Kinetics and Mechanism of Catalysis by Proteolytic Enzymes

A COMPARISON OF THE KINETICS OF HYDROLYSIS OF SYNTHETIC SUBSTRATES BY BOVINE α - AND β -TRYPSIN

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Several esters of the α -*N*-toluene-*p*-sulphonyl and α -*N*-benzoyl derivatives of *S*-(3-amino-propyl)-L-cysteine and the methyl ester of *S*-(4-aminobutyl)-*N*-toluene-*p*-sulphonyl-L-cysteine were synthesized. The kinetics of hydrolysis of these and esters of the α -*N*-toluene-*p*-sulphonyl and α -*N*-benzoyl derivatives of L-arginine, L-lysine, *S*-(2-aminoethyl)-L-cysteine and esters of γ -guanidino-L- α -toluene-*p*-sulphonamidobutyric acid and α -*N*-toluene-*p*-sulphonyl-L-homoarginine by α - and β -trypsin were compared. On the basis of values of the specificity constants ($k_{\text{cat.}}/K_m$), the two enzymes display similar catalytic efficiency towards some substrates. In other cases α -trypsin is less efficient than β -trypsin. It is possible that α -trypsin possesses greater molecular flexibility than β -trypsin.

Schroeder & Shaw (1968) separated bovine trypsin into two active components, α - and β -trypsin, by ion-exchange chromatography. They showed that β -trypsin consists of a single polypeptide chain, whereas α -trypsin contains two chains as a result of the cleavage of the Lys(131)-Ser(132) peptide bond. [This is the Lys(145)-Ser(146) bond by the numbering scheme

proposed by Hartley *et al.* (1965).] The present work was carried out to determine the effect of this structural difference on the kinetics and specificity of α -trypsin compared with β -trypsin. Ester substrates with a variety of structures (Ia, Ib, IIa, IIb, IIIa, IIIb) were used. *S*-(2-Aminoethyl)-L-cysteine is an isostere of L-lysine and it has been shown that esters of

α -N-acyl derivatives of *S*-(2-aminoethyl)-L-cysteine are good substrates for trypsin (Elmore *et al.*, 1967). Substrates with side chains longer than that of lysine were obtained by converting L-cysteine into other *S*-(ω -aminoalkyl)-L-cysteine derivatives. Derivatives of L-homoarginine and γ -guanidino-L- α -aminobutyric acid provided substrates with side chains that are respectively longer and shorter than that of L-arginine. Attempts to synthesize esters of *S*-(ω -guanidinoalkyl)-*N*-toluene-*p*-sulphonyl-L-cysteines (compounds IV) were unsuccessful, although several potential intermediates were obtained.

Experimental

Materials

Trypsin, twice-crystallized, salt-free (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), was separated into two active forms, α - and β -trypsin, by the method of Schroeder & Shaw (1968). Other samples of α - and β -trypsin were kindly given by Dr. E. Shaw. Pyridine was purified by heating with KMnO_4 under reflux followed by distillation over P_2O_5 . *N*-Toluene-*p*-sulphonyl-L-cysteine was prepared by the method of Dadić *et al.* (1961). α -*N*-Toluene-*p*-sulphonyl-L-lysine methyl ester hydrochloride (Ia; $\text{R} = \text{CH}_3$, $\text{X}^- = \text{Cl}^-$) was prepared by the method of Barrass & Elmore (1957). α -*N*-Toluene-*p*-sulphonyl-L-lysine 2-propyl ester hydrochloride [Ia; $\text{R} = (\text{CH}_3)_2\text{CH}$, $\text{X}^- = \text{Cl}^-$], α -*N*-benzoyl-L-lysine methyl ester toluene-*p*-sulphonate (Ib; $\text{R} = \text{CH}_3$, $\text{X}^- = p\text{-CH}_3\text{-C}_6\text{H}_4\text{-SO}_3^-$), α -*N*-benzoyl-L-lysine cyclohexyl ester toluene-*p*-sulphonate (Ib; $\text{R} = \text{C}_6\text{H}_{11}$, $\text{X}^- = p\text{-CH}_3\text{-C}_6\text{H}_4\text{-SO}_3^-$), *S*-(2-aminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine methyl ester hydrochloride (IIa; $n = 2$, $\text{R} = \text{CH}_3$, $\text{X}^- = \text{Cl}^-$), *S*-(2-aminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine *n*-propyl ester hydrochloride (IIa; $n = 2$, $\text{R} = \text{CH}_3\text{-CH}_2\text{-CH}_2$, $\text{X}^- = \text{Cl}^-$), *S*-(2-aminoethyl)-*N*-benzoyl-L-cysteine methyl ester hydrochloride (IIb; $n = 2$, $\text{R} = \text{CH}_3$, $\text{X}^- = \text{Cl}^-$) and *S*-(2-aminoethyl)-*N*-benzoyl-L-cysteine cyclohexyl ester toluene-*p*-sulphonate (IIb; $n = 2$, $\text{R} = \text{C}_6\text{H}_{11}$, $\text{X}^- = p\text{-CH}_3\text{-C}_6\text{H}_4\text{-SO}_3^-$) were prepared by the method of Elmore *et al.* (1967). *S*-(3-Aminopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine hydrochloride (IIa; $n = 3$, $\text{R} = \text{H}$, $\text{X}^- = \text{Cl}^-$) was synthesized by the method of Jameson *et al.* (1973). α -*N*-Toluene-*p*-sulphonyl-L-arginine methyl ester hydrochloride (IIIa; $n = 3$, $\text{R} = \text{CH}_3$, $\text{X}^- = \text{Cl}^-$) was prepared by the method of Bergmann *et al.* (1939). α -*N*-Benzoyl-L-arginine cyclohexyl ester hydrochloride (IIIb; $n = 3$, $\text{R} = \text{C}_6\text{H}_{11}$, $\text{X}^- = \text{Cl}^-$) was prepared by the method of Curragh & Elmore (1964); the corresponding methyl ester hydrochloride was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. α -*N*-Toluene-*p*-sulphonyl-L-homoarginine methyl ester hydrochloride (IIIa;

$n = 4$, $\text{R} = \text{CH}_3$, $\text{X}^- = \text{Cl}^-$), α -*N*-toluene-*p*-sulphonyl-L-homoarginine cyclohexyl ester hydrochloride (IIIa; $n = 4$, $\text{R} = \text{C}_6\text{H}_{11}$, $\text{X}^- = \text{Cl}^-$) and α -*N*-toluene-*p*-sulphonyl-L-arginine cyclohexyl ester hydrochloride (IIIa; $n = 3$, $\text{R} = \text{C}_6\text{H}_{11}$, $\text{X}^- = \text{Cl}^-$) were prepared by the method of Baines *et al.* (1964). Methyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate hydrochloride (IIIa; $n = 2$, $\text{R} = \text{CH}_3$, $\text{X}^- = \text{Cl}^-$) was prepared by the method of Baird *et al.* (1965). *p*'-Nitrophenyl *p*-guanidinobenzoate was prepared by the method of Chase & Shaw (1967) and the 4-methylumbelliferyl ester was prepared by the method of Jameson *et al.* (1973).

*Ethyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate hydrochloride* (IIIa; $n = 2$, $\text{R} = \text{CH}_3\text{-CH}_2$, $\text{X}^- = \text{Cl}^-$). γ -Guanidino-L- α -toluene-*p*-sulphonamidobutyric acid was esterified with ethanol saturated with dry HCl. Recrystallized from ethanol-ether, the *ester hydrochloride* had m.p. 130–131°C, $[\alpha]_D^{+19.4}$ (*c* 4 in ethanol) (Found: C, 44.3; H, 6.0; N, 14.9. $\text{C}_{14}\text{H}_{23}\text{ClN}_4\text{O}_4\text{S}$ requires C, 44.4; H, 6.1; N, 14.8%).

3-Benzylloxycarbonylaminopropyl iodide. 3-Bromopropylamine hydrobromide (22g) and NaHCO_3 (8.5g) were dissolved in water. The solution was cooled in an ice bath and benzyl chloroformate (18ml) was added with stirring; 1M-NaOH (100ml) was added in ten portions during the reaction. When the solution gave a negative ninhydrin reaction, it was extracted with ethyl acetate and the extract was washed with 1M- H_2SO_4 , then with water and dried (with MgSO_4). The solution was evaporated to dryness and NaI (15g) was added. The mixture was dissolved in acetone (300ml) and heated under reflux for 1 h. After removal of NaBr, the filtrate was evaporated under reduced pressure. The resulting oil was dissolved in ethyl acetate, washed with water and dried (with MgSO_4). Evaporation gave a highly lachrymatory oil which was used without further purification.

S-(3-Benzylloxycarbonylaminopropyl)-L-cysteine. This compound (63%) was synthesized from L-cysteine and 3-benzylloxycarbonylaminopropyl iodide by a method similar to that described by Lindley (1959) for the preparation of *S*-(2-benzylloxycarbonylaminopropyl)-L-cysteine. After recrystallization from water, it had m.p. 210–212°C (decomp.) (Found: C, 53.9; H, 6.2; N, 9.1. $\text{C}_{14}\text{H}_{20}\text{N}_2\text{O}_4\text{S}$ requires C, 53.8; H, 6.5; N, 9.0%).

S-(3-Benzylloxycarbonylaminopropyl)-*N*-benzoyl-L-cysteine. *S*-(3-Benzylloxycarbonylaminopropyl)-L-cysteine (6.2g) was suspended in a mixture of ethanol (220ml) and water (110ml) at 0°C. The solution was adjusted to pH 11 and benzoyl chloride (5.6g) was added dropwise with addition of NaOH solution at this pH (in a pH-stat). When a test for free amino groups (ninhydrin) was negative, the pH was adjusted to 7 and the solution was evaporated under reduced pressure to 50–70ml, acidified to pH 2 and extracted

with ethyl acetate. The extract was washed with water and dried (with MgSO_4). Addition of benzylamine and ether caused the *benzylammonium salt* (86%) to crystallize. After recrystallization from ethanol-ether, it had m.p. 141–142°C (Found: C, 63.8; H, 6.1; N, 8.0. $\text{C}_{28}\text{H}_{33}\text{N}_3\text{O}_5\text{S}$ requires C, 64.2; H, 6.4; N, 8.0%).

S-(3-Aminopropyl)-*N*-benzoyl-L-cysteine. *S*-(3-Benzyloxycarbonylamino)propyl - *N* - benzoyl - L - cysteine benzylammonium salt (6.4g) was acidified with dilute acid. *S*-(3-Benzyloxycarbonylamino)propyl)-*N*-benzoyl-L-cysteine was extracted with ethyl acetate and the extract was washed with water and dried (with MgSO_4). After evaporation under reduced pressure, the residual oil was treated with 50% (w/v) HBr in acetic acid (6ml) containing a small quantity of phenol to remove free bromine. The solution was warmed to 50°C for 10min and then evaporated under reduced pressure. Trituration with dry ether caused the *hydrobromide* (78%) to crystallize. After recrystallization from propan-1-ol-ether, it had m.p. 194–196°C (Found: C, 43.4; H, 5.4; N, 8.0. $\text{C}_{13}\text{H}_{19}\text{BrN}_2\text{O}_3\text{S}$ requires C, 43.0; H, 5.3; N, 7.7%). The hydrobromide (2g) was dissolved in ethanol (20ml) and ether was added until the solution was faintly cloudy. Tri-*n*-butylamine was added dropwise until the solution was alkaline to moist Phenol Red indicator paper. Addition of ether caused the *product* (67%) to crystallize. It was recrystallized by suspension in hot ethanol with dropwise addition of water until it just dissolved. Crystallization began on cooling and was completed by the addition of ether, when it had m.p. 252–254°C (decomp.) (Found: C, 53.3; H, 6.6; N, 9.5. $\text{C}_{13}\text{H}_{18}\text{N}_2\text{O}_3\text{S} \cdot \frac{1}{2}\text{H}_2\text{O}$ requires C, 53.6; H, 6.6; N, 9.6%).

S-(3-Aminopropyl)-*N*-benzoyl-L-cysteine *methyl ester hydrochloride* (IIb; $n = 3$, $R = \text{CH}_3$, $X^- = \text{Cl}^-$). *S*-(3-Aminopropyl)-*N*-benzoyl-L-cysteine (1.6g) was esterified with methanol saturated with dry HCl. The solution was evaporated under reduced pressure and the residue was dissolved in methanol and purified by filtration through a column (2cm $\times$ 3cm) of neutral alumina. The solution was evaporated and the *product* (71%) was crystallized from methanol-ether when it had m.p. 132–134°C, $[\alpha]_D^{25} -60.8 \pm 0.2^\circ$ (c 4 in ethanol) (Found: C, 50.7; H, 6.4; N, 8.2. $\text{C}_{14}\text{H}_{21}\text{ClN}_2\text{O}_3\text{S}$ requires C, 50.5; H, 6.4; N, 8.4%).

S-(3-Aminopropyl)-*N*-benzoyl-L-cysteine *cyclohexyl ester toluene-p-sulphonate* (IIb; $n = 3$, $R = \text{C}_6\text{H}_{11}$, $X^- = p\text{-CH}_3\text{-C}_6\text{H}_4\text{-SO}_3^-$). *S*-(3-Aminopropyl)-*N*-benzoyl-L-cysteine (0.65g) was esterified by the azeotropic distillation of a mixture of cyclohexanol (50ml) and benzene in the presence of toluene-*p*-sulphonic acid (0.5g). Benzene was added dropwise to the distillation mixture during 5h. Addition of ether caused the *product* (81%) to crystallize. After recrystallization from propan-2-ol-ether, it had m.p. 113–115°C, $[\alpha]_D^{20} -33.3 \pm 0.1^\circ$ (c 3.94 in ethanol)

(Found: C, 57.9; H, 7.1; N, 5.1. $\text{C}_{26}\text{H}_{36}\text{N}_2\text{O}_6\text{S}_2$ requires C, 58.2; H, 6.8; N, 5.2%).

Alkyl ester hydrochlorides of S-(3-aminopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine. *S*-(3-Aminopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine hydrochloride (2g) was esterified by the requisite alcohol saturated with dry HCl. After evaporation, the ester hydrochlorides were recrystallized first from a mixture of the alcohol corresponding to the ester and ether and then from propan-2-ol-ether. The *methyl ester hydrochloride* (IIa; $n = 3$, $R = \text{CH}_3$, $X^- = \text{Cl}^-$) (81%) had m.p. 122–123°C, $[\alpha]_D^{25} +25.3 \pm 0.4^\circ$ (c 4 in ethanol) (Found: C, 43.6; H, 6.0; N, 7.5. $\text{C}_{14}\text{H}_{23}\text{ClN}_2\text{O}_4\text{S}_2$ requires C, 43.9; H, 6.0; N, 7.3%); the *n*-propyl ester hydrochloride (IIa; $n = 3$, $R = \text{CH}_3\text{-CH}_2\text{-CH}_2$, $X^- = \text{Cl}^-$) (79%) had m.p. 128–129°C, $[\alpha]_D^{23} +28.0 \pm 0.3^\circ$ (c 4 in ethanol) (Found: C, 46.9; H, 6.7; N, 7.0. $\text{C}_{16}\text{H}_{27}\text{ClN}_2\text{O}_4\text{S}_2$ requires C, 46.8; H, 6.6; N, 6.8%); the *2*-propyl ester hydrochloride [IIa; $n = 3$, $R = (\text{CH}_3)_2\text{CH}$, $X^- = \text{Cl}^-$] (91%) had m.p. 144–145°C, $[\alpha]_D^{20} +30.5 \pm 0.4^\circ$ (c 4 in ethanol) (Found: C, 46.6; H, 6.5; N, 7.0. $\text{C}_{16}\text{H}_{27}\text{ClN}_2\text{O}_4\text{S}_2$ requires C, 46.8; H, 6.6; N, 6.8%).

4-Benzyloxycarbonylaminobutan-1-ol. 4-Aminobutan-1-ol (8.9g) was dissolved in ice-cold water and the pH of the solution was adjusted to 11 by the addition of 2M-NaOH. Benzyl chloroformate (20g) was added dropwise during 1h and the pH was kept at 11 (in a pH-stat). Excess of benzyl chloroformate was removed by adding some glycine and stirring for a time. The solution was extracted with ethyl acetate and the extract was washed with dilute acid, water and then dried (with MgSO_4). Addition of light petroleum (b.p. 60–80°C) caused the *product* (63%) to crystallize. After recrystallizations from propan-2-ol-water and chloroform-light petroleum (b.p. 60–80°C), it had m.p. 80–81°C (Found: C, 64.6; H, 7.6; N, 6.2. $\text{C}_{12}\text{H}_{17}\text{NO}_3$ requires C, 64.6; H, 7.7; N, 6.3%).

4-Benzyloxycarbonylaminobutyl naphthalene-2'-sulphonate. 4-Benzyloxycarbonylaminobutan-1-ol (8.8g) was dissolved in pure dry pyridine (30ml) and benzene (30ml). A portion (20ml) of the solvent was distilled off to remove any residual traces of water. The solution was cooled in ice-salt mixture and naphthalene-2-sulphonyl chloride (8.8g) was added. The solution was stored at 0°C for 24h then ice-cold water (150ml) and ethyl acetate (50ml) were added. The organic layer was washed with 2M- H_2SO_4 (3 $\times$ 50ml), ice-cold NaHCO_3 (2 $\times$ 50ml) and ice-cold water (50ml) and dried (with MgSO_4). Evaporation of the solvent and the addition of light petroleum (b.p. 60–80°C) caused the *product* (81%) to crystallize. It was recrystallized from chloroform-light petroleum (b.p. 40–60°C), when it had m.p. 41–43°C (Found: C, 64.1; H, 5.7; N, 3.3. $\text{C}_{22}\text{H}_{23}\text{NO}_5\text{S}$ requires C, 63.9; H, 5.6; N, 3.4%).

4-Benzyloxycarbonylaminobutyl iodide. 4-Benzyloxycarbonylaminobutyl naphthalene-2'-sulphonate

(8.3 g) and NaI (3 g) were heated under reflux in acetone (100 ml) for 1 h, cooled and the precipitate of sodium naphthalene-2-sulphonate was removed by filtration. Evaporation of the solvent and addition of light petroleum (b.p. 40–60°C) gave the *product* (80%), which had m.p. 35–37°C after treatment with decolorizing charcoal and recrystallization from ethyl acetate–light petroleum (b.p. 40–60°C) (Found: C, 43.5; H, 4.8; N, 4.3. $C_{12}H_{16}INO_2$ requires C, 43.4; H, 4.8; N, 4.2%).

S-(4-Benzoyloxycarbonylaminoethyl)-L-cysteine. This compound (81%) was prepared by a method similar to that of Lindley (1959) for the synthesis of *S*-(2-benzoyloxycarbonylaminoethyl)-L-cysteine. After recrystallization from water, it had m.p. 205–207°C (decomp.) (Found: C, 55.5; H, 7.0; N, 8.6. $C_{15}H_{22}N_2O_4S$ requires C, 55.2; H, 6.8; N, 8.6%).

S-(4-Benzoyloxycarbonylaminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine. *S*-(4-Benzoyloxycarbonylaminoethyl)-L-cysteine (5.3 g) was suspended in water (200 ml) and dissolved by the addition of 2M-NaOH. A solution of toluene-*p*-sulphonyl chloride (3.1 g) in ether (50 ml) was added and the mixture was stirred and maintained at pH 11 by the addition of 2M-NaOH (in a pH-stat). When a test for free amino groups was negative (ninhydrin), the solution was extracted with ether (3 × 100 ml) and acidified to pH 2. The resulting oil was extracted with ethyl acetate and the extract was washed with water and dried (with $MgSO_4$). Addition of light petroleum (b.p. 40–60°C) caused the *product* (76%) to crystallize. It was recrystallized from ethyl acetate–light petroleum (b.p. 40–60°C) and then had m.p. 145–147°C (Found: C, 55.2; H, 6.0; N, 5.8. $C_{22}H_{28}N_2O_6S_2$ requires C, 55.0; H, 5.9; N, 5.8%).

S-(4-Aminobutyl)-*N*-toluene-*p*-sulphonyl-L-cysteine. *S*-(4-Benzoyloxycarbonylaminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine (2 g) was dissolved in 50% (w/v) HBr in acetic acid (2 ml), which contained a small amount of phenol. The solution was gently warmed to 50°C for 15 min and then triturated with sodium-dried ether (30 ml) and the ether layer was decanted. The residual oil was triturated with more ether (4 × 30 ml) and dissolved in water (20 ml). Adjustment of the pH to 7 caused the *product* (69%) to precipitate. After recrystallization from water, it had m.p. 262–264°C (decomp.) (Found: C, 48.7; H, 6.3; N, 8.1. $C_{14}H_{22}N_2O_4S_2$ requires C, 48.5; H, 6.4; N, 8.1%).

S-(4-Aminobutyl)-*N*-toluene-*p*-sulphonyl-L-cysteine methyl ester toluene-*p*-sulphonate (IIa; $n = 4$, $R = CH_3$, $X^- = p-CH_3-C_6H_4-SO_3^-$). *S*-(4-Aminobutyl)-*N*-toluene-*p*-sulphonyl-L-cysteine (3.5 g) and toluene-*p*-sulphonic acid (2.3 g) were dissolved in methanol. A mixture of benzene–methanol (1:1, v/v) was added dropwise while the solution was slowly distilled to remove water azeotropically. After 6 h, the solution was evaporated under reduced pressure.

The residual oil was dissolved in methanol and the *product* (72%) was caused to crystallize by the addition of ether. After recrystallization from methanol–ether, it had m.p. 141–142°C, $[\alpha]_D^{20} +16.5 \pm 0.1^\circ$ (c 4 in methanol) (Found: C, 49.7; H, 6.1; N, 5.2. $C_{22}H_{32}N_2O_7S_3$ requires C, 49.6; H, 6.1; N, 5.3%).

S-(2-Guanidinoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine (IV; $n = 2$). *S*-(2-Aminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine (9.6 g) and *O*-methylisourea hydrogen sulphate (10.9 g) were suspended in water (150 ml). Then 5M-NaOH was added until the amino acid just dissolved (pH 11.5) and the solution was left for 48 h. The resulting precipitate was collected and recrystallized from boiling water. The filtrate was adjusted to pH 10.5 and kept at 0°C overnight. More *product* was precipitated, collected and recrystallized. The pH of the filtrate was lowered to 9.5 and the sequence was repeated. The precipitates formed in the pH range 10.5–11.5 were shown to be homogeneous by paper chromatography in butan-1-ol–acetic acid–water (4:1:5, by vol.). The precipitate formed in the pH range 9.5–10.5 was usually slightly contaminated with starting material but could be purified by two or three recrystallizations from boiling water. The *product* (42%) had m.p. 220–222°C (decomp.) (Found: C, 43.1; H, 5.7; N, 15.6. $C_{13}H_{20}N_4O_4S_2$ requires C, 43.3; H, 5.6; N, 15.5%).

S-(3-Guanidinopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine (IV; $n = 3$). This compound was prepared from *S*-(3-aminopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine by a similar procedure to that used for the previous compound, except that a slightly lower pH (approx. 11) was required to dissolve the amino acid initially. The various fractions were checked for purity by paper chromatography, and any fraction that was contaminated with starting material was recrystallized two or three times from boiling water. Any fraction that was still impure after this treatment was rejected. The *product* (48%) had m.p. 238–240°C (decomp.) (Found: C, 44.6; H, 6.0; N, 15.0. $C_{14}H_{22}N_4O_4S_2$ requires C, 44.9; H, 5.9; N, 15.0%).

S-(2-Nitroguanidinoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine. *S*-(2-Aminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine (3.2 g) and 1-nitro-2-methylisourea (1.5 g) were suspended in water (50 ml). The pH was raised to 11.5 and the clear solution was left for 24 h. The solution was extracted with ethyl acetate (2 × 25 ml) and acidified to pH 2.5. The resultant oil was extracted into ethyl acetate (2 × 25 ml) and the extract was washed with water and dried (with $MgSO_4$). Addition of light petroleum (b.p. 60–80°C) caused the *product* (82%) to crystallize. After recrystallizations from ethyl acetate–light petroleum (b.p. 60–80°C) and ethanol–water, it had m.p. 142–143°C (Found: C, 38.6; H, 4.7; N, 17.3. $C_{13}H_{19}N_5O_6S_2$ requires C, 38.5; H, 4.7; N, 17.3%).

S-(2-Nitroguanidinoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine methyl ester. *S*-(2-Nitroguani-

dinoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine (1.5 g) and toluene-*p*-sulphonic acid (0.2 g) were dissolved in methanol. A mixture of benzene-methanol (1:1, v/v) was added dropwise while the solution was slowly distilled to remove water azeotropically. After 4 h, the solution was evaporated to dryness and the resulting oil was dissolved in ethyl acetate. The ethyl acetate solution was extracted with NaHCO_3 solution, washed with water and dried (with MgSO_3). The addition of light petroleum (b.p. 60–80°C) caused the ester (86%) to crystallize. It had m.p. 89–91°C after recrystallization from ethyl acetate–light petroleum (b.p. 60–80°C) (Found: C, 39.9; H, 5.0; N, 17.0. $\text{C}_{14}\text{H}_{21}\text{N}_5\text{O}_6\text{S}_2$ requires C, 40.0; H, 5.0; N, 15.7%).

S-(3-Cyanopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine. A solution of *N*-toluene-*p*-sulphonyl-L-cysteine (5.5 g) and 3-cyano-1-iodopropane (5.8 g) in a mixture of methanol (100 ml) and water (100 ml) was warmed to 45°C, made alkaline to phenolphthalein and flushed with N_2 until a test portion no longer gave a positive test for the thiol group with 5,5'-dithiobis-(2-nitrobenzoic acid). The solution was twice extracted with ethyl acetate, then acidified to pH 2.5. The resulting oil was extracted into ethyl acetate and the extract was washed with water and dried (with MgSO_4). Addition of light petroleum (b.p. 60–80°C) caused the product (87%) to crystallize. After recrystallizations from benzene and from aq. ethanol, it had m.p. 96–98°C (Found: C, 48.8; H, 5.3; N, 8.4. $\text{C}_{14}\text{H}_{18}\text{N}_2\text{O}_4\text{S}_2$ requires C, 49.1; H, 5.3; N, 8.2%).

S-(3-Cyanopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine methyl ester. *S*-(3-Cyanopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine (3.4 g) in methanol (15 ml) was treated with a slight excess of diazomethane in ether. The solvent was removed under reduced pressure and the residual oil was dissolved in ethyl acetate. The solution was washed with saturated NaHCO_3 , then with water and dried (with MgSO_4). Addition of light petroleum (b.p. 60–80°C) caused the ester (85%) to crystallize with m.p. 45–47°C (Found: C, 50.2; H, 5.5; N, 7.9. $\text{C}_{15}\text{H}_{20}\text{N}_2\text{O}_4\text{S}_2$ requires C, 50.5; H, 5.7; N, 7.9%).

Reduction of S-(3-cyanopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine derivatives. Reduction of the cyclohexylammonium, tri-*n*-butylammonium, cetyltrimethylbenzylammonium and cetyltrimethylammonium salts of *S*-(3-cyanopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine with diborane was carried out by the method of Brown & Subba Rao (1960). The salts were prepared in solution by the addition of the calculated amount of the particular amine or quaternary base to a solution of *S*-(3-cyanopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine in dry tetrahydrofuran. The solution was saturated with diborane at 0°C and left for 5–24 h. The excess of diborane was destroyed by the addition of ethanol and the solution was acidified with 11 M-HCl. Addition of ether caused

the products to crystallize. After recrystallization from ethanol-ether, they had similar melting points and elementary compositions. Their i.r. spectra did not contain a bond in the carbonyl-stretching region. The product in each case was 3-(4'-aminobutylthio)-2-toluene-*p*-sulphonamidopropan-1-ol, since it was also formed by reduction of *S*-(3-cyanopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine methyl ester by using diborane. The alcohol had m.p. 107–108°C (Found: C, 45.3; H, 6.6; N, 7.5. $\text{C}_{14}\text{H}_{25}\text{ClN}_2\text{O}_3\text{S}_2$ requires C, 45.6; H, 6.8; N, 7.6%).

Methods

The steady-state kinetics of hydrolysis of esters were followed by means of a pH-stat and recorder (Baines *et al.*, 1964). Initial velocities, kinetic and activation constants were computed by FORTRAN programs on the basis of statistical procedures described by Elmore *et al.* (1963). The operational molarity of trypsin solutions was determined either spectrophotometrically with *p*'-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1967) or spectrofluorimetrically with 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson *et al.*, 1973). The latter method was preferred because of its higher sensitivity and consequent economy of enzyme. Substrates bearing the α -*N*-toluene-*p*-sulphonyl group were found to display substrate activation (Trowbridge *et al.*, 1963; Elmore *et al.*, 1967) particularly with α -trypsin. Consequently, care was taken not to exceed substrate concentrations when this phenomenon became significant. Visual examination of Lineweaver–Burk plots and computation of weighted deviations of experimental points from the Lineweaver–Burk plot were useful indications of the upper limit of substrate concentrations which could be safely used. $\text{p}K_1$ and $\text{p}K_2$ values were obtained by computing the weighted linear regressions of k_{cat} on $[\text{H}^+]$ and on $1/[\text{H}^+]$ respectively.

Scheme 1. Kinetic constants for the hydrolysis of various esters

E, Ester; S, S', substrates; P, P', products. For further details see the text.

Table 1. Values of $k_{cat.}$, K_m and $k_{cat.}/K_m$ for the hydrolysis of esters of α -N-benzoyl and α -N-toluene-p-sulphonyl derivatives of basic amino acids by α -trypsin at pH 8.0 and 25.0°C in 0.1M-NaCl

Values are means $\pm$ s.d. [S] ₀ , Initial substrate concentrations, [E] ₀ , initial ester concentrations.						
Substrate	$k_{cat.}$ (s ⁻¹)	$10^5 K_m$ (M)	$10^{-3} k_{cat.}/K_m$ (M ⁻¹ ·s ⁻¹)	$10^5 [S]_0$ (M)	$10^2 [S]_0/[E]_0$	No. of runs
(Ia; R = CH ₃ , X ⁻ = Cl ⁻)	88.0 $\pm$ 1.9	3.95 $\pm$ 0.18	2200	0.4 - 400	20-1000	20
(Ia; R = (CH ₃) ₂ CH, X ⁻ = Cl ⁻)	82.3 $\pm$ 1.9	9.02 $\pm$ 0.31	910	1.0 - 60	20-1000	10
(Ib; R = CH ₃ , X ⁻ = p-CH ₃ -C ₆ H ₄ -SO ₃ ⁻)	20.3 $\pm$ 0.5	1.74 $\pm$ 0.06	1200	0.2 - 50	5- 20	16
(Ib; R = C ₆ H ₁₁ , X ⁻ = p-CH ₃ -C ₆ H ₄ -SO ₃ ⁻)	22.5 $\pm$ 0.3	1.24 $\pm$ 0.03	1800	0.6 - 20	10- 500	12
(IIa; n = 2, R = CH ₃ , X ⁻ = Cl ⁻)	37.4 $\pm$ 0.5	54.6 $\pm$ 1.3	68	5 - 800	30- 400	12
(IIa; n = 2, R = CH ₃ -CH ₂ -CH ₃ , X ⁻ = Cl ⁻)	22.1 $\pm$ 0.3	21.0 $\pm$ 0.6	110	5 - 800	30- 400	16
(IIa; n = 3, R = CH ₃ , X ⁻ = Cl ⁻)	5.49 $\pm$ 0.21	249 $\pm$ 17	2.2	80 -2000	200-3000	9
(IIa; n = 3, R = CH ₃ -CH ₂ -CH ₃ , X ⁻ = Cl ⁻)	2.47 $\pm$ 0.08	143 $\pm$ 10	1.7	60 -2000	200-3000	7
(IIa; n = 4, R = CH ₃ , X ⁻ = p-CH ₃ -C ₆ H ₄ -SO ₃ ⁻)	0.718 $\pm$ 0.061	96.1 $\pm$ 37.0	0.75	90 - 800	500-6000	4
(IIb; n = 2, R = CH ₃ , X ⁻ = Cl ⁻)	8.80 $\pm$ 0.11	11.0 $\pm$ 0.4	80	1 - 800	40-3000	10
(IIb; n = 2, R = C ₆ H ₁₁ , X ⁻ = p-CH ₃ -C ₆ H ₄ -SO ₃ ⁻)	8.72 $\pm$ 0.07	1.42 $\pm$ 0.03	610	0.8 - 50	20-1000	15
(IIb; n = 3, R = CH ₃ , X ⁻ = Cl ⁻)	3.79 $\pm$ 0.50	329 $\pm$ 65	1.2	50 - 800	100-1000	11
(IIb; n = 3, R = C ₆ H ₁₁ , X ⁻ = p-CH ₃ -C ₆ H ₄ -SO ₃ ⁻)	3.89 $\pm$ 0.06	12.5 $\pm$ 0.8	31	8 - 400	100- 500	8
(IIIa; n = 2, R = CH ₃ , X ⁻ = Cl ⁻)	58.9 $\pm$ 0.8	132 $\pm$ 3	45	10 - 800	40-1000	10
(IIIa; n = 2, R = CH ₃ -CH ₂ , X ⁻ = Cl ⁻)	48.0 $\pm$ 0.8	191 $\pm$ 5	25	10 - 800	40-1000	7
(IIIa; n = 3, R = CH ₃ , X ⁻ = Cl ⁻)	91.5 $\pm$ 4.4	1.00 $\pm$ 0.10	9100	0.2 - 40	30-1000	44
(IIIa; n = 3, R = C ₆ H ₁₁ , X ⁻ = Cl ⁻)	87.2 $\pm$ 0.9	0.568 $\pm$ 0.015	15000	0.3 - 20	7- 600	16
(IIIa; n = 4, R = CH ₃ , X ⁻ = Cl ⁻)	5.73 $\pm$ 0.03	121 $\pm$ 2	4.7	20 - 800	50-1000	12
(IIIa; n = 4, R = C ₆ H ₁₁ , X ⁻ = Cl ⁻)	5.79 $\pm$ 0.08	102 $\pm$ 3	5.6	20 - 600	50-1000	7
(IIIb; n = 3, R = CH ₃ -CH ₂ , X ⁻ = Cl ⁻)	23.3 $\pm$ 0.2	0.508 $\pm$ 0.012	4600	0.15- 40	20- 200	38
(IIIb; n = 3, R = C ₆ H ₁₁ , X ⁻ = Cl ⁻)	23.7 $\pm$ 0.3	0.301 $\pm$ 0.011	7900	0.25- 20	10- 600	14

Table 2. Values of k_{cat} , K_m and k_{cat}/K_m for the hydrolysis of esters of α -N-benzoyl and α -N-toluene-p-sulphonyl derivatives of basic amino acids by β -trypsin at pH 8.0 and 25.0°C in 0.1 M-NaCl

Substrate	Values are means $\pm$ s.d.					No. of runs
	k_{cat} (s^{-1})	$10^5 K_m$ (M)	$10^{-3} k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)	$10^5 [S]_0$ (M)	$10^3 [S]_0/[E]_0$	
(Ia; R = CH ₃ , X' = Cl ⁻)	122 $\pm$ 1	4.82 $\pm$ 0.18	2500	1 - 50	30-300	13
(Ia; R = (CH ₃) ₂ CH, X' = Cl ⁻)	119 $\pm$ 4	7.11 $\pm$ 0.46	1700	2 - 60	40-200	12
(Ib; R = CH ₃ , X' = p-CH ₃ -C ₆ H ₄ -SO ₃ ⁻)	25.0 $\pm$ 0.8	1.63 $\pm$ 0.17	1500	0.6 - 20	9-300	13
(Ib; R = C ₆ H ₁₁ , X' = p-CH ₃ -C ₆ H ₄ -SO ₃ ⁻)	27.3 $\pm$ 0.4	0.806 $\pm$ 0.024	3400	0.3 - 50	6-200	24
(IIa; n = 2, R = CH ₃ , X' = Cl ⁻)	113 $\pm$ 3	26.3 $\pm$ 1.1	430	1 - 200	20-400	11
(IIa; n = 2, R = CH ₃ -CH ₂ -CH ₂ , X' = Cl ⁻)	102 $\pm$ 2	18.7 $\pm$ 0.7	550	1 - 200	20-400	13
(IIa; n = 3, R = CH ₃ , X' = Cl ⁻)	16.2 $\pm$ 0.3	22.7 $\pm$ 1.0	71	4 - 600	20-1000	8
(IIa; n = 3, R = CH ₃ -CH ₂ -CH ₂ , X' = Cl ⁻)	8.86 $\pm$ 0.09	9.00 $\pm$ 0.29	98	4 - 800	50-1000	11
(IIa; n = 4, R = CH ₃ , X' = p-CH ₃ -C ₆ H ₄ -SO ₃ ⁻)	0.774 $\pm$ 0.004	28.3 $\pm$ 0.5	2.7	10 - 800	5-1000	9
(IIb; n = 2, R = CH ₃ , X' = Cl ⁻)	35.0 $\pm$ 0.6	10.4 $\pm$ 0.4	340	1 - 50	20-200	12
(IIb; n = 2, R = C ₆ H ₁₁ , X' = p-CH ₃ -C ₆ H ₄ -SO ₃ ⁻)	33.9 $\pm$ 0.8	2.07 $\pm$ 0.10	1600	0.6 - 50	10-1000	17
(IIb; n = 3, R = CH ₃ , X' = Cl ⁻)	6.46 $\pm$ 0.06	26.6 $\pm$ 0.5	24	5 - 300	60-800	15
(IIb; n = 3, R = C ₆ H ₁₁ , X' = p-CH ₃ -C ₆ H ₄ -SO ₃ ⁻)	6.64 $\pm$ 0.01	11.9 $\pm$ 0.7	56	5 - 100	80-400	8
(IIIa; n = 2, R = CH ₃ , X' = Cl ⁻)	54.3 $\pm$ 1.5	66.7 $\pm$ 3.4	81	10 - 800	100-1000	12
(IIIa; n = 2, R = CH ₃ -CH ₂ , X' = Cl ⁻)	39.9 $\pm$ 0.6	83.5 $\pm$ 2.5	48	10 - 800	100-1000	10
(IIIa; n = 3, R = CH ₃ , X' = Cl ⁻)	106 $\pm$ 1	0.762 $\pm$ 0.013	14000	0.1 - 40	3-1000	43
(IIIa; n = 3, R = C ₆ H ₁₁ , X' = Cl ⁻)	99.0 $\pm$ 1.1	0.577 $\pm$ 0.014	17000	0.2 - 50	5-1000	38
(IIIa; n = 4, R = CH ₃ , X' = Cl ⁻)	6.66 $\pm$ 0.12	42.8 $\pm$ 1.4	16	8 - 500	10-1000	13
(IIIa; n = 4, R = C ₆ H ₁₁ , X' = Cl ⁻)	4.95 $\pm$ 0.38	33.4 $\pm$ 6.0	15	10 - 800	8-1000	27
(IIIb; n = 3, R = CH ₃ -CH ₂ , X' = Cl ⁻)	24.8 $\pm$ 0.4	0.355 $\pm$ 0.013	7000	0.12-40	5-1000	27
(IIIb; n = 3, R = C ₆ H ₁₁ , X' = Cl ⁻)	24.5 $\pm$ 0.4	0.144 $\pm$ 0.007	17000	0.06-20	2-500	47

Results and Discussion

Kinetic constants (Scheme 1) for the hydrolysis of esters of α -*N*-toluene-*p*-sulphonyl-L-lysine (Ia), α -*N*-benzoyl-L-lysine (Ib), *S*-(ω -aminoalkyl)-*N*-toluene-*p*-sulphonyl-L-cysteine (IIa; $n = 2, 3$ or 4), *S*-(ω -aminoalkyl)-*N*-benzoyl-L-cysteine (IIb; $n = 2$ or 3), γ -guanidino-L- α -toluene-*p*-sulphonamidobutyric acid (IIIa; $n = 2$), α -*N*-toluene-*p*-sulphonyl-L-arginine (IIIa; $n = 3$), α -*N*-toluene-*p*-sulphonyl-L-homoarginine (IIIa; $n = 4$) and α -*N*-benzoyl-L-arginine (IIIb; $n = 3$) by α - and β -trypsin respectively are given in Tables 1 and 2. Trypsin normally hydrolyses peptide bonds involving the carbonyl groups of L-lysine and L-arginine. Consequently, an examination of the kinetic constants for the hydrolysis of derivatives of these amino acids by α - and β -trypsin serves as a reference for comparing the kinetic results for the other synthetic substrates. For reactions catalysed by β -trypsin, the k_{cat} values for the esters of *N*-toluene-*p*-sulphonyl derivatives of L-lysine [Ia; $R = \text{CH}_3$ and $(\text{CH}_3)_2\text{CH}$, $X^- = \text{Cl}^-$] and L-arginine (IIIa; $n = 3$, $R = \text{CH}_3$, $X^- = \text{Cl}^-$ and $R = \text{C}_6\text{H}_{11}$, $X^- = p\text{-CH}_3\text{-C}_6\text{H}_4\text{-SO}_3^-$) are very similar (approx. $100\text{--}120\text{s}^{-1}$). They are higher than the values we quoted previously (Baines *et al.*, 1964; Elmore *et al.*, 1967), presumably because at that time we were using unpurified trypsin and because we were not determining the operational molarity of trypsin solutions. The k_{cat} values are also some four times higher than those for the α -*N*-benzoyl derivatives of L-lysine (Ib; $R = \text{CH}_3$ and C_6H_{11} , $X^- = p\text{-CH}_3\text{-C}_6\text{H}_4\text{-SO}_3^-$) and L-arginine (IIIb; $R = \text{CH}_3\text{-CH}_2$ and C_6H_{11} , $X^- = \text{Cl}^-$). For reactions catalysed by α -trypsin, the k_{cat} values for the foregoing substrates are only slightly lower than those observed when β -trypsin effects hydrolysis. The k_{cat} values are essentially independent of the ester group within each series, and this is consistent with a mechanism in which the rate-limiting step is the deacylation of a common acyl-enzyme and hence $k_{\text{cat}} \approx k_{+3}$. The K_m values for the α -*N*-benzoyl derivatives are lower than those for the α -*N*-toluene-*p*-sulphonyl derivatives. This is probably not an indication that the α -*N*-benzoyl derivatives are more firmly bound to the enzyme but more likely stems from the lower k_{+3} values for these substrates. Since $K_m = k_{+3}(k_{-1} + k_{+2})/k_{+1}(k_{+2} + k_{+3})$ and deacylation is rate-determining ($k_{+2} \gg k_{+3}$) then an increase in k_{+3} will increase K_m values and vice versa (Curragh & Elmore, 1964). The higher k_{cat} values of the α -*N*-toluene-*p*-sulphonyl derivatives compared with those of the α -*N*-benzoyl derivatives possibly reflect, at least in part, the superior electron-withdrawing nature of the sulphonamido group, which makes the ester bond more susceptible to nucleophilic attack by a water molecule. Brot & Bender (1969) have suggested that the specificity constant, k_{cat}/K_m , is a better indication of the 'goodness' of the substrate than

either k_{cat} or K_m values alone. It is noteworthy therefore that the specificity constants of the L-arginine derivatives are approx. 5–10 times higher than those of the corresponding L-lysine derivatives.

The remaining results given in Tables 1 and 2 are for derivatives of synthetic amino acids, and one of these, *S*-(2-aminoethyl)-L-cysteine, is an isostere of L-lysine in which $-\text{CH}_2-$ has been replaced by $-\text{S}-$ in the side chain. This change affects the orientation of the side chain because of the slightly different bond angles and bond lengths. There is little difference between the k_{cat} values for the hydrolysis of the methyl esters of *S*-(2-aminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine (IIa; $n = 2$, $R = \text{CH}_3$, $X^- = \text{Cl}^-$) and α -*N*-toluene-*p*-sulphonyl-L-lysine (Ia; $R = \text{CH}_3$, $X^- = \text{Cl}^-$) by β -trypsin. Surprisingly, the k_{cat} value for the hydrolysis of *S*-(2-aminoethyl)-*N*-benzoyl-L-cysteine methyl ester (IIb; $n = 2$, $R = \text{CH}_3$, $X^- = p\text{-CH}_3\text{-C}_6\text{H}_4\text{-SO}_3^-$) is higher than that for the lysine derivative (Ib; $R = \text{CH}_3$, $X^- = p\text{-CH}_3\text{-C}_6\text{H}_4\text{-SO}_3^-$) with β -trypsin as catalyst. Nevertheless the lysine derivatives have higher specificity constants than the isosteres. Values of k_{cat} for the hydrolysis of the *N*-benzoyl and *N*-toluene-*p*-sulphonyl derivatives of *S*-(2-aminoethyl)-L-cysteine by β -trypsin are higher than those observed for reactions catalysed by α -trypsin. In addition, the k_{cat} values for the hydrolysis of two esters of *S*-(2-aminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine by α -trypsin are different. Deacylation is therefore not rate-determining and presumably k_{+2} and k_{+3} are similar in magnitude for these substrates. The K_m values for the hydrolysis of individual esters of *S*-(2-aminoethyl)-*N*-benzoyl- and -*N*-toluene-*p*-sulphonyl-L-cysteine by α - and β -trypsin are similar in magnitude. In view of the differences in k_{cat} values mentioned above, however, the specificity constants are greater with β - than with α -trypsin as catalyst. For derivatives of *S*-(2-aminoethyl)-L-cysteine (IIa, b; $n = 2$), the K_m values are decreased when the ester group is more hydrophobic. The effect is most marked when the cyclohexyl and methyl esters (IIb; $n = 2$, $R = \text{C}_6\text{H}_{11}$ or CH_3) are compared. The value of k_{+2} is almost certainly lower for the cyclohexyl ester than for the methyl ester and, provided that $k_{+3} < k_{-1}$, this would be expected to lead to an increase in K_m values. Since the opposite result was observed, this implies that binding of the substrate is improved as the ester group is made more hydrophobic. The possibility that $k_{+3} > k_{-1}$, which would lead to a decrease in K_m values as k_{+2} decreased, is considered most unlikely, since the step governed by k_{-1} involves no covalency change and is probably diffusion-controlled.

Not surprisingly, k_{cat} values are decreased as the chain length of the substrate is increased. This is particularly noticeable for the *N*-toluene-*p*-sulphonyl derivatives (IIa; $n = 2\text{--}4$, $R = \text{CH}_3$). Similar relationships hold for hydrolyses catalysed by both α - and

β -trypsin. The K_m values are mostly elevated as the chain length is increased, but there are two notable exceptions. For the hydrolysis of the two compounds (IIa; $n = 2$ or 3 , $R = CH_3$) by β -trypsin, the K_m values are not significantly different, whereas for the hydrolysis of the two compounds (IIa; $n = 3$ or 4 , $R = CH_3$) by α -trypsin the K_m value is surprisingly lower for the substrate with the longer side chain. In spite of these slight anomalies, the specificity constants decrease with chain length in every case.

For the hydrolysis of esters of α -*N*-toluene-*p*-sulphonyl derivatives of ω -guanidino acids (IIIa; $n = 2-4$, $R = CH_3$, $X^- = Cl^-$), the value of k_{cat} is decreased when the side chain is shorter ($n = 2$) or longer ($n = 4$) than that of *L*-arginine, but the effect is less marked for the smaller substrate. The K_m values are much higher when $n = 2$ or 4 , especially when α -trypsin is the catalyst, and consequently the *L*-arginine derivative has much the highest specificity constant.

The variation of k_{cat} with pH at 25°C for the hydrolysis of α -*N*-benzoyl-*L*-arginine cyclohexyl ester by α - and β -trypsin was compared. The apparent pK_a values are listed in Table 3, showing that the values for β -trypsin are slightly lower than for α -trypsin. The lower of the two apparent pK_a values for each enzyme is attributed to the charge relay system involving Asp(90)-His(46)-Ser(183) [Asp(102)-His(57)-Ser(195) by the numbering scheme of Hartley *et al.* (1965)], and the higher apparent pK_a value for each enzyme probably arises from the ionization of the α -amino group of the *N*-terminal isoleucine residue. The lower apparent pK_a values are similar to that reported by Gutfreund (1955) for the same substrate. The higher

apparent pK_a values are similar to that determined by Béchet (1965) for the hydrolysis of α -*N*-toluene-*p*-sulphonyl-*L*-arginine methyl ester by trypsin. In both of these earlier studies, it is probable that the enzyme consisted of a mixture of α - and β -trypsins. Since both apparent pK_a values for each enzyme probably refer to cationic acids, it is possible that the slightly higher pK_a values for α -trypsin indicate that they are in a more polar environment than the corresponding groups in β -trypsin. This could be the case if α -trypsin has a looser structure which is more accessible to water molecules.

The activation constants for reactions catalysed by α - and β -trypsin at pH 8.0 were compared. Values for the energy, enthalpy and entropy of activation for the hydrolysis of α -*N*-benzoyl- and α -*N*-toluene-*p*-sulphonyl-*L*-lysine methyl esters are given in Table 4. The values of the energy and enthalpy of activation are similar in magnitude for reactions catalysed by α - and β -trypsin. In contrast, the value of the entropy of activation is significantly different, at least for the former substrate. It is possible that the more negative value of $\Delta S^\ddagger$ associated with the reaction catalysed by α -trypsin reflects greater flexibility in the ground state of the acylated derivative of the α -enzyme compared with the corresponding derivative of β -trypsin. If this is the case, the formation of the activated complex involving α -trypsin would require a greater degree of organization. Thus the higher apparent pK_a values of groups involved in the catalytic process, the more negative values of the entropy of activation for α -trypsin-catalysed reactions and the greater tendency of α -trypsin to undergo autolysis all point to a greater flexibility in this enzyme compared with β -trypsin.

Some aspects of the chemical syntheses of substrates and intermediates deserve mention. First, an improvement in the preparation of the *N*-benzoyl derivatives of *L*-arginine, ϵ -*N*-benzyloxycarbonyl-*L*-lysine and *S*-(ω -benzyloxycarbonylaminoalkyl)-*L*-cysteine was achieved by the use of ethanol-water (2:1, v/v) as the reaction solvent. This led to the formation of the by-product, ethyl benzoate, by ethanolysis rather than benzoic acid by hydrolysis and the purification of the products was simplified as a result. Secondly, we had hoped to synthesize a

Table 3. Effect of pH on the hydrolysis of *N*-benzoyl-*L*-arginine cyclohexyl ester hydrochloride (IIIa; $n = 3$, $R = C_6H_{11}$, $X^- = Cl^-$) by α - and β -trypsin at 25°C

Values are means $\pm$ S.D. with the no. of runs in parentheses.

	α -Trypsin	β -Trypsin
pK_1	6.24 ± 0.02 (21)	6.15 ± 0.03 (14)
pK_2	10.36 ± 0.09 (21)	10.10 ± 0.02 (14)

Table 4. Activation constants for the hydrolysis of α -*N*-benzoyl-*L*-lysine methyl ester toluene-*p*-sulphonate (Ib; $R = CH_3$, $X^- = p-CH_3-C_6H_4-SO_3^-$) and α -*N*-toluene-*p*-sulphonyl-*L*-lysine methyl ester hydrochloride (Ia; $R = CH_3$, $X^- = Cl^-$) by α - and β -trypsin at pH 8.0

Values are means $\pm$ S.D. with the number of runs in parentheses.

	Ib; $R = CH_3$, $X^- = p-CH_3-C_6H_4-SO_3^-$		Ia; $R = CH_3$, $X^- = Cl^-$	
	α -Trypsin (4)	β -Trypsin (5)	α -Trypsin (10)	β -Trypsin (11)
E_a (kJ·mol ⁻¹)	43.6 ± 1.3	48.4 ± 0.3	41.9 ± 2.1	44.4 ± 0.8
$\Delta H^\ddagger$ (kJ·mol ⁻¹)	41.1 ± 1.3	45.9 ± 0.3	39.4 ± 2.1	41.9 ± 0.8
$\Delta S^\ddagger$ (kJ·mol ⁻¹ ·°C ⁻¹)	-83.3 ± 3.8	-64.9 ± 0.8	-76.2 ± 8.4	-65.3 ± 2.5

range of substrates containing the *S*-(ω -gaunidinoalkyl)-*N*-toluene-*p*-sulphonyl-L-cysteinyl residue (IV). Although the *S*-(2-guanidinoethyl) and *S*-(3-guanidinopropyl) derivatives of *N*-toluene-*p*-sulphonyl-L-cysteine (IV; $n = 2$ or 3) could be synthesized, no crystalline ester salts could be isolated. *S*-(2-Nitroguanidinoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine methyl ester was successfully synthesized but the nitro group could not be removed. Finally, our first attempt to synthesize *S*-(4-aminobutyl)-*N*-toluene-*p*-sulphonyl-L-cysteine involved the preparation and reduction of *S*-(3-cyanopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine. Various salts of *S*-(3-cyanopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine were subjected to reduction by diborane (Brown & Subba Rao, 1960). Unfortunately, not only the cyano group but also the carboxylate group was reduced. The latter reaction was unexpected, since it has been reported (Brown & Subba Rao, 1960) that salts of carboxylic acids are unaffected by diborane.

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