Peptide Synthesis. Part 4.† Solid-phase Syntheses of Peptides Related to Gastrin

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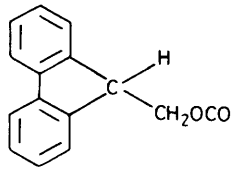
Details are given of the synthesis of the tetradecapeptide amide [12-leucine]human minigastrin I and its des-1-tryptophan analogue using solid-phase methods based on polar polydimethylacrylamide supports. Exposure to acidic reagents during the synthesis was minimised by use of N_α-fluorenylmethoxycarbonyl-amino-acids and t-butyl-based side-chain protecting groups. High yields of readily purified minigastrin analogues were obtained showing the full biological activity of the natural hormone.

The generation of molecules with the high biological activity characteristic of natural hormones has provided much of the impetus for peptide synthesis. Both classical (solution) and solid-phase methods have been fruitful and have done much to advance understanding of the relationship between chemical structure and biological effect. The introduction of solid-phase methods [1] was a particularly important development. It provided an element of speed and simplicity which was lacking in the more rigorous discipline of classical organic chemistry. Biochemists, pharmacologists, and others with little previous experience in chemical synthesis were in many instances able to produce easily and rapidly peptide preparations with high biological potency, though increased understanding of the processes involved in conventional solid-phase synthesis would now suggest that many of the early products must have been impure. In this present paper we describe recent studies in the gastrin series using modern methods of solid-phase synthesis. We believe that these methods [2-4] have been substantially optimised with regard to the reaction conditions employed, and that the scope of solid-phase synthesis has thus been widened. In these experiments, particular emphasis has been placed on the search for and identification of impurities, and understanding of the processes leading to their formation.

Some amino-acid sequences were clearly not accessible by the early solid-phase methods. The antral hormone gastrin is a case in point. Although gastrins of several species were isolated,[5] sequenced,[6] and synthesised by solution methods [7] in the early 1960's when the then novel solid-phase technique was being widely and vigorously applied, no significant solid-phase synthesis of gastrin was apparently reported until the present work [8] and one other recent investigation.[9] The reason for this is easy to see. Since its inception, solid-phase peptide synthesis has been based almost exclusively on protecting group combinations which require strongly acidic conditions for their removal. The method which has come to be adopted as the standard 'Merrifield technique' [10] utilises t-butoxycarbonyl groups for the protection of α-amino-functions and various benzyl ester, ether, or urethane derivatives for amino-acid side chains and the peptide-resin linkage. The former require, *e.g.* trifluoroacetic acid or hydrogen chloride for their cleavage which is repeated at every cycle of amino-acid addition; the latter require even more vigorous conditions, typically liquid hydrogen fluoride or hydrogen bromide in trifluoroacetic acid, although this is carried out only once. Many amino-acids, protected or otherwise, are known to be unstable to acidic treatment of this vigour. The indole ring of tryptophan derivatives is highly sensitive to acid-catalysed degradation; serious side reactions occur when benzyl esters of glutamic acid are cleaved by liquid hydrogen fluoride,[12] and benzyl aspartate may similarly be cleaved with cyclisation or isomerisation.[13] The products of acid-catalysed cleavage reactions are themselves not innocuous; benzyl cations may react with the nucleophilic sulphur atom of methionine [14] or substitute (or rearrange intramolecularly) into the phenolic ring of tyrosine.[15] These amino-acids are of frequent occurrence in peptide hormones generally, and constitute no less than 12 out of the 17 residues of porcine gastrin (1a).



(3)

Glp·Gly·Pro·Trp·X·Glu$_5$·Ala·Tyr·Gly·Trp·Y·Asp·Phe·NH$_2$

(1)

H·Trp·X·Glu$_5$·Ala·Tyr·Gly·Trp·Y·Asp·Phe·NH$_2$

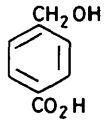
(2)

a, X = Y = Met; b, X = Leu, Y = Met; c, X = Y = Leu
Glp = pyroglutamyl

In Part 3 of this series [16] we described two comparative syntheses of the opiate peptide β-endorphin, using in one a protecting group combination not dissimilar to that of the standard Merrifield technique, and in the other a newer combination which minimised acid treatment during the course of the synthesis. The results were clearly in favour of the latter. A four-fold increase in yield was obtained and the crude product was of much improved purity. The new protecting group combination utilised base-labile fluorenylmethoxycarbonyl protecting groups (3) for α-amino-functions together with t-butyl or similarly very acid labile *p*-alkoxybenzyl groups for side chain and carboxy terminal (resin linkage) groups.[3] The repeated acid treatments which characterise the Merrifield technique are thus dispensed with and the very vigorous final treatment is replaced with one single cleavage with milder acid. In the experiments described, these protecting groups were used advantageously in combination with a polar, polyamide support,[2] but this is not a necessary association and they may also be used with more conventional polystyrene supports.[11] The gastrin sequence with its assembly of difficult, acid-sensitive amino-acids provided a further demanding test of this resin and protecting group system. We now describe our recent experiments which for the first time make hormones of the gastrin type readily available by solid-phase synthesis.

Several forms of gastrin have been isolated from antral

† Part 3, preceding paper.



(4)

mucosa or from carcinoma tissue.[17] The original gastrin (1), now known as little gastrin, is a heptadecapeptide amide showing some species variation (*e.g.*, porcine gastrin = 1a; human gastrin = 1b) but characterised by the strictly conserved carboxy terminal Trp·Met·Asp·Phe·NH_2 sequence. This last tetrapeptide amide contains all the information required for expression of biological activity.[18] It is found also in the related gastrointestinal hormone, cholecystokinin,[19] and even in a range of peptides from the skin of amphibia.[20] More recently, both longer and shorter forms of (1) have been identified in biological tissue. Minigastrin (2) comprises the carboxy terminal 14 residues of (1)[21] (it was originally thought[22] to contain only 13 residues), and big gastrin consists of (1) extended at the amino-terminus by a further 17 residues.[23] All the gastrins apparently exist naturally in two forms, in one of which the tyrosine residue bears a sulphate ester group.

For our first synthetic objective, we selected the des-1-tryptophan [12-leucine] analogue of minigastrin. Replacement of methionine by leucine in gastrin sequences is without effect on biological activity.[24] It was a convenient simplification in the present work, avoiding the frequent problem of oxidation during the synthesis of methionine peptides. The synthetic methods used are, however, equally applicable to sulphur containing amino-acids (see, for example, refs. 3, 16). The des-1-tryptophan sequence was selected because of the availability of a highly purified authentic sample prepared[25] by solution synthesis. Later the work was extended to the full minigastrin sequence (2c), and in a separate synthesis to [15-leucine] little gastrin (1c) itself. Future publications will describe synthesis of the latter and of little gastrin extended at the carboxy terminus.

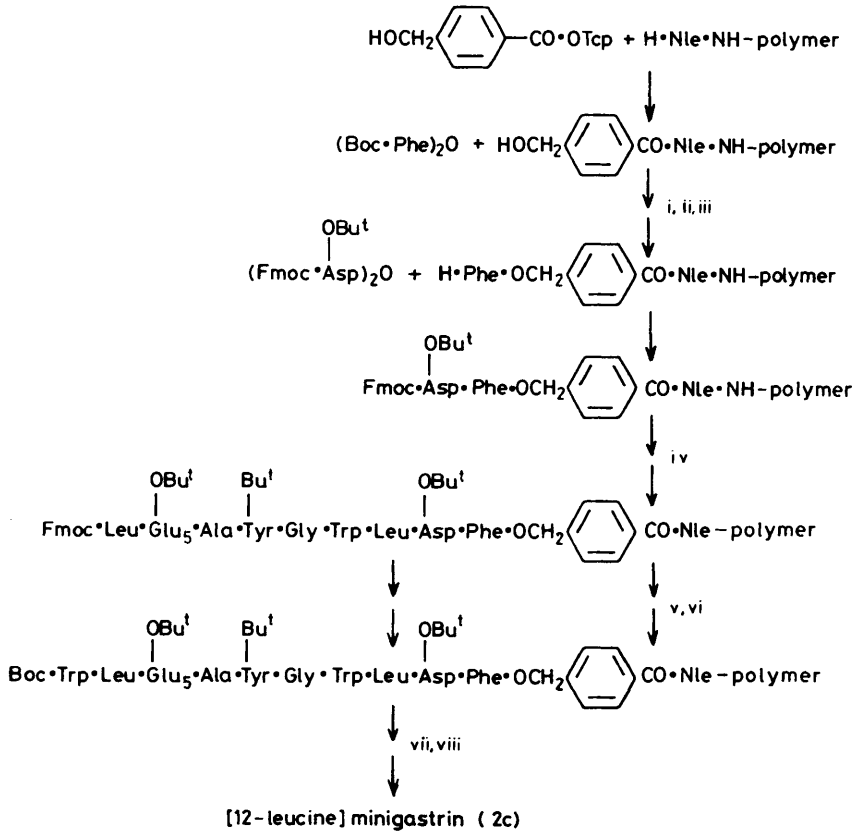
All the gastrins are carboxy terminal amides. In our polyamide solid-phase method, amides are easily prepared through ammonolysis of benzyl esters of the resin-bound linkage agent (4). Since this was one of the first applications of fluorenylmethoxycarbonyl (Fmoc) amino-acids in combination with this linkage agent, it was appropriate to study in preliminary model experiments the formation and stability of resin-bound esters of (4). The resin used was our usual beaded copolymer of dimethylacrylamide, ethylene bisacrylamide, and acryloylsarcosine methyl ester, functionalised by reaction with ethylenediamine.[2] Following our normal practice, a permanently bound internal reference amino-acid (norleucine) was first introduced, followed by the trichlorophenyl ester of (4). Completeness of this and of all subsequent amide bond-forming steps was verified qualitatively by the sensitive ninhydrin test.[26] t-Butoxycarbonylphenylalanine was esterified to the hydroxymethyl resin using the preformed symmetrical anhydride[27] in the presence of 4-dimethylaminopyridine.[28] Amino-acid analysis after only 5.5 min reaction indicated complete acylation. In contrast, acylation with Boc-glycine in the absence of basic catalyst was extraordinarily slow. Thus after a 4 h reaction period with Boc-glycine anhydride (60-fold excess), the resin sample was washed and further acylated with Boc-phenylalanine anhydride in the presence of 4-dimethylaminopyridine. Amino-acid analysis (Phe : Gly : Nle = 0.95 : 0.03 : 1.00) showed that only *ca.* 3% of the hydroxy-groups had been initially acylated. This result is of importance because it establishes that free hydroxy-groups on the resin which might arise through cleavage of peptide-resin esters are, under normal conditions of synthesis, unlikely to function as starting points for the growth of new peptide chains using symmetrical anhydride activation in the absence of catalysts.* In the event, the ester bond between Boc-glycine and resin-bound (4) proved to be very stable to the basic conditions used for the repetitive cleavage of fluorenylmethoxycarbonyl derivatives. After treatment with 20% piperidine in dimethylformamide for 24 h (equivalent to 144 deprotection cycles), 94% of the glycine was retained on the resin. This corresponds to 0.04% cleavage per cycle.

Fluorenylmethoxycarbonylamino-acids were prepared by the general procedures already described.[29,30] t-Butyl side chain derivatives were prepared by hydrogenolysis of the corresponding benzyloxycarbonyl compounds, followed by acylation with fluorenylmethyl chloroformate. The purity of protected amino-acids proved to be of crucial importance in obtaining reproducibly high yields in acylation reactions. Large excesses of acylating species are customarily employed in solid-phase synthesis (in the present instance six-fold excesses), and fast reacting contaminants may have effects disproportionate to their relative molar proportions. This effect has been noted also in solid-phase oligonucleotide synthesis.[32] All Fmoc-amino-acids were rigorously purified and checked for purity by m.p., t.l.c. and n.m.r. Derivatives of γ-t-butyl glutamate and of *O*-t-butyltyrosine initially required chromatography on silica before satisfactory solid-phase incorporations were achieved, but latterly recrystallisation was effective.

Assembly of the minigastrin sequence followed that outlined in the Scheme. The first amino-acid residue (phenylalanine) was esterified as its Boc-derivative to the previously functionalised resin, avoiding any possible problems due to the slight lability[3] of Fmoc-derivatives to the basic catalyst (4-dimethylaminopyridine) used. Subsequent experience has now shown that this is not a generally necessary precaution. Pre-formed symmetrical anhydrides were used for this and all succeeding acylation reactions. Anhydride formation utilised dicyclohexylcarbodi-imide in methylene chloride solution, but following the general principles we have established for polyamide solid-phase synthesis, all resin reactions (acylation and deprotection) were carried out exclusively in the more polar solvent dimethylformamide.† After acidic cleavage of the Boc-protected phenylalanyl resin, all subsequent amino-acids were incorporated as Fmoc-derivatives. Intermediate Fmoc-peptide resins were deprotected with 20% piperidine in dimethylformamide. As indicated above, acidic side chains of glutamic and aspartic acids and the phenolic group of tyrosine were masked as t-butyl esters or ethers. Tryptophan residues were side chain unprotected. The resin sample was divided at the dodecapeptide stage, synthesis of the tridecapeptide sequence being completed by addition of (terminating) Boc-leucine, and of the tetradecapeptide ([12-leucine]minigastrin) by addition of Fmoc-leucine and then Boc-tryptophan. Amino-acid incorporation was rapid throughout as judged by the ninhydrin reaction. Negative tests were obtained after 10—15 min and no repeated acylation steps were necessary. Resin samples were with-

* On the other hand, it should be noted that attempts to use side chain unprotected serine or threonine residues in the polyamide Fmoc-t-butyl procedure have thus far not been successful (E. Atherton, unpublished; *cf.* ref. 31).

† On other occasions we have used dimethylacetamide as the general solvent for polyamide-based synthesis, but its principal advantage (increased stability of Fmoc-amino-acids[33]) now appears to be outweighed by the disadvantage of slower reaction rates. In the gastrin series, both solvents give satisfactory results.



Scheme. Assembly of the [12-leucine]minigastrin sequence. In the synthesis of the des-l-tryptophan analogue, the terminating leucine residue was added as its t-butoxycarbonyl derivative. *Reagents:* i, dimethylaminopyridine; ii, HCl–AcOH; iii, Pr^i_2NEt; iv, 11 cycles of deprotection and Fmoc-amino-acid addition; v, piperidine–DMF; vi, $(Boc.Trp)_2O$; vii, CF_3CO_2H; viii, MeOH–NH_3

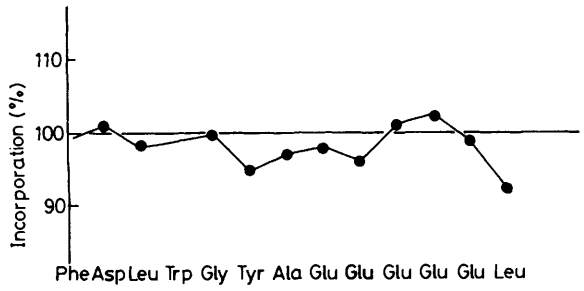


Figure 1. Amino-bound incorporation as determined by analysis of intermediate resin-bound peptides after addition of 1, 2, 8, 9, 10, 11, 13, and 14 residues of the minigastrin sequence

drawn at intervals and analysed for amino-acid content (apart from tryptophan). The incorporation curve (Figure 1) reflects previous experience in the analysis of resin-bound peptides (see, for example, refs. 2, 33). Later examination of the crude detached peptides (see below) failed to provide any evidence for detectable levels of failure sequences. The departure of the incorporation curve from theoretical must therefore arise largely from the vagaries of hydrolysis and amino-acid analysis of peptide-resins, exacerbated by the cumulative errors of multiple residues. Such curves are really informative (in a negative sense) only when they show major (catastrophic) departures from quantitative incorporation. We judged the data of Figure 1 to indicate the likelihood of satisfactory assembly, with only incorporation of the terminal eucine residue questionable.

Some preliminary experiments were carried out to determine optimum conditions for the deprotection and detachment of the resin-bound tridecapeptide. The high proportion of side chain t-butyl ester groups strongly suggested acidic cleavage prior to ammonolysis. The ability to cleave all acid-labile protecting groups without detachment of the peptide from the resin is an advantageous feature of the linkage agent (4). A trial cleavage using 90% aqueous trifluoroacetic acid, neutralisation of the washed resin with di-isopropylethylamine, and then ammonolysis gave on analytical h.p.l.c., major and (earlier eluting) minor (*ca.* 20%) peaks, together with some lesser (2—5%) later eluting impurities. The early, more polar, contaminant was separable (more acidic) on anion-exchange chromatography, gave a correct amino-acid analysis identical with that of the major product, and was tentatively identified as the C-terminal free-acid tridecapeptide. Its formation was entirely eliminated when the peptide resin was thoroughly dried before the ammonolysis step. The less polar minor impurities were not separable from the major product by ion-exchange chromatography. Variation of the acidic cleavage conditions by incorporation of scavengers (10% anisole, 5% methionine, 5% tryptophan, or 10% water) did not significantly affect the proportions of the minor contaminants,* but curtailment of the ammonolysis time increased one later eluting peak. This product was isolated in pure form

* This result is probably largely attributable to retention of the peptide on the resin during de-t-butylation. Reaction of t-butyl cations with tryptophan residues was much more serious in the absence of scavengers when similar reactions were carried out in solution (E. Brown, unpublished).

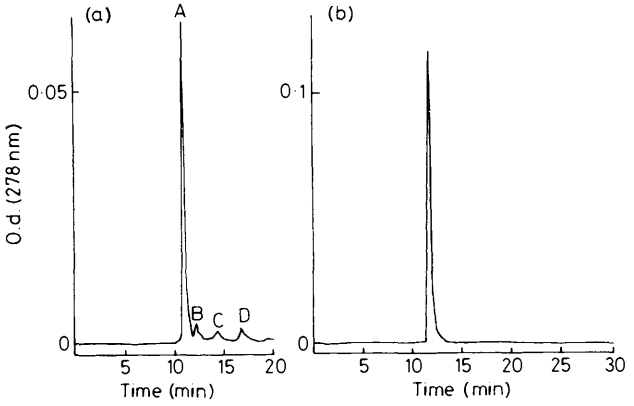


Figure 2. H.p.l.c. on μ-Bondapak C_{18} of (a) crude, and (b) purified des-1-tryptophan[12-leucine]minigastrin

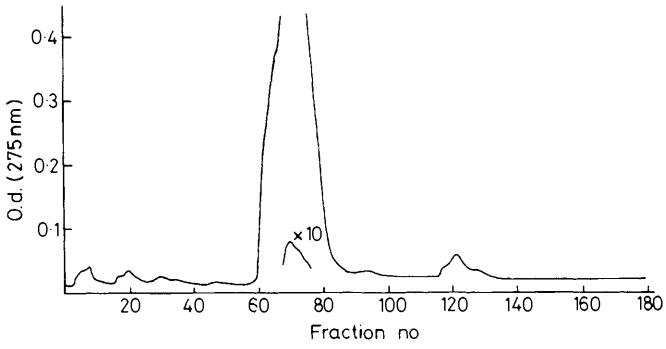


Figure 3. Anion-exchange chromatography of crude des-1-tryptophan[12-leucine]minigastrin on diethylaminoethyl cellulose

by preparative h.p.l.c. It also gave a correct amino-acid analysis for the tridecapeptide sequence after both enzymic and acidic hydrolysis. Its structure was assigned as the corresponding C-terminal methyl ester on the basis of its less polar (h.p.l.c.) but isoelectric (ion-exchange chromatography) relationship to the major product, its identical amino-acid composition, and partial conversion to the foregoing free acid during manipulation (Sephadex chromatography) in aqueous solution. Its formation was almost completely suppressed by prolonging the ammonlysis step from 3 to 18 h, although detachment of the peptide from the resin support was 94% complete after the shorter time.

The tridecapeptide-resin was cleaved on a preparative scale using reaction conditions determined from the foregoing experiments. Residual resin analysis showed that 95% of the peptide had been detached although there were some manipulative losses and only 75% of peptide amide was recovered in the filtrate. The product contained only minor impurities at this stage (h.p.l.c., Figure 2a). It was subjected to anion-exchange chromatography on diethylaminoethyl cellulose (Figure 3) and then on Sephadex G25, but little or no purification was achieved, h.p.l.c. giving an almost unchanged elution profile. Recovery from the two columns was nearly quantitative. The overall yield from fully protected peptide resin was 74% at this stage, and the amino-acid analysis was good. Removal of the remaining impurities was achieved by semi-preparative h.p.l.c. giving the major product in 59% yield (analytical h.p.l.c., Figure 2b).* The elution position was identical with that of authentic tridecapeptide amide prepared by solution synthesis and generously provided by Professor E. Wünsch.

In a later experiment, partition chromatography [25] was used for the purification of the final product, but this was less effective and recovery from the column (45%) was also less than with h.p.l.c. (80%).

All the minor impurities from the foregoing preparative h.p.l.c. separation were also collected. That corresponding to peak C had the same amino-acid compositions as the major peak A, and corresponded with the product already identified as the terminal methyl ester derivative. Peak D also had the same amino-acid composition when determined after acidic hydrolysis, but enzymic hydrolysis showed that it was deficient in tryptophan. This region is more complex in the h.p.l.c. profile of the crude tetradecapeptide amide whose sequence contains two tryptophan residues. Modification of tryptophan by t-butylation has been observed in solution synthesis of the same sequence by Wünsch and his colleagues,[25] and it is likely that peak D has similar origins. Peak B also has the correct amino-acid composition when determined after acidic hydrolysis but is deficient in phenylalanine on enzymic cleavage. α→β Isomerisation of the adjacent aspartyl residue was a possible explanation, although we have previously shown that this does not normally occur to a detectable extent with t-butyl esters under the conditions of polyamide based solid-phase synthesis [3] and is not supported by the amino-acid analysis. The recent demonstration [34] that slight racemisation of the C-terminal residue may occur under the conditions for attachment to hydroxymethyl-resins now provides strong presumptive evidence for this identification. Such racemisation is of no great significance in the present work where the resulting diastereoisomer is separable, but it could be significant in other cases. Conditions have been described for its minimisation.[34]

Isolation and purification of the synthetic tetradecapeptide (2c) ([12-leucine]minigastrin I) was initially carried out in a similar manner to the foregoing. t-Butyl groups were cleaved from the resin-bound peptide with aqueous trifluoroacetic acid, the resin thoroughly dried, and the peptide detached by prolonged ammonolysis with methanolic ammonia. The crude product (h.p.l.c., Figure 4a) obtained in 83% yield with good amino-acid analysis was chromatographed successively on DEAE cellulose and Sephadex G25. It was eluted from both columns as a single peak with recoveries of 85 and 97% respectively. The purity as judged by analytical h.p.l.c. was unchanged. As before, the trace contaminants were best removed by preparative h.p.l.c., giving purified [12-leucine]-human minigastrin I (h.p.l.c., Figure 4b) in an overall yield of 33% based on the fully protected resin-bound tetradecapeptide. As in the tridecapeptide case, the impurity peaks were also collected and were tentatively identified as carboxy-terminal diastereoisomer and t-butylated tryptophan derivatives.

It was evident during these last experiments that having eliminated the formation of deamidated product through careful drying of the resin before ammonolysis, little if any purification was achieved by ion-exchange or gel filtration chromatography. Some final investigations were therefore carried out on the direct h.p.l.c. purification of the resin-cleavage product. In two experiments, overall yields of 49 and

* It is appreciated that analytical h.p.l.c. using the same column packing and eluting media as that used in preparative separation is not to be taken as an additional criterion of absolute purity but only of the efficiency of the separation process. Independent evidence of purity is provided by t.l.c. and paper electrophoresis (Experimental section), and particularly be enzymic degradation and comparison (finger printing) with highly purified authentic [25] material (see later).

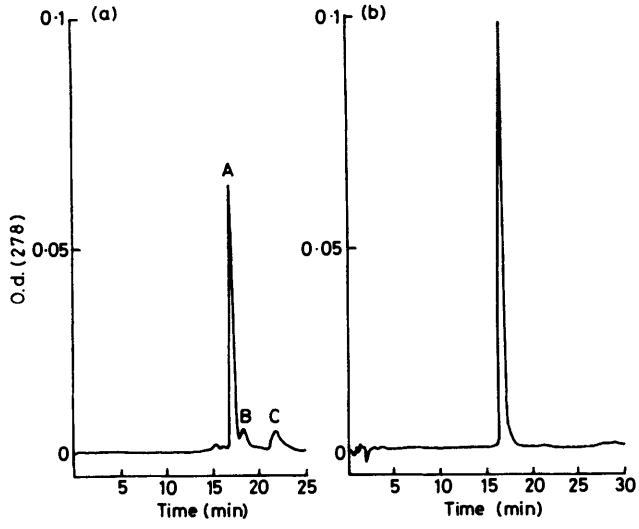


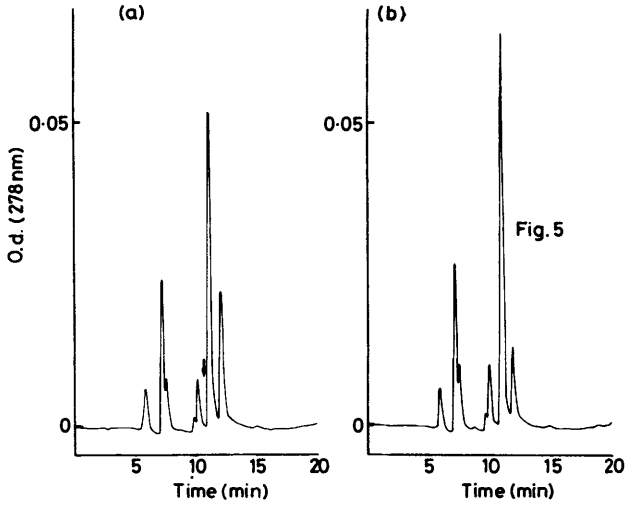
Figure 4. H.p.l.c. of (a) crude, and (b) purified [12-leucine]minigastrin

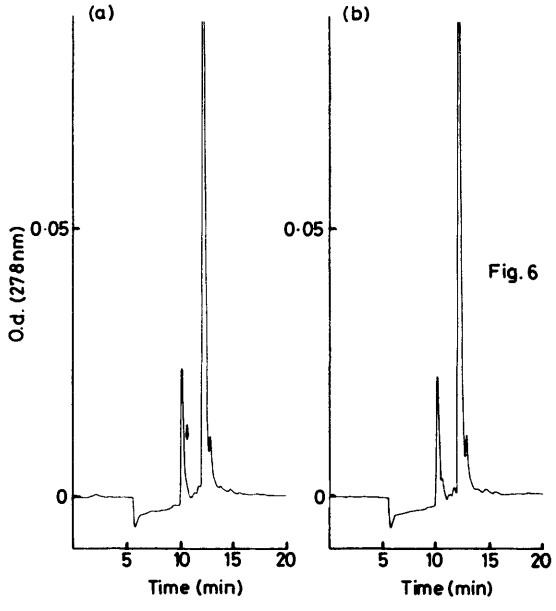
51% were obtained of material apparently identical in purity with that obtained by the earlier longer procedure.

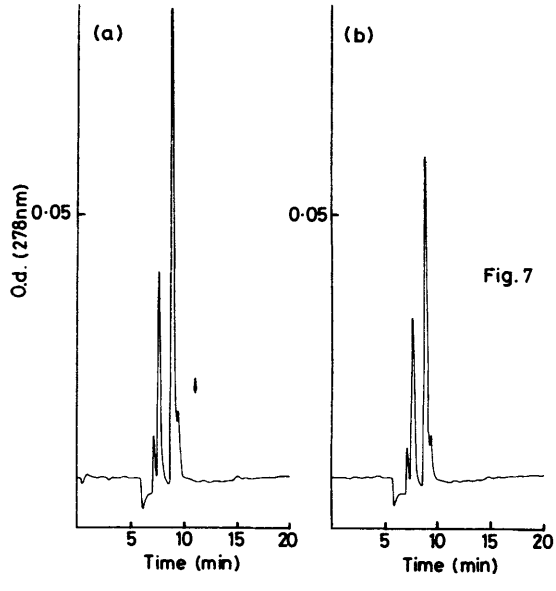
The high yields obtained in both these syntheses demanded that purity be carefully established. The availability of authentic tridecapeptide amide prepared by solution methods in which both the intermediates and final product were subjected to rigorous purification prompted careful comparison with the solid-phase product. H.p.l.c., t.l.c., and electrophoretic behaviour of the two materials were identical. A more rigorous comparison was clearly desirable, however, and for this ' finger printing ' methods were selected with h.p.l.c. replacing the older paper or thin layer chromatographic techniques. In separate experiments, the solution and solid-phase synthesised peptides were subjected to limited proteolysis by chymotrypsin, *Staphylococcus aureus* protease V8, and thermolysin. Aliquots of the various digests were removed at time intervals and the patterns of h.p.l.c. peaks (Figures 5—7) compared. The close similarity of the two sets of chromatograms is striking.

The experiments described above provide, in our view, ample justification for the belief that polar support systems in combination with mildly removed protecting groups constitute useful additions to the methods of solid-phase synthesis. The high yield and purity of the synthetic products are notable. No evidence was seen for the formation of recognisable failure sequences, even though partial omission of any one of the six acidic residues would have led to contaminants almost certainly separable by ion-exchange chromatography. t-Butylation of indole groups remains a significant side reaction but is minimised by carrying out the deprotection reaction on resin-bound peptide.

As expected, the trideca- and tetradeca-peptide amides were equiactive in stimulating gastrin secretion in the conscious dog, and were also equiactive with the same molar amount of natural human little gastrin I (heptadecapeptide amide).







Figures 5—7. Representative h.p.l.c. separations of enzyme digests of (1) solid-phase synthesised, and (b) authentic [25] des-1-tryptophan[12-leucine]minigastrin. Figure 5, chymotrypsin after 40 h; Figure 6, *Staph. aureus* V8 after 24 h; and Figure 7, thermolysin after 69 min. The elution position of the starting tridecapeptide amide is indicated in (a)

Experimental

The following procedures for solvent and reagent purification or preparation were rigorously followed. Dimethylformamide (DMF), fractionally distilled under reduced pressure through a 12 in Fenske column packed with steel gauze coils. Acetic acid, distilled from chromium trioxide (20 g/l). 2-Methylbutan-2-ol, fractionally distilled from anhydrous potassium carbonate. Dichloromethane, distilled from phosphorus pentoxide less than 48 h before use. Piperidine, distilled from potassium hydroxide. Di-isopropylethylamine, distilled from ninhydrin and then from potassium hydroxide. Diaminoethane, distilled from potassium hydroxide. 1.5 M-Hydrogen chloride in acetic acid was prepared by passing dry (H_2SO_4) hydrogen chloride through acetic acid at room temperature until the appropriate weight gain was obtained. Dicyclohexylcarbodiimide, distilled under reduced pressure (oil pump) using a N_2 bleed. 1-Hydroxybenzotriazole, recrystallised from hot water. Resin-samples for amino-acid analysis were taken *after* removal of the N-terminal protecting group and were hydrolysed *in* redistilled 6M-hydrochloric acid containing phenol (*ca.* 5 mg/ml) at 110 °C/18 h.

Preparation of Symmetrical Anhydrides.—The protected amino-acid was dissolved in dichloromethane (2.8 ml/mmole). If necessary, the minimum amount of DMF was added dropwise to achieve a clear solution. A solution of dicyclohexylcarbodi-imide (0.5 equiv.) in dichloromethane (5.6 ml/mmol) was added and the mixture stirred at room temperature for approximately 10 min. Precipitated urea was removed by filtration and the filtrate evaporated to dryness. The residue was dissolved in the appropriate volume (*ca.* 15 ml/g resin) of DMF and added immediately to the amino-resin.

Solid-phase Synthesis Procedures.—Preparation of the polydimethylacrylamide resin (sarcosine content 0.33 mequiv./g) has been described previously.[2] All synthetic operations were carried out in a glass cell of the Merrifield type [1] attached to a wrist action shaker. Washing procedures were facilitated through the use of a simple manual PTFE valving system, or with the aid of a modified [2] Beckman Model 990 Peptide Synthesiser. Standard reaction and wash cycles: A (Fmoc group cleavage and anhydride coupling): DMF, 5 × 1 min; 20% piperidine/DMF, 3 + 7 min; DMF, 10 × 1 min; coupling, 60—120 min; DMF, 5 × 1 min. B (Boc group cleavage and coupling): 2-methylbutan-2-ol, 5 × 2 min; acetic acid, 5 × 2 min; 1.5M-HCl/acetic acid, 5 + 25 min; acetic acid, 5 × 2 min; 2-methylbutan-2-ol, 5 × 2 min; DMF, 10 × 2 min; 10% di-isopropylethylamine/DMF, 3 × 2 min; DMF, 5 × 2 min; coupling, 60—120 min; DMF, 5 × 2 min. C (base wash): DMF, 5 × 2 min, 10% di-isopropylethylamine/DMF, 3 × 2 min; DMF, 5 × 2 min. D (t-butyl group cleavage): 90% aqueous trifluoroacetic acid, 5 + 25 min under N_2; acetic acid, 5 × 1 min; 2-methyl-butan-2-ol, 5 × 1 min; DMF, 5 × 1 min, washed on a filter with dichloromethane and then ether, and dried *in vacuo* over P_2O_5 for 4 h. The ninhydrin colour test for residual amino-groups [26] was performed as follows. A sample of resin (*ca.* 5 mg) removed from the reaction cell immediately prior to addition of the acylating component was washed on a sintered filter with DMF and then dichloromethane and transferred to a small test tube. A similar resin sample was removed usually 15—20 min after addition of the acylating species and washed on a sinter with DMF, 10% di-isopropylethylamine/DMF, DMF, and dichloromethane. Two drops each of (1) ninhydrin (0.5 g) in ethanol (10 ml); (2) phenol (80 g) in ethanol (20 ml); and (3) 0.001M aqueous KCN (2 ml) and pyridine (98 ml) were added to the resin samples which were then heated at 100 °C for 5 min. Acylation was considered complete if the second sample gave a pale straw coloured supernatant solution and the unacylated resin gave a very deep blue solution. (Some unacylated amino-resins terminating in, for example, proline residues give only pale coloured beads or solutions under these conditions, and the results of the test should then be interpreted with caution.)

Hydroxymethylbenzoyl-L-norleucyl-polydimethylacrylamide Resin.—The dimethylacrylamide ethylene biscrylamide acryloylsarcosine methyl ester co-polymer [2] (0.5 g, 0.16 mequiv. sarcosine) was treated with diaminoethane (15 ml) for 18.5 h with occasional shaking, filtered, and washed with 1% aqueous KCl (20 × 1 min), water (5 × 2 min) (the washings at this stage gave no colour with ninhydrin), dioxan (5 × 5 min),* DMF (10 × 2 min), 10% di-isopropylethylamine/DMF (3 × 2 min), and DMF (5 × 2 min). A solution of the symmetrical anhydride (0.9 mmol) of fluorenylmethoxycarbonyl-L-norleucine in DMF (7.5 ml) was added to the drained resin and shaken for 60 min. The ninhydrin test was negative. The resin was washed with DMF (5 × 2 min) and the Fmoc group cleaved using cycle A. 2,4,5-Trichlorophenyl 4-hydroxymethylbenzoate [3] (298 mg, 0.9 mmol) and 1-hydroxybenzotriazole (138 mg, 0.9 mmol) were added at the coupling step. The ninhydrin test was negative after overnight reaction (18 h) and the resin washed using cycle C.

Rates of Esterification of Hydroxymethylbenzoyl-resin.—The foregoing resin (0.1 mmol) was esterified with the symmetrical anhydride (0.6 mmol) of Boc-L-phenylalanine in the presence of 4-dimethylaminopyridine (732 mg, 0.6 mmol) in DMF (5 ml). Resin samples were removed for amino-acid analysis after 5.5 and 125 min [Found: Phe, 1.00; Nle, 1.00 (5.5 min), and Phe, 1.01; Nle, 1.00 (125 min)]. In a second experiment, a smaller sample of resin (*ca.* 0.01 mmol) was treated with the symmetrical anhydride (0.6 mmol) of Boc-glycine in DMF (5 ml) for 4 h, washed (cycle C), and further treated with the symmetrical anhydride (0.6 mmol) of Boc-phenylalanine and 4-dimethylaminopyridine (732 mg, 0.6 mmol) in DMF (5 ml). A sample was taken for amino-acid analysis after 2 h (Found: Gly, 0.03; Phe, 0.95; Nle, 1.00).

Stability of the Esterified Hydroxymethyl-resin to Piperidine.—A sample (0.06 mmol) of hydroxymethylbenzoyl-L-leucyl resin (prepared as for the foregoing norleucyl derivative) was acylated with the symmetrical anhydride (0.36 mmol) of Boc-glycine and 4-dimethylaminopyridine (44 mg, 0.36 mmol) in DMF (6 ml) for 6 h and then washed (cycle C) (Found: Gly, 0.99; Leu, 1.00). The resin was treated with 20% piperidine/DMF with samples removed and washed after 3 and 24 h [Found: (3 h) Gly, 0.98; Leu, 1.00; (24 h) Gly, 0.94; Leu, 1.00].

Solid-phase Synthesis of [12-*Leucine*]*Human Minigastrin I and Des*-1-*tryptophan*-[12-*Leucine*]*Human Minigastrin* I.—The hydroxymethylbenzoylnorleucyl-polydimethylacrylamide resin was prepared as described above. This resin (0.16 mmol) was esterified with the symmetrical anhydride (0.9 mmol) of Boc-phenylalanine (all amino-acids were of the L-configuration) in DMF (10 ml) for 2 h. The ninhydrin test was negative for this and all subsequent acylation reactions. A sample was removed for amino-acid analysis at this stage and subsequently as indicated in Table 1. The resin was washed, the butoxycarbonyl group cleaved, and the neutral-

* In more recent experiments, these aqueous and dioxan washings have been replaced by additional washings of DMF.[2]

Table 1. Amino-acid analysis of intermediate peptide-resin samples

Sample No.	Glu	Ala	Tyr	Gly	Trp	Leu	Asp	Phe	Nle
1								1.00	0.90
								1.00	0.89
2							0.98	1.00	1.02
8	1.00	0.96	0.92	1.00	—	0.97	1.01	0.98	1.07
	0.97	0.96	0.95	1.00	—	1.03	1.01	1.00	1.11
9	1.94	0.97	0.95	1.00	—	1.02	1.01	1.01	1.06
	1.95	0.96	0.94	1.00	—	1.02	1.01	1.00	1.05
10	2.96	0.98	0.97	1.00	—	0.98	1.01	0.98	1.05
	2.96	0.98	0.96	1.00	—	0.97	1.00	0.98	1.04
11	3.97	0.95	0.94	1.00	—	0.94	1.01	0.92 *	1.00
	4.00	0.96	0.95	1.00	—	0.94	1.00	0.93 *	1.01
13	5.00	0.98	0.93	1.00	—	1.90	1.00	0.98	1.03
	5.04	0.97	0.94	1.00	—	1.91	1.01	0.98	1.04
14	4.95	0.96	0.93	1.00	—	1.85	1.00	0.97	0.95
	4.90	0.95	0.93	1.00	—	1.95	1.00	0.96	1.04

* Upon re-analysis = 1.00.

Table 2. Amino-acid analysis of des-1-tryptophan[12-leucine]-minigastrin I and contaminants separated by h.p.l.c. (*cf.* Figure 2a). Hydrolysis conditions: (a) 5.5M-HCl containing phenol, 18 h, 120 °C (* 5.5M-HCl containing 2.5% thioglycolic acid, 18 h, 120 °C); (b) amino peptidase-M, 10 units/mg substrate, 37 °C, 5 h

	Peak A		Peak B		Peak C		Peak D	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Asp	1.00	0.98	1.04	0.82		0.94	1.00	0.86
Glu	5.14	5.10	5.10	5.16	5.09	5.11	5.08	5.44
Gly	0.98	2.98	1.00	1.00	1.00	0.98	1.08	1.00
Ala	0.98	1.02	1.00	1.05	1.14	1.01	0.99	1.10
Leu	1.97	1.97	1.94	1.99	1.99	1.96	1.93	1.92
Tyr	0.96	1.01	0.96	1.05	1.05	1.01	0.95	1.02
Phe	0.97	1.00	0.97	0.42	1.06	1.00	0.97	1.11
Trp	0.97 *	1.18		1.04		1.15	0.93 *	0.0

ised amino-resin acylated with the symmetrical anhydride (0.9 mmol) of fluorenylmethoxycarbonyl-β-t-butyl aspartate in DMF (10 ml) for 1 h (reaction cycle B). Thereafter, the following fluorenylmethoxycarbonylamino-acids were added in similar manner using reaction cycle A for the acylation times indicated: leucine (70 min); tryptophan (85 min); glycine (60 min); *O*-t-butyltyrosine (60 min); alanine (60 min); γ-t-butylglutamate (60, 60, 60, 75, and 75 min) (five successive residues). The resin sample was then divided into approximately equal batches. To one was added Boc-leucine (90 min) and the resin washed (cycle C); to the other was added Fmoc-leucine (90 min), and then Boc-tryptophan (60 min), and the resin washed (cycle C). Both resins were then finally washed with dichloromethane, ether, and then dried. The resin loadings were 0.167 mmol Gly/g (tridecapeptide) and 0.161 mmol Gly/g (tetradecapeptide).

*Isolation of Des-*1*-tryptophan*[12*-Leucine*]*Minigastrin I.*—A sample of the dried tridecapeptide resin (99 mg, 16.7 μmol Gly) was deprotected with trifluoroacetic acid using reaction cycle D (including drying of the resin over phosphorus pentoxide *in vacuo*), swollen in DMF (15 min), and washed with DMF (5 × 1 min), 10% di-isopropylethylamine (3 × 1 min), and DMF (5 × 1 min). The drained resin was transferred to a round-bottomed flask, a solution (60 ml) of ammonia in methanol saturated at 0 °C was added and the flask sealed and set aside for 16 h at room temperature. The resin was filtered, washed with methanol (Found: Gly, 0.045; Nle, 1.00), and the combined filtrate and washings evaporated (Found: Asp, 1.04; Glu, 5.29; Gly, 1.00; Ala, 0.91; Leu, 2.06; Tyr, 1.00; Phe, 1.07; total glycine, 12.46 μmol, 75.4%), h.p.l.c., Figure 2a. Part (12.2 μmol) of this product was applied to a column of diethylaminoethyl cellulose (Whatman DE52, 11.5 × 1 cm diam.) and eluted at a flow rate of 1 ml/min with a linear gradient of 0.01—0.5M-aqueous ammonium hydrogencarbonate pH 8.1, 500 ml in each mixing chamber. The peptide eluted as a single peak between 300 and 415 ml (Figure 3). The freeze-dried product was desalted on Sephadex G25 eluted with 0.1M-ammonia (Found: Asp, 1.01; Glu, 5.24; Gly, 1100; Ala, 1.01; Leu, 2.01; Tyr, 1.01; Phe, 1.00; total glycine 11.89 μmol). Part of the product was further purified by reverse-phase semi-preparative h.p.l.c. Aliquots containing *ca.* 1 μmol of a solution of the synthetic gastrin (4.32 μmol) in 0.01M-aqueous ammonia (2 ml) were applied to a column of μ-Bondapak C_{18}. Reservoir A contained 0.01M-aqueous ammonium acetate, pH 4.5; reservoir B contained 90% MeCN and 10% of buffer A. The gastrin was eluted with a concave gradient from 0—30% B over 20 min at 2 ml/min. All the peaks which emerged from the column were collected separately and corresponded with those of Figure 2a (see Table 2). Des-Trp[1]-[Leu[12]]minigastrin I was recovered in overall 59% yield from the protected resin-bound peptide. It was identical with authentic material [25] on h.p.l.c., t.l.c. in butan-1-ol–acetic acid–pyridine–water (60 : 6 : 24 20) (R_F 0.19) (single spot), and paper electrophoresis at 3 kV and pH 6.5 streaking to R_{Asp} 0.62. In parallel experiments, aliquots of the solid phase (A) and solution synthesised (B) [25] gastrins were digested with chymotrypsin, *Staphylococcus aureus* protease V8, and thermolysin as follows: α-chymotrypsin (4 mg) was dissolved in 0.01M-ammonium acetate (6 ml), and to portions of this enzyme solution was added gastrin (A) (0.4 mg in 0.12 ml) and gastrin (B) (0.72 mg in 0.22 ml) (enzyme : substrate ratio 1 : 5). The mixtures were incubated at 25 °C and aliquots (10 μl) removed at various time intervals and compared by h.p.l.c. (*e.g.* Figure 5 at 40 h). *S. aureus* protease V8 (78 μg) was similarly dissolved in 0.05M-ammonium hydrogencarbonate (2 ml), pH 7.9; gastrin (A) (0.114 mg) was added to an aliquot (61 μl) of this solution, and gastrin (B) (0.23 mg) to 123 μl (enzyme : substrate ratio 1 : 50). Both mixtures were incubated at 25 °C and aliquots (20 μl) removed at timed intervals (h.p.l.c., *e.g.* Figure 6 at 24 h). Thermolysin (0.4 mg) was dissolved in 0.05M-Tris acetate buffer (2 ml) pH 8.0, 0.01M in $CaCl_2$, and this enzyme solution (0.2 ml) diluted to 2 ml by addition of further buffer. Gastrin (A) (0.123 mg) was dissolved in 66 μl, and gastrin B (0.46) in 246 μl of this solution (enzyme: substrate ratio 1 : 1.00), the mixtures incubated at 25 °C and aliquots (20 μl) withdrawn at timed inter-

Table 3. Conditions as Table 2

	Peak A		Peak B		Peak C	
	(a)	(b)	(a)	(b)	(a)	(b)
Asp	1.01	0.97	1.06	0.91	1.01	0.85
Glu	5.05	4.89	5.28	4.87	5.11	4.65
Gly	1.00	0.92	1.09	1.00	1.07	1.05
Ala	0.99	0.96	1.04	1.01	1.01	1.02
Leu	1.99	1.83	1.95	1.80	1.87	1.85
Tyr	0.98	0.96	0.97	0.98	0.92	1.01
Phe	0.98	1.00	1.00	0.43	1.00	1.00
Trp	1.97 *	2.31		1.95		1.41

vals (h.p.l.c., *e.g.* Figure 7 at 69 min). H.p.l.c. was on μ-Bondapak C_{18} using a gradient of 0—90% MeCN in 0.01M-NH_4OAc over 20 min at 2 ml/min.

Isolation of [12-*Leucine*]*Minigastrin I.*—A sample of the dried tetradecapeptide resin (75 mg, 12.1 μmol) was deprotected and the peptide cleaved from the resin essentially as in the foregoing experiment. Residual resin analysis (Gly, 0.055; Nle, 1.00) indicated that 94.5% had been cleaved. The filtrate and washings were combined and evaporated giving 10.04 μmol (83%) of peptide (Found: Asp, 1.00; Glu, 5.02; Gly, 1.00; Ala, 0.95; Leu, 1.94; Tyr, 0.95; Phe, 1.02), h.p.l.c. Figure 4a. Part (9.83 μmol) of the crude tetradecapeptide amide was applied to a column of diethylaminoethyl cellulose (Whatman DE52, 14 × 1 cm diam.) and eluted with a linear gradient of 0.01—1.0M ammonium hydrogencarbonate, pH 8.1 at a flow rate of 1 ml/min. The product (8.53 μmol, 87%) eluted as a single peak at 414—536 ml (Found: Asp, 1.00; Glu, 5.24; Gly, 1.00; Ala, 1.01; Leu, 1.96; Tyr, 0.96; Phe, 0.97). Part (8.47 μmol (96.5%) (Found: Asp, 1.00; Glu, 5.17; Gly, 0.98; Ala, 0.99; Leu, 1.95; Tyr, 0.96; Phe, 0.96). A sample (3.81 μmol) of this product was applied in four batches to an analytical μ-Bondapak C_{18} column and eluted with a convex gradient of 0—35% solvent B over 20 min at 2 ml/min (see above for solvent composition). The tetradecapeptide amide 1.79 μmol, 47%, was recovered from the main peak (h.p.l.c., Figure 4b; amino-acid analysis, Table 3); overall yield 32.8%.

In a later experiment, the tetradecapeptide amide was detached from the resin as before, and chromatographed directly on μ-Bondapak C_{18} under the same conditions as above except that 1.37 μmol was applied to two analytical columns connected in series. The purified peptide (0.90 μmol, 51% overall) (Found: Asp, 1.02; Glu, 5.24; Gly, 1.00; Ala, 0.99; Leu, 2.03; Tyr, 0.97; Phe, 1.00) was identical on analytical h.p.l.c. with material purified as above.

Acknowledgements

We are grateful to Professor R. A. Gregory for the biological assays, and to Professor E. Wünsch for a generous gift of highly purified des-1-tryptophan[12-leucine]minigastrin I.

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Received 24*th May* 1982; *Paper* 2/852