes, this means a 0.5 nm-long hydrophobic group bearing a positive charge at its end for binding (Mares-Guia & Shaw, 1965), and, if present, a bond that is cleaved very slowly or not at all. Such a bond has the advantage that it is possible to build groups that interact with enzyme on each of its sides.

When arginine in Tos-Arg-OMe is replaced by homoarginine, a thrombin inhibitor is obtained which is not perceptibly hydrolysed by the enzyme (Curragh & Elmore, 1964). In the course of our investigations we observed that Tos-Har-OMe inhibits thrombin clotting activity just about as strongly as Tos-Arg-OMe (cf. Fig. 3) and that homoarginine oligomers, unlike their behaviour with trypsin (Rigbi *et al.*, 1974), are neither substrates nor inhibitors of thrombin (M. Right, unpublished work). Thus the homoarginine residue does not appear to make for powerful thrombin inhibition.

Weinstein & Doolittle (1972) prepared compounds containing the non-cleavable arginine-sarcosine bond, which resembles the four cleavable arginine-glycine bonds of fibrinogen (Magnusson, 1971), and found that Tos-Arg-Sar inhibited thrombin clotting activity, though less than Tos-Arg-OMe.

The *p*-guanidino-L-phenylalanine residue seems to meet the requirements for thrombin inhibition better than the homoarginine residue or the arginine-sarcosine bond. K_m values for Tos-Gph-OMe and

Tos-Arg-OMe are only slightly different (Table 3). k_{cat} , however, is lower for Tos-Gph-OMe than for Tos-Arg-OMe by an order of magnitude. In contrast with thrombin, trypsin hydrolyses Tos-Gph-OMe about twice as fast as Tos-Arg-OMe, whereas, again, K_m for both substrates is about the same (Table 1). Thus for trypsin, Tos-Gph-OMe is an excellent substrate, whereas for thrombin it is a very poor one and a potent inhibitor of clotting, amidase and esterase activity. The differences in the catalytic rate constants of thrombin and trypsin, for Tos-Gph-OMe compared with Tos-Arg-OMe, bring out the importance of the P₁ position in determining the selectivity for a substrate or quasi-substrate among trypsin-like enzymes (Chase & Shaw, 1969; Glover *et al.*, 1973; Wong & Shaw, 1974, 1976).

The finding that the inhibition constant K_i is lower than K_m by about two orders of magnitude, and increases with inhibitor concentration (Table 3 and Fig. 6) calls for an explanation. Apparently, besides binding at the catalytic site, with a K_m of about 0.1 mM, the enzyme binds inhibitor more strongly at multiple additional sites. Hence K_i at low inhibitor concentrations is considerably smaller than K_m . As the inhibitor concentration increases, binding sites become saturated in the order of increasing K_i , which is the measured K_i at that concentration. The hyperbolic variation of K_i with inhibitor concentration suggests saturation.

It should be possible to substitute Gph for Arg in peptides resembling the *N*-terminal part of fibrinogen chains. Presumably the Gph side chain would bind to thrombin, but the peptide bond on its carbonyl side would not be hydrolysed by the enzyme. This and the presence of specific side chains on either side of the susceptible bond should make for even stronger inhibition than by Tos-Gph-OMe. In this connexion the work of Lobo *et al.* (1976) on peptide substrates of thrombin and trypsin is of particular interest.

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