Peptide Synthesis. Part 5. Solid-phase Synthesis of [15-Leucine]Little Gastrin

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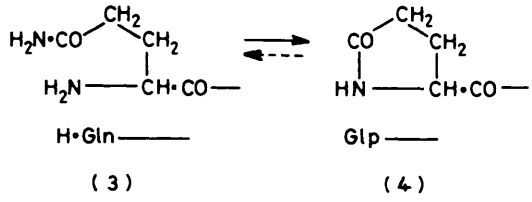
A procedure is described for the solid-phase synthesis of gastrin peptides exemplified by [15-leucine]little gastrin. The method makes use of a polar polyamide resin support and stepwise addition of fluorenylmethoxycarbonylamino-acid derivatives. Treatment with acidic reagents is minimised, obviating the need for side-chain protection of tryptophan and eliminating acid-catalysed side reactions at the multiple glutamyl residues. Formation of the terminal pyroglutamyl residue was achieved through a glutaminyl intermediate.

In a previous paper in this series,[1] we described the solid phase synthesis of the leucine analogue (1b) of human minigastrin I (1a) and of its shortened des-1-tryptophan derivative. These syntheses established that the novel solid phase methods we have developed over recent years [2-4] were applicable to the difficult amino-acid sequences presented by the gastrin series of hormones. Hitherto, gastrins had apparently not been accessible by solid-phase synthesis techniques based on conventional polystyrene supports and acid-labile (t-butoxycarbonyl and benzyl) protecting groups,† presumably owing largely to the high content of amino-acids which in protected or free form are known to undergo acid-catalysed side reactions.[1] The methods successfully employed utilised polar polydimethylacrylamide resins [2] and a protecting group combination which minimised acid treatment,[3] *i.e.* base-labile fluorenylmethoxycarbonylamino-acids [6] with side chains protected as t-butyl esters or ethers as appropriate. No protecting groups were necessary on the particularly acid-sensitive tryptophan residues. Exceptionally high yields of the minigastrin analogue (1b) and of its des-1-tryptophan derivative were obtained with the full biological potency of natural little gastrin I.‡

H.Trp.Leu.Glu_5.Ala.Tyr.Gly.Trp.X.Asp.Phe.NH_2
(1a) X = Met; (1b) X = Leu
Y.Gly.Pro.Trp.Leu.Glu_5.Ala.Tyr.Gly.Trp.X.Asp.Phe.NH_2
(2a) Y = Glp, X = Met; (2b) Y = Glp, X = Leu;
(2c) Y = Gln, X = Leu

The gastrin family [9] includes hormones or prohormones of substantially greater length than the minigastrins. Thus porcine little gastrin I (2a), the first characterised,[10] contains 17 amino-acids terminating at its amino-terminus in a pyroglutamyl (Glp) residue. The latter is evidently formed by cyclisation of terminal glutamine during processing or isolation, since the presumed hormone, big gastrin,[9] contains glutamine at this position and is extended by a further 17 residues. This paper describes our synthetic studies on [15-leucine]human little gastrin (2b). To permit possible future extension into the prohormone region, the 17-residue sequence (2c) was assembled with *N*-terminal glutamine, and we include observations on the separation of glutaminyl (3) and pyroglutamyl peptides (4) and their interconversion. Where pyroglutamyl derivatives are the main synthetic objectives, however, direct incorporation of this residue has proved superior in other cases.[11]



(3) (4)

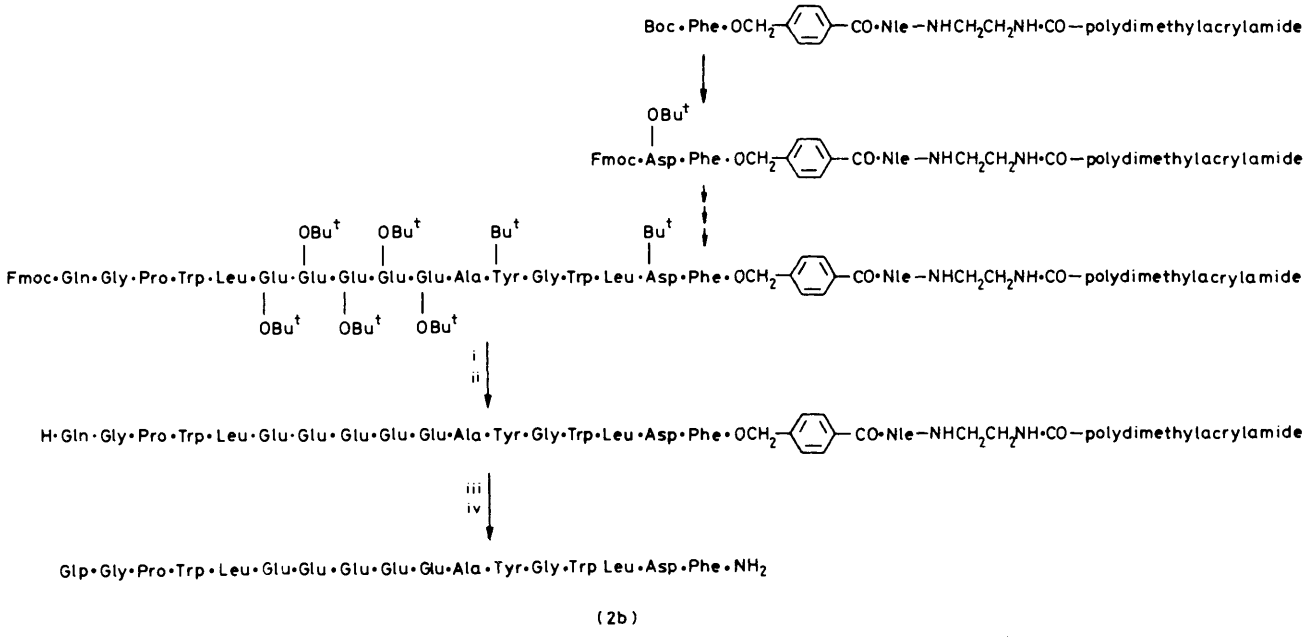
The initial steps (Scheme) in the synthesis of (2b) followed those of the earlier minigastrin synthesis [1] and are listed only briefly here. Polydimethylacrylamide-resin functionalised with methoxycarbonyl groups [2] was converted into the amino-resin with excess of ethylenediamine. An internal reference-spacer amino-acid was introduced by acylation with Fmoc-norleucine anhydride and the Fmoc-group cleaved with 20% piperidine.[3] All acylation and deprotection reactions in the assembly utilised dimethylacetamide as the only solvent. Completeness of acylation was verified by qualitative ninhydrin tests on resin samples. The peptide-resin reversible linkage agent, 4-hydroxymethylbenzoic acid, was added as its activated trichlorophenyl active ester [1,3] in the presence of catalyst hydroxybenzotriazole. The first amino-acid of the gastrin sequence (phenylalanine) was esterified to the resin as its t-butoxycarbonyl symmetrical anhydride derivative in the presence of 4-dimethylaminopyridine (1 equiv.). §

Use of the t-butoxycarbonyl protecting group in this step is indicated because of the slight lability of Fmoc groups to 4-dimethylaminopyridine.[3] There are no other acid-sensitive groups attached to resin at this stage. After cleavage of the Boc group using anhydrous hydrogen chloride in acetic acid,[2] the following 15 residues were added as fluorenylmethoxycarbonyl derivatives, again using the symmetrical anhydride procedure. Intermediate Fmoc-peptides were deprotected with piperidine in dimethylformamide. A sample of peptide resin was removed after addition of 13 residues, enabling comparison with previously prepared [1] tridecapeptide amide after protecting group and resin cleavage. The synthesis of little gastrin was continued by sequential addition of tryptophyl, prolyl, and glycyl residues, and then terminated with Fmoc-glutamine *p*-nitrophenyl ester in the presence of hydroxy-

† Coy [5] has recently described synthetic studies in the gastrin series using very acid-labile *o*-nitrophenylsulphenylamino-acid derivatives.

‡ Replacement of methionine by leucine has no effect on the biological activity of gastrins.[7] It was adopted as a simplifying measure avoiding the problems of oxidation frequently encountered in the synthesis of methionyl peptides, but the methods employed are equally applicable in the methionyl series (*cf.* refs. 3, 8).

§ Following the recognition that slight racemisation of protected amino-acids may ensue from the use of 4-dimethylaminopyridine in esterification reactions,[12] our current practice uses less pyridine derivative (0.1 equiv.) in the presence of *N*-methylmorpholine.[12]



Scheme. Synthesis of [15-leucine]human little gastrin. *Reagents:* i, 20% piperidine, DMA; ii, 90% aq. trifluoroacetic acid; iii, NH_3, MeOH; iv, 20% aq. acetic acid

Table 1. Amino-acid composition of resin-bound peptides referred to Phe = 1.00. The norleucine (Nle) residue is an internal reference amino-acid directly attached to the polydimethylacrylamide resin

Residue no.	17	10—17	9—17	8—17	7—17	6—17	1—17
Nle	1.07	1.09	1.10	1.11	1.08	1.13	1.07
Phe	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Asp		1.01	1.02	1.02	1.01	1.00	0.94
Leu		0.99	1.01	1.00	1.00	2.02	1.99
Gly		1.00	1.01	1.00	1.01	1.01	1.97
Tyr		1.00	1.00	1.01	1.00	1.00	1.00
Ala		1.01	1.01	1.01	1.02	1.01	1.06
Glu		1.19	2.11	3.14	4.18	5.27	6.18
Pro							1.02

benzotriazole. This last was the only amino-acid coupling not performed by the symmetrical anhydride procedure. Resin samples were removed after every deprotection step and 10—15 min into each acetylation reaction. The latter gave in every case no colour with ninhydrin and no repeated acylation reactions were judged necessary. Samples were additionally removed at steps 1, 8, 9, 10, 11, 13, and 17, enabling (after the assembly was complete) a full picture of amino-acid incorporation to be built up. Very satisfactory results were obtained (Table 1). The near constancy of the ratio of the internal reference amino-acid (norleucine) to those of the sequence proper indicated little if any loss of peptide chains from the resins during the acylation and deprotection reactions.

At least partial conversion of the *N*-terminal glutamine residue into its cyclised, pyroglutamyl form [(3) ⟶ (4)] was to be expected during the acidic and basic treatments required to liberate the free heptadecapeptide amide from the resin, and initial studies on the isolation of the synthetic product concentrated on this aspect. The fluorenylmethoxycarbonyl group protecting the glutamine amino terminal residue was first cleaved with piperidine in the usual manner, followed by removal of all seven t-butyl groups with aqueous trifluoroacetic acid. The carefully dried resin * was then subjected to ammonolysis with methanolic ammonia. Anion-exchange chromatography at pH 8.1 of the total crude product gave the elution profile depicted in Figure 1. Analytical h.p.l.c. of the main peak resolved only minor impurities (Figure 2b). The virtual absence of failure or truncated sequence in the crude product was to be expected from the excellent amino-acid incorporation figures which were obtained throughout (Table 1), but the lack of evidence for the presence of both glutaminyl (2c) and pyroglutamyl (2b) peptide amides warranted further investigation. At pH's significantly greater than the p*K* of the terminal amino-group, the two forms would be isoelectric and probably not separable by ion-exchange chromatography. Relative behaviour on reverse phase h.p.l.c. was less predictable but would be expected to be pH-dependent although the nominal pH of the elution system (4.5) was already substantially below the p*K* of near neutrality to be expected for the amino-group in the terminal glutaminyl in (2c) residue (4). In the event, changes in pH of the eluting buffers enabled resolution of glutaminyl and pyroglutamyl peptides on both ion-exchange and reverse phase chromatography. Thus at pH 3.5, h.p.l.c. of the ion-exchange chromato-

* This drying is essential in order to avoid partial formation of the free terminal carboxylic acid groups during ammonolytic detachment from the resin.[1]

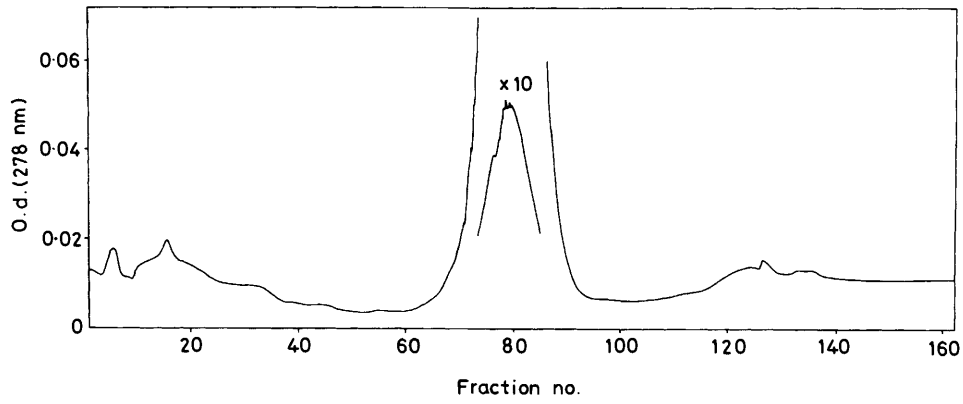


Figure 1. Anion-exchange chromatography of total crude heptadecapeptide amide on diethylaminoethyl cellulose. For conditions see text

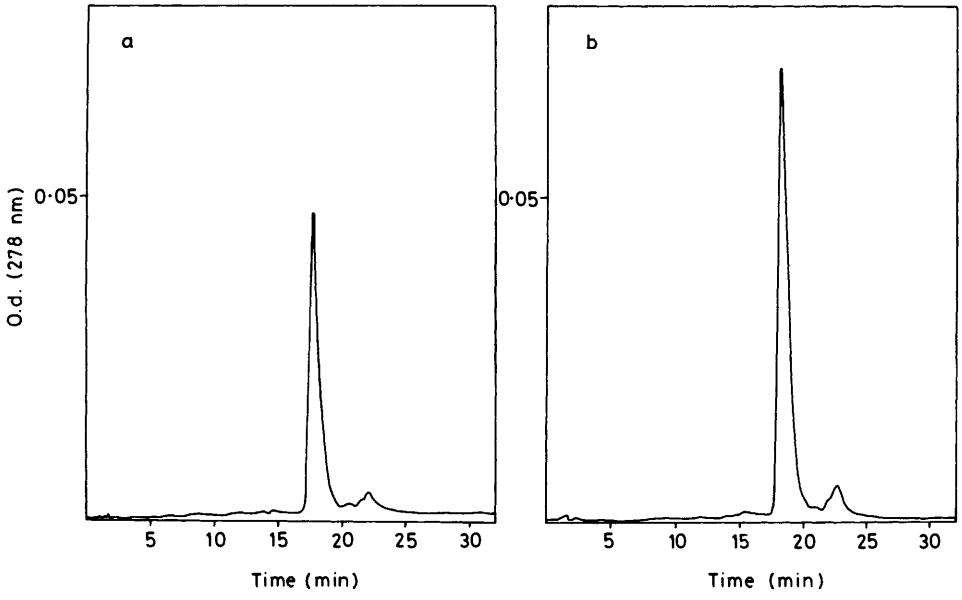


Figure 2. H.p.l.c. on μ-Bondapak C_{18} at pH 4.5 of (a) total crude heptadecapeptide amide, and (b) principal peak from anion-exchange chromatography (Figure 1). Linear gradient of 20—50% MeCN in 0.1M-NH_4OAc, pH 4.5 during 30 min

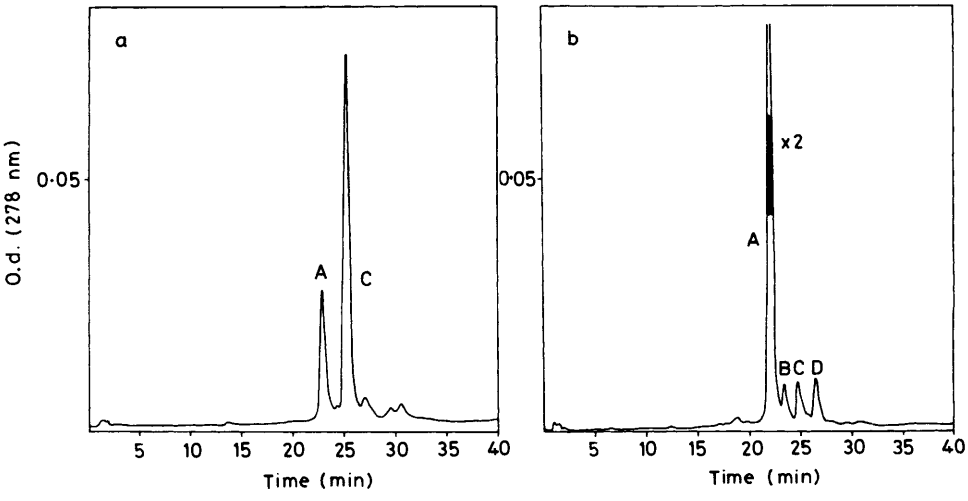


Figure 3. H.p.l.c. on μ-Bondapak C_{18} at pH 3.5 of (a) principal peak from anion-exchange chromatography (Figure 1) of crude heptadecapeptide amide (*cf.* Figure 2b), and (b) after cyclisation in 20% acetic acid. For identification of peaks see text. Conditions: linear gradient of 20—50% MeCN in 0.1M-NH_4OAc, pH 3.5 during 30 min

graphed product revealed two main peaks (Figure 3a) of which the smaller, A (*ca.* 25%), corresponded exactly in elution position with that of authentic [13] [15-leucine]human little gastrin (2c). The larger, C, of the two peaks was presumed to be the corresponding glutaminyl peptide amide (2b), subsequently confirmed by enzymic degradation (see below). With the aid of h.p.l.c. at reduced pH, interconversion of the two peptide amides could now be studied. The pyroglutamyl derivative gave no trace of glutaminyl [*i.e.* (4) ⟶ (3)] peptide after treatment with methanolic ammonia for 18 h, conditions typically used for ammonolysis of the peptide resin. This confirms the general feasibility of direct addition of pyroglutamyl residues when peptide amides terminating in this amino-acid are the sole synthetic objective. Conversion of glutaminyl into the pyroglutamyl form was slowly achieved in the presence of weakly acidic catalysts.[14] It was nearly complete after treatment with 20% acetic acid for 64 h at 30 °C (h.p.l.c.; Figure 3b). Following this treatment, the peptide mixture was subjected to anion-exchange chromatography at a reduced pH of 6.5. The elution profile (Figure 4) showed separation of an earlier emerging, less negatively charged species X, presumably protonated residual glutaminyl peptide. At this stage, the main peak Y still showed the presence (Figure 5a) of two very minor impurities. These were

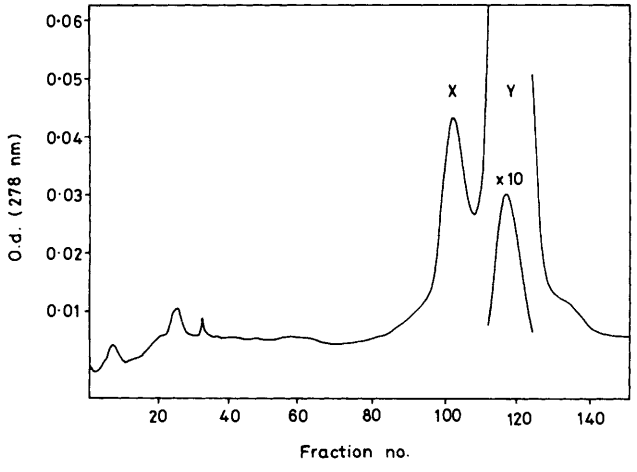


Figure 4. Anion-exchange chromatography of cyclised heptadecapeptide amide (Figure 3b) on diethylaminoethyl cellulose. For conditions see text

in turn removed by semi-preparative reverse phase h.p.l.c. to give the final, highly purified [15-leucine]human little gastrin I (Figure 5b) in an overall yield of 32%. It was identical on both h.p.l.c. and t.l.c. with an authentic sample prepared by solution synthesis,[13] generously provided by Professor E. Wunsch. ' Fingerprints ' prepared by partial proteolysis of the two synthetic products confirmed their identity (Figure 6). The heptadecapeptide amide was equiactive with the same molar concentration of natural human little gastrin I when continuously infused into conscious dogs equipped with gastric cannulae.

The structure of the intermediate glutaminyl peptide was confirmed, and suggestive evidence obtained for the structures of the minor impurities B and D (Figure 3b), by comparative acidic and enzymatic hydrolysis of the various peptide fractions together with authentic [15-leucine]little gastrin [13] (Table 2). The preparation of amino-peptidase-M used evidently had hydrolase activity for pyroglutamyl peptides, as authentic [15-leucine]little gastrin was extensively hydrolysed and gave analytical figures similar to the corresponding acidic hydrolysate but with reduced glutamic acid (Table 2, columns 1 and 2). The same result was obtained with the present solid-phase-synthesised product (columns 3 and 4). The putative glutaminyl intermediate (peak C, Figure 3) likewise gave reduced glutamic acid on enzymatic hydrolysis, but also liberated nearly one residue of glutamine (columns 7 and 8). The minor impurity (peak B, Figure 3b) was incompletely hydrolysed by the enzyme preparation with very much reduced liberation of aspartic acid and phenylalanine (columns 5 and 6). This is consistent with earlier observations regarding slight racemisation of the *C*-terminal phenylalanine residue in minigastrin synthesis.[1] We have recently defined conditions for attachment of terminal residues to hydroxymethyl solid-phase supports which minimise this racemisation.[14] The second minor impurity (peak D, Figure 3b) shows reduced tryptophan on enzymic hydrolysis (columns 9 and 10), suggesting that, as in the minigastrin series, some attachment of t-butyl cations occurs on either one of the two tryptophan residues. The small extent of this side reaction is notable, especially in view of the very large proportion of t-butyl protected residues (7 out of 17) present in the peptide resin. It is probable that the t-butyl cations formed in the side-chain deprotection step are rapidly excluded or washed from the resin matrix, giving advantage to the two-step cleavage procedure used in the present work. In similar deprotection reactions in solution, very substantial quantities of t-butylated derivatives appear to be formed.[15]

The sample of peptide resin removed after addition of only

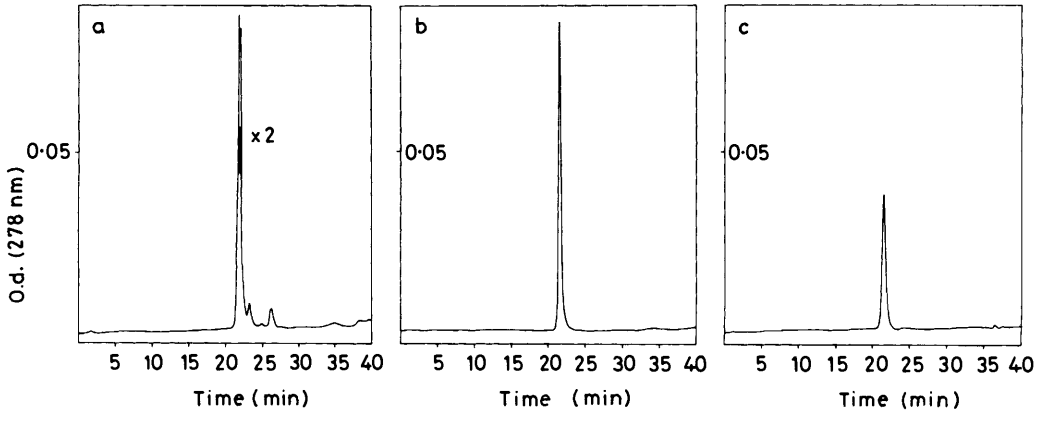


Figure 5. H.p.l.c. on μ-Bondapak C_{18} at pH 3.5 of (a) anion-exchange purified cyclised heptadecapeptide amide (Figure 4, peak Y); (b) the same after reversed phase semi-preparative h.p.l.c. purification; and (c) authentic [13] [15-leucine]human little gastrin I. Conditions as Figure 3

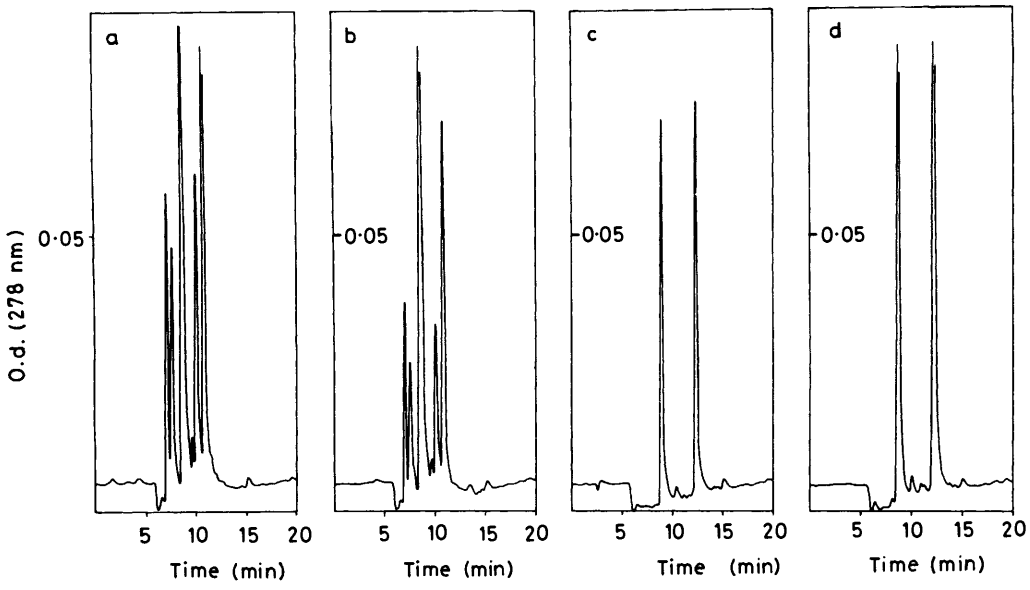


Figure 6. Comparative h.p.l.c. ' fingerprints ' of enzymic digests of solid-phase synthetic (a, c) and authentic [13] (b, d) [15-leucine]human little gastrin I using chymotrypsin (a, b) and *Staphylococcus aureus* protease V8 (c, d) (*e.g.* ref. 1)

Table 2. Amino-acid analysis of the fractions produced on h.p.l.c. of heptadecapeptide amide. Conditions: (a) constant boiling HCl containing phenol, 18 h, 120 °C; (b) aminopeptidase-M 10 units/mg substrate, 37 °C, 5 h

	[Leu[15]]Little gastrin I				[Gln[1],Leu[15]]LG I					
	Authentic		Synthetic (peak A)		Peak B		Peak C		Peak D	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Asp	0.97	0.93	0.96	1.03	1.00	0.15	1.00	0.93	0.98	0.96
Gln								0.92		
Glu	6.40	5.11	6.34	5.23	6.15	4.42	6.41	5.25	6.41	5.33
Pro	1.11	1.15	1.04	0.96	1.01		1.05	1.18	1.00	0.99
Gly	2.02	1.75	2.03	1.79	2.05	0.98	2.00	1.84	2.07	1.73
Ala	0.99	1.05	1.06	1.04	1.00	1.09	0.98	1.02	1.02	1.10
Leu	2.01	1.95	2.03	1.98	1.97	2.00	1.98	1.90	2.03	1.94
Tyr	0.97	0.98	1.00	0.97	0.95	1.12	0.98	0.98	0.98	1.00
Phe	1.00	1.00	1.00	1.00	1.00	0.07	1.00	1.00	1.00	1.00
Trp		2.35		2.31		2.1		2.14		1.37

13 amino-acid residues was similarly deprotected and cleaved with methanolic ammonia (Figure 7a). It yielded after direct h.p.l.c. purification des-1-tryptophan[12-leucine]human minigastrin I (52%), identical on h.p.l.c. (Figure 7b) and t.l.c. with an authentic sample [15] and with that prepared previously by solid-phase synthesis.[1]

Experimental

The general procedures for solvent and reagent preparation and purification given in Part 4 [1] were rigorously applied. Procedures for solid-phase synthesis using a polydimethylacrylamide support were as described previously,[1] except that dimethylacetamide (DMA) was used in place of dimethylformamide (DMF).

Solid-phase Synthesis of [15-*Leucine*]*Human Little Gastrin* (1b).—The polydimethylacrylamide resin [2] (0.254 g, containing 0.10 mmol of sarcosine) was treated with 1,2-diaminoethane (*ca.* 10 ml) for 20 h [1] and, after being thoroughly washed, the resin was acylated with preformed Fmoc-norleucine anhydride [1] (0.59 mmol) in DMA (5 ml) for 1 h. The incorporation of norleucine was 0.25 mequiv./g. 2,4,5-Trichlorophenyl 4-hydroxymethylbenzoate (0.60 mmol) was coupled in the presence of 1-hydroxymethylbenzotriazole (0.59 mmol) in DMA (5 ml) for 20 h.[1] To the washed resin was added solutions of Boc-Phe anhydride (0.59 mmol), DMA (1.25 ml), and 4-dimethylaminopyridine (0.59 mmol) in DMA (1.25 ml). After 2 h the resin was washed, deprotected with 1.5M-HCl–AcOH and the hydrochloride salt neutralised with 10% di-isopropylethylamine–DMA (cycle B).[1] The following 12 *N*-protected amino-acid anhydrides (0.59 mmol) were added in DMA (5 ml) and shaken for the times given before deprotection using the standard Fmoc procedure [2] (wash cycle 1): [1] Fmoc.Asp.(OBu[t]).OH, 17.5 h; Fmoc-Leu.OH, 60 min; Fmoc.Trp.OH, 72 min: Fmoc.Gly.OH, 108 min; Fmoc.Tyr.(Bu[t]).OH, 60 min; Fmoc.Ala.OH, 140 min; Fmoc.Glu.(OBu[t]).OH, 60 min; Fmoc-Glu.(OBu[t]).OH, 65 min; Fmoc.Glu.(OBu[t]).OH, 110 min; Fmoc.Glu.(OBu[t]).-OH, 60 min; Fmoc.Glu.(OBu[t]).OH, 16 h; Fmoc.Leu.OH, 75 min. A sample of resin (*ca.* 35 mg) was removed for comparison with tridecapeptide amide (3b) prepared earlier.[1] The loading of phenylalanine on the resin at the tridecapeptide stage was 0.154 mequiv./g. For amino-acid analysis data of selected intermediate peptide-resins, see Table 1.

The remaining resin was deprotected and acylated with the

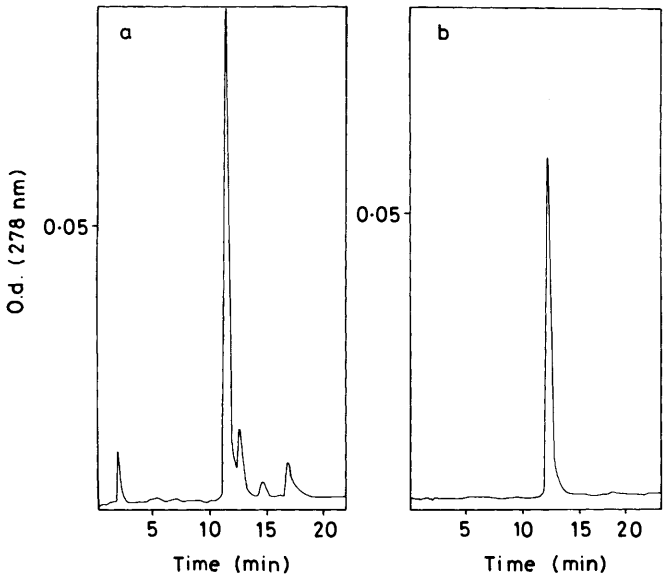


Figure 7. Analytical h.p.l.c. on μ-Bondapak C_{18} at pH 4.5 of des-Trp[1][12-leucine]human minigastrin I, (a) total crude product; (b) after semi-preparative reverse phase h.p.l.c. purification. Conditions: 20—40% MeCN in 0.1M-NH_4OAc during 20 min

N-protected amino-acid anhydrides (0.59 mmol) of Fmoc.-Trp.OH, 60 min; Fmoc.Pro.OH, 90 min; and Fmoc.Gly.OH, 60 min. The final residue was added by the active ester procedure with Fmoc.Gln.ONp (0.59 mmol) in the presence of 1-hydroxybenzotriazole (0.59 mmol) in DMA (7 ml) for 60 min. The washed resin was stored at 5 °C under nitrogen until required. Samples of resin were removed for qualitative ninhydrin tests after every amino-acid addition and deprotection step. The final loading of phenylalanine on the resin was 0.135 mequiv./g.

A sample of heptadecapeptide-resin was deprotected with piperidine in DMA (cycle A),[1] washed with CH_2Cl_2 and diethyl ether and then kept *in vacuo* for 1 h. The dried resin (20.1 mg, 2.71 μmol) was washed as follows under nitrogen 90% aqueous TFA, 1 × 5 min and 1 × 25 min; AcOH, 5 × 1 min; AmOH,* 5 × 1 min; DMA, 5 × 1 min; CH_2Cl_2, 2 × 1 min; diethyl ether, 2 × 1 min; DMA, 5 × 1 min; CH_2Cl_2, 2 × 1 min; diethyl ether, 2 × 1 min. The resin was dried *in vacuo* for 2 h, and after swelling in DMA (15 min) it was washed with DMA, 5 × 1 min; 10% di-isopropylethylamine–DMA, 3 × 1 min; DMA, 5 × 1 min.

The swollen resin was treated with methanolic ammonia (*ca.* 12 ml, saturated at 0 °C) in a sealed flask for 17 h. The suspension was filtered and the resin washed with methanol (Asp:Nle = 0.08; 9% residual peptide). The filtrate and methanol washings were combined and evaporated to dryness to yield peptide (2.42 μmol, 89.3%) (Found: Phe, 1.04; Asp, 1.00; Leu, 2.01; Gly, 1.93; Tyr, 0.98; Ala, 1.04; Glu, 6.32; Pro, 0.95). This product (2.27 μmol) was applied to a column of diethylaminoethyl cellulose (Whatman DE-52, 10.2 × 1 cm diam.) and eluted with a linear gradient of 0.01—0.8M-aqueous ammonium hydrogen carbonate, pH 8.1 at 1 ml/min. The product eluted as a single peak (Figure 1) at 198—270 ml (1.77 μmol; 78.0% recovery) (Found: Phe, 1.01; Asp, 1.00; Leu, 2.03; Gly, 1.89; Tyr, 0.97; Ala, 1.06; Glu, 6.09; Pro, 0.94). Analytical h.p.l.c. (Figure 3a) indicated this product contained a mixture of pyroglutamyl peptide amide (2b) (25%) and glutaminyl peptide amide (2c) (75%) (see later for enzymic characterisation of this material). The product (1.601 μmol) was discovered in 20% aqueous acetic acid (20 ml), kept at 30 °C for 64 h under argon, and then evaporated. This product (1.58 μmol) (analytical h.p.l.c. Figure 3b), was applied to a column of diethylaminoethyl cellulose (Whatman DE-52, 12.5 × 1 cm) and eluted with a linear gradient of 0.01—1.0M-ammonium acetate, pH 6.5 at 1 ml/min (Figure 4). The glutaminyl heptadecapeptide (2c) eluted between 285 and 315 ml and the pyroglutamyl heptadecapeptide (2b) eluted between 330 and 381 ml (1.03 μmol, 65% recovery) † (Found: 1.00; Asp, 0.97; Leu, 2.02; Gly, 2.03; Tyr, 1.00; Ala, 1.05; Glu, 6.40; Pro, 1.07), analytical h.p.l.c. (Figure 5a). Part (0.613 μmol) of this product was further purified by μ-Bondapak C_{18} using a convex gradient of 0—35% B during 25 min followed by a linear gradient of 35—100% during 10 min at 2 ml/min (solvent A = 0.01M-ammonium acetate, pH 4.5; solvent B = 90% acetonitrile and 10% solvent A). The heptadecapeptide amide (2b) (0.43 μmol, 70% recovery, overall yield 32.3%) was obtained (Found: Phe, 1.00; Asp, 0.96; Leu, 2.03; Gly, 2.03; Tyr, 1.00; Ala, 1.06; Glu, 6.34; Pro, 1.04). This product was identical with authentic [13] (2b) on t.l.c. (butanol–acetic acid–pyridine–water, 60 : 6 : 20 : 24), R_F 0.23; analytical h.p.l.c. on μ-Bondapak C_{18}, 20—50% acetonitrile during 30 min at 2 ml/min in 0.01M-ammonium acetate, pH 4.5 (retention time = 16.4 min) and 0.01M-ammonium acetate, pH 3.5 (retention time = 21.5 min) (Figure 5b). Analytical h.p.l.c. ' fingerprint ' analysis after limited proteolysis with α-chymotrypsin (Figure 6a, b), *S. aureus* protease V8 (Figure 6c, d), and thermolysin was used to compare this product with authentic 15-leucine little gastrin.[13] For conditions see ref. 1.

Des-1-tryptophan[12-leucine]human Minigastrin I.—The sample of intermediate peptide-resin containing the protected tridecapeptide (1b) was deprotected with 20% piperidine–DMA (1 × 3 min, 1 × 7 min), washed with DMA (10 × 0.5 min), CH_2Cl_2 (2 × 0.5 min), diethyl ether (2 × 0.5 min), and dried *in vacuo* (P_2O_5) for 2 h. The dried resin (27.5 mg, 4.24 μmol) was deprotected with 90% aqueous TFA, dried, neutralised with 10% di-isopropylethylamine–DMA and cleaved from the resin with methanolic ammonia for 16 h in the same way as described above for the heptadecapeptide (2b). Amino-acid analysis was performed on the residual resin (Asp:Nle = 0.054, 94% cleavage). The filtrate and methanol washings were combined and evaporated to dryness to yield peptide (3.74 μmol, 88.2%) (Found: Phe, 1.00; Asp. 0.98; Leu, 1.94; Gly, 0.97; Tyr, 0.95; Ala, 1.05; Glu, 5.17), analytical h.p.l.c. (Figure 7), prepared in the preparation described above. The peptide (2.93 μmol) was purified by semi-preparative h.p.l.c. on μ-Bondapak C_{18} using a convex gradient of 0—30% B during 20 min at 2 ml/min [solvent A and B as above for the semi-preparative h.p.l.c. purification of heptadecapeptide amide (2b)]. The tridecapeptide amide (1b) (1.73 μmol, 59% recovery, overall yield 52%) was identical with authentic (1b) on t.l.c. (butanol–acetic acid–pyridine–water, 60 : 6 : 20 : 24), one spot, R_F 0.19; paper electrophoresis at 3 kV at pH 6.5 (streaking from origin); analytical h.p.l.c. on μ-Bondapak C_{18}, 20—50% acetonitrile during 30 min at 2 ml/min (retention time = 12 min, 0.01M-ammonium acetate, pH 4.5; retention time = 16.4 min, 0.01M-ammonium acetate, pH 3.8).

* AmOH = 2-Methylbutan-2-ol (t-amyl alcohol).

† The yield was lowered by some physical loss.

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