

# Synthesis and Catalytic Properties of Peptides containing Amino-acids involved in the Active Centres of Hydrolytic Enzymes

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Seven oligopeptides of the type indicated in the title have been synthesised and found to catalyse the hydrolysis of *p*-nitrophenyl acetate with a catalytic activity of *ca.* 30–50% of that of imidazole.

BOTH the amino-acid sequence in the region of the 'active serine' residue and the sequence near the histidine residue of the 'active site' are similar in such hydrolytic enzymes as chymotrypsin, trypsin, and elastase.<sup>1</sup> This similarity in primary structure suggests that the tertiary structures could also be similar, in which case the amino-acid residues comprising the active centres of these enzymes would be in similar conformations.<sup>2</sup> The histidine sequence, which is far removed from the active serine sequence in the primary structures of these enzymes, contains a second histidine residue close to the first one, and a mechanism of action of  $\alpha$ -chymotrypsin has been proposed<sup>3</sup> in which both histidine residues participate in the catalytic process.

Many relatively small peptides containing the amino-acid residues corresponding to the active centres of enzymes have been synthesised and studied as 'hydrolase models'<sup>4</sup> in comparison with  $\alpha$ -chymotrypsin and imidazole.<sup>5,6</sup> In most cases the observed catalytic activity is significant but low, probably owing to the inability of the molecule to realise a conformation similar to that of the active centre of a real enzyme.

Histidine and serine residues are usually contained in such enzyme models. In the present investigation the peptides (D)–(G), each containing one serine and two histidine residues, and the smaller peptides (A)–(C) have been prepared and used as hydrolase models in comparison with chymotrypsin and imidazole. Molecular models indicate that a molecule containing one serine and two histidine residues could show considerable flexibility if there are two amino-acid residues between the two histidines and also between the serine and the first histidine unit. To separate the serine and histidine in this way glycine was chosen, in order to avoid complications resulting from side-chain interactions or steric hindrance.

Gly-His-Gly (A)

Phe-Gly-His-Gly (B)

Gly-His-Gly-Gly-His-Gly (C)

Ser-Gly-Gly-His-Gly-Gly-His-Gly (D)

Asp-Ser-Gly-Gly-His-Gly-Gly-His-Gly-OEt (E)

Ser-Gly-Gly-His-Gly-Gly-His-Gly-Asp (F)

Gly-Asp-Ser-Gly-Gly-His-Gly-Gly-His-Gly-OEt (G)

In addition to serine, histidine, and glycine, aspartic acid is included in the nonapeptides (E) and (F), and the decapeptide (G) contains the sequence around the active serine residue of some proteolytic enzymes, *i.e.* Gly-Asp-

Ser-Gly-Gly. A glycine residue was included at the end of the peptide chain to give the molecule still more flexibility.<sup>7</sup> The tetrapeptide (B) was prepared in order to test the influence of the phenylalanine<sup>8</sup> residue on the catalytic activity of the tripeptide (A).

The synthetic route to the peptides (A)–(G) is shown in Schemes 1 and 2. The starting material *N*-benzyl-oxycarbonylglycyl-L-histidylglycine ethyl ester (I) was prepared by a slight modification of a literature method.<sup>9</sup> Saponification of the protected hexapeptide (V) and octapeptide (VII) proved difficult, so the catalytic hydrogenolysis was performed first.<sup>10</sup> Compounds (X) and (E) in the crystalline state proved insoluble even in dimethylformamide; for coupling purposes, these compounds were therefore used in the oily state, *i.e.* before crystallization.

Peptides (A)–(G) showed a catalytic activity for the hydrolysis of *p*-nitrophenyl acetate (*p*-NPA) (expressed by the catalytic coefficient  $k_2^{(4a)}$ ) of *ca.* 30–50% of that of imidazole (see Table). First-order kinetics were

Hydrolysis of *p*-nitrophenyl acetate in 0.2M-phosphate buffer (pH 7.7) containing 2.7% dioxan (v/v) at 27–28 °C; [*p*-nitrophenyl acetate] = 32  $\mu\text{mol l}^{-1}$

Catalyst	[His] $\times 10^4$ / $\text{mol l}^{-1}$	$k_1 \times 10^4$ / $\text{min}^{-1}$	$k_2/\text{l mol}^{-1}$ $\text{min}^{-1}$
None		42	
Imidazole	4.5	186	32
(A)	4.4	104	14
(B)	4.1	84	10
(C)	4.4	183	16
(D)	1.5	76	11
(D)	2.25	93	11
(D)	4.5	136	10
(E)	4.4	145	12
(F)	4.7	124	9
(G)	1.48	75	11
(G)	4.5	134	10

<sup>4</sup> Cf. *e.g.* (a) E. Katchalski, G. D. Fasman, E. Simon, F. R. Blout, F. R. N. Gurd, and W. L. Koltun, *Arch. Biochem. Biophys.*, 1960, **88**, 361; K. D. Kopple, D. E. Nitechi, *J. Amer. Chem. Soc.*, 1961, **83**, 4103; C. G. Overberger, T. St. Pierre, N. Vorschheimer, and S. Yaroslavsky, *J. Amer. Chem. Soc.*, 1963, **85**, 3513; F. Schneider, *Z. physiol. Chem.*, 1967, **348**, 1034; G. Losse and H. Weddige, *Annalen*, 1964, **678**, 148; J. Sheehan, G. Bennett, and J. Schneider, *J. Amer. Chem. Soc.*, 1966, **88**, 3455; W. H. Carr, *Diss. Abs.*, 1968, **B28** (7), 2711; K. Nakajima and K. Okawa, *Bull. Chem. Soc. Japan*, 1973, **46**, 1811; M. Fridkin and H. J. Goren, *European J. Biochem.*, 1974, **41**, 273; (b) I. Photaki, V. Bardakos, A. W. Lake, and J. Lowe, *J. Chem. Soc. (C)*, 1968, 1860; (c) I. Photaki and S. Moschopedis, *Experientia*, 1969, **25**, 903.

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<sup>9</sup> R. F. Fischer and R. R. Whetstone, *J. Amer. Chem. Soc.*, 1954, **56**, 5078.

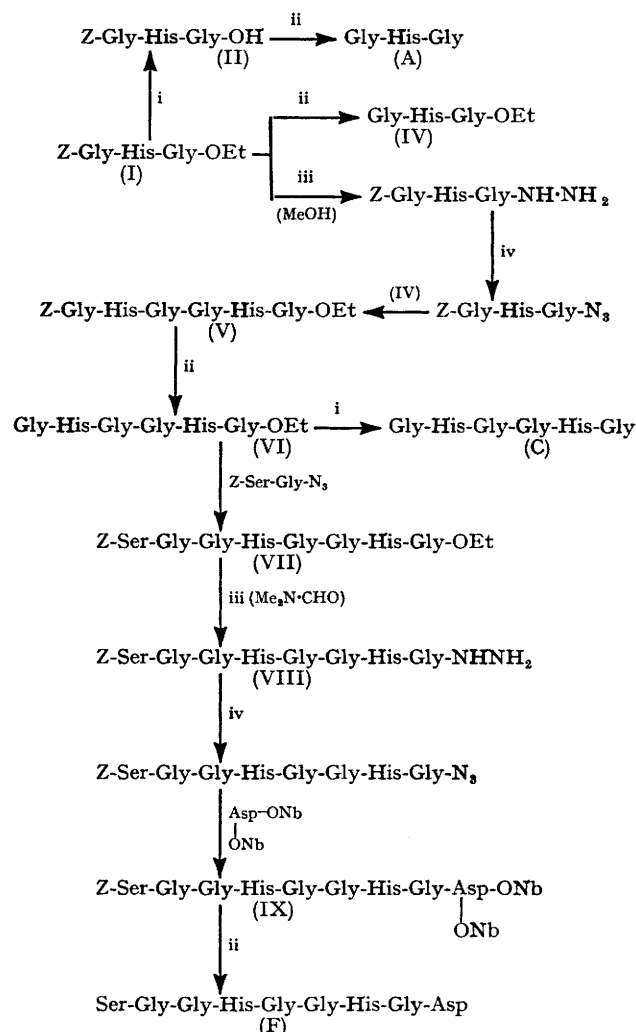
<sup>10</sup> R. Schwyzler, B. Iselin, H. Kappeler, B. Riniker, W. Rittel, and H. Zuber, *Helv. Chim. Acta*, 1958, **41**, 1287.

<sup>1</sup> M. L. Bender and F. J. Kezdy, *Ann. Rev. Biochem.*, 1965, **34**, 49.

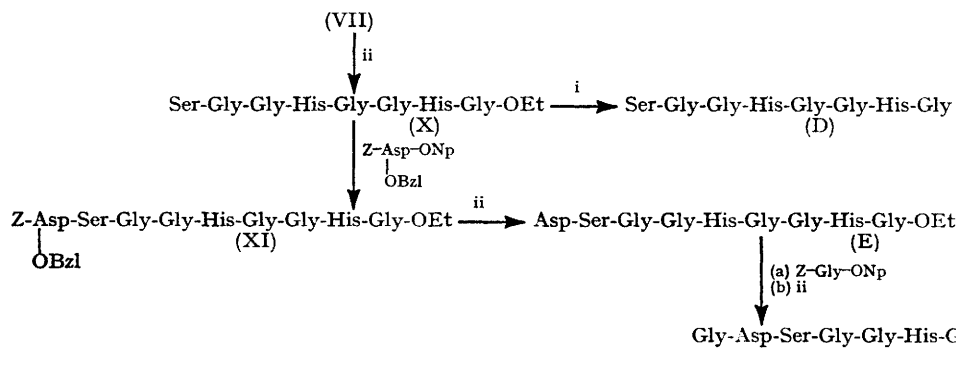
<sup>2</sup> A. Williams, *Quart. Rev.*, 1969, **23**, 1.

<sup>3</sup> B. S. Hartley in 'Structure and Activity of Enzymes,' ed. T. W. Goodwin, J. I. Harris, and B. S. Hartley, Academic Press, 1964, p. 47.

observed from *ca.* 10% to *ca.* 70% completion of the reaction. The liberation of *p*-nitrophenol was followed



SCHEME 1 Reagents: *i*, N-NaOH added to aqueous or alcoholic solution; *ii*, Pd-H<sub>2</sub> in AcOH containing water or water and an alcohol; *iii*, NH<sub>2</sub>NH<sub>2</sub>; *iv*, H<sup>+</sup>-NaNO<sub>2</sub>; Nb = *p*-nitrobenzyl



SCHEME 2 Reagents: *i*, N-NaOH added to aqueous or alcoholic solution; *ii*, Pd-H<sub>2</sub> in AcOH containing water

by u.v. spectrometry at 400 nm. Peptides (D) and (G) were tested at various concentrations. The molar

\* For details of Supplementary Publications see Notice to Authors No. 7, *J.C.S. Perkin I*, 1975, Index issue.

concentrations of the peptides (A)–(G) were calculated on the basis of histidine residues. The catalytic coefficient *k*<sub>2</sub> of peptides (D)–(G) would be doubled, but still lower than that of imidazole itself, if the concentrations of the peptides were calculated in terms of a mechanism involving both histidine residues.<sup>2</sup>

A change in optical density at 245 nm, indicating the probable formation and decomposition of an *N*<sup>im</sup>-acetyl peptide intermediate,<sup>5</sup> was observed in the case of the tripeptide (A) when used at a concentration of  $5 \times 10^{-3}$  mol l<sup>-1</sup> in the presence of *p*-NPA ( $1.2 \times 10^{-4}$  mol l<sup>-1</sup>). The peptides (D)–(G) containing two histidine residues could not be compared since solutions in this range of concentration could not be prepared.

Finally, the possibility of hydrolysis of *p*-NPA by nucleophilic attack by the α-NH<sub>2</sub> group<sup>11</sup> was excluded by electrophoresis of mixtures where the substrate concentration was greater (10 ×) than that of the peptide catalyst. After 2–4 days incubation only peptide with free α-NH<sub>2</sub> group was detected.

A comparison of the *k*<sub>2</sub> values obtained for various synthetic peptides, including those reported here, does not produce any general conclusions which could help to predict the catalytic ability of a new peptide. Before any more new peptides are synthesised as esterase models, more information about their tertiary structure in relation to that of the enzymes is needed.

#### EXPERIMENTAL

The whole Experimental section is available as Supplementary Publication No. SUP 21 631 (17 pp., 1 microfiche).<sup>\*</sup> Only key experiments are also described here.

Anhydrous solvents were used for the coupling reactions. M.p.s were taken for samples in capillary tubes. Before analysis, compounds were dried over P<sub>2</sub>O<sub>5</sub> at room temperature under high vacuum; microanalyses were performed at the Analytical Laboratory of F. Hoffman-La Roche and Co., Basle, Switzerland, under the direction of Dr. A. Dirscherl, or by A. Bernhardt, Elbach, Germany.

Amino-acid analyses were carried out at the Nuclear Research Centre 'Demokritos', Aghia Paraskevi Attikis,

Greece. *R<sub>F</sub>* Values refer to t.l.c. on Kieselgel G (Fluka) containing 13% Gibs in the following solvent systems:

<sup>11</sup> W. L. Koltun and F. R. N. Gurd, *J. Amer. Chem. Soc.*, 1959, **81**, 301.

(proportions by volume): (1) butan-1-ol-acetic acid-water (6:2:2); (2) butan-1-ol-acetic acid-water (4:1:1); (3) butan-1-ol-acetic acid-water-pyridine (30:6:24:20); (4) phenol-water (3:1); (5) chloroform-methanol (4:1); (6) propan-1-ol-33% ammonium hydroxide (67:33). Substances with a free amino-group were detected with ninhydrin, and *N*-protected derivatives with iodine. Histidine-containing compounds were also detected by use of the Pauly reagent.<sup>12</sup> For paper electrophoresis, an LKB 3 276 apparatus was used, with Schleicher and Schüll 2 043-B paper, or a high-voltage Pherograph-L Hormuth apparatus, with MN-Düren paper. The following solvent systems were employed: (a) acetic acid (5.7 ml)-pyridine (8 ml)-water (to 1 l) (pH 4.9); (b) acetic acid (18.25 ml)-pyridine (8 ml)-water (to 1 l) (pH 3.9); (c) 0.5*N*-acetic acid; (d) acetic acid-formic acid (pH 1.85).<sup>7</sup> Unless otherwise stated compounds moved as single bands toward the cathode. Neutralization equivalents were found by titration with perchloric acid in acetic acid.<sup>13</sup>

Optical rotations were measured with a Perkin-Elmer automatic polarimeter (1 dm cell). Kinetics were followed with a Zeiss RPQ 20A spectrophotometer.

**Glycyl-L-histidylglycine Ethyl Ester (IV) Acetate.**—The *N*-protected tripeptide ester (I) (4 g, 0.0092 mol) in 90% acetic (130 ml) was hydrogenated at room temperature and pressure during 1 h over palladium-charcoal (10%; 0.5 g). The mixture was then filtered and washed with water. The filtrate was evaporated to dryness *in vacuo*. The excess of acid was removed by addition and evaporation of ethyl alcohol several times, and the residue was extracted with ethyl alcohol giving the ester acetate (2.3 g, 70%), m.p. 161° [(2 g, 60%), m.p. 163–165° after trituration with hot acetone-nitrile],  $[\alpha]_D^{24} - 8.9^\circ$  (*c* 2 in *N*-HCl),  $R_F$  (3) 0.6, neutralization equiv. 361 (calc. 357) (Found: C, 46.8; H, 6.6; N, 19.4.  $C_{12}H_{19}N_5O_4 \cdot AcOH$  requires C, 46.9; H, 6.8; N, 19.5%).

**Glycyl-L-histidylglycine (A) Acetate.**—A suspension of compound (II) (1.5 g, 0.0039 mol) in water (31 ml), methanol (62 ml), and acetic acid (3.5 ml) was hydrogenated as described for compound (IV). The residue was dissolved in methanol and precipitated with ether to give the crude acetate (1.1 g, 90%), m.p. 217° [218–219° after recrystallization from water (recovery 60%)],  $[\alpha]_D^{24} - 10.5^\circ$  (*c* 1 in 0.1*N*-NaOH),  $R_F$  (4) 0.4; paper electrophoresis (350 V; 3 h) in system (a) (Found: C, 41.95; H, 5.85; N, 22.1.  $C_9H_{14}N_5O_4 \cdot AcOH$  requires C, 41.8; H, 5.7; N, 22.1%).

***N*-Benzyloxycarbonylglycyl-L-histidylglycylglycyl-L-histidylglycine Ethyl Ester (V).**—(a) Compound (I) (1.3 g, 0.003 mol) was dissolved in methyl alcohol (10 ml) by gently warming. The solution was allowed to cool to room temperature and hydrazine hydrate (1.2 ml) was added. The crystalline *N*-benzyloxycarbonylglycyl-L-histidylglycyl hydrazide (III) formed was isolated after 24 h; m.p. 200–201° after boiling in methanol (yield 1 g, 95%),  $[\alpha]_D^{24} - 15^\circ$  (*c* 1 in *N*-HCl),  $R_F$  (3) 0.8; (lit.,<sup>9</sup> m.p. 199–201°).

(b) A solution of the hydrazide (III) (1.25 g, 0.003 mol) in *N*-hydrochloric acid (12 ml) and dimethylformamide (30 ml) was treated at –5 °C with a cold aqueous *M*-sodium nitrite (3.3 ml). After 5 min the ester acetate (IV) (1.071 g, 0.003 mol) was added to the resulting solution of the azide, followed by triethylamine (2.3 ml; pH of the mixture *ca.* 7). The mixture was stirred at 0 °C for 2 h and left at 4 °C for

24 h. The precipitated product was filtered off, washed with a little dimethylformamide, water, 0.5*M*-boric acid, and water, and dried *in vacuo* ( $P_2O_5$  and NaOH); m.p. 245°, unchanged after recrystallization by addition of water to a solution in dimethylformamide; yield 1.6 g (80%),  $[\alpha]_D^{24} - 20^\circ$  (*c* 1 in *N*-HCl),  $R_F$  (3) 0.8 (Found: C, 52.6; H, 5.4; N, 20.4.  $C_{30}H_{38}N_{10}O_9$  requires C, 52.8; H, 5.6; N, 20.5%).

**Glycyl-L-histidylglycylglycyl-L-histidylglycine (C) Acetate.**—The ester (VI) acetate (0.6 g, 0.001 mol) was dissolved by gentle heating in 85% ethanol (13 ml). *N*-Sodium hydroxide (2.8 ml) was added and the solution was stirred for 2.5 h at room temperature then acidified with *N*-hydrochloric acid (2.8 ml). The precipitated crystalline product was recrystallized from water; yield 0.27 g (48%), m.p. >230°,  $[\alpha]_D^{20} - 11^\circ$  (*c* 1 in *N*-HCl),  $R_F$  (4) 0.5; paper electrophoresis in system (a) or (b) (1 500 V; 2 h) and (d) (350 V; 3 h); neutralization equiv. 547 (calc. 582) (Found: C, 45.0; H, 5.5; N, 24.4.  $C_{20}H_{30}N_{10}O_7 \cdot AcOH$  requires C, 45.36; H, 5.9; N, 24.0%).

**L-Serylglycylglycyl-L-histidylglycylglycyl-L-histidylglycine (D) Acetate.**—Compound (X) (0.74 g, 0.001 mol) was dissolved in hot water (15 ml). *N*-Sodium hydroxide (5.6 ml) was added at room temperature; after 3 h a small amount of undissolved material was filtered off, and the solution was acidified with *N*-hydrochloric acid (5.6 ml). The clear solution was passed through a column of Dowex 50 W ( $H^+$ ), which was washed with water until chloride ions were completely removed. The peptide was eluted with 3% ammonium hydroxide. The eluate and washings were concentrated *in vacuo*. Water was added and evaporated off repeatedly and the peptide was precipitated with ethyl alcohol. It was collected by centrifugation; yield 0.5 g (69%), m.p. 233°–234° (from water; recovery 80%),  $[\alpha]_D^{20} - 5.4^\circ$  (*c* 1 in *N*-HCl),  $R_F$  (4) 0.3; paper electrophoresis (350 V; 2.5 h) in system (d) and (1 500 V; 2 h) in system (a); amino acid analysis: His, 2; Ser, 0.755; Gly, 5; neutralization equiv. 727 (calc. 725) (Found: C, 44.5; H, 5.8; N, 23.15.  $C_{25}H_{36}N_{12}O_{10} \cdot AcOH$  requires C, 44.7; H, 5.6; N, 23.2%).

**L-Aspartyl-L-serylglycylglycyl-L-histidylglycylglycyl-L-histidylglycine Ethyl Ester (E).**—The protected peptide ester (XI) (3 g, 0.003 mol) was hydrogenated in 50% acetic acid (150 ml) for 3 h over palladium-charcoal (1.4 g). The mixture was filtered and evaporated (0.5–1 mmHg) and the oily residue was dissolved in ethyl alcohol and precipitated with ethyl acetate. The crystalline material obtained was purified by dissolving in water and precipitating with acetone; yield 2.3 g (90%), m.p. 233–235°,  $[\alpha]_D^{24} - 13.9^\circ$  (*c* 1 in *N*-HCl),  $[\alpha]_D^{24} - 8.7^\circ$  (*c* 1 in glacial AcOH); paper electrophoresis (1 500 V; 2 h) in system (b); amino-acid analysis: His, 1.88; Ser, 0.93; Gly, 5; Asp, 1.11 (Found: C, 46.1; H, 5.7; N, 21.5.  $C_{31}H_{46}N_{13}O_{13} \cdot AcOH$  requires C, 45.7; H, 5.7; N, 21.0%).

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