An Efficient General Method for the Synthesis of Gastrins

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Summary Use of a polyamide-based solid phase method has permitted rapid and efficient synthesis of 13-, 14-, and 17-amino-acid residue gastrin peptide amides.

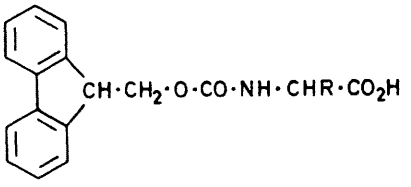
THE gastrin family of peptide hormones has taken on renewed importance following their discovery in brain and other nervous tissue.[1] In addition to their well recognised actions on alimentary tract structures, they or their *C*-terminal fragments are now seen to have possible neurotransmitter roles. Several gastrins have been isolated from natural sources, of which human little gastrin I (**1a**) and its shorter minigastrin analogue (**2a**) are characteristic. Larger, presumed prohormones, in which the sequence of (**1a**) (with *N*-terminal glutamine in place of pyroglutamic acid) is further extended at its amino-terminus, are also known. The amino-acid compositions of the gastrins, especially the high content of glutamic acid and tryptophan residues, are particularly unfavourable for conventional solid phase synthetic methods which involve strongly acidic conditions. Little solid phase synthesis of these types of compound has been recorded[2] and in general synthetic gastrins and their analogues have been available only from laborious solution syntheses. We now report rapid and efficient procedures for the solid phase synthesis of the 17- and 14-residue leucine analogues† (**1b**) and (**2b**), and the des-1-tryptophan analogue of the latter. Problems inherent in the application of conventional procedures have been overcome by the use of polar polydimethylacrylamide-based supports,[3] and particularly of base-labile N^{α}-fluorenylmethoxycarbonyl (Fmoc) amino-acid derivatives (**3**).[4] The methods described herein appear to provide a general route to the gastrins.

Glp.Gly.Pro.Trp.Leu.Glu.Glu.Glu.Glu.Glu.Ala.Tyr.Gly.-Trp.X.Asp.Phe.NH_2

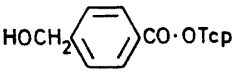
(**1**) Glp = pyroglutamic acid

H.Trp.Leu.Glu.Glu.Glu.Glu.Glu.Ala.Tyr.Gly.Trp.X.Asp.-Phe.NH_2

(**2**)
a; X = Met
b; X = Leu



(**3**)



(**4**)

Tcp = 2,4,5-trichlorophenyl

Cross-linked, methoxycarbonyl functionalised polydimethylacrylamide resin[3]‡ was converted into primary amine by reaction with excess of ethylenediamine. An internal reference-spacer amino-acid was incorporated by acylation with Fmoc-norleucine anhydride and cleavage of the Fmoc group with 20% piperidine (10 min), followed by addition of the reversible linkage agent (**4**) in the presence of hydroxybenzotriazole. All reactions were carried out in dimethylacetamide and six-fold excesses of acylating species were used to ensure rapid and near-quantitative

† Replacement of methionine by leucine in the gastrin series leads to biologically fully active products (J. J. Mendive, G. W. Kenner, and R. C. Sheppard, *J. Chem. Soc.*, 1968, 761). The synthetic methods described are, however, equally applicable for the synthesis of methionyl peptides.

‡ This resin sample was a commercial preparation provided by Dr. A. Loffet of UCB Bioproducts, Peptide Department, Brussels, Belgium.

reaction The peptide sequence was then elaborated using initially t-butoxycarbonyl (Boc) phenylalanine anhydride in the presence of catalyst (dimethylaminopyridine) to establish the peptide–resin ester bond (Found Phe, 1 00, Nle = norleucine, 1·06), cleavage of the Boc-group with 1 5M HCl–AcOH, and sixteen cycles of Fmoc-amino-acid addition and deprotection Symmetrical anhydrides were used for acylation in all cases except the last (glutamine), which was introduced as its *p*-nitrophenyl ester in the presence of hydroxybenzotriazole Glutamine was chosen as the *N*-terminal residue rather than pyroglutamic acid, present in little gastrin, so as to permit possible future extension into the prohormone series Side-chain carboxy-groups of aspartic and glutamic acid residues were protected as t-butyl esters, and tyrosine as its t-butyl ether The synthesis proceeded smoothly with excellent amino-acid incorporation at each stage as indicated by amino-acid analysis of intermediate and final resins (Found Asp, 0 94, Glu, 6 18, Pro, 1 02, Gly, 1 97, Ala, 1 06, Leu, 1·99, Tyr, 1 00, Phe, 1·00, Nle, 1 07), and by results of purification studies below Little if any loss of peptide from the resin occurred during the synthesis as shown by the constancy of the Phe : Nle ratios

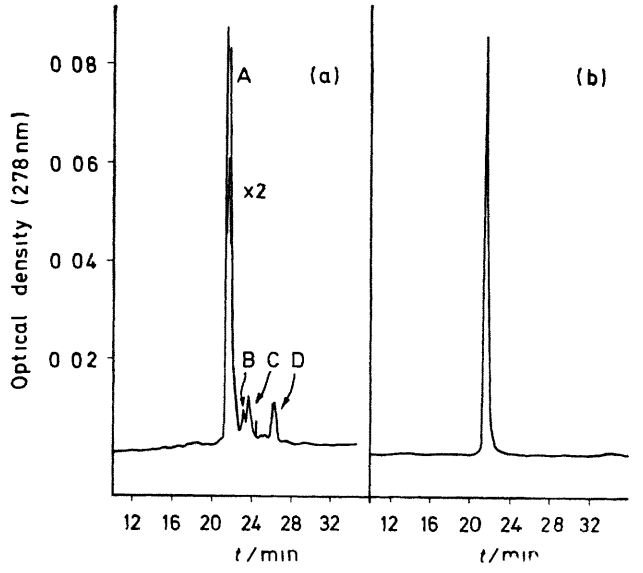


FIGURE H p l c of synthetic 15-leucine human little gastrin I (a) Total crude product after ammonolysis and cyclisation in aq acetic acid Uncyclised glutamine peptide is present in peak C, peak D is probably due to a t butylated indole derivative of peptide A (b) Purified final product Analytical h p l c conditions linear gradient of 18—45% MeCN in 0 01M NH_4OAc, pH 3 5, over 30 min , flow rate 2 ml min^{-1} μ-Bondapak C_{18} column Resolution is strongly pH-dependent, and at pH 4 5 the terminal glutamine peptide (peak C) coalesces with the pyroglutamyl peptide (peak A)

All the side-chain t-butyl derivatives were cleaved by treatment with 90% aq trifluoroacetic acid (30 min) and the free peptide detached by ammonolysis in saturated methanolic ammonia for 22 h Residual resin analysis indicated removal of 91% of the peptide At this stage the product consisted (h p l c) of a mixture of the 17-residue *N*-terminal glutamine peptide amide and its cyclised pyroglutamyl analogue (**1b**) (*ca* 5 : 1) Cyclisation was brought nearly to completion (*ca* 95%) by treatment with 20% acetic acid (30 °C, 64 h under Ar) (Figure a) Residual glutaminyl peptide (peak C) was removed by ion-exchange chromatography on DEAE cellulose DE52 (linear gradient of 0 01—1M NH_4OAc, pH 6·5), and remaining impurities (peaks B and D)§ were removed by preparative h p l c The yield of purified [15-leucine] human little gastrin I (**1b**) (Figure b), identical by h p l c and t l c with an authentic sample,[6] was 32% A second isolation gave 28% (Found Asp, 1·00, Glu, 6·37, Pro, 1 03, Gly, 1·97, Ala, 0 98, Leu, 2·03, Tyr, 1·00, Phe, 1·00)

A resin sample removed after addition of thirteen residues was deprotected and cleaved as above Purification by preparative h p l c provided des-1-tryptophan[11-leucine] human minigastrin I (52%) (Found Asp, 1 00, Glu, 5 35, Gly, 1 00, Ala, 1·03, Leu, 2·04, Tyr, 1·01, Phe, 1·00), identical with an authentic sample,[7] and with that from an earlier solid-phase preparation This last synthesis was similar to the foregoing except that a different resin sample[3] was used, part of the resin was terminated with Boc-leucine at thirteen residues, and the remainder with Fmoc-leucine and then Boc-tryptophan at 14 residues The thirteen-residue synthesis yielded des-1-tryptophan[11-leucine] human minigastrin I (59%) (Found Asp, 1·00, Glu, 5·14, Gly, 0·98, Ala, 0 98, Leu, 1·97, Tyr, 0·96, Phe, 0·97, Trp, 0 97) which was compared rigorously with classically synthesised material,[7] *inter alia* by h p l c fingerprinting of three different enzymic digests No significant differences were found All the trace impurities in the crude product separable by h p l c were isolated and subjected to amino-acid analysis All had the expected compositions for the full sequence apart from tryptophan which was not determined By-products were tentatively identified as the tridecapeptide methyl ester (*ca* 2%), the corresponding free acid (2 1%), and a t-butylated tryptophan[5] derivative (6 3%) The fourteen-residue synthesis yielded, after similar deprotection, cleavage, and h p l c purification, [12-leucine] human minigastrin (**2b**) (52%) (Found Asp, 1 02, Glu, 5 24, Gly, 1·00, Ala, 0 99, Leu, 2·03, Tyr, 0·97, Phe 1 00, Trp, 2 03) All three peptides were subjected to bioassay in conscious dogs equipped with gastric cannulae and were equiactive with the same molar quantity of natural human little gastrin I in stimulating gastric secretion

The efficiency of amino-acid incorporation and the relative freedom from side reactions in these syntheses is noteworthy The former is attributable to the use of a polar, polyamide support which permits all the reactions to be carried out under optimum conditions in a good solvating medium for both peptide and polymer The latter is ascribed to the mildness of the overall synthesis which is made possible by an N^{α}-fluorenylmethoxycarbonyl-ω-t-butyl protecting-group strategy Serious side reactions are commonly observed with peptide sequences containing tryptophyl- or benzyl-protected glutamyl, aspartyl, and

§ Impurities B (3 9%) and D (5 5%) (Figure a) were isolated and gave amino-acid analyses almost identical to that of the main product Tryptophan was not determined, but their u v absorptions are consistent with equal tryptophan content for all components in the crude mixture Formation of various t-butylated indole derivatives during acidic cleavage of t-butyl esters and ethers is well established in the gastrin series [5]

tyrosyl residues under the strongly acidic conditions of conventional solid phase synthesis.

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