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THE SYNTHESIS OF AN α-AZAORNITHINE DERIVATIVE AND ITS REACTION WITH TRYPSIN

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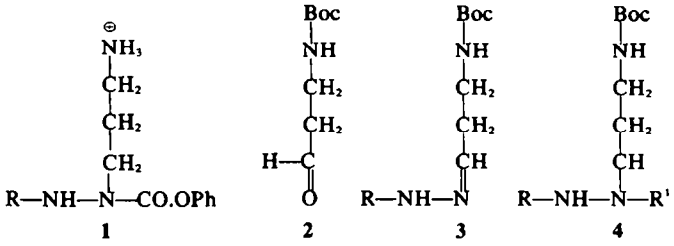
Abstract—N^{α}-Benzoyl-α-azaornithine phenyl ester has been synthesised as the trifluoroacetate. In aqueous solution the ester salt is stable at pH 3 but decomposes at higher pH values. The analogue ester inhibits trypsin rapidly, and chymotrypsin much more slowly, by acylation at the active site. Spontaneous deacylation of the α-azaacyl-trypsin occurs with a first-order rate constant of $2{\cdot}1 \times 10^{-4}\ s^{-1}$ at 23·8° and pH 7·6.

Evidence presently available indicates that both trypsin and chymotrypsin function by similar mechanisms.[1,2] In the trypsin- or chymotrypsin-catalysed hydrolysis of a carboxylic acid derivative, such as an ester or an amide, the hydroxyl group of the active site serine residue first becomes acylated. This acyl-enzyme is subsequently hydrolysed very rapidly. Recently, from this laboratory we[3] reported evidence that when chymotrypsin reacts with N-acetyl-α-azaphenylalanine phenyl ester, the deacylation is extremely slow, so that in effect the analogue functions as an active-site-directed irreversible inhibitor of chymotrypsin.

We wished to extend this work to trypsin and here report the synthesis of N^{α}-benzoyl-α-azaornithine phenyl ester (1; R=C_6H_5CO–) (phenyl N^2 - benzoyl - N^1 - (3 - aminopropyl) - carbazate) and the reaction of 1 with trypsin.

The specificity of trypsin is such that the hydrolysis of amino-acid derivatives carrying basic (i.e. positively-charged at neutral pH) side-chains is favoured.[1] In the first instance, since N^{α}-tosyl- and N^{α}-benzoyl-derivatives of arginine, lysine and ornithine esters are good substrates for trypsin, we examined the synthesis of two α-azaornithine esters (1; R=C_6H_5CO– and R=tosyl).



1 2 3 4

This route followed a general method often used for the preparation of α-azamino-acid derivatives (e.g. H.-J. Hess *et al.*,[4] Kurtz and Niemann[5]) which involves the preparation and subsequent reduction of a hydrazone followed by the reaction of the hydrazide produced with an aryl or alkyl chloroformate. The synthesis reported here, however, represents, so far as we are aware, the first synthesis of an α-aza analogue with a side-chain carrying a reactive functional group other than the relatively unreactive carboxamido-group of α-azaasparagine.[6]

3-Amino-1-propanol was converted into the N-t-butyloxycarbonyl (Boc)—derivative by a standard method. Oxidation to the corresponding aldehyde (2) was accomplished by the use of chromium (6) oxide:dipyridine complex dissolved in dichloromethane (see Ratcliffe and Rodehorst[7]), the use of acidic conditions thus being avoided. The tosylhydrazone (3; R=tosyl) of this aldehyde proved to be resistant to hydrogenation and attempts to reduce the imino-group using sodium borohydride were also unsuccessful, a reductive decomposition apparently occurring instead. Caglioti *et al.*,[8] have reported such a decomposition when other tosylhydrazones have been treated with sodium borohydride. However, the benzoylhydrazone (3; R=C_6H_5CO) was reduced by catalytic hydrogenation to the hydrazide (4; R=C_6H_5CO, R^1=H). This was then converted into the phenyl carbazate (4; R=C_6H_5CO, R^1=CO_2Ph) by treatment with phenyl chloroformate.

Removal of the Boc-group using trifluoracetic acid gave the α-aza-ornithine derivative (1; R=C_6H_5CO–) as the trifluoroacetate, which was converted into the picrate for characterisation.

As indicated by TLC, N^{α}-benzoyl-α-azaornithine phenyl ester trifluoroacetate was stable in aqueous solution at pH 3 for several weeks at 4°. At pH 6·2 or 7·6 the analogue decomposed to give two new ninhydrin positive components. Phenol was also produced.

This instability in neutral solutions contrasts strongly with the relative stability of N-acetyl-α-azaphenylalanine phenyl ester[3] under similar conditions.

It is possible that the decomposition of the α-azaornithine derivative (1, R=C_6H_5CO–) might be due to some intramolecular process involving the unprotonated side chain amino-group. Investigations into the nature of this decomposition process are under way.

However, despite its instability in aqueous solution, N^{α}-benzoyl-α-azaornithine phenyl ester trifluoroacetate had a sufficiently long life-time to react with trypsin at pH 6·2 and 7·6 at 24°.

Figure 1 shows how the activity of trypsin varied with time on treatment with N^{α}-benzoyl-α-azaornithine phenyl ester at pH 4·6, 6·2 and 7·6. At pH 4·6 no

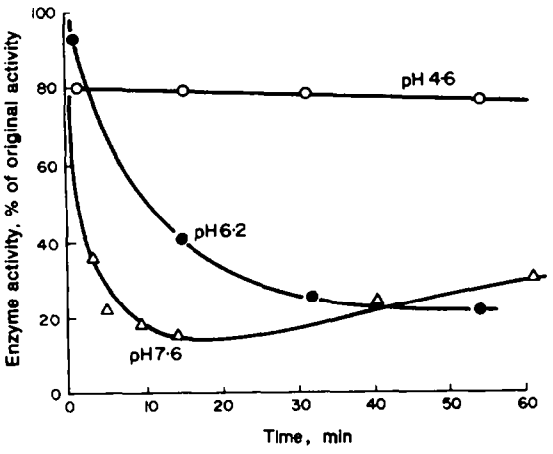


Fig. 1. Variation with time of activity of trypsin treated with N-benzoyl-α-azaornithine phenyl ester. Trypsin (4·8 nmol, in buffer, 1·0 ml) was treated with N-benzoyl-α-azaornithine phenyl ester (1·0 μmol in water 0·10 ml) at 24°, as described in the Experimental.

progressive loss of activity was observed. At pH 6·2 and 7·6 there occurred a rapid decrease followed by a gradual regeneration of activity (e.g. Fig. 2). Both the loss and the regeneration of activity were more rapid at pH 7·6 than 6·2. By analogy with previous work on the reaction of chymotrypsin with the *p*-nitrophenyl[9] and phenyl[3] esters of N-acetyl-α-azaphenylalanine, the rapid decrease in tryptic activity is due to the acylation of the serine residue at the active site to give N^{α} - benzoyl - α - azaornithyl - trypsin. The regeneration of activity is a result of the spontaneous deacylation of this acyl-trypsin. A large excess of analogue was used in these experiments and the fact that activity began to return after a short time indicated that most of the excess analogue had been destroyed within this time. In agreement with this was the observation that pre-incubation of the analogue at pH 7·6 at 24° for 15 min resulted in a lower degree of inhibition.

From plots of log (percentage inactive enzyme remaining) against time, it was possible to calculate first-order rate constants for the reactivation process. Slopes were calculated by a least squares procedure. The rate constants were found to be $1{\cdot}0 \times 10^{-4}\ s^{-1}$ (pH 7·6, 24°) and $2{\cdot}5 \times 10^{-5}\ s^{-1}$ (pH 6·2, 24°).

An attempt to isolate N^{α} - benzoyl - α - aza - ornithyl - trypsin free of inhibitor and by-products gave a preparation which possessed an activity of 23·5% relative to a control preparation of trypsin and which therefore contained a large amount of unacylated enzyme. Deacylation at pH 7·6 and 23·8° of this sample gave a value of $2{\cdot}1 \times 10^{-4}\ s^{-1}$ for the first-order rate constant which is in fair agreement with the value of $1{\cdot}0 \times 10^{-4}\ s^{-1}$ obtained when no isolation procedure had been carried out. Indeed the slightly lower value for the latter may be attributed to the presence of excess aza analogue.

These rate constants are of the same order of magnitude as that determined for the deacylation of N - acetyl - α - aza - phenylalanyl - chymotrypsin.[3]

The acyl-trypsin was unstable at 4° at pH 7·6 and regained activity to the level of 53% after 20 hr (Table 1). This is in contrast to N - acetyl - α - azaphenylalanyl - chymotrypsin[3] which was stable at pH 7·0 at 4° for at least 16 hr.

N^{α}-Benzoyl-α-azaornithine phenyl ester reacted with chymotrypsin, causing loss of activity but the reaction was, not unexpectedly, considerably slower than its

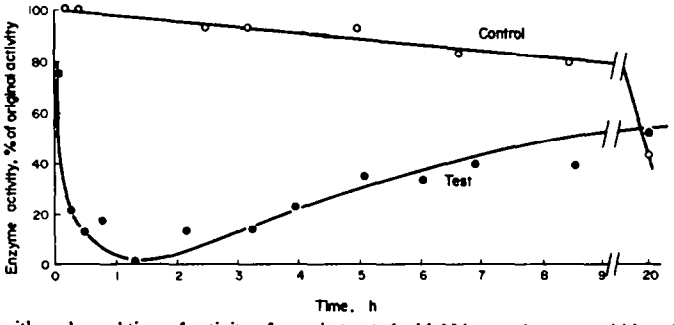


Fig. 2. Variation with prolonged time of activity of trypsin treated with N-benzoyl-α-azaornithine phenyl ester at pH 6·2. Trypsin (3·74M in 0·05M phosphate buffer, pH 6·2) was treated with N-benzoyl-α-azaornithine phenyl ester (1·06 mM) at 24°C.

Table 1. Preparation and reactivation of N^{α}-benzoyl-α-azaornithyl-trypsin. The acyl-trypsin was isolated by gel filtration at 4°C and stored at this temperature. The specific activities of this preparation and of suitable controls were determined 1 and 20 hr after isolation

Time when Assayed (h)	Description of Sample	Specific Activity (% of Control A)
1	(i) Preparation (containing acyl-trypsin)	23.5
1	(ii) Control A (unchromatographed trypsin)	100
1	(iii) Control B (chromatographed trypsin)	96
20	(iv) Preparation; stored at 4°C	53.0
20	(v) Control A; stored at 4°C	100
20	(vi) Control B; stored at 4°C	99

reaction with trypsin, and than the reaction of the azaphenylalanine derivative with chymotrypsin.[3] For example, the reaction of 1·55 μmol of analogue with chymotrypsin (4·9 nmol) in 1·1 ml. of pH 6·2 buffer at 25° gave a progressive loss of activity reaching approx. 45% after 40 min.

EXPERIMENTAL

Trypsin (EC 3.4.21.4) (2 × crystallised; ethanol precipitate, essentially salt-free) and chymotrypsin (EC 3.4.21.1) [3 × crystallised; prepared free of autolysis products and contaminants by the method of Yapel *et al.*[10]] were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Human serum albumin for protein standard curves was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. TLC was carried out by using Merck "Kieselgel G nach Stahl" and the following solvent systems: A, butanol-acetic acid-water (4:1:5, by vol); B, hexane-ethyl acetate (1:1, by vol); C, ethanol-chloroform (1:24, by vol); D, ethanol-chloroform (1:9, by vol); components were detected by using iodine vapour, ninhydrin or the chlorine spray of Pan and Dutcher.[11]

Proteinase activities of trypsin and chymotrypsin preparations were determined by the casein method of Laskowski,[12] modified to allow the concentration of hydrolysis products in the supernatant after precipitation by trichloroacetic acid to be determined by using the Folin-Ciocalteu reagent in a manner similar to that described by Anson.[13] It was established for the enzyme assays that over the range required the method was linear with respect to both time and enzyme concentration. Protein concentrations were determined by the method of Lowry *et al.*[14]

3-Amino-1-propanol was obtained from Koch-Light Laboratories Ltd. The molecular weights of trypsin and chymotrypsin were assumed to be 23 280 and 25 310,[15] respectively.

N - Boc - 3 - amino - 1 - propanol. A soln of t-butylazidoformate[16] (23·9 g; 0·168 mol), 3-amino-1-propanol (12·6 g; 0·168 mol) and triethylamine (33·9 g; 0·334 mol) in a mixture of dioxane (120 ml) and water (50 ml) was heated at 50° for 20 hr and then extracted with ether. The ether extracts were washed with 0·5M citric acid soln and water, dried and evaporated to give the *protected alcohol* as an orange oil (16·4 g). TLC: chlorine positive: R_{f_A} 0·87, R_{f_B} 0·19. IR λ_{max} (CH_2Cl_2): 1700 (C=O), 3450 (N–H) and 3620 cm^{-1} (OH). NMR ($CDCl_3$): 4·56 (broad t; 1 proton: N–H), 6·12 (broad s; 1 proton: O–H), 6·42 (t; 2 protons: $-CH_2^c-CH_2^d-OH$, $J_{c,d} = 6$ c/s), 6·82 (q; 2 protons: $-NH^b-CH_2^a-CH_2^c$, $J_{a,b} = J_{a,c} = 6$ c/s), 8·37 (quintet; 2 protons: $-CH_2-CH_2-CH_2-$) and 8·56 τ (s; 9 protons: t-butyl). The protected alcohol was characterised as its *O-3,5-dinitrobenzoyl derivative* (N - Boc - 3 - amino - 1 - propyl 3,5-dinitrobenzoate), m.p. 126–127° (Found: C, 48·8; H, 5·6; N, 11·4. $C_{15}H_{19}N_3O_8$ requires: C, 48·5; H, 5·7; N, 11·3%).

Boc-3-aminopropanal. To a soln of CrO_3 (analytical reagent, 13·7 g; 137 mmol) and dry pyridine (21·5 g; 272 mmol) in purified[7] dichloromethane (340 ml) was added, after 15 min, a soln of N - Boc - 3 - amino - 1 - propanol (4·0 g; 23 mmol) in a small volume of purified dichloromethane. A black tar precipitated and after 20 min at room temp, the dichloromethane was decanted off and the tar washed with ether. The organic solns were combined and washed with 0·5 M citric acid and water, dried and evaporated to give the aldehyde as an oil (2·9 g). TLC iodine positive; R_{f_B} 0·40 (intense), R_{f_B} 0·22 (faint) and R_{f_B} 0·66 (faint). IR λ_{max} (CH_2Cl_2): 1720 (C=O), 2730 (O=C–H) and 3450 cm^{-1} (N–H). NMR ($CDCl_3$): 0·14 (s; 1 proton: $-CH_2^b-CO-H^a$, $J_{a,b} = 0$ c/s), 4·90 (broad s: 1 proton: $-NH-CH_2-$), 6·52 (q; 2 protons: $-NH-CH_2^c-CH_2^b-$, $J_{c,b} = 6$ c/s), 7·26 (t; 2 protons: $-CH_2^c-CH_2^b-CO-H$) and 8·58 τ (s; 9 protons: t-butyl).

Boc-3-aminopropanal tosylhydrazone. Tosylhydrazide[17] (1·41 g; 7·56 mmol) was added to Boc-3-aminopropanal (1·31 g; 7·57 mmol) in benzene (100 ml) and the soln left at room temp for 15 hr and then evaporated to give a solid residue. TLC R_{f_B} 0·27 (major; chlorine and iodine positive) and R_{f_B} 0·53 (minor; iodine positive and chlorine negative). The residue was chromatographed on silica gel using an ethanol-chloroform system of increasing polarity and the major component isolated and identified as the *hydrazone* (yield 50%). Recrystallisation was achieved from chloroform-hexane, m.p. 144–145°. (Found: C, 52·8; H, 6·5; N, 12·0; S, 9·6. $C_{15}H_{23}N_3O_4S$ requires: C, 52·8; H, 6·8; N, 12·3; S, 9·4%); IR λ_{max} (nujol): 1680 (C=O), 3080 and 3330 cm^{-1} (N–H). NMR (CD_3CN): 2·5 (m; 4 protons: aromatic), 2·82 (t; 1 proton: $-CH_2^b-CH^a=N-$, $J_{a,b} = 3$ c/s), 4·80 (broad t; 1 proton: $-NH^a$), 6·86 (q; 2 protons: $-NH^a-CH_2^b-$, $J_{a,b} = J_{b,c} = 6$ c/s), 7·60 (s; 3 protons: $Ph-CH_3$), 7·73 (m: 2 protons: $-CH_2-CH_2-CH=N-$) and 8·62 τ (s; 9 protons: t-butyl).

Treatment of Boc-3-aminopropanal tosylhydrazone with sodium borohydride. A mixture of $NaBH_4$ (1·50 g; 39·7 mmol) and the hydrazone (0·44 g; 1·29 mmol) in EtOH (35 ml) was left at room temp for 2 hr. Water (100 ml) was added and 16 hr later 6M HCl was added to adjust the pH to 8. The soln was extracted with ether and the extracts dried and evaporated to give an unknown white solid (0·32 g). TLC R_{f_B} 0·34 and 0·53 (both chlorine positive and ninhydrin and iodine negative). Mass spectrum: top mass = 169.

Boc-3-aminopropanal benzoylhydrazone. Benzoylhydrazide[18] (1·23 g; 9·05 mmol) was added to Boc-3-aminopropanal (1·57 g; 9·08 mmol) in EtOH (30 ml) and the soln left at room temp for 20 hr and then evaporated to give a brown oil. A chloroform soln of the oil was washed with 0·5M citric acid and water, dried and evaporated to give a pink solid. TLC: iodine positive; R_{f_C} 0·24 (major) and 0·38 (minor). The product was chromatographed on silica gel using an ethanol-chloroform system of increasing polarity and the major component isolated and identified as the *hydrazone*, m.p. 145–148°. IR λ_{max} (nujol): 1685 and 1650 (C=O) and 3350 and 3240 cm^{-1} (N–H). The NMR spectrum of this compound ($CDCl_3$) was unusual; all the absorptions were very broad and no splittings could be seen. However the chemical shifts and peak areas were consistent with the expected structure.

N-Benzoyl-N²-(3-Boc-amidopropyl)-hydrazide. Unchromatographed boc-3-amidopropanal benzoylhydrazone (1·31 g) in EtOH (100 ml) was hydrogenated over 10% Pd-C (0·40 g) for 22 hr at room temp and atmospheric pressure. The soln was filtered and evaporated to give a light brown residue (1·18 g). TLC: iodine positive; R_{f_C} 0·23 (faint), 0·34 (intense), 0·39 (faint) and 0·47 (faint). The product was chromatographed on silica gel using an ethanol-chloroform system of increasing polarity and the major component was isolated and identified as the *hydrazide*. Recrystallisation from chloroform-hexane gave colourless crystals, m.p. 111–113°. (Found: C, 61·8; H, 7·9; N, 14·3. $C_{15}H_{23}N_3O_3$ requires: C, 61·4; H, 7·8; N, 14·3%); IR λ_{max} (Nujol): 1690 and 1635 (C=O), 3370, 3300 and 3220 cm^{-1} (N–H). NMR ($CDCl_3$): 1·48 (s; 1 proton: $-NH^a-NH^1-CO$), 2·4 (m; 6 protons: aromatic + NH^a–), 5·03 (broad t; 1 proton: $NH^a-CH_2^b$–), 6·9 (q; 2 protons: $NH^a-CH_2^b-CH_2^c$–, $J_{a,b} = 6{\cdot}0$, $J_{b,c} = 6{\cdot}5$ c/s), 7·17 (t; 2 protons; $CH_2^c-CH_2^d-NH^a$–, $J_{c,d} = 6{\cdot}5$ c/s), 8·42 (q; 2 protons: $CH_2^b-CH_2^c-CH_2^d$, $J_{b,c} = J_{c,d} = 6{\cdot}5$ c/s) and 8·7 τ (s; 9 protons: t-butyl).

N^a-Benzoyl-N^a-Boc-α-azaornithine phenyl ester (phenyl N^2-benzoyl-N^1-(3-Boc-amidopropyl)-carbazate). N^2-Benzoyl-N^1-(3-Boc-amidopropyl)-hydrazide (chromatographically pure) (0·70 g; 2·39 mmol) in EtOAc (125 ml) containing triethylamine (0·24 g; 2·36 mmol) was treated at 0° with a soln of phenyl chloroformate (0·38 g; 2·43 mmol) in EtOAc (2 ml). The mixture was stirred at 0° for 1·5 hr then washed with water, 0·1M Na_2CO_3, 0·5M citric acid and water, dried and evaporated to give a brown oil. TLC: iodine positive: R_{f_C} 0·33 (faint), 0·38 (faint), 0·44 (faint) and 0·52 (intense). The product was chromatographed on silica gel using an ethanol-chloroform system of increasing polarity and the major component was isolated and identified as the *phenyl ester*, m.p: softened at 25°, shrunk at 41°. (Found: C, 64·3; H, 6·7; N, 10·3. $C_{22}H_{27}N_3O_5$ requires: C, 64·0; H, 6·5; N, 10·2%); IR λ_{max} (nujol): 1660–1740 (C=O) and 3300 cm^{-1} (N–H). NMR ($CDCl_3$): 0·55 (broad s; 1 proton: N–H), 2·6 (m; 10 protons; aromatic), 4·95 (broad t; 1 proton; N–H), 6·25 (broad t; 2 protons: $-CH_2^b-CH_2^a-N-NH-$, $J_{a,b} = 6{\cdot}5$ c/s), 6·75 (q; 2 protons: $-CO-NH^d-CH_2^c-CH_2^b-$, $J_{c,d} = J_{c,b} = 6{\cdot}6$ c/s), 8·15 (quintet; 2 protons: $-CH_2^c-CH_2^b-CH_2^a-$) and 8·70 τ (s; 9 protons: t-butyl).

N^a-Benzoyl-α-azaornithine phenyl ester trifluoroacetate (phenyl N^2-benzoyl-N^1-(3-aminopropyl) carbazate trifluoroacetate salt). A soln of N^a-benzoyl-N^a-Boc-α-azaornithine phenyl ester (chromatographically pure) (34 mg) in trifluoroacetic acid

(2·0 ml) was left at room temp for 1·5 hr and then evaporated to dryness leaving an oil. TLC: (i) aqueous soln at pH 3: R_{f_A} 0·71 (ninhydrin positive), (ii) aqueous soln at pH 7: R_{f_A} 0·61 and 0·55 (both ninhydrin positive). Phenol was also formed R_{f_D} 0·36 (iodine positive). The product was characterised as its *picrate derivative* (N$^{\alpha}$-benzoyl-α-azaornithine phenyl ester picrate), m.p. 167–169°. (Found: C, 51·0; H, 4·1; N, 15·5. $C_{23}H_{22}N_6O_{10}$ requires: C, 51·0; H, 4·1; N, 15·5%); IR λ_{max} (nujol): 1710 and 1665 cm^{-1} (C=O).

Enzyme studies

The reaction of N$^{\alpha}$-benzoyl-α-azaornithine phenyl ester trifluoroacetate with trypsin at pH 4·6, 6·2 and 7·6. A soln of trypsin (4·8 nmol) in 0·05M sodium acetate buffer, pH 4·6 (1·00 ml) was incubated at 24°C with N$^{\alpha}$-benzoyl-α-azaornithine phenyl ester trifluoroacetate (1·0 μmol) in water (0·10 ml). Tryptic activity was estimated periodically by the casein assay using an assay incubation period of 5 min at a temperature of 24°. Simultaneously, a control experiment was carried out in which water only was added to the enzyme soln. Similar experiments were performed using 0·05M-sodium phosphate buffer, pH 6·2 and 7·6 in place of the sodium acetate buffer. A similar experiment was performed in which the N$^{\alpha}$-benzoyl-α-azaornithine phenyl ester trifluoroacetate solution was kept at pH 7·6 at 24° for 15 min before being added to the enxyme solution.

Preparation of N$^{\alpha}$-benzoyl-α-azaornithyl-trypsin. To trypsin (18·9 nmol) in 0·5M-sodium phosphate buffer, pH 7·6 (0·2 ml) was added an aqueous soln of N$^{\alpha}$-benzoyl-α-azaornithine phenyl ester trifluoroacetate (0·40 ml; 5·0 μmol). The mixture was incubated at 25° for 15 min and then chromatographed at 4° on a column (10 × 200 mm) of Sephadex G-25 equilibrated with 0·05M-sodium phosphate buffer, pH 7·6. The protein fractions eluted were pooled and stored at 4°. The specific activity was determined by the casein and Lowry *et al.*[14] assays. A control preparation was obtained in the same way except that water was used in place of the analogue solution.

Reactivation at 23·8° at pH 7·6. Samples of deactivated and control trypsin were incubated at 23·8° for several hr. Aliquots were removed at intervals and assayed for tryptic activity by the casein assay using an assay incubation period of 5 min at a temperature of 23·8°.

The reaction of N$^{\alpha}$-benzoyl-α-azaornithine phenyl ester trifluoroacetate with chymotrypsin. A soln of α-chymotrypsin (4·9 nmol) in 0·05M-sodium phosphate buffer, pH 6·2 (1·00 ml) was incubated at 25° with N$^{\alpha}$-benzoyl-α-azaornithine phenyl ester trifluoroacetate (1·55 μmol) in water (0·10 ml). Chymotryptic activity was estimated periodically by the casein assay using an assay incubation period of 10 min at a temperature of 25°. A control experiment was conducted in which water alone was added to the enzyme soln.

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