Polypeptides. Part IV.[1] Syntheses of Human Gastrin (HI)

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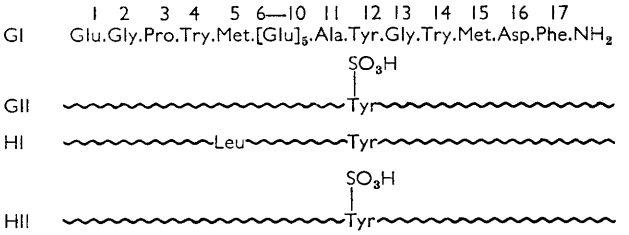
Syntheses of human gastrin (HI), a heptadecapeptide hormone, are described which proceed by coupling of derivatives of the tridecapeptide (1—13) or undecapeptide (3—13) sequences of the molecules with the *C*-terminal tetrapeptide amide (14—17) or its t-butyl ester. A feature of these syntheses is the application of a procedure which permits the rapid synthesis of the tri- and un-decapeptide fragments in high overall yield, and which is of general application in peptide synthesis.

THE gastrins are now recognised as a group of polypeptide hormones which are released in the gastro-intestinal tract of man and certain other animal species, and which have pronounced physiological actions on (*a*) secretory cells of the stomach and pancreas (in particular, they stimulate the release of hydrochloric acid from the parietal cells of the stomach), and (*b*) the musculature of the stomach and intestine. In Part III of this Series,[1] recent work leading to the isolation, characterisation, and synthesis of two hog gastrins (gastrins I and II, now referred to as GI and GII) was summarised, and details were given of the synthesis of the *C*-terminal tetrapeptide amide sequence, its optical isomers, and its acylated derivatives. Subsequently, two human gastrins (HI and HII) were isolated,[2] their structures were determined,[3] and HI was synthesised,[4] and tentative structures for bovine, ovine, and canine gastrins were proposed.[5] The gastrins are all closely related heptadecapeptides. In hog and human gastrins, the amino-acid residues at positions 1—4, 6—11, and 13—17 are the same. GI and HI have tyrosine at position 12, whereas GII and HII have tyrosine-*O*-sulphate; GI and

[1] Part III, (Miss) J. M. Davey, A .H. Laird, and J. S. Morley, *J. Chem. Soc.* (*C*), 1966, 555.
[2] R. A. Gregory, H. J. Tracy, and M. I. Grossman, *Nature* 1966, **209**, 583.
[3] P. H. Bentley, G. W. Kenner, and R. C. Sheppard, *Nature*, 1966, **209**, 583.
[4] J. Beacham, P. H. Bentley, R. A. Gregory, G. W. Kenner, J. K. MacLeod, and R. C. Sheppard, *Nature*, 1966, **209**, 586.
[5] J. Beacham, P. H. Bentley, G. W. Kenner, J. J. Mendive, and R. C. Sheppard, Proc. Eighth European Peptide Symposium Noordwijk-on-sea, The Netherlands, September 1966 (North Holland Publishing Co., 1967, p. 235.

GII have methionine at position 5, whereas HI and HII have leucine (see Figure).



The finding that the biological activity of the gastrins, on both secretory cells and musculature, resides in the *C*-terminal tetrapeptide amide sequence,[6,7] enabled us to investigate structure–function relationships on a scale not hitherto possible with polypeptide hormones. About 500 analogues of the tetrapeptide amide were prepared [8] and the results obtained by testing certain of these analogues in the conscious dog [7] or anaesthetised rat [8,9] have been summarised. We have recently devoted some attention to the tridecapeptide head (1—13) of the gastrins. Although this large part of the molecule has apparently little influence on the biological activity, we wished to find out if the same situation applied in extended analogues, *i.e.*, heptadecapeptides related to the gastrins by a single amino-acid change in the 'active' tetrapeptide amide stump. For the synthesis of these extended analogues, we used derivatives of the undecapeptide (3—13) and tridecapeptide (1—13) sequences of human gastrin (HI). These were prepared in quantity and successfully coupled with a number of the tetrapeptide analogues prepared in the course of the main investigation. We also effected efficient couplings of these fragments with the gastrin *C*-terminal tetrapeptide amide (14—17) and its t-butyl ester, which led to syntheses of human gastrin (HI); this aspect of our work is the subject of the present Paper.

The t-butoxycarbonylundecapeptide penta-t-butyl ester (3—13) (XXVIIA) and the tridecapeptide penta-t-butyl ester (1—13) (XXX) were synthesised stepwise (Scheme 1) by a procedure we have recently used widely for peptide synthesis. The procedure has enabled the rapid synthesis, in high overall yield, of a variety of pure peptides; in the present case the synthesis of the tridecapeptide penta-t-butyl methyl ester (1—13) (XXXII) was completed in 5 weeks in overall yield (based on glycine methyl ester) of 45%. Generalised comments and a description of the procedure are therefore appropriate.

Use of active esters of single amino-acid residues for the stepwise synthesis of peptide chains was first advocated by Bodanszky and du Vigneaud.[10] Our own experience in the use of the common coupling reactions and protecting groups supports their opinions and emphasises two features. First, the method employing active esters generally proceeds quantitatively without side reactions when functional groups in the side-chain of the growing peptide chain are suitably protected. Second, quantitative selective removal (by hydrogenolysis) of the benzyloxycarbonyl group in the presence of t-butoxycarbonyl, t-butyl (as ester or *O*-ether), or tosyl groups has frequently been possible. The combination of benzyloxycarbonyl for *N*-protection and these other groups for side-chain protection enables the synthesis of peptide chains containing all the common amino-acids with the exception of methionine and cystine (the presence of sulphur-containing amino-acids generally hinders hydrogenation). Thus, with only this exception, a peptide fragment may often be synthesised quantitatively by the stepwise active ester method with these protecting groups. It follows that the only purification problem is the removal of the alcoholic or phenolic component liberated from the active ester and of any excess active ester used at each coupling stage. Our procedure may be generalised as follows.

On *day* 1, a benzyloxycarbonyl(Z)-peptide is hydrogenated in 90% aqueous acetic acid at room temperature and atmospheric pressure in the presence of 5% palladised carbon (usually 0·5 g./10 mmoles). Removal of the Z-group is often complete in 1—2 hr.; we usually continue hydrogenation for 5—6 hr. The solution is filtered (Kieselguhr) and evaporated and the resulting acetate is dried by several azeotropic distillations with benzene. The purity of the product is estimated by t.l.c. If the visual assessment of purity is satisfactory (our experience is that this is always so, in accordance with the arguments that we have advanced; purification would otherwise be necessary), the acetate (sometimes collected with ether, sometimes used directly) is dissolved in the minimum volume of dimethylformamide (DMF) and treated with an excess (10—20% in the early stages) of an active ester of the Z-derivative of the next amino-acid (with side-chain protection where appropriate) and triethylamine (0·6—0·75 equiv.). We normally use 2,4,5-trichlorophenyl esters (OCP);[11] *p*-nitrophenyl esters (ONP) [10] or hydroxy succinimide esters(OSu) [12] are also convenient in some cases. For side-chain protection we use t-butoxycarbonyl (in the case of Lys, Orn, etc.), Bu^t (Ser and Thr), OBu^t (Asp and Glu), benzyl (Tyr), Z (His), and tosyl (Arg). The reaction mixture is then set aside overnight at 4° or at room temperature. Extended reaction times are rarely necessary until about ten amino-acids have been incorporated into the growing peptide chain.

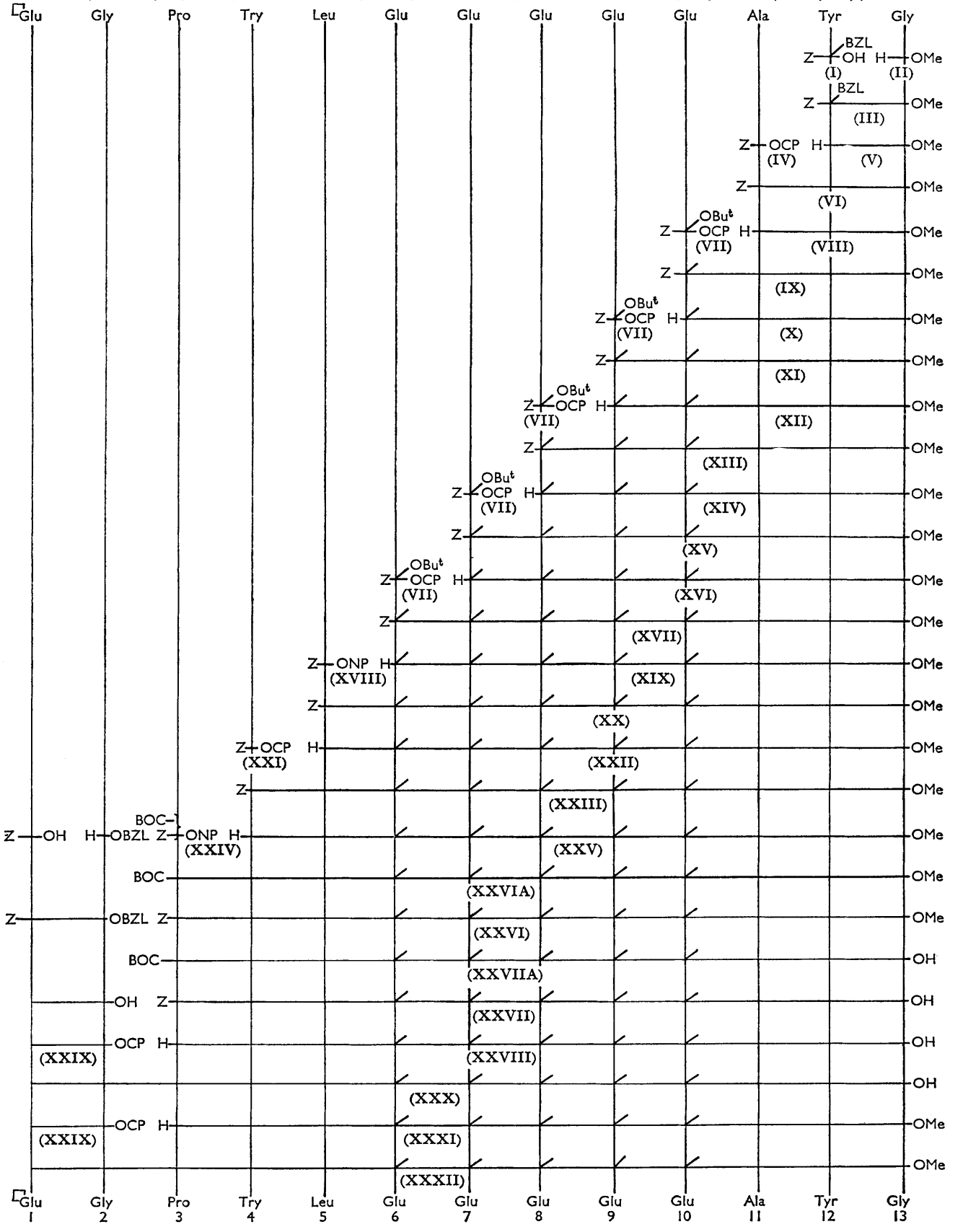
On *day* 2, the reaction mixture is checked to ensure that there is no free amino-constituent present (usually

[6] H. J. Tracey ad R. A. Gregory, *Nature*, 1964, **204**, 935.
[7] J. S. Morley, H. J. Tracy, and R. A. Gregory, *Nature*, 1965, **207**, 1356.
[8] J. S. Morley, Proc. Thirtysixth International Congress Ind. Chem., Brussels, September 1966 (in the press).
[9] J. S. Morley, Proc. Eighth European Peptide Symposium, Noordwijk-on-sea, The Netherlands, September 1966 (North Holland Publishing Co., 1967, p. 226.
[10] M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, 1959, **81**, 5688.
[11] J. Pless and R. A. Boissonnas, *Helv. Chim. Acta*, 1963, **46**, 1609.
[12] G. W. Anderson, J. E. Zimmerman, and F. Callahan, *J. Amer. Chem. Soc.*, 1963, **85**, 3039.

Scheme 1

Synthesis of the tridecapeptide penta-t-butyl ester (I—13)

(Z = Benzyloxycarbonyl; BOC = t-Butoxycarbonyl; BZL = Benzyl; CP = 2,4,5-Trichlorophenyl; NP = p-nitrophenyl)



with ninhydrin reagent, alone, or in conjunction with t.l.c.) and worked up by an appropriate procedure. The products encountered in the first few stages of the synthesis (derivatives of small peptides) may be taken up in ethyl acetate or methylene chloride and washed successively with water, sodium hydrogen carbonate, water, 10% citric acid, and water; the residue is dried, evaporated, and freed from 2,4,5-trichlorophenol (easily soluble in ether and ethyl acetate, and fairly soluble in cyclohexane) or *p*-nitrophenol by crystallisation or trituration. In some early cases, and as the peptide chain grows, the products are insoluble in many common solvents. A convenient procedure in these cases is to add ether or ethyl acetate followed by ice–water; the solid product is collected and washed thoroughly with ethyl acetate and/or ether, water, and ether. The resulting Z-peptide is examined by t.l.c., purified if necessary (this has not yet been required), and dried *in vacuo* overnight. Purity is subsequently checked by quantitative acid hydrolysis followed by analysis of amino-acids in the hydrolysate.

On *day* 3, the procedure of day 1 is repeated. It is thus normally possible to introduce one new amino-acid into the growing peptide chain every two days.

For the present synthesis (Scheme 1), purity was assessed by t.l.c. at each stage with eight solvent systems. In the chromatography of the eleven products (V), (VIII), (X), (XII), (XIV), (XVI), (XIX), (XXII), (XXV), (XXVIII), and (XXXI)] obtained by hydrogenolysis, single spots were produced with ninhydrin and acid permanganate in all cases, with Ehrlich's reagent when tryptophan was present [(XXV), (XXVIII), and (XXXI)], and with chlorine–potassium iodide–tolidine in cases when this method was also used. The sterically hindred couplings between Z-Glu(OBu^t)-OCP(VII) and the tri- and tetra-t-butyl esters [(XIV) and (XIV)] required 2 days at room temperature for completion.* Otherwise all couplings, including that between Z-Leu-ONP (XVIII) and the penta-t-butyl ester (XIX), were complete after overnight reaction at room temperature. In the t.l.c. of the eleven Z- [(III), (VI), (IX), (XI), (XIII), (XV), (XVII), (XX), (XXIII), (XXVI), and (XXVII)] and two t-butoxycarbonyl(BOC)- [(XXVIA) and (XXVIIA)] peptide derivatives and of the tridecapeptide derivatives (XXX) and (XXXII), acid permanganate and the chlorine–potassium iodide–tolidine method were used in all cases; Ehrlich's reagent was employed when tryptophan was present [(XXIII), (XXVI), (XXVIA), (XXVII), (XXVIIA), (XXX), and (XXXII)]. Single spots were always revealed. Quantitative amino-acid analysis of acid hydrolysates of these fifteen peptide derivatives gave excellent figures for each amino-acid. The optical purity of the undecapeptide penta-t-butyl ester (XXVIII) was confirmed (after removal of the t-butyl groups) by amino-acid analysis of leucine aminopeptidase and carboxypeptidase digests.

Partial saponification of the tridecapeptide penta-t-butyl methyl ester (3—13) (XXXII) in dioxan or 2-ethoxyethanol was attempted under a variety of conditions, but the best yield of the required acid (XXX) never exceeded 25%. The difficulties seemed largely due to the insolubility of the ester, though opening of the pyroglutamyl ring may also be a complicating factor. However, conditions for the quantitative partial saponification of the Z- (XXVI) and BOC- (XXVIA) undecapeptide penta-t-butyl methyl esters in 75% aqueous dioxan (with 5 equiv. of sodium hydroxide at 20—25° for 1 hr.) were readily found, to give crystalline Z- (XXVII) and BOC- (XXVIIA) undecapeptide penta-t-butyl esters. Hydrogenolysis of the former (XXVII) smoothly removed the Z-group, and the product (XXVIII), with L-pyroglutamylglycine 2,4,5-trichlorophenyl ester (XXIX), and triethylamine (1 equiv.), gave the tridecapeptide penta-t-butyl ester (XXX) in high yield.

In completing the synthesis of human gastrin (Schemes 2 and 3), we were not faced with racemisation problems (glycine at 13), but we anticipated difficulties in purifying the products derived from coupling the tridecapeptide penta-t-butyl ester (1—13) (XXX) or t-butoxycarbonyl-undecapeptide penta-t-butyl ester (3—13) (XXVIIA) with the tetrapeptide amide (14—17) (XXXIV). All methods, with the exception of the active ester method, seemed likely to yield mixtures of peptide acids of similar chain-length which would be difficult to separate. On the other hand, our earlier work [1] had shown that active ester couplings with the tetrapeptide amide (XXXIV) (in the presence of tertiary base) often proceeded quantitatively, and the method seemed less likely to yield acidic contaminants. We prepared a pure 2,4,5-trichlorophenyl ester (XXXIII) of the t-butoxycarbonylundecapeptide penta-t-butyl ester (3—13) (XXVIIA) in high yield from the acid (1 mole), trichlorophenol (5 moles), and *NN'*-dicyclohexylcarbodi-imide (5 moles) in dimethylformamide (8 days at 4°), which did in fact couple smoothly with the tetrapeptide amide (XXXIV) in the presence of triethylamine (1 equiv.) to yield a crude t-butoxycarbonylpentadecapeptide penta-t-butyl ester amide (3—17) (XXXV) (Scheme 2). The butoxycarbonyl and butyl ester protecting groups of the crude product were removed with trifluoroacetic acid, and the synthesis of human gastrin (HI) (XL) was completed by coupling the resulting pentadecapeptide amide (XXXVI) with L-pyroglutamylglycine 2,4,5-trichlorophenyl ester (XXIX) in 90% aqueous dimethylformamide in the presence of triethylamine (6 equiv.).

An alternative approach (Scheme 3) to avoid the purification difficulties seemed to be the use of a t-butyl ester of the tetrapeptide amide (14—17) (XXXVIII).

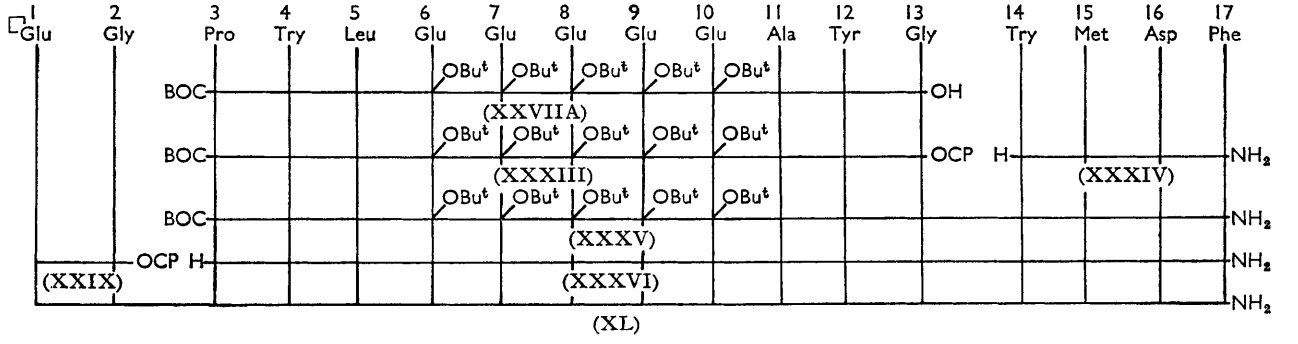
* The synthesis, as far as the octapeptide penta-t-butyl methyl ester (XIX), has previously been described by Anderson, Kenner, MacLeod, and Sheppard,[13] who found that the latter coupling was incomplete even after 10 days at 35°; the discrepency is, we feel, accounted for by the marked catalytic influence of the 0·25 equivalents of acetic acid present in our reaction mixture, but not that of Anderson *et al.* (for the general catalytic influence of acetic acid in reactions involving active esters see ref. 24).

[13] J. C. Anderson, G. W. Kenner, J. K. MacLeod, and R. C. Sheppard, *Tetrahedraon*, 1966, Suppl. 8, 39.

Couplings of the tridecapeptide (1—13) (XXX) or undecapeptide derivative (3—13) (XXVIIA) with this ester would generate a neutral peptide from which 1—13 or 3—13 acidic contaminants could be more easily removed. This was the case. Coupling of the components by the mixed anhydride method (with pivaloyl chloride) gave gastrin hexa-t-butyl ester (XXXIX) and

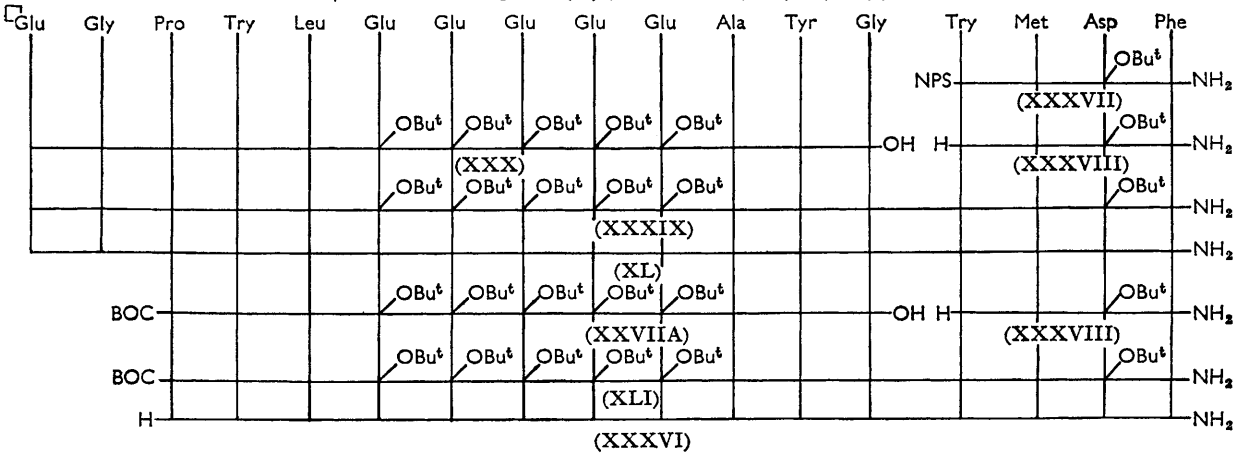
Scheme 2

Synthesis of human gastrin (HI)



Scheme 3

Synthesis of human gastrin (HI) (NPS = *o*-nitrophenylsulphenyl)



the t-butoxycarbonylpentadecapeptide derivative (3—17) (XLI) in good yield; both were readily purified by precipitation at pH 9—10. Treatment of the former with trifluoroacetic acid gave human gastrin HI; the latter gave the pentadecapeptide amide (3—17) (XXXVI), identical with that obtained as described in Scheme 2. The t-butyl ester of the tetrapeptide amide (14—17) (XXXVIII) was prepared by treating the *o*-nitrophenylsulphenyl (NPS) derivative (XXXVII) [1] with 4-chloro-2-nitrothiophenol in pyridine. Our use of 4-chloro-2-nitrothiophenol arose from the observation that the NPS-derivative (XXXVII) resisted the action of thiophenol or thioglycollic acid under the generalised cleavage conditions described by Scoffone and his co-workers [14] (an observation since confirmed by Professor Scoffone). An alternative preparation of the t-butyl ester (XXXVIII), supplied by Professor Scoffone, involved cleavage of the NPS-derivative (XXXVII) with 80% aqueous thioglycollic acid.

The synthetic samples of human gastrin HI had the full biological activity of the gastrins. Chemical purity was established by amino-acid analysis of acid hydrolysates, column and thin-layer chromatography, and electrophoresis.

EXPERIMENTAL

The general explanations given in Part III [1] apply. Thin-layer plates were run in triplicate and separately sprayed with acid potassium permanganate, sodium hypochlorite–potassium iodide–tolidine, and ninhydrin (see Part II [15] for details of these reagents and of the solvent systems A—H). In the case of tryptophan containing peptides, the permanganate-developed plates were subsequently sprayed with Ehrlich's reagent (the sensitivity of Ehrlich's reagent was increased by this procedure, and the development of colour was rapid). In the case of benzyloxycarbonylpeptide derivatives, which did not reveal spots after ninhydrin spraying, colour spots arose after permanganate-developed plates were sprayed

[14] A. Fontana, F. Marchiori, L. Moroder, and E. Scoffone, *Tetrahedron Letters*, 1966, 2985.
[15] P. H. Bentley and J. S. Morley, *J. Chem. Soc.* (*C*), 1966, 60.

with ninhydrin. This was a useful supplementary test. Unless otherwise stated, quotation of an R_F value implies a single visible spot. Hydrogenations were carried out at atmospheric pressure and room temperature, with 5% palladised carbon as catalyst and magnetic or mechanical stirring; on completion of the reaction, the filtered (Kieselguhr) solution was evaporated in a rotary evaporator, and the residue was dried by several azeotropic distillations with benzene. For microanalysis samples were dried for 18 hr. *in vacuo*, at 25° (NaOH) in the case of acetates, and at 80° (P_2O_5) in other cases.

Preparation of Intermediates.—*α-Benzyl N-benzyloxycarbonyl-L-glutamate.* A stirred solution of *N*-benzyloxycarbonyl-L-glutamic acid (281 g., 1 mole) in dimethylformamide (250 ml.) was treated at 15—20° with triethylamine (140 ml., 1 mole) followed by benzyl bromide (132 ml., 1·1 mole). The temperature was kept at 20—25° overnight, and the mixture was then diluted with ice–water (2 l.) and extracted with ethyl acetate (3 × 750 ml.). The combined extracts were washed with water (2 × 300 ml.), dried, evaporated to *ca.* 1 l., and then treated below 25° with dicyclohexylamine (220 ml., 1·1 mole). The solid (458 g., 83%) was collected, washed with ethyl acetate, and gave the dicyclohexylammonium salt (265 g., 48%), m. p. 163—164° [from ethanol (1·5 l.)] (lit.,[16] 70% yield, m. p. 162—164°). A suspension of this salt in ethyl acetate (1·5 l.) was treated with 3·5N-hydrogen chloride in ethyl acetate (274 ml., 2 equiv.) and the mixture was stirred at 20—25° for 1·5 hr. and then filtered. The filtrate was washed with water (3 × 100 ml.), dried, and evaporated, and the residue was triturated with light petroleum, to give the α-ester (164 g., 44% overall), m. p. 96·5—98·5° (lit.,[17] 97—98°).

α-Benzyl γ-t-butyl N-benzyloxycarbonyl-L-glutamate. The above α-ester (111·3 g., 0·3 mole), methylene chloride (600 ml.), isobutene (300 ml.), and concentrated sulphuric acid (3 ml.), were stirred together in a stainless-steel autoclave at room temperature for 5 days. The solution was neutralised with 2N-sodium carbonate (240 ml.) and the excess of isobutene was removed in a cold trap. The resulting emulsion was set aside overnight, and the organic layer was separated, washed with N-sodium carbonate and water (4 times), dried, and evaporated. The residue solidified under light petroleum, to yield the diester (118·3 g., 92·5%), m. p. 49—51·5°, R_{FE} 0·85, R_{FF} 0·82, R_{FH} 0·80 (minor, slow-running components also detected) (lit., m. p. 46—48,[18] 48—49° [19]).

α-(2,4,5-Trichlorophenyl) γ-t-butyl N-benzyloxycarbonyl-L-glutamate (VII). A stirred solution of the above diester (42·7 g., 0·1 mole) in acetone (300 ml.) was treated dropwise during 15 min. at 15—17° with N-sodium hydroxide (110 ml.); then most of the acetone was removed during 15 min. in a rotary evaporator (bath at 25°). The solution was extracted with ether (2 × 50 ml.), then acidified at 0° to pH 4 with N-hydrochloric acid (110 ml.), and extracted with ethyl acetate (1 × 250 ml., 2 × 100 ml.). The water-washed and dried extracts were evaporated to *ca.* 250 ml., then treated with 2,4,5-trichlorophenol (21·8 g., 0·11 mole) followed, at 0°, by a solution of *NN'*-dicyclohexylcarbodi-imide (21·7 g., 0·105 mole) in ethyl acetate (50 ml.). The mixture was kept at 4° for 16 hr. then filtered from dicyclohexylurea (20·2 g., 90%) and evaporated. Crystallisation of the solid residue from cyclohexane (300 ml.) gave needles of the active ester (34·8 g., 68% based on diester), m. p. 108—109° (lit., 107—108, [11] 111—112° [13]). In a subsequent run on three times the above scale in which the sodium hydroxide was added during 30 min. and the acetone was removed during 1 hr., the yield was unaffected.

O-Benzyl-L-tyrosine. A solution of L-tyrosine (362 g., 2 moles) in 2N-sodium hydroxide (2 l.) was treated with copper sulphate pentahydrate (249·6 g., 1 mole) in water (1 l.). The mixture was stirred at 20—25° for 10 min.; methanol (6 l.), followed by benzyl bromide (250 ml., 2·1 moles), was added and vigorous stirring was continued at 22—25° for 1·5 hr. The blue copper complex was collected and washed with water (3 × 1 l.), methanol (2 × 1 l.), and ether (2 × 1 l.). The air-dried product (435 g., 72%) was stirred at 40—50° for 15 min. with concentrated hydrochloric acid (400 ml.) and water (4 l.). The white hydrochloride was collected, ground with 2N-hydrochloric acid, collected again, stirred at 40—50° with concentrated hydrochloric acid (200 ml.) and water (2 l.), collected again, and then stirred for 15 min. with sodium acetate (4 moles) and water (2 l.) at 80—90°. The crude *O*-benzyl-L-tyrosine (366·4 g., 67%), m. p. 224—226° (decomp.; darkened and sintered at 205°) (lit.,[20] 223°) was collected, washed with water, and dried *in vacuo* at 50°; the compound crystallised from 90% aqueous acetic acid, but further purification was unnecessary.

N-Benzyloxycarbonyl-O-benzyl-L-tyrosine (I). Crude *O*-benzyl-L-tyrosine (135·5 g., 0·5 mole,) in N-sodium hydroxide (500 ml.), water (2 l.), and acetone (50 ml.), was carbobenzoxylated at pH 11 in the usual manner by simultaneous dropwise addition of benzyloxycarbonyl chloride (0·5 mole) and 2N-sodium hydroxide (250 ml.) at 20—25° (reaction complete in 2—3 hr.). The mixture was heated rapidly to 40—45° (to dissolve the separated sodium salt), then immediately added to a stirred mixture of concentrated hydrochloric acid (100 ml.) and ice (sufficient to maintain a temperature of 0—15°). The product was collected and gave needles of the tyrosine derivative (151·1 g., 75%), m. p. 112—113° (from ethyl acetate–cyclohexane) (lit.,[20] 82% yield, m. p. 116·5°).

N-Benzyloxycarbonyl-O-benzyl-L-tyrosine 2,4,5-trichlorophenyl ester. The above tyrosine derivative (144·5 g., 0·355 mole), 2,4,5-trichlorophenol (76·5 g., 0·39 mole), and *NN'*-dicyclohexylcarbodimide (77 g., 0·373 mole) were stirred at 4° for 16 hr. The mixture was filtered from dicyclohexylurea (79·4 g., 96%) and evaporated; the residue gave the *active ester* (174 g., 83%), m. p. 108—110° [from ethyl acetate–light petroleum (1 : 2; 600 ml.)] (Found: C, 61·6; H, 4·2; N, 2·4. $C_{30}H_{24}Cl_3NO_5$ requires C, 61·7; H, 4·15; N, 2·4%).

N-Benzyloxycarbonyl-L-pyroglutamylglycine benzyl ester. (*a*) Glycine benzyl ester toluene-*p*-sulphonate (29·7 g., 88 mmoles), methylene chloride (200 ml.), and a solution of potassium carbonate (24·4 g., 176 mmoles) in water (200 ml.) were shaken together in a separating funnel. The organic layer and two extracts of the aqueous layer with methylene chloride (each 100 ml.) were combined, dried ($MgSO_4$), and evaporated below 25° to *ca.* 200 ml. *N*-Benzyloxycarbonyl-L-pyroglutamic acid (21·04 g., 80 mmoles) was added with stirring, followed, at 0°, by a solution of *NN'*-dicyclohexylcarbodi-imide (18·13 g., 88 mmoles) in methylene chloride

[16] G. H. L. Nefkens and R. J. F. Nivard, *Rec. Trav. chim.*, 1964, **83**, 199.
[17] E. Klieger and H. Gibian, *Annalen*, 1962, **655**, 195.
[18] R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, 1961, **44**, 1991.
[19] E. Schröder and K. Klieger, *Annalen*, 1964, **673**, 196.
[20] E. Wünsch, G. Fries, and A. Zwick, *Chem. Ber.*, 1958, **91**, 542.

(50 ml.). The mixture was stirred at 0° for 1 hr. and then at 20—25° for 2 hr. The solution was filtered from dicyclohexylurea and washed with water, saturated aqueous sodium hydrogen carbonate, water, 10% aqueous citric acid, and water, then dried ($MgSO_4$ and activated alumina), and evaporated. The solid (31 g.) obtained by trituration of the residue with ether gave needles of the dipeptide derivative (24·7 g., 75%), m. p. 116—118° [from ethanol (150 ml.)], $[\alpha]_D^{24}$ −22·2° (*c* 1·05 in dimethylformamide) (lit.,[21] m. p. 113—115°) (Found: C, 64·3; H, 5·3; N, 6·9. Calc. for $C_{22}H_{22}N_2O_6$: C, 64·4; H, 5·4; N, 6·8%).

(*b*) A solution of *N*-benzyloxycarbonyl-L-pyroglutamic acid *p*-nitrophenyl ester (16·8 g., 43·5 mmoles), glycine benzyl ester toluene-*p*-sulphonate (17·6 g., 52·2 mmoles), and triethylamine (7 ml., 50 mmoles) in dimethylformamide (100 ml.) was kept at 22—25° for 16 hr. Ether (300 ml.) followed by water (200 ml.) was added; the resulting solid was collected and gave the dipeptide derivative (14·37 g., 81%), m. p. 116—118° [from ethanol (100 ml.)].

L-*Pyroglutamylglycine*. A solution of the above dipeptide derivative (14·37 g., 35 mmoles) in methanol (200 ml.) was hydrogenated for 4 hr. in the presence of catalyst (3·5 g.). The water-soluble acid (100% yield), m. p. 167—169° (unchanged after crystallisation from methanol–ethyl acetate) (lit., 165—167,[22] 162—166°[23]), was characterised as its dicyclohexylammonium salt, which separated as needles, m. p. 199—200° (decomp.), from methanol–ether (Found: C, 61·8; H, 9·1; N, 11·6. $C_7H_{10}N_2O_4,C_{12}H_{23}N$ requires C, 62·1; H, 9·0; N, 11·4%).

L-*Pyroglutamylglycine* 2,4,5-*trichlorophenyl ester* (XXIX). A solution of the above acid (6·51 g., 35 mmoles) in dimethylformamide (200 ml.) was evaporated *in vacuo* to 80 ml. (to remove any traces of water), then treated at 0° with 2,4,5-trichlorophenol (7·59 g., 38·5 mmoles) followed by *NN'*-dicyclohexylcarbodi-imide (7·65 g., 36·75 mmoles) in dimethylformamide (20 ml.). The mixture was kept at 4° for 1 day, then filtered from dicyclohexylurea (6·46 g., 82%) and diluted with ether (100 ml.) and ice–water (400 ml.) The solid was collected, washed with water and ether, and gave prismatic needles of the active ester (7·24 g., 57%), m. p. 179—180° [from ethanol (150 ml.)], R_{FE} 0·17, R_{FE} 0·64, R_{FE} 0·59, $[\alpha]_D^{25}$ −15·5° (*c* 1·09 in dimethylformamide); amino-acids in 16 hr. acid hydrolysate of 1 μ mole: glu 1·01, gly 1·00 μ mole (Found: C, 42·7; H, 3·0; N, 7·7. $C_{13}H_{11}Cl_3N_2O_4$ requires C, 42·7; H, 3·0; N, 7·7%).

Other active esters. The following were prepared by literature methods: *N*-benzyloxycarbonyl-L-alanine 2,4,5-trichlorophenyl ester (80%), m. p. 102—103° (lit.,[11] 104°), *N*-benzyloxyloxycarbonyl-L-leucine *p*-nitrophenyl ester (81%), m. p. 92—93° (lit.,[10] 95°), *N*-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (92%), m. p. 95—96° (lit.,[10] 94—96°), and *N*-benzyloxycarbonyl-L-tryptophan 2,4,5-trichlorophenyl ester (77%), m. p. 114—115° (lit.,[11] 115°). N-*t-Butoxycarbonyl-L-proline* 2,4,5-*trichlorophenyl ester*, prepared by the general method of Pless and Boissonnas,[11] was a colourless solid (95% yield), m. p. 52—54° (Found: C, 48·8; H, 4·7; N, 3·5. $C_{16}H_{18}Cl_3NO_4$ requires C, 48·6; H, 4·6; N, 3·55%).

N-*Benzyloxycarbonyl*-(O-*benzyl*)-L-*tyrosylglycine Methyl Ester* (III).—(*a*) Glycine methyl ester hydrochloride (12·5 g., 0·1 mole), methanol (25 ml.), and triethylamine (14 ml., 0·1 mole) were stirred together at 0° for 2 min. A solution of *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosine 2,4,5-trichlorophenyl ester (58·5 g., 0·1 mole) in dimethylformamide (120 ml.) was added and the mixture was then stirred at 4° for 16 hr. The solid obtained by addition of ice–water (1 l.) gave the *dipeptide derivative* (41·3 g., 87%), m. p. 128—130° [from methanol (350 ml.)], R_{FD} 0·80, R_{FE} 0·70, R_{FF} 0·86, R_{FG} 0·24, R_{FH} 0·76, $[\alpha]_D^{24}$ −23·1° (*c* 0·96 in dimethylformamide).

(*b*) A vigorously stirred mixture of *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosine (I) (134 g., 0·33 mole), glycine methyl ester hydrochloride (41·5 g., 0·33 mole), and methylene chloride (500 ml.) was treated at 0—5° with triethylamine (46·1 ml., 0·33 mole) followed by *NN'*-dicyclohexylcarbodiimide (68·1 g., 0·364 mole). It was then stirred at 4° overnight. The residue was filtered off and washed with methylene chloride (3 × 300 ml.), and the combined filtrate and washings were washed successively with water, N-sodium hydrogen carbonate, water, 10% aqueous citric acid, and water, then dried and evaporated. The residue gave the dipeptide derivative (119 g., 76%), m. p. 126—127° [from methanol (1·5 l.)].

L-*Tyrosylglycine Methyl Ester Acetate and Hydrochloride* (V).—The dipeptide derivative (III) (41·3 g., 86·5 mmoles) in 90% aqueous acetic acid was hydrogenated for 5 hr. over 8·6 g. of catalyst. The product was obtained as an oil, R_{FA} 0·45, R_{FC} 0·67, R_{FD} 0·52, R_{FE} 0·16, R_{FF} 0·35, R_{FG} 0·0, R_{FH} 0·29, which was used directly. A hygroscopic hydrochloride, m. p. 92—95° (decomp.), was obtained by adding dry ethereal hydrogen chloride to a solution of the acetate in ethanol.

N-*Benzyloxycarbonyl-L-alanyl-L-tyrosylglycine Methyl Ester* (VI).—A solution of the acetate (V) (from hydrogenolysis of 86·5 mmoles of the benzyloxycarbonyl compound), *N*-benzyloxycarbonyl-L-alanine 2,4,5-trichlorophenyl ester (38·4 g., 95·19 mmoles), and triethylamine (9·7 ml., 69·2 mmoles) in dimethylformamide (150 ml.) was kept at 4° for 16 hr. Ice–water (1 l.) was added and the mixture was extracted with methylene chloride (1 × 500 ml., 2 × 250 ml.). The extracts were washed with 5% aqueous citric acid (2 × 75 ml.) and water (4 × 100 ml.), then dried and evaporated. The tripeptide derivative (34·2 g., 86%), m. p. 146—149°, was obtained after trituration of the residue with ether; a portion (0·5 g.) separated from ethyl acetate–light petroleum (1 : 2, 9 ml.) in gelatinous crystals (0·453 g.), m. p. 149—151°, R_{FA} 0·75, R_{FC} 0·80, R_{FD} 0·72, R_{FE} 0·45 (tailed), R_{FF} 0·78, R_{FH} 0·70, $[\alpha]_D^{26}$ −17·3° (*c* 1·0 in dimethylformamide) [lit.,[13] m. p. 151—152·5°, $[\alpha]_D^{22}$ − 17·9° (*c* 2·4 in dimethylformamide)].

L-*Alanyl-L-tyrosylglycine Methyl Ester and Acetate Hydrate* (VIII).—The tripeptide derivative (VI) (29·7 g., 65 mmoles) was hydrogenated for 5 hr. in 90% aqueous acetic acid over 3·3 g. of catalyst. The *acetate* (24·9 g., 100%) separated from a mixture of acetic acid (100 ml.) and ethyl acetate (200 ml.) as needles, m. p. 138—139°, R_{FA} 0·43, R_{FC} 0·47, R_{FD} 0·46, R_{FE} 0·05, R_{FF} 0·34, R_{FG} 0·0, $[\alpha]_D^{26}$ +11·5° (*c* 1·0 in dimethylformamide) (Found: C, 51·4; H, 6·6; N, 10·2. $C_{15}H_{21}N_3O_5,C_2H_4O_2$ requires C, 50·9; H, 6·8; N, 10·5%). The derived *base* separated from ethyl acetate or propan-2-ol as needles, m. p. 152—153° (Found C, 55·9; H, 6·55; N, 12·8. $C_{15}H_{21}N_3O_5$ requires C, 55·7; H, 6·55; N, 13·0%). The hydrochloride [13] is described as a glass.

N-*Benzyloxycarbonyl*-(γ-*t-butyl*)-L-*glutamyl-L-alanyl-L-*

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tyrosylglycine Methyl Ester (IX).—A solution of the base (VIII) (1·29 g., 4 mmoles), α-(2,4,5-trichlorophenyl) γ-t-butyl *N*-benzyloxycarbonyl-L-glutamate (2·275 g., 4·4 mmoles), and acetic acid (0·06 ml., *ca.* 1 mmole) in dimethylformamide (10 ml.) was kept at 4° for 16 hr. and then at 22—24° for 1 day. Ether (25 ml.) followed by ice–water (50 ml.) was added, and the solid was collected and washed with water and ether, to give the tetrapeptide derivative (2·32 g., 93%), m. p. 163—164° (from acetonitrile or ethyl acetate), R_{FA} 0·92, R_{FC} 0·90, R_{FE} 0·44, R_{FF} 0·85, R_{FH} 0·82, $[\alpha]_D^{26}$ −14·3° (*c* 1·0 in dimethylformamide), amino-acid ratios in 16 hr. acid hydrolysate: ala 1·00, glu 2·01, gly 0·99 [lit.,[13] m. p. 152—154·5°, $[\alpha]_D^{22}$ −15·7° (*c* 1·8 in dimethylformamide)] (Found: C, 59·6; H, 6·6; N, 8·7. Calc. for $C_{32}H_{42}N_4O_{10}$: C, 59·8; H, 6·6; N, 8·7%). In a subsequent experiment, the acetate (7·768 g., 20 mmoles), active ester (22 mmoles), and triethylamine (2·10 ml., 15 mmoles) were used in dimethylformamide (30 ml.); the yield was unaffected.

(γ-t-Butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester Acetate (X).—The tetrapeptide derivative (IX) (1·929 g., 3 mmoles) in 90% aqueous acetic acid (25 ml.) was hydrogenated for 5 hr. over 0·15 g. of catalyst. The acetate was obtained as a crystalline solid, m. p. 118—120° after air-drying, R_{FA} 0·49, R_{FC} 0·54, R_{FD} 0·69, R_{FE} 0·09, R_{FF} 0·37, R_{FH} 0·32, $[\alpha]_D^{26}$ −17·6° (*c* 0·94 in dimethylformamide), m. p. unchanged by recrystallisation from acetic acid–ethyl acetate (Found: C, 54·6; H, 7·1; N, 9·6. $C_{24}H_{36}N_4O_8,C_2H_4O_2$ requires C, 54·9; H, 7·1; N, 9·85%). In a subsequent experiment (16·5 mmoles) 90% aqueous acetic acid (137 ml.) and 0·82 g. of catalyst were used.

N-*Benzyloxycarbonyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester* (XI).—A solution of the acetate (X) (from hydrogenolysis of 3 mmoles of the benzyloxycarbonyl compound), α-(2,4,5-trichlorophenyl) γ-t-butyl *N*-benzyloxycarbonyl-L-glutamate (1·86 g., 3·6 mmoles), and triethylamine (0·28 ml., 2 mmoles) in dimethylformamide (15 ml.) was kept at 4° for 16 hr. Ether (20 ml.) was added, and the solution was added to a mixture of ice–water (80 ml.) and ether (20 ml.), to yield the pentapeptide derivative (2·370 g., 95%), m. p. 172·5—174·5°, R_{FA} 0·90, R_{FC} 0·89, R_{FE} 0·42, R_{FF} 0·78, R_{FH} 0·75, $[\alpha]_D^{26}$ −17·9° (*c* 1·07 in dimethylformamide), amino-acid ratios in 98 hr. acid hydrolysate: ala 1·01, glu 2·02, gly 0·98 [lit.,[13] m. p. 170—171°, $[\alpha]_D^{23}$ −17·1° (*c* 1·6 in dimethylformamide)]. The compound crystallised from ethyl acetate in needles, m. p. 177—178°. The yield was identical in a subsequent experiment (16·5 mmoles) with dimethylformamide (50 ml.) as solvent.

(γ-t-Butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester Acetate Hydrate (XII).—The pentapeptide derivative (XI) (1·656 g., 2 mmoles) in 90% aqueous acetic acid (27·5 ml.) was hydrogenated for 5 hr. over 0·1 g. of catalyst. The *acetate* was obtained as a crystalline solid, m. p. 204—210° (sintering and partial melting at 70°) after air-drying, R_{FA} 0·66, R_{FC} 0·78, R_{FE} 0·15, R_{FF} 0·64, R_{FH} 0·49, $[\alpha]_D^{26}$ −7·7° (*c* 0·96 in dimethylformamide) (Found: C, 54·1; H, 7·3; N, 9·0. $C_{33}H_{51}N_5O_{11},C_2H_4O_2,H_2O$ requires C, 54·4; H, 7·4; N, 9·1%). In a subsequent experiment (9·6 mmoles), 90% acetic acid (110 ml.) and 0·5 g. of catalyst were used.

N-*Benzyloxycarbonyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester* (XIII).—A solution of the acetate (XII) (from hydrogenolysis of 2 mmoles of the benzyloxycarbonyl compound), α-(2,4,5-trichlorophenyl) γ-t-butyl *N*-benzyloxycarbonyl-L-glutamate (1·240 g., 2·4 mmoles), and triethylamine (0·21 ml., 1·5 mmole) in dimethylformamide (25 ml.) was kept at 4° for 18 hr. and then at 22—24° for 1 day. Ether (50 ml.), followed by ice–water (50 ml.) and more ether (50 ml.), was added, to give the hexapeptide derivative (1·902 g., 94%), m. p. 197—198° (decomp.), R_{FA} 0·92, R_{FC} 0·92, R_{FE} 0·60, R_{FF} 0·79, R_{FH} 0·83, $[\alpha]_D^{26}$ −19·9° (*c* 1·0 in dimethylformamide), amino-acid ratios in 16 hr. acid hydrolysate: ala 1·03, glu 3·10, gly 0·98 [lit.,[13] m. p. 182—184° (decomp.), $[\alpha]_D^{27}$ −21·7° (*c* 1·1 in dimethylformamide)]. In a subsequent experiment (9·5 mmoles), the yield was 88%.

(γ-t-Butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester Acetate Hydrate (XIV).—The hexapeptide derivative (XIII) (1·787 g., 1·76 mmole) in 90% aqueous acetic acid (33 ml.) was hydrogenated for 6 hr. over 0·1 g. of catalyst. The *acetate* was obtained as a crystalline solid, m. p. 212—214° (decomp.) (sintering at 170—180°), R_{FA} 0·82, R_{FC} 0·82, R_{FE} 0·19, R_{FF} 0·74, R_{HF} 0·53, $[\alpha]_D$ −13·0° (*c* 1·0 in dimethylformamide) (Found: C, 55·0; H, 7·4; N, 8·75. $C_{42}H_{66}N_6O_{14},C_2H_4O_2,H_2O$ requires C, 55·2; H, 7·6; N, 8·8%). In a subsequent experiment (8·3 mmoles), 90% aqueous acetic acid (100 ml.) and 0·42 g. of catalyst were used.

N-*Benzyloxycarbonyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester* (XV).—A solution of the acetate (XIV) (from hydrogenolysis of 1·76 mmole of benzyloxycarbonyl compound), α-(2,4,5-trichlorophenyl) γ-t-butyl *N*-benzyloxycarbonyl-L-glutamate (1·09 g., 2·11 mmoles), and triethylamine (0·185 ml. 1·32 mmole) in dimethylformamide (25 ml.) was kept at 23—25° for 2 days. Ether (50 ml.) was added, and the solution was added to a mixture of ice–water (100 ml.) and ether (50 ml.), to yield the heptapeptide derivative (1·935 g., 92%), m. p. 220—221° (decomp.), R_{FA} 0·90, R_{FB} 0·92, R_{FE} 0·46, R_{FF} 0·83, R_{FH} 0·76, $[\alpha]_D^{26}$ −21·4° (*c* 0·97 in dimethylformamide), amino-acid ratios in 16 hr. acid hydrolysate: ala 1·02, glu 4·00, gly 0·98 [lit.,[13] m. p. 204—207° (decomp.), $[\alpha]_D^{27}$ −19·8° (*c* 1·1 in dimethylformamide)]. In a subsequent experiment (8·3 mmoles), with dimethylformamide (80 ml.) as solvent, the yield was 93%.

(γ-t-Butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester Acetate Hydrate (XVI).—The heptapeptide derivative (XV) (1·85 g., 1·55 mmole) in 90% aqueous acetic acid (44 ml.) was hydrogenated for 6 hr. over 0·1 g. of catalyst. The *acetate* was a crystalline solid, m. p. 235—237° (decomp.), R_{FA} 0·85, R_{FB} 0·95, R_{FC} 0·85, R_{FE} 0·24, R_{FF} 0·69, R_{FH} 0·64, $[\alpha]_D^{25}$ −18·1° (*c* 1·03 in dimethylformamide) (Found: C, 55·4; H, 7·4; N, 8·6. $C_{51}H_{81}N_7O_{17},C_2H_4O_2,H_2O$ requires C, 55·6; H, 7·7; N, 8·6%). In a subsequent experiment (7·6 mmoles) 90% aqueous acetic acid (165 ml.) and 0·4 g. of catalyst were used.

N-*Benzyloxycarbonyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester* (XVII).—A solution of the acetate (XVI) (from hydrogenolysis of 1·55 mmoles of benzyloxycarbonyl compound), α-(2,4,5-trichlorophenyl) γ-t-butyl *N*-benzyloxycarbonyl-L-glutamate (0·96 g., 1·86 mmole), and triethylamine (0·16 ml., 1·16 mmole) in dimethylformamide (10 ml.) was kept at 23—25° for 2 days. Ether (15 ml.) was added, and the

solution was added to a mixture of ice–water (50 ml.) and ether (35 ml.), to yield the octapeptide derivative (1·940 g., 91%), m. p. 240—242° (decomp.), R_{FD} 0·79, R_{FE} 0·37, R_{FF} 0·81, R_{FG} 0·0, R_{FH} 0·63, $[\alpha]_D^{26}$ −17·2° (*c* 1·0 in dimethylformamide), amino-acids in 16 hr. acid hydrolysate of 1 μ mole: ala 1·07, glu 5·00, gly 1·04 μ mole [lit.,[13] m. p. 239—241°, $[\alpha]_D^{27}$ −18·3° (*c* 1·2 in dimethylformamide)]. In a subsequent experiment (7·6 mmoles), with dimethylformamide (50 ml.) as solvent, the yield was 94%.

(γ-t-Butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester Acetate (XIX).—The octapeptide derivative (XVII) (1·80 g., 1·3 mmole) in 90% aqueous acetic acid (44 ml.) was hydrogenated for 6 hr. over 0·1 g. of catalyst. The *acetate* was a crystalline solid, m. p. 229—231° (decomp.), R_{FA} 0·84, R_{FC} 0·83, R_{FE} 0·27, R_{FF} 0·70, R_{FH} 0·69, $[\alpha]_D^{26}$ −14·5° (*c* 1·0 in dimethylformamide) (Found: C, 56·4; H, 7·7; N, 8·7. $C_{60}H_{96}N_8O_{20},C_2H_4O_2$ requires C, 56·9; H, 7·7; N, 8·6%). In a subsequent experiment (7·1 mmoles), 90% aqueous acetic acid (220 ml.) and 0·35 g. of catalyst were used.

N-Benzyloxycarbonyl-L-leucyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester Hydrate (XX).—A solution of the acetate (XIX) (from hydrogenolysis of 1·27 mmole of benzyloxycarbonyl compound), *N*-benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester (XVIII) (0·59 g., 1·52 mmole), and triethylamine (0·11 ml., 0·785 mmole) in dimethylformamide (10 ml.) was kept at 23—25° for 18 hr. Ethyl acetate (10 ml.) was added, and the resulting solution was added to a mixture of ice–water (25 ml.) and ether (25 ml.), to yield the nonapeptide derivative (1·910 g., 100%), m. p. 260—261°, R_{FA} 0·94, R_{FB} 0·91, R_{FC} 0·76, R_{FD} 0·83, R_{FE} 0·37 (streaked), R_{FF} 0·78, R_{FG} 0·0, R_{FH} 0·70, $[\alpha]_D^{26}$ −17·5° (*c* 0·96 in dimethylformamide), amino-acids in 16 hr. acid hydrolysate of 1 μ mole: ala 1·00, glu 4·87, gly 0·96, leu 0·82 μ mole (Found: C, 58·2; H, 7·4; N, 8·1. $C_{74}H_{113}N_9O_{23},H_2O$ requires C, 58·6; H, 7·65; N, 8·3%). In a subsequent experiment (7·1 mmoles) with triethylamine (0·825 ml., 5·89 mmoles), and dimethylformamide (50 ml.) as solvent, the yield was 92%.

L-Leucyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl)-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester Acetate Hydrate (XXII).—The nonapeptide derivative (XX) (1·877 g., 1·25 mmole) in 90% aqueous acetic acid (44 ml.) was hydrogenated at 23—25° for 5 hr. over 0·1 g. of catalyst. The *acetate* was a crystalline solid, m. p. 259—260° (decomp.), R_{FA} 0·90, R_{FB} 0·83, R_{FC} 0·85, R_{FD} 0·69, R_{FE} 0·19, R_{FF} 0·73, R_{FH} 0·56, $[\alpha]_D^{26}$ −11·2° (*c* 0·99 in dimethylformamide) (Found: C, 56·4; H, 7·7; N, 8·8. $C_{66}H_{107}N_9O_{21},C_2H_4O_2,H_2O$ requires C, 56·7; H, 7·9; N, 8·75%). In a subsequent experiment (6·37 mmoles), 90% aqueous acetic acid (165 ml.) and 0·4 g. of catalyst were used.

N-Benzyloxycarbonyl-L-tryptophyl-L-leucyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester Hydrate (XXIII).—A solution of the acetate (XXII) (from hydrogenolysis of 1·20 mmole of benzyloxycarbonyl derivative), *N*-benzyloxycarbonyl-L-tryptophan 2,4,5-trichlorophenyl ester (XXI) (0·776 g., 1·5 mmole), and triethylamine (0·132 ml., 0·94 mmole) in dimethylformamide (20 ml.) was kept at 23—25° for 18 hr. Ethyl acetate (20 ml.), followed by ice–water (40 ml.) was added, to yield the *decapeptide derivative* (1·738 g., 86%), m. p. 248—250° (decomp.), R_{FA} 0·93, R_{FB} 0·90, R_{FC} 0·75, R_{FD} 0·79, R_{FE} 0·39 (streaked), R_{FF} 0·77, R_{FG} 0·0, R_{FH} 0·68, $[\alpha]_D^{26}$ −14·2° (*c* 1·01 in dimethylformamide), amino-acid ratios in 16 hr. acid hydrolysate: ala 1·01, glu 4·96, gly 1·01, leu 0·84, tyr 0·94 (Found: C, 59·8; H, 7·2; N, 8·9. $C_{85}H_{123}N_{11}O_{24},H_2O$ requires C, 60·0; H, 7·4; N, 9·05%). In a subsequent experiment (6·37 mmoles), with dimethylformamide (80 ml.) as solvent, the yield was 87%.

L-Tryptophyl-L-leucyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester Acetate Hydrate (XXV).—The decapeptide derivative (XXIII) (1·696 g., 1·01 mmoles) in 90% aqueous acetic acid (55 ml.) was hydrogenated at 23—25° for 5 hr. over 0·1 g. of catalyst. The *acetate* was a crystalline solid, m. p. 249—251° (decomp.) (sintering at 221°), R_{FA} 0·82, R_{FB} 0·88, R_{FC} 0·77, R_{FD} 0·60, R_{FE} 0·18 (tailed), R_{FF} 0·78, R_{FH} 0·52, $[\alpha]_D^{26}$ −19·1° (*c* 1·0 in dimethylformamide), amino-acid ratios in 16 hr. acid hydrolysate; ala 1·02, leu 0·89, glu 5·5, gly 1·01, tyr 1·01; in 7 day alkaline hydrolysate: ala 1·02, leu 0·83, glu 4·94, gly 1·04, try 0·86, tyr 0·94 (Found: C, 58·1; H, 7·8; N, 9·4. $C_{77}H_{117}N_{11}O_{22},C_2H_4O_2,H_2O$ requires C, 58·3; H, 7·6; N, 9·5%). In a subsequent experiment (5·5 mmoles), 90% aqueous acetic acid (220 ml.) and 0·3 g. of catalyst were used.

N-Benzyloxycarbonyl-L-prolyl-L-trytophyl-L-leucyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester Hydrate (XXVI).—A solution of the acetate (XXV) (from hydrogenolysis of 1 mmole of benzyloxycarbonyl compound), *N*-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (Z-Pro-ONP) (0·44 g., 1·2 mmole), and triethylamine (0·105 ml., 0·75 mmole) in dimethylformamide (15 ml.) was stirred at 23—25° for 2 days. Ethyl acetate (15 ml.), followed by ice–water (40 ml.), was added, to yield the *undecapeptide derivative* (1·530 g., 86%), m. p. 250—251° (decomp.), R_{FA} 0·91, R_{FB} 0·90, R_{FC} 0·74, R_{FD} 0·79, R_{FE} 0·41, R_{FF} 0·75, R_{FG} 0·0, R_{FH} 0·64, $[\alpha]_D$ −17·7° (*c* 1·0 in dimethylformamide), amino-acid ratios in 16 hr. acid hydrolysate: ala 1·03, leu 0·92, glu 5·10, gly 0·99, pro 0·85, tyr 1·10; in 64 hr. acid hydrolysate: ala 1·02, leu 0·96, glu 5·18, gly 1·01, pro 0·97 (Found: C, 59·7; H, 7·1; N, 9·2. $C_{90}H_{130}N_{12}O_{25},H_2O$ requires C, 60·1; H, 7·4; N, 9·35%).

L-Prolyl-L-tryptophyl-L-leucyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester Acetate Hydrate (XXXI).—A solution of the undecapeptide derivative (XXVI) (0·895 g., 0·5 mmole) in hot acetic acid (40 ml.) was treated at 60° with a suspension of 0·1 g. of catalyst in water (4 ml.). The mixture was then hydrogenated for 5·5 hr. (temperature initially 50° dropping to 25° during 1 hr.). The *acetate* was a crystalline solid, m. p. 243—245° (decomp.), R_{FA} 0·77, R_{FB} 0·90, R_{FC} 0·88. R_{FD} 0·63, R_{FE} 0·0, R_{FF} 0·71 (tailed), R_{FG} 0·0, R_{FH} 0·47 to origin, $[\alpha]_D^{26}$ −7·3° (*c* 1·0 in dimethylformamide) (Found: C, 57·9; H, 7·4; N, 9·5. $C_{82}H_{124}N_{12}O_{23},C_2H_4O_2,H_2O$ requires C, 58·5; H, 7·6; N, 9·75%).

L-Pyroglutamylglycyl-L-prolyl-L-tryptophyl-L-leucyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine Methyl Ester (XXXII).—A solution of the acetate (XXXI) (from hydrogenolysis of 0·481 mmole of benzyloxycarbonyl compound), L-pyroglutamylglycine 2,4,5-trichlorophenyl ester (XXIX) (0·264 g., 0·721 mmole)

(50% excess), and triethylamine (0·05 ml., 0·357 mmole) in dimethylformamide (10 ml.) was kept at 25—26° for 4 days. Ethyl acetate (20 ml.), followed by ice–water (40 ml.), was added, to yield the *tridecapeptide derivative* (0·787 g., 90%), m. p. 246—247° (decomp.), R_{FA} 0·86, R_{FB} 0·92, R_{FC} 0·74, R_{FD} 0·73, R_{FF} 0·67, R_{FH} 0·43 (tailed), $[\alpha]_D$ −23·2° (*c* 0·93 in dimethylformamide), amino-acid ratios in 16 hr. acid hydrolysate: ala 1·04, glu 6·02, gly 1·76, leu 0·88, pro 0·86, tyr 0·96; in 64 hr. acid hydrolysate: ala 1·03, glu 6·15, gly 1·91, leu 0·96, pro 1·01, tyr 0·93; in 14 day alkaline hydrolysate: ala 1·12, glu 6·05, gly 1·91, leu 0·89, pro 1·02, try 0·93, tyr 0·98 (Found: C, 57·8; H, 7·1; N, 10·4. $C_{89}H_{132}N_{14}O_{26},H_2O$ requires C, 58·3; H, 7·4; N, 10·7%).

N-*Benzyloxycarbonyl*-L-*prolyl*-L-*tryptophyl*-L-*leucyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-((γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-L-*alanyl*-L-*tyrosylglycine Hydrate* (XXVII).—The corresponding methyl ester (XXVI) (0·356 g., 0·2 mmole) in boiling dioxan (80 ml.) [freshly purified by passage through alumina (Woelm, activity 1)] and water (20 ml.) was rapidly cooled to 22°, then treated with N-sodium hydroxide (1·00 ml.). The solution was kept for 45 min. at 22—24°, then evaporated for 15 min. in a rotary evaporator (bath at 40—45°). At the end of the evaporation the internal temperature was 24° and the volume remaining was 25 ml. The solution was made up to 40 ml. with ice and acidified to pH 3 with 20% aqueous citric acid. The *acid* (0·353 g., 100%) was collected and washed 6 times with water by centrifugation, and dried by lyophilisation, R_{FA} 0·86, R_{FB} 0·87, R_{FC} 0·56, R_{FD} 0·69, R_{FH} 0·35, R_{FF} 0·62 (single spots with permanganate or Ehrlich reagent), m. p. 237—239° (decomp.) $[\alpha]_D^{26}$ −17·6° (*c* 0·96 in dimethylformamide), +54·4° (*c* 0·96 in 0·1N-sodium hydroxide), amino-acid ratios in 16 hr. acid hydrolysate: ala 1·01, glu 5·15; gly 1·00, leu 0·88, pro 0·87, tyr 0·97 (Found: C, 59·2; H, 7·2; N, 9·3. $C_{89}H_{128}N_{12}O_{25},H_2O$ requires C, 59·9; H, 7·35; N, 9·4%).

L-*Prolyl*-L-*tryptophyl*-L-*leucyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-L-*alanyl*-L-*tyrosylglycine* (XXVIII).—A solution of the acid (XXVII) (2·70 g., 1·53 mmole) in 90% aqueous acetic acid (300 ml.) was hydrogenated at 23—25° for 5 hr. over 0·1 g. of catalyst. The *undecapeptide penta-t-butyl ester* (100% yield), which decomposed with partial melting at 245—247°, R_{FA} 0·74, R_{FB} 0·77, R_{FC} 0·25, R_{FD} 0·64, R_{FF} 0·81 (tailed to origin), R_{FH} 0·0, was obtained after drying the product over sodium hydroxide *in vacuo*, amino-acid ratios in 16 hr. acid hydrolysate: ala 1·05, glu 5·40, gly 1·01, leu 0·95, pro 0·96, tyr 0·99; in 64 hr. alkaline hydrolysate: ala 1·06, glu 5·48, gly 0·99, leu 1·00, pro 0·97, try 0·88.

L-*Pyroglutamylglycycl*-L-*prolyl*-L-*tryptophyl*-L-*leucyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-L-*alanyl*-L-*tyrosylglycine* (XXX).—A solution of the undecapeptide penta-t-butyl ester (XXVIII) (from hydrogenolysis of 1·53 mmole of benzyloxycarbonyl compound), L-pyroglutamylglycine 2,4,5-trichlorophenyl ester (XXIX) (1·12 g., 3·06 mmoles), and triethylamine (0·215 ml., 1·53 mmole) in dimethylformamide (150 ml.) was stirred at 23—25° for 6 days. Ice–water (150 ml.) was added, and the solution was acidified to pH 2 with aqueous citric acid. The solid was collected and washed once with 0·1N-hydrochloric acid, thrice with water, twice with chloroform, and four times with ether by centrifugation. Crystallisation from ethanol (80 ml.) yielded the *tridecapeptide penta-t-butyl ester* (2·29 g., 83%), m. p. 234—236° (decomp.), R_{FA} 0·79, R_{FB} 0·79, R_{FC} 0·25, R_{FD} 0·61, R_{FE} 0·0, R_{FF} 0·66 (tailed), R_{FG} 0·0, R_{FH} 0·0, $[\alpha]_D^{24}$ −10·8° (*c* 0·93 in dimethylformamide), amino-acid ratios in 16 hr. acid hydrolysate: ala 1·02, glu 6·26, gly 1·82, leu 0·93, pro 0·92, tyr 1·01 (Found: C, 58·3; H, 7·1; N. 10·6. $C_{88}H_{130}N_{14}O_{26}$ requires C, 58·7; H, 7·3; N, 10·9%).

N-*t*-*Butoxycarbonyl*-L-*prolyl*-L-*tryptophyl*-L-*leucyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-L-*alanyl*-L-*tyrosylglycine Methyl Ester Hydrate* (XXVIA).—This *compound* (90% yield), prepared in a manner similar to that described for the corresponding *N*-benzyloxycarbonyl derivative (XXVI) (from *N*-t-butoxycarbonyl-L-proline 2,4,5-trichlorophenyl ester instead of *N*-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester), had m. p. 237—239° (decomp.), R_{FA} 0·87, R_{FB} 0·86, R_{FC} 0·80, R_{FD} 0·76, R_{FE} 0·46, R_{FF} 0·85, R_{FG} 0·0, R_{FH} 0·66 (Found: C, 58·8; H, 7·3; N, 9·3. $C_{87}H_{132}N_{12}O_{25},H_2O$ requires C, 59·2; H, 7·65; N, 9·5%).

N-*t*-*Butoxycarbonyl*-L-*prolyl*-L-*tryptophyl*-L-*leucyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-L-*alanyl*-L-*tyrosylglycine Hydrate* (XXVIIA) *and* 2,4,5-*Trichlorophenyl Ester* (XXXIII).—A solution of the methyl ester (XXVIA) (1·53 g., 0·875 mmole) in hot dioxan (200 ml.) [freshly purified by passage through alumina (Woelm, activity 1)] and water (40 ml.) was rapidly cooled to 25° then treated with N-sodium hydroxide (4·38 ml., 5 equiv.). The solution was kept at 22—24° for 1 hr., then evaporated for 15 min. in a rotary evaporator (bath at 40—45°, internal temperature 24° at end of evaporation). The evaporated solution (*ca.* 100 ml.) was acidified to pH 3 with ice–water and 20% aqueous citric acid, to give the *acid* (XXVIIA) (1·395 g., 92%), m. p. 231—233° (decomp.), R_{FA} 0·84, R_{FB} 0·79, R_{FC} 0·39 R_{FD} 0·63, R_{FE} 0·20 (tailed to origin), R_{FF} 0·79, R_{FG} 0·0, R_{FH} 0·60, amino-acid ratios in 16 hr. acid hydrolysate: ala 1·01, glu 5·43, gly 1·01, leu 0·98, pro 0·94, tyr 0·98; in 64 hr. alkaline hydrolysate: ala 1·04, glu 4·92, gly 1·00, leu 0·86, try 0·86, tyr 0·98, which was collected and washed well with water by centrifugation (Found: C, 58·8; H, 7·4; N, 9·7. $C_{86}H_{130}N_{12}O_{25},H_2O$ requires C, 59·1; H, 7·6; N, 9·6%).

A solution of this acid (0·865 g., 0·5 mmole) in pure dry dimethylformamide (200 ml.) was evaporated to *ca.* 45 ml. at 10 mm., then treated with 2,4,5-trichlorophenol (0·495 g., 2·5 mmoles) followed by a solution of *NN'*-dicyclohexylcarbodi-imide (0·513 g., 2·5 mmoles) in pure dry dimethylformamide (5 ml.). The mixture was kept at 4° for 6 days and then at 23—25° for 2 days. Saturated aqueous sodium hydrogen carbonate (5 drops) followed by ether (50 ml.) and ice–water (50 ml.) was added, and the solid (0·828 g.), m. p. 245° (decomp.), was collected and washed well with ether and water. It contained *NN'*-dicyclohexylurea but was otherwise pure, R_{FA} 0·90, R_{FC} 0·89, R_{FD} 0·81, R_{FE} 0·60 (tailed), R_{FH} 0·82. The pure *active ester* (XXXIII) separated as needles from aqueous acetic acid (Found: C, 57·6; H, 7·0; Cl, 5·4; N, 8·7. $C_{92}H_{131}Cl_3N_{12}O_{25}$ requires C, 57·8; H, 6·9; Cl, 5·6; N, 8·8%).

N-*t*-*Butoxycarbonyl*-L-*prolyl*-L-*tryptophyl*-L-*leucyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-(γ-*t*-*butyl*)-L-*glutamyl*-L-*alanyl*-L-*tyrosylglycyl*-L-*tryptophyl*-L-*methionyl*-L-*aspartyl*-*phenylalanine Amide* (*BOC*-3—17 *Penta-t-butyl Ester*) (XXXV).—A solution of the undecapeptide (3—13)

2,4,5-trichlorophenyl ester (XXXIII), (382 mg., 0·2 mmole), L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide (XXXIV) trifluoroacetate [1] (142 mg., 0·2 mmole), and triethylamine (56 μ l., 0·4 mmole) was stirred at 22—24° under nitrogen for 11 days. The mixture was warmed to 40° and then added to a mixture of ice (5 g.) and N-hydrochloric acid (1 ml.). The solid was collected and washed by centrifugation with ice-cold 0·5N-hydrochloric acid (6 ml.), water (6 × 6 ml.), ethanol (3 × 4 ml.), and ether (peroxide-free; 4 × 7 ml.), then dried at 25° *in vacuo*, to yield the crude t-butoxycarbonyl penta-t-butyl ester (XXXV) (0·271 g., 63%). Analysis of this product (amino-acid ratios in acid hydrolysate: asp 0·74, glu 5·24, pro 0·86, gly 0·99, ala 1·01, met 0·73, leu 0·95, tyr 0·99, phe 0·74) indicated about 75% purity.

N-t-Butoxycarbonyl-L-prolyl-L-tryptophyl-L-leucyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycyl-L-tryptophyl-L-methionyl-(β-t-butyl)-L-aspartyl-L-phenylalanine Amide (*BOC-3—17 Hexa-t-butyl Ester*) (XLI).—(*a*) A solution of the undecapeptide (3—13) acid (XXVIIA) (0·875 g., 0·5 mmole) in pure dry dimethylformamide (100 ml.) was evaporated to *ca.* 30 ml. at 10 mm., then treated at −10° with stirring with triethylamine (70 μ l., 0·5 mmole) followed by freshly distilled pivaloyl chloride (64·6 μ l., 0·525 mmole). The mixture was stirred at −10° for 10 min., then L-tryptophyl-L-methionyl-(β-t-butyl)-L-aspartyl-L-phenylalanine amide (0·653 g., 1 mmole) (100% excess) was added and stirring was continued at ambient temperature for 18 hr. The solution was cooled to 0° and 50% aqueous potassium carbonate (0·5 ml.) was added, followed immediately by ice–water (50 ml.). The solid was collected and washed by centrifugation with water (2 × 80 ml.), 0·2 N-hydrochloric acid (80 ml.), water (4 × 80 ml.), ethanol (3 × 40 ml.), and ether (peroxide-free; 4 × 40 ml.), to yield the crude *t-butoxycarbonyl hexa-t-butyl ester* (925 mg. 78%), m. p. 239—244° (effervescence), R_{FA} 0·92, R_{FB} 0·95, R_{FC} 0·87, R_{FD} 0·65, R_{FE} 0·0, R_{FF} 0·80, R_{FH} 0·71, amino-acid ratios in acid hydrolysates: asp 0·98, glu 5·46, pro 0·94, gly 1·00, ala 1·04, met 1·00, leu 1·01, tyr 1·00, phe 1·00.

(*b*) A solution of the undecapeptide (3—13) 2,4,5-trichlorophenyl ester (XXXIII) (191 mg., 0·1 mmole) and L-tryptophyl-L-methionyl-(β-t-butyl)-L-aspartyl-L-phenylalanine amide (66 mg., 0·1 mmole) in pure dimethylformamide (1 ml.) was stirred at 23—25° for 4 days. Additional dimethylformamide (1 ml.) was then added and stirring was continued at 23—25° for a further 6 days. The mixture was warmed to 40° (to dissolve the solid which separated), then ice–water (6 ml.) and N-hydrochloric acid were added (to pH 1). The solid was collected and washed by centrifugation with ice-cold 0·5N-hydrochloric acid (6 ml.), water (6 × 6 ml.), ethanol (2 × 5 ml.), and ether (peroxide-free; 4 × 8 ml.), then dried at 25° *in vacuo*, to yield the t-butoxycarbonyl hexa-t-butyl ester (116 mg.). On t.l.c. this behaved like the product of (*a*). Amino-acid ratios in acid hydrolysates: asp 0·87, glu 5·16, gly 0·99, ala 1·00, met 0·81, leu 0·87, tyr 1·01, phe 0·84. A small sample was dissolved rapidly in hot acetic acid and the solution was cooled to yield needles of the pure compound, m. p. 240—244° (effervescence), amino-acids in acid hydrolysate of 1·06 μ mole: asp 1·04, glu 5·32, gly 1·05, ala 1·06, leu, 0·96, met 0·92, tyr 1·05, phe 1·03 μ mole).

L-Prolyl-L-tryptophyl-L-leucyl-L-glutamyl-L-glutamyl-L-glutamyl-L-glutamyl-L-glutamyl-L-alanyl-L-tyrosylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine Amide and Trifluoroacetate (3—17) (XXXVI).—(*a*) The t-butoxycarbonyl hexa-t-butyl ester (XLI) (870 mg.) [prepared by method (*a*)] was dissolved at 15—20° under nitrogen in trifluoroacetic acid (25 ml.). The solution was kept at 23—25° for 1 hr. and then evaporated at room temperature and lyophilised. The product was suspended in 0·05M-ammonium hydrogen carbonate (5 ml.) and concentrated aqueous ammonia was added at 0—5° until all the solid had dissolved. The resulting solution (pH 8) was centrifuged from a small amount of brown amorphous solid and then applied to a G-25 Sephadex column (60 × 3·3 cm.) (previously equilibrated with 0·05M-ammonium hydrogen carbonate). Fractions (14·5 ml.) were collected every 20 min. and optical density was recorded continuously with a Uvicord instrument (type 8301A). The main peak (tubes 19—33) was dried by lyophilisation to yield the *pentadecapeptide amide ammonium salt hydrate* as a light solid (1·29 g.), amino-acid ratios in 16 hr. acid hydrolysate: ala 1·06, asp 0·98, leu 1·02, glu 5·5, gly 1·0, met 0·96, phe 0·96, pro 0·93, tyr 1·03. The recovery of amino-acids (52%) in the acid hydrolysate suggested that this salt was hydrated with its own weight of water, and agreed with the recovery of the derived acid. A solution of the ammonium salt (250 mg.) in ice-cold water (40 ml.) was acidified to pH 3 with N-hydrochloric acid (no effervescence). The precipitate was collected, washed five times with water by centrifugation and dried (conc. H_2SO_4) at 0·1 mm., to yield the *pentadecapeptide amide* (126 and 127 mg. in successive experiments) (51% based on ammonium salt, 84% based on crude butoxycarbonylpentadecapeptide penta-t-butyl ester), m. p. 243—245° (effervescence), R_{FA} 0·46, R_{FB} 0·56, R_{FC} 0·14, R_{FD} 0·26, R_F 0·52 in chloroform–methanol–17% aqueous ammonia (20 : 20 : 9), $E_{2\cdot2}$ 0·0, $E_{6\cdot2}$ 0·46 × glu (tailed to origin), $E_{8\cdot8}$ 0·71 × glu, $[\alpha]_D^{25}$ −16·5° (*c* 0·95 in dimethylformamide), amino-acids in 18 hr. acid hydrolysate of 1 μ mole: ala 1·04, asp 0·99, leu 1·03, glu 5·4, gly 1·01, met 0·97, phe 0·97, pro 0·96, tyr 1·03 μ mole, $\lambda_{min.}$ (in 0·05M-ammonium hydrogen carbonate) 250·5 mμ (ε 5270), $\lambda_{max.}$ 280·5 (11,800) and 288·5 (9650).

(*b*) The t-butoxycarbonyl hexa-t-butyl ester (73 mg.) [prepared by method (*b*)] was similarly cleaved with trifluoroacetic acid (3 ml.). Trituration of the evaporated solution with dry ether (peroxide-free) gave the trifluoroacetate (72 mg.), which was collected and washed several times with ether by centrifugation. The product was used in the synthesis of human gastrin (HI) [method (*b*)] without further purification.

(*c*) The crude t-butoxycarbonyl penta-t-butyl ester (XXXV) (170 mg.) was similarly cleaved with trifluoroacetic acid (5 ml.), to yield the trifluoroacetate (140 mg.), which was used in the synthesis of human gastrin (HI) [method (*b*)] without further purification.

Human Gastrin (*HI*) *Hexa-t-butyl Ester* (XXXIX).—A solution of L-pyroglutamylglycyl-L-prolyl-L-tryptophyl-L-leucyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-(γ-t-butyl)-L-glutamyl-L-alanyl-L-tyrosylglycine (XXX) (1·260 g., 0·7 mmole) in dimethylformamide (100 ml.) [freshly-distilled and purified immediately prior to use by passage through a column of alumina (Woelm, activity 1)] was evaporated to 30 ml. at 10 mm., then treated at −20° with stirring with triethylamine (98 μ l., 0·7 mmole) followed by freshly-distilled pivaloyl chloride (95 μ l., 0·77 mmole). The mixture was stirred at −20 to −15° for 15 min., then L-tryptophyl-

L-methionyl-(β-t-butyl)-L-aspartyl-L-phenylalanine amide (XXXVIII) (0·840 g., 1·4 mmole) was added and stirring was continued at ambient temperature for 18 hr. The solution was cooled to 0° and 50% aqueous potassium carbonate (5 ml.), followed immediately by ice–water (100 ml.), was added. The solid was collected and washed by centrifugation with water (5 × 80 ml.) (final pH 7), ice-cold 0·2N-hydrochloric acid (80 ml.), ice-cold 0·1N-hydrochloric acid (80 ml.), water (5 × 80 ml.), ethanol (1 × 60 ml., 2 × 40 ml.) and ether (4 × 80 ml.). It was then dried at 25° *in vacuo* for 48 hr., to yield *gastrin* (*HI*) *hexa-t-butyl ester* (XXXIX) (1·331 g., 78%), m. p. 237—240° (effervescence), R_{FA} 0·81; R_{FB} 0·82; R_{FC} 0·80 (tailed), R_{FD} 0·71; R_{FE} 0·0; R_{FF} 0·71; R_{FG} 0·0, amino-acid ratios in acid hydrolysate: asp 1·03, glu 5·9, gly 1·88, ala 1·05, met 0·95, leu 0·96, tyr 0·95, phe 1·01.

Human Gastrin (*HI*) (XL).—(*a*) Gastrin (HI) hexa-t-butyl ester (XXXIX) (40 mg.) was added at 20—22° under nitrogen to trifluoroacetic acid (2 ml.). The solution was stirred at 22—24° for 1 hr. and then evaporated at room temperature and lyophilised. The pale brown solid residue was triturated at 0° with 0·05M-ammonium hydrogen carbonate (0·5 ml.), and concentrated aqueous ammonia was added at 0° until all the solid dissolved. The pale yellow solution was centrifuged from a small amount of gelatinous precipitate and then applied to a G-25 Sephadex column (75 × 0·9 cm.) (previously equilibrated with 0·05M-ammonium hydrogen carbonate). Fractions (0·7 ml.), were collected every 7·5 min. and their optical density was measured at 280 mμ. The main peak (tubes 59—72) was dried by lyophilisation to yield human gastrin (HI) ammonium salt (21 mg.) R_{FA} 0·75, R_{FB} 0·73, R_{FC} 0·22 (tailed), R_{FD} 0·51 (tailed), R_F 0·59 in chloroform–methanol–17% aqueous ammonia (20 : 20 : 9), $E_{2.2}$ 0·0, $E_{6.2}$ 0·54 × glu, amino-acid ratios in 16 hr. acid hydrolysate: asp 1·06, glu 6·15, pro 0·94, gly 1·91, ala 1·09, met 0·92, leu 0·88, tyr 0·97, phe 0·96. In each of two repeat experiments, 660 mg. of the hexa-t-butyl ester was used, the reaction product was applied to a G-25 Sephadex column (60 × 3·3 cm.), fractions (16 ml.) were collected every 20 min., and optical density was recorded continuously with a Uvicord instrument (type 8301A). The yields from the main peak (tubes 15—22) were 344 and 431 mg.

(*b*) A solution of L-prolyl-L-tryptophyl-L-leucyl-L-glutamyl-L-glutamyl-L-glutamyl-L-glutamyl-L-glutamyl-L-alanyl-L-tyrosylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide trifluoroacetate (3—17) (XXXVI) (50 mg., 24 μ mole) in pure dimethylformamide (1 ml.) was treated with triethylamine (23·5 μ l., 168 μ mole, 7 equiv.) and the precipitate was dissolved by adding water (0·1 ml.) at 0°. L-Pyroglutamylglycine 2,4,5-trichlorophenyl ester (XXIX) (44 mg., 120 μ mole) (5 equiv.) was added, and the mixture was stirred at 23—25° for 10 days. The solution was cooled to 0°, acidified with N-hydrochloric acid (0·5 ml.), and immediately diluted with ice–water (5 ml.). The solid was collected and washed by centrifugation with ice–cold 0·5N-hydrochloric acid (5 ml.), water (6 × 8 ml.), ethanol (2 × 8 ml.), chloroform (2 × 8 ml.), and ether (peroxide-free; 4 × 8 ml.), then dried at 25° *in vacuo*, to yield a solid (49 mg.). This was chromatographed on G-25 Sephadex as in (*a*), to yield, from the major peak, pure (electrophoresis, t.l.c.) gastrin (HI) ammonium salt, amino-acid ratios in acid hydrolysate: asp 0·94, glu 6·00, pro 0·93, gly 2·03, ala 0·97, met 0·90, leu 0·86, tyr 0·98, phe 0·95.

L-*Tryptophyl*-L-*methionyl*-(β-*t*-*butyl*)-L-*aspartyl*-L-*phenylalanine Amide* (XXXVIII).—(*a*) A solution of *o*-nitrophenylsulphenyl-L-tryptophyl-L-methionyl-(β-t-butyl)-L-aspartyl-L-phenylalanine amide (XXXVII)[1] (2·015 g., 2·5 mmoles) and 4-chloro-2-nitrothiophenol (0·719 g., 3·8 mmoles) in dry pyridine (10 ml.) was kept at 22—24° for 2 hr. The solid (1·68 g.) obtained by the addition of dry ether (100 ml.) was shaken for 5 min. at 4° in a Vibromixer with 0·1N-hydrochloric acid (25 ml.), the mixture was filtered, and the filtrate was neutralised at 0° with sodium hydrogen carbonate. The residue from the filtration was digested in the same manner with 0·1N-hydrochloric acid (6 × 25 ml.) with filtration and neutralisation of the filtrates as before. The solid in the combined neutralised filtrates was then collected, washed six times with water by centrifugation, and dried at 25° *in vacuo*, to yield the *tetrapeptide amide t-butyl ester* (0·661 g., 40%), m. p. 171—172·5°, R_{FA} 0·78, R_{FC} 0·80, R_{FD} 0·71, R_{FF} 0·14, R_{FF} 0·70. This separated from ethyl acetate as needles, m. p. 167—168°, $[\alpha]_D^{25}$ −20° (*c* 1·7 in dimethylformamide), +1·9° (*c* 2·1 in methanol) (Found: C, 61·0; H, 7·1; N, 12·8. $C_{33}H_{44}N_6O_6S$ requires C, 60·6; H, 6·8; N, 12·9%).

(*b*) (Based on method supplied by Professor E. Scoffone). The *o*-nitrophenylsulphenyl derivative (XXXVII)[1] (4·03 g., 5 mmoles) was dissolved at 15—20° in 80% aqueous thioglycollic acid (25 ml.) and the solution was kept at 20—24° for 15 min. Dry ether (200 ml.) was then added, and the thioglycollate (2·944 g., 78%), m. p. 175° (effervescence), was collected and washed well with ether. A solution of this salt in cold dimethylformamide (25 ml.) was treated with ether (100 ml.) followed, at 0°, by 50% aqueous potassium carbonate (5 ml.), and ice–water (200 ml.). The solid was collected, washed well with water and ether, and dried *in vacuo* at 25°, to yield the tetrapeptide amide t-butyl ether (2·150 g., 66% overall), m. p. 167—168° [from ethyl acetate (500 ml.)].

[7/067 *Received, January 18th,* 1967]