666. *Polypeptides. Part V.*[1] *The Synthesis of Some Acetylserine Peptides Related to the Active Centre of Chymotrypsin.*

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The two isomeric pairs of peptides, *O*-acetyl-DL-seryl-glycyl-glycine (V) and *N*-acetyl-DL-seryl-glycyl-glycine (VI), and L-aspartyl-*O*-acetyl-L-seryl-glycyl-glycine (X) and *O*-L-aspartyl-*N*-acetyl-L-seryl-glycyl-glycine (XI), have been synthesised for comparison with degradation products of acetylchymotrypsin. All four peptides are stable at pH 2·0, but at pH 7·8 the *O*-acetyl-tripeptide (V) is converted into its isomeride, and the tetrapeptide (XI) is broken down into aspartic acid and the tripeptide (VI).

Although there is now abundant degradative evidence [2] to show that the reactive serine residue of the active centre of chymotrypsin is present in the sequence

–Gly·Asp·Ser·Gly·Gly·Pro·Leu–,

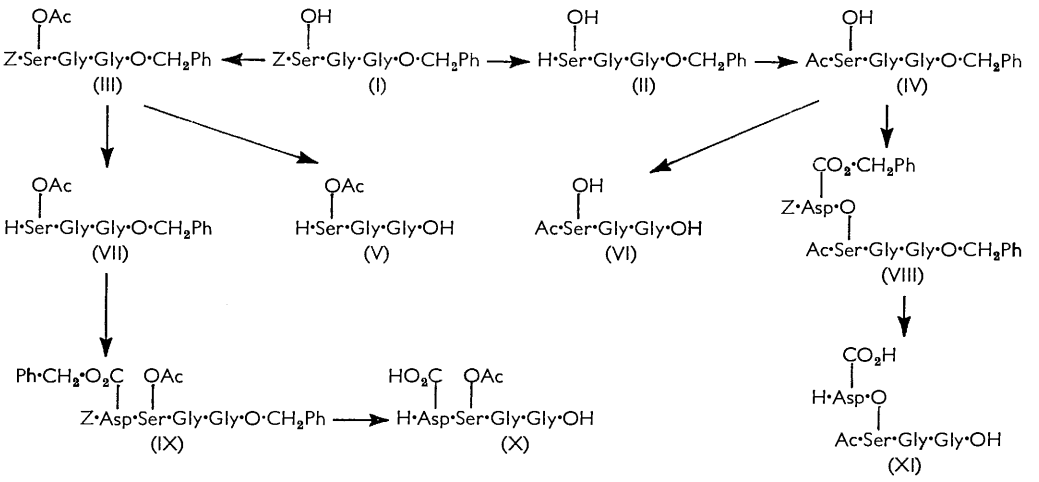
and that similar sequences surround the reactive serines of the active centres of other esterases,[3] synthetic confirmation for this is lacking. Oosterbaan and Van Adrichem [4] recently obtained five peptides of acetylserine by enzymic degradation of acetylchymotrypsin; [5] the largest of these contains the sequence Gly·Asp·Ser·Gly·Gly·Pro·Leu [6] and it is reasonable to suppose that the other four contain the shorter sequences Ser·Gly·Gly·Pro·Leu, Asp·Ser·Gly·Gly, Gly·Asp·Ser·Gly, and Ser·Gly·Gly. The position of the acetyl group in these peptides is, however, not clear and the electrophoretic behaviour [4] of the smallest of them is such as to suggest that it is *N*-acetyl-seryl-glycyl-glycine (VI) rather than the *O*-acetyl-peptide (V).

On the basis of the oxazoline hypothesis [7] concerning the nature of the reactive serine in chymotrypsin and other esterases, the hydrolysis of acetylchymotrypsin might well give rise to *N*-acetyl-*O*-peptides, such as (XI), as well as, or instead of, the isomeric *O*-acetyl-*N*-peptides, such as (X), which are the most likely products on the basis of the alternative histidine-activation hypothesis.[8] We have, therefore, undertaken the synthesis

[1] Part IV, Rydon and Smith, *J.*, 1956, 3642.
[2] Oosterbaan, Kunst, and Cohen, *Biochim. Biophys. Acta*, 1955, **16**, 299; Turba and Gundlach, *Biochem. Z.*, 1955, **327**, 186; Schaffer, Simet, Harshman, Engle, and Drisko, *J. Biol. Chem.*, 1957, **225**, 197; Oosterbaan, Kunst, Van Rotterdam, and Cohen, *Biochim. Biophys. Acta*, 1958, **27**, 549, 556.
[3] Dixon, Go, and Neurath, *Biochim. Biophys. Acta*, 1956, **19**, 193; Oosterbaan, Jansz, and Cohen, *ibid.*, 1956, **20**, 402; Dixon, Kauffman, and Neurath, *J. Amer. Chem. Soc.*, 1958, **80**, 1260.
[4] Oosterbaan and Van Adrichem, *Biochim. Biophys. Acta*, 1958, **27**, 423.
[5] Balls and Wood, *J. Biol. Chem.*, 1956, **219**, 245.
[6] Jansz, Berends, and Oosterbaan, *Rec. Trav. chim.*, 1959, **78**, 876.
[7] Porter, Rydon, and Schofield, *Nature*, 1958, **182**, 927; Rydon, *ibid.*, p. 928.
[8] For references see Davies and Green, *Adv. Enzymol.*, 1958, **20**, 283.

of a number of acetylated and phosphorylated serine peptides related to the active centre of chymotrypsin and now report the synthesis of four such peptides, *viz.*, (V), (VI), (X), and (XI); comparison of these synthetic peptides with those isolated from acetylchymotrypsin is reported elsewhere.[9]

The synthetic scheme was as pictured (the abbreviations are those used by Goodman



and Kenner[10]). We chose to introduce the serine residue initially as the *N*-benzyloxycarbonyl derivative with the side-chain hydroxyl unprotected and to attach the *N*- and *O*-acetyl groups later, partly because of the greater danger of racemisation when using *N*-acetylserine in coupling reactions[11] and partly because we had some difficulty in preparing the intermediates, *O*-acetyl-*N*-benzyloxycarbonyl-[12] and *N*-acetyl-serine,[13] required for the alternative, more direct, approach by the published procedures.

The protected tripeptide (I), the key intermediate for the syntheses, was prepared in satisfactory yield by coupling *N*-benzyloxycarbonylserine with glycyl-glycine benzyl ester, dicyclohexylcarbodi-imide being used in chloroform containing acetonitrile to repress the formation of acylurea;[14] catalytic hydrogenolysis gave seryl-glycyl-glycine. Selective removal of the benzyloxycarbonyl group from the protected tripeptide (I) was carried out by using hydrogen bromide in nitromethane[15] rather than in acetic acid,[16] to avoid the *O*-acetylation to be expected with the latter solvent.[14,17] The resulting benzyl ester (II) was then selectively *N*-acetylated with thioacetic acid,[14] and the resulting *N*-acetyl compound (IV) was catalytically hydrogenolysed to the required *N*-acetylseryl-glycyl-glycine (VI). For the preparation of the isomeric *O*-acetylserylglycyl-glycine (V), the protected tripeptide (I) was *O*-acetylated with acetic anhydride in pyridine, and the product (III) was subjected to catalytic hydrogenolysis.

For the preparation of the tripeptides (V) and (VI), containing only one asymmetric centre, DL-serine was used as starting material, the DL-products sufficing for our purpose. In the case of the tetrapeptides (X) and (XI), which contain two asymmetric centres, it was necessary, in order to avoid the formation of mixtures of diastereoisomerides, to use

[9] Benoiton, Cohen, Oosterbaan, Rydon, and Van Adrichem, *Nature*, in the press.
[10] Goodman and Kenner, *Adv. Protein Chem.*, 1957, **12**, 465.
[11] North and Young, *Chem. and Ind.*, 1955, 1597.
[12] Frankel and Halmann, *J.*, 1952, 2735.
[13] Synge, *Biochem. J.*, 1939, **33**, 1924; Wagner-Jauregg and Short, *Chem. Ber.*, 1956, **89**, 253.
[14] Guttmann and Boissonnas, *Helv. Chim. Acta*, 1958, **41**, 1852.
[15] Albertson and McKay, *J. Amer. Chem. Soc.*, 1953, **75**, 5323.
[16] Ben-Ishai and Berger, *J. Org. Chem.*, 1952, **17**, 1564.
[17] Sheehan, Goodman, and Hess, *J. Amer. Chem. Soc.*, 1956, **78**, 1367.

L-serine and L-aspartic acid as starting materials. Accordingly, the above syntheses were repeated, with L-serine, as far as the intermediates (III) and (IV). Treatment of the former with hydrogen bromide in nitromethane [15] gave the *O*-acetyl benzyl ester (VII); the product was contaminated with some de-acetylated material and was, therefore, re-acetylated with hydrogen chloride in acetic acid [17] before proceeding with the synthesis. Condensation of β-benzyl *N*-benzyloxycarbonyl-L-aspartate with the benzyl ester (VII), by means of dicyclohexylcarbodi-imide in pyridine, afforded the protected tetrapeptide (IX) which, on catalytic hydrogenolysis, gave the required *O*-acetyl-tetrapeptide (X). The isomeric *N*-acetyl-tetrapeptide (XI) was prepared similarly by condensing the *N*-acetyl benzyl ester (IV) with β-benzyl *N*-benzyloxycarbonyl-L-aspartate, and hydrogenolysing the resulting protected tetrapeptide (VIII).

As a preliminary to the comparison of the synthetic acetyl-peptides with those isolated from acetylchymotrypsin, it was desirable to study the stability of the former under the conditions used in the preparation of the latter, *viz.*, pH 2·0 for 16 hr. at 20° and pH 7·8 for 16 hr. at 37°. All four synthetic acetyl-peptides were stable under the acidic conditions, but this was not true of the alkaline conditions. At pH 7·8, at 37°, *N*-acetylseryl-glycyl-glycine (VI) and aspartyl-*O*-acetylseryl-glycyl-glycine (X) were stable, but *O*-acetylseryl-glycyl-glycine (V) was almost completely converted into the *N*-acetyl compound (VI) within 9 hr., while *O*-aspartyl-*N*-acetylseryl-glycyl-glycine (XI) was rapidly hydrolysed to aspartic acid and *N*-acetylseryl-glycyl-glycine (VI). At the beginning of our work, the only case known to us of the shift of an acyl group from oxygen to nitrogen in a serine derivative was that of *O*-glycylserine; [18] recently, however, similar shifts have been observed with *O*-acetylseryl-tyrosine [14] and *O*-acetylserine.[19] The lability, under mildly alkaline conditions, of the *O*-peptide bond in the tetrapeptide (XI) is not altogether surprising in view of the observation [20] that a number of *N*-acylated *O*-peptides undergo ammonolysis of the *O*-peptide linkage with dilute aqueous ammonia.

Experimental

N-Benzyloxycarbonylseryl-glycyl-glycine Benzyl Ester (I).—(i) *N*-Benzyloxycarbonyl-DL-serine [14,21] (7·18 g.), suspended in chloroform (70 ml.) containing triethylamine (5·2 ml.), was shaken overnight with glycyl-glycine benzyl ester toluene-*p*-sulphonate [22] (14·2 g.) and dicyclohexylcarbodi-imide (7·6 g.) in acetonitrile (70 ml.). Next day, the mixture was filtered and the solid washed with chloroform and extracted with boiling ethyl acetate (250 ml.); evaporation of the extract afforded the required product (7 g., 53%). A further 4 g. (30%) was obtained from the filtered mixture by evaporation to dryness under reduced pressure, dissolution of the residue in hot ethyl acetate, washing of the filtered extract with, successively, N-hydrochloric acid, N-potassium hydrogen carbonate, and water, drying, and evaporation. Recrystallisation of the combined products from water (300 ml./g.) gave the pure DL-*peptide ester* (8·5 g., 64%), m. p. 134—135° (Found: C, 59·8; H, 6·0; N, 8·8. $C_{22}H_{25}N_3O_7$ requires C, 59·6; H, 5·7; N, 9·5%).

(ii) The L-compound, prepared similarly from *N*-benzyloxycarbonyl-L-serine [14] in 60% yield, had m. p. 151—152° (from water), $[\alpha]_D^{23}$ −4·2° (*c* 2·0 in acetic acid) (lit.,[23] m. p. 151—153°) (Found: C, 59·8; H, 5·5; N, 9·1%).

DL-*Seryl-glycyl-glycine.*—*N*-Benzyloxycarbonyl-DL-seryl-glycyl-glycine benzyl ester (1·6 g.) in 75% aqueous t-butyl alcohol [22] (100 ml.) was shaken in hydrogen over 5% palladised charcoal. When the uptake of hydrogen was complete, the mixture was warmed to dissolve the precipitated peptide, filtered from the catalyst, and evaporated to dryness. Recrystallisation from aqueous ethanol gave the *tripeptide* (600 mg., 75%), m. p. 196—200° (decomp.) (Found: C, 38·7; H, 6·1; N, 18·8. $C_7H_{13}N_3O_5$ requires C, 38·4; H, 6·0; N, 19·2%).

[18] Moore, Dice, Nicolaides, Westland, and Wittle, *J. Amer. Chem. Soc.*, 1954, **76**, 2884.
[19] Narita, *J. Amer. Chem. Soc.*, 1959, **81**, 1751.
[20] Botvinik, Avaeva, Konovalova, and Ostoslavskaya, *Zhur. obshchei Khim.*, 1957, **27**, 1910.
[21] Baer and Maurukas, *J. Biol. Chem.*, 1955, **212**, 25.
[22] Crofts, Markes, and Rydon, *J.*, 1959, 3610.
[23] Baer, Maurukas, and Clarke, *Canad. J. Chem.*, 1956, **34**, 1182; *J. Biol. Chem.*, 1957, **228**, 181.

Seryl-glycyl-glycine Benzyl Ester (II).—(i) A stream of hydrogen bromide was passed through a suspension of *N*-benzyloxycarbonyl-DL-seryl-glycyl-glycine benzyl ester (3 g.) in nitromethane (40 ml.); the starting material dissolved during the first 5 min., the product beginning to separate shortly after. After 30 min., anhydrous ether (120 ml.) was added, and the mixture was shaken and then decanted. The solid residue was washed thrice more by decantation with ether and then recrystallised, from ethanol–ether and then from ethanol, affording the DL-*hydrobromide* (2·05 g., 78%), m. p. 174—175° (Found: C, 42·9; H, 5·0; N, 10·6. $C_{14}H_{20}BrN_3O_5$ requires C, 43·1; H, 5·2; N, 10·8%).

(ii) The L-*hydrobromide*, prepared similarly in 70% yield, had m. p. 181·5—183° (from ethanol), $[\alpha]_D^{22}$ +19·6° (*c* 1·3 in H_2O) (Found: C, 43·3; H, 5·0; N, 10·5%).

N-*Acetylseryl-glycyl-glycine Benzyl Ester* (IV).—(i) DL-Seryl-glycyl-glycine benzyl ester hydrobromide (1·2 g.), suspended in acetonitrile (50 ml.), was treated with triethylamine (0·6 ml., 1·5 mols.), and the mixture shaken to bring about dissolution. Thioacetic acid (0·6 ml.) was added and the mixture kept at room temperature; a little more thioacetic acid (5 drops) was added after the second day. After three days, the solid product (0·7 g., 65%) was collected and washed with ether, a further amount (0·24 g., 22%) being obtained by evaporating the filtrate and washings to dryness and triturating the residue with acetonitrile. Recrystallisation from acetonitrile gave the DL-*acetyl-ester* (0·86 g., 80%), m. p. 137—139° (Found: C, 54·2; H, 6·25; N, 11·9. $C_{16}H_{21}N_3O_6$ requires C, 54·7; H, 6·0; N, 12·0%).

(ii) The L-*ester*, prepared similarly in 75% yield, had m. p. 156—157°, $[\alpha]_D^{23}$ −9·3° (*c* 2·0 in MeOH) (Found: C, 54·0; H, 6·0; N, 11·9%).

N-*Acetyl*-DL-*seryl-glycyl-glycine* (VI).—The DL-benzyl ester (IV) (500 mg.) was hydrogenated over 5% palladised charcoal in 50% aqueous t-butyl alcohol. Filtration, evaporation, and recrystallisation from methanol gave the *acetyl-tripeptide* (270 mg., 80%), m. p. 179—181° (Found: C, 41·2; H, 5·8; N, 16·4. $C_9H_{15}N_3O_6$ requires C, 41·4; H, 5·8; N, 16·1%).

O-*Acetyl*-N-*benzyloxycarbonylseryl-glycyl-glycine Benzyl Ester* (III).—(i) Acetic anhydride (0·37 g.) was added, in four portions, with shaking and ice-cooling, during 30 min. to *N*-benzyloxycarbonyl-DL-seryl-glycyl-glycine benzyl ester (1·0 g.) in anhydrous pyridine (15 ml.). The solution was warmed on a boiling water-bath for 5 min., cooled, and poured into ice-water (20 ml.). After an hour, the product was collected, triturated with cold 2N-hydrochloric acid, washed with water, and dried in a vacuum-desiccator. Recrystallisation of the crude product (0·97 g., 89%) from ethanol gave the DL-*compound* (0·87 g., 80%), m. p. 109—111°, raised to 112—113° by further recrystallisation (Found: C, 58·9; H, 5·6; N, 9·4. $C_{24}H_{27}N_3O_8$ requires C, 59·4; H, 5·6; N, 8·7%).

(ii) The L-*compound*, prepared similarly, but at room temperature, in 75% yield, had m. p. 105—107°, $[\alpha]_D^{22}$ +2·9° (*c* 2·0 in acetic acid) (Found: C, 58·5; H, 5·6; N, 8·8%).

O-*Acetyl*-DL-*seryl-glycyl-glycine* (V).—*O*-Acetyl-*N*-benzyloxycarbonyl-DL-seryl-glycyl-glycine benzyl ester (1·9 g.) was hydrogenated over 5% palladised charcoal in 75% aqueous t-butyl alcohol (100 ml.). The product, isolated in the usual manner, was recrystallised from aqueous ethanol, affording the O-*acetyl-peptide* (0·75 g., 75%), m. p. 165—166° (Found: C, 41·7; H, 5·6; N, 15·7. $C_9H_{15}N_3O_6$ requires C, 41·4; H, 5·8; N, 16·1%).

O-*Acetylseryl-glycyl-glycine Benzyl Ester* (VII).—(i) *O*-Acetyl-*N*-benzyloxycarbonyl-DL-seryl-glycyl-glycine benzyl ester (1·0 g.) was treated with hydrogen bromide in nitromethane as described for the un-acetylated compound. The product was recrystallised from ethanol–ether and then from nitromethane, affording the DL-*ester hydrobromide* (0·68 g., 77%), m. p. 144—146° (Found: N, 10·45. $C_{16}H_{22}BrN_3O_6$ requires N, 9·7%); hydrogenolysis, carried out as usual, gave an 83% yield of *O*-acetyl-DL-seryl-glycyl-glycine, chromatographically indistinguishable from material prepared in the alternative way.

(ii) The L-ester hydrobromide, prepared similarly in 82% yield, was found by paper chromatography to contain a substantial amount of de-acetylated material. It was, accordingly, dissolved in acetic acid, and the solution was saturated with hydrogen chloride. After 24 hr. at room temperature, the solution was evaporated to dryness under reduced pressure and the evaporation thrice repeated with addition of ethanol. Trituration of the residue with light petroleum (b. p. 40—60°) and recrystallisation from ethanol gave a product which was chromatographically homogeneous.

O-α-L-*Aspartyl*-N-*acetyl*-L-*seryl-glycyl-glycine* (XI).—Dicyclohexylcarbodi-imide (430 mg.) was added to a solution of *N*-acetyl-L-seryl-glycyl-glycine benzyl ester (750 mg.) and β-benzyl

N-benzyloxycarbonyl-L-aspartate [24] (760 mg.) in anhydrous pyridine (8 ml.). Next day, the precipitated dicyclohexylurea (400 mg., 90%) was removed and the filtrate poured into ice-water (100 ml.). After 3 hr. the product was collected and triturated successively with 2N-hydrochloric acid, 10% aqueous sodium hydrogen carbonate, and water. Recrystallisation of the crude product (1000 mg., 69%), m. p. 156—159°, from ethanol (75 ml.) gave N-*acetyl*-O-(N-*benzyloxycarbonyl*-α-L-*aspartyl*)-L-*seryl-glycyl-glycine dibenzyl ester* (VIII) (800 mg., 55%), m. p. 166—168°, $[\alpha]_D^{22}$ −27·8° (*c* 2·0 in dimethylformamide); the analytical sample, further recrystallised from acetonitrile, had m. p. 169—170° (Found: C, 60·6; H, 5·3; N, 7·6. $C_{35}H_{38}N_4O_{11}$ requires C, 60·9; H, 5·5; N, 8·1%).

This compound (775 mg.) was hydrogenated over 5% palladised charcoal in slightly aqueous ethanol for 48 hr., more catalyst being added at the end of the first 24 hr. Working up as usual, followed by recrystallisation from aqueous ethanol, gave the required N-*acetyl-tetrapeptide tetrahydrate* (230 mg., 46%), m. p. 157—160° (decomp.), $[\alpha]_D^{20}$ +3·7° (*c* 3·1 in H_2O) (Found: C, 35·0; H, 6·0; N, 12·1. $C_{13}H_{20}N_4O_9,4H_2O$ requires C, 34·8; H, 6·3; N, 12·5%).

α-L-*Aspartyl*-O-*acetyl*-L-*seryl-glycyl-glycine* (X).—*O*-Acetyl-L-seryl-glycyl-glycine benzyl ester hydrohalide (900 mg.) was condensed with β-benzyl *N*-benzyloxycarbonyl-L-aspartate (910 mg.) as described above for the *N*-acetyl compound. The crude product (900 mg., 74%), m. p. 117—120°, recrystallised from ethanol, affording N-*benzyloxycarbonyl*-α-L-*aspartyl*-O-*acetyl*-L-*seryl-glycyl-glycine dibenzyl ester* (IX) (550 mg., 45%), m. p. 132—134°, $[\alpha]_D^{22}$ −24·2° (*c* 1·2 in acetonitrile) (Found: C, 59·7; H, 5·4. $C_{35}H_{38}N_4O_{11}$ requires C, 60·9; H, 5·5%).

This compound (400 mg.) was hydrogenated for 48 hr. over 5% palladised charcoal in aqueous methanol. The product (220 mg., 85%), isolated as usual, was a glass which did not crystallise; it was purified by slow evaporation of an aqueous solution, with frequent additions of ethanol. Two such treatments gave the chromatographically homogeneous O-*acetyl-tetrapeptide tetrahydrate*, decomp. *ca.* 156°, $[\alpha]_D^{20}$ +11·1° (*c* 1·5 in H_2O) (Found: C, 35·6; H, 5·9; N, 12·4. $C_{13}H_{20}N_4O_9,4H_2O$ requires C, 34·8; H, 6·3; N, 12·5%).

Chromatographic Data.—The following R_F values are for ascending chromatograms on Whatman No. 1 filter paper. Spots were detected with ninhydrin and by the chlorine–starch–iodide procedure.[25]

	H·Ser·Gly·Gly·OH	(V)	(VI)	(X)	(XI)
Bu^tOH–H·CO_2H–H_2O (70 : 15 : 15)	0·28	0·40	0·51	0·32	0·32
Bu^nOH–AcOH–H_2O (4 : 1 : 5)	0·22	0·32	0·40	0·25	0·24
Phenol saturated with 10% sodium citrate	0·43	0·65	0·73	0·49	0·49

Stability of the Acetyl-peptides.—(i) *At* pH 2·0. Dilute aqueous solutions of the four acetyl-peptides were brought to pH 2·0 with hydrochloric acid and kept at 37° for 16 hr. In each case, paper chromatography revealed no change.

(ii) *At* pH 7·8. Dilute aqueous solutions of the four acetyl-peptides in 0·1M-phosphate buffer of pH 7·8 were kept at 37°; samples were removed for paper chromatography from time to time. No change was detected with *N*-acetylseryl-glycyl-glycine (up to 48 hr.) or with aspartyl-*O*-acetylseryl-glycyl-glycine (up to 16 hr.). After 9 hr., *O*-acetylseryl-glycyl-glycine had been converted almost entirely into *N*-acetylseryl-glycyl-glycine. Under these conditions *O*-aspartyl-*N*-acetylseryl-glycyl-glycine was rapidly converted into *N*-acetylseryl-glycyl-glycine and aspartic acid (R_F 0·16 in phenol saturated with 10% sodium citrate solution).

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[25] Rydon and Smith, *Nature*, 1952, **169**, 922.