Continuous-flow Solid-phase Synthesis of a 74-Peptide Fragment Analogue of Human β-Chorionic Gonadotropin

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The linear dimer of a triacontaheptapeptide corresponding to the *C*-terminal of the β-subunit of human chorionic gonadotropin has been synthesized by the continuous-flow Fmoc-polyamide solid-phase method. The *S*-protected peptide was obtained in good yield after purification, suggesting that this synthesis procedure may be generally applicable for the assembly of large sequences. Potential problems with the method are discussed with reference to those experienced in this assembly.

The development of a peptide vaccine against the placental hormone, human chorionic gonadotropin (hCG), provides an immunological approach to fertility control in the human female.[1,2] The β-subunit of hCG possesses an extended unique carboxy terminal segment of 30 residues which is not present in the structurally related glycoprotein hormones luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH).[3] Synthetic peptide antigens corresponding to this extended region should result in antibodies which are specific for hCG and which do not cross-react with the pituitary hormones. Indeed, extensive studies in the baboon[4–6] using a vaccine comprised of a 37-peptide synthetic fragment of the β-subunit of hCG conjugated to a protein carrier have demonstrated that this approach is viable and Phase I clinical trials of this vaccine in the human have been successfully completed.[7]

In an attempt to improve the immunogenicity of the synthetic peptide and to avoid the use of protein carriers we have been investigating the use of polymers of the unique *C*-terminal peptide segment of h-βCG. In this report we describe the synthesis of a linear dimer of the carboxy terminal 37 peptide, the primary structure of which is shown in Figure 1. We chose to use the continuous-flow fluoren-9-yl methoxycarbonyl (Fmoc)-polyamide method of solid-phase synthesis[8] so as to enable us to evaluate critically the effectiveness of this procedure for the chemical assembly of a large peptide. This technique was recently employed for the successful synthesis of an acid-labile 37-residue peptide corresponding to the carboxy terminal region of baboon β-CG.[9]

The linear dimer of h-βCG (109–145) [hereafter designated as h-βCG(*1–74*)†] was assembled (Scheme) on a low-loading composite poly(dimethylacrylamide)-macroporous Kieselguhr support[10] using a semi-automatic computer-controlled synthesizer. Preliminary conversion of the methoxycarbonyl-functionalized resin into its amino form was achieved by treatment with excess of ethylenediamine. A permanent internal reference residue (glycine‡) was then introduced using the Fmoc-amino acid anhydride procedure. After cleavage of the Fmoc group by brief (10 min) treatment with 20% piperidine in dimethylformamide (DMF), the reversible peptide-resin linkage agent (4-hydroxymethylphenoxyacetic acid) was coupled as its 2,4,5-trichlorophenyl ester in the presence of catalyst (1-hydroxybenzotriazole). Three-fold molar excesses of acylating species were employed and reactions were complete after 30 min as determined by the 2,4,6-trinitrobenzenesulphonic acid colour test[11] for free amine.

1 *5* *10* *15*
Thr-Cys-Asp-Asp-Pro-Arg-Phe-Gln-Asp-Ser-Ser-Ser-Ser-Lys-Ala-

20 *25* *30*
Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Ser-

35 *40* *45*
Asp-Thr-Pro-Ile-Leu-Pro-Gln-Thr-Cys-Asp-Asp-Pro-Arg-Phe-Gln-

50 *55* *60*
Asp-Ser-Ser-Ser-Ser-Lys-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-

65 *70*
Ser-Arg-Leu-Pro-Gly-Pro-Ser-Asp-Thr-Pro-Ile-Leu-Pro-Gln

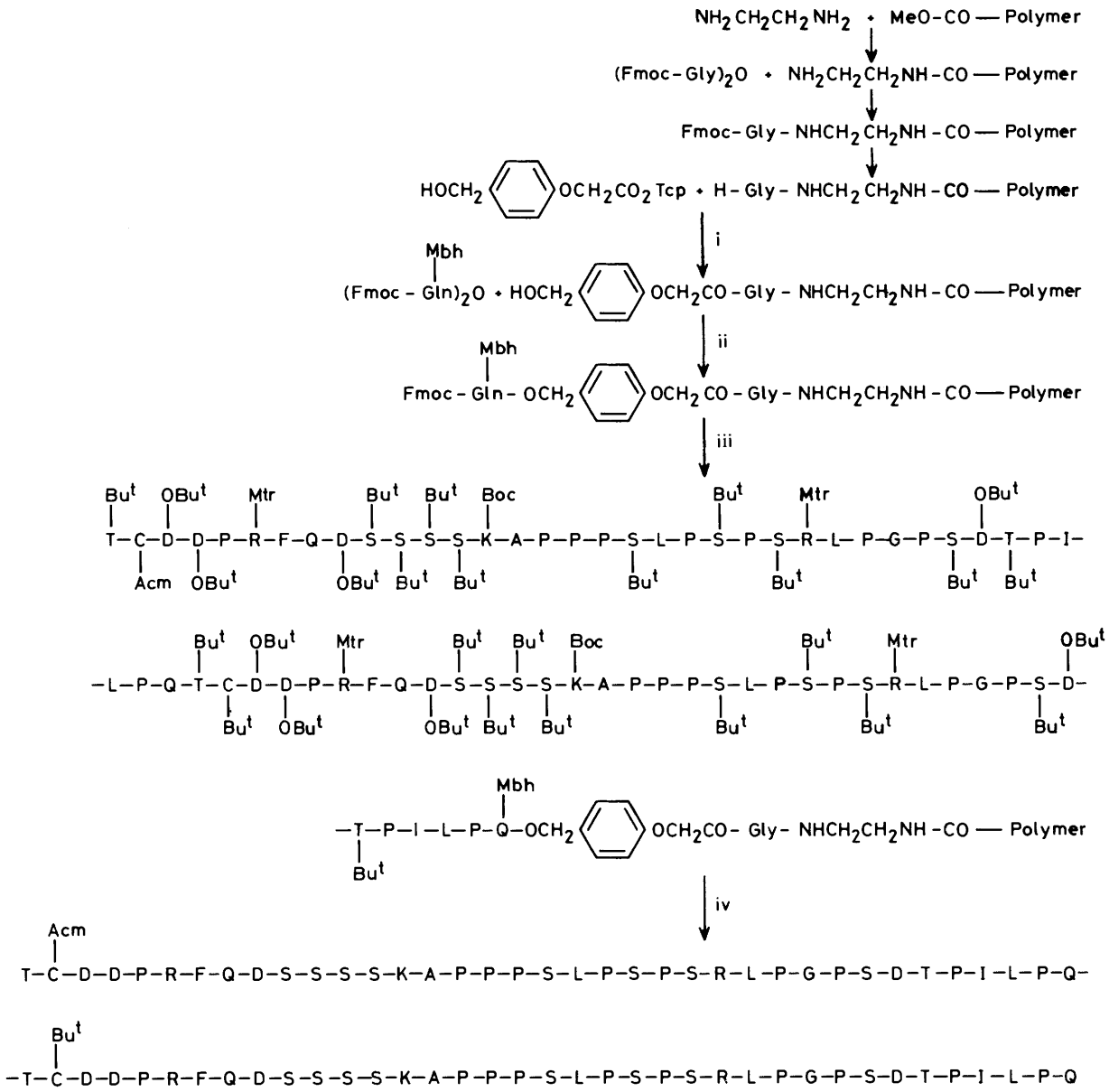
Figure 1. Amino acid sequence of h-βCG(*1–74*)

Esterification of the first amino acid of the sequence to the resin-bound linkage agent was carried out using the preformed symmetrical anhydride of Fmoc-glutamine with its carboxamide sidechain temporarily protected by the trifluoroacetic acid (TFA)-labile 4,4′-dimethoxybenzhydryl (Mbh) group.[12] The use of this group prevents *N,N′*-dicyclohexylcarbodiimide-mediated dehydration of the carboxamide function during amino acid anhydride formation and subsequent incorporation of the modified residue. Esterification of the preformed symmetrical anhydride of Fmoc-Gln(Mbh)-OH is catalysed by the presence of 4-dimethylaminopyridine (DMAP). The reaction conditions were similar to those previously found to effect a good degree of incorporation of this bulky derivative, *viz.* a double coupling each of 1 h duration using fresh reagents for each reaction.[9] Amino acid analysis of a sample of the resulting resin indicated that *ca.* 94% incorporation of Gln was achieved (Table 1). Residual hydroxy groups were left unblocked as it is known that these do not normally affect the success of Fmoc-polyamide solid-phase synthesis.[13]

The remaining seventy-three amino acids of the peptide sequence were assembled in a routine manner according to the Scheme. Three-fold molar excesses of activated N^{α}-Fmoc-amino acid were used throughout the synthesis. Most amino

† Terminology for linear peptide polymers is not well defined and can be cumbersome. We have chosen, for reason of simplicity, to use the format h-βCG(*1–74*) with the numbers in italics indicating the length of the peptide. It does not mean residues 1 to 74 inclusive of the β-subunit of hCG.

‡ The use of a reference amino acid that does not occur within the sequence being synthesized is obviously much preferred so as to enable easier interpretation of peptidyl resin amino acid analysis results. Norleucine is an ideal example (see ref. 14). However, its use in this assembly was precluded by its presence as internal standard in the amino acid analyser-developing buffer.



Scheme. Solid-phase synthesis of h-βCG(*1–74*). The polymeric support was Kieselguhr-supported poly(dimethylacrylamide). *Reagents:* i, 1-hydroxybenzotriazole; ii, DMAP; iii, 73 cycles of deprotection (20% piperidine in DMF) and acylation; iv, (a) 20% piperidine in DMF; (b) 95% TFA–5% scavenger

acid derivatives were coupled as their preformed symmetrical anhydrides.[14] Fmoc-Arg(Mtr) anhydride (Mtr = 4-methoxy-2,3,6-trimethylbenzenesulphonyl) was coupled in the presence of an equivalent amount of catalyst, 1-hydroxybenzotriazole, in order to enhance its rate of acylation which can otherwise be slow due to the instability of the derivative.[15] Fmoc-Gln and Fmoc-Lys(Boc) (Boc = butoxycarbonyl) were both coupled as their *p*-nitrophenyl esters also in the presence of 1-hydroxybenzotriazole.[16] Each coupling of Fmoc-Pro anhydride onto a Pro residue was permitted to proceed for 1 h instead of the allotted time of 30 min. This prudent action was necessitated by the inappropriateness of amine colour tests for this residue. The coupling of all other residues was monitored by the 2,4,6-trinitrobenzenesulphonic acid test and was found to be negative at the end of the allotted time. The first Cys residue was coupled with its sidechain protected by the t-butyl group. This function is stable to TFA and remains attached to the thiol under the usual conditions of Fmoc-polyamide peptide synthesis.[17,18] In contrast, however, the second Cys residue was coupled as its *S*-acetamidomethyl derivative. This endowed the required degree of flexibility enabling the selective removal of this group and simultaneously to form a disulphide dimer of the peptide if so desired.[19] Aliquots of peptide-resin were removed during the course of the synthesis for amino acid analysis and the results were generally acceptable (Table 1). Qualitative feedback was also provided by continuous spectrophotometric monitoring (307 nm) of the reaction column effluent during the assembly.[20,21] Typical acylation and deprotection traces were obtained which also indicated satisfactory progress in the assembly.

However, *ca.* two-thirds of the way into the synthesis [from residue *29* (Pro)] and for three cycles [up to and including residue *26* (Leu)], unexpected and significant swelling of the resin was observed. This led to dense packing of the reaction

Table 1. Assembly of h-βCG(*1–74*). Amino acid analysis of peptidyl resins with rounded values in parentheses

	74	*64–74*	*58–74*	*38–74*	*32–74*	*16–74*	*1–74*	*14–74*R	*1–74*R
Asp		1.04(1)	1.12(1)	3.94(4)	3.91(4)	4.94(5)	7.79(8)	5.12(5)	7.50(8)
Thr		1.02(1)	1.08(1)	1.63(2)	2.60(3)	2.65(3)	3.25(4)	2.97(3)	3.75(4)
Ser		1.15(1)	3.16(3)	8.39(8)	8.34(8)	12.29(12)	16.53(16)	12.78(12)	16.67(16)
Glu	0.93(1)	1.07(1)	1.09(1)	1.99(2)	2.96(3)	3.04(3)	3.95(4)	3.22(3)	4.22(4)
Pro		3.91(4)	5.79(6)	10.30(10)	12.38(12)	18.89(19)	20.67(20)	18.48(19)	20.35(20)
Gly[a]	1.00(1)	2.23(2)	2.24(2)	2.55(2)	2.64(2)	3.71(3)	3.83(3)	3.68(3)	3.97(3)
Ala				1.16(1)	1.05(1)	1.18(1)	2.08(2)	1.97(2)	2.04(2)
Cys[a]				(1)	(1)	(1)	(1)	(1)	(2)
Ile		0.88(1)	0.82(1)	0.92(1)	1.85(2)	1.91(2)	1.99(2)	1.87(2)	2.00(2)
Leu		0.94(1)	1.82(2)	3.00(3)	4.15(4)	6.07(6)	6.33(6)	5.92(6)	6.12(6)
Phe				0.88(1)	0.96(1)	1.11(1)	1.81(1)	1.00(1)	1.86(2)
Lys				0.99(1)	0.97(1)	1.01(1)	1.91(2)	1.91(2)	2.00(2)
Arg			0.91(1)	1.80(2)	1.84(2)	2.21(3)	2.83(4)	2.79(3)	3.49(4)

The column heading indicates stage of assembly for each analytical sample. Columns *14–74*R and *1–74*R are from the repeat assembly of the peptide using the original h-βCG(*26–74*)-resin. [a] Gly is reference amino acid. [b] Cys values not determined.

column and production of resin 'fines' which together resulted in generation of increasing pump back-pressure and leakage of solvent from the top column end-fitting. The lower column end-fitting frit was replaced on three occasions during this period because of blockage caused by the fines. As the rigid Kieselguhr support comprises pores of greater than several thousand Angstroms in diameter,[10,22] it should, in theory, be able to accommodate significant swelling of the poly(dimethylacrylamide) resin. It is likely, then, that the aforementioned observed swelling of the solid support is due to that of an outer coat layer of the poly(dimethylacrylamide), at least in the particular batch of resin used on this occasion. It was necessary to alleviate the pressure generation within the reaction column by removing approximately one-quarter of the peptide-resin. This portion [h-βCG(*26–74*)-resin] was washed well and dried *in vacuo* (0.23 g).

The remainder of the peptide-resin was washed on a sintered glass funnel with both non-polar and then polar solvents in order to remove any 'fines' which had accumulated both within and without the resin beds. The peptide-resin was transferred to the reaction column and synthesis then resumed with the N^{α}-Fmoc removal of residue *26* (Leu). Continuous u.v. monitoring of this step showed the deprotection to be unusually slow and for this reason it was permitted to continue for a total of 20 min. Coupling of the next residue (Arg) was initially believed to be complete after 30 min as assessed by the amine colour test and the synthesis was continued according to the Scheme. However, 20 min later it was observed that the test had yielded a weakly positive result and the corresponding u.v. trace of the Fmoc-piperidine adduct from the N^{α}-deprotection of this residue was of a much smaller size than the previous peak. It was then evident that rates of reaction were much slower as a consequence of either the non-polar solvent washing previously carried out on the peptide resin or because of steric hindrance caused by the inability of the resin to support a large peptide assembly. However, there was no further evidence of inefficient coupling or deprotection during the remainder of the synthesis, nor were additional complications encountered or observed. U.v. traces and amine colour tests continued to give good results although the latter became progressively slower to develop. Amino acid analyses of the final peptide-resin and that of h-βCG(*25–74*) did, however, suggest that low incorporation of residue *25* had been obtained (Table 1).

Cleavage and deprotection of an aliquot of this material was effected by an extended treatment with TFA in the presence of anisole and phenol.[9] Under these conditions, little acid-mediated degradation of the peptide at sites of Asp–Pro bonds is observed. The use of anisole as scavenger ensures optimum quenching of the carboxy terminal residue Mbh group. Extent of peptide-resin cleavage was greater than 98% as measured by analysis of the residual resin. Most of the scavengers were conveniently removed by extraction with ethyl acetate. The crude di-*S*-protected peptide was desalted by gel filtration (89.0%; h.p.l.c., Figure 2; analysis, Table 2) and then purified by semi-preparative h.p.l.c. to afford a product (0.91 μmol, 9.5%) which had a high degree of homogeneity (Figure 3). However, amino acid analysis of this material again showed the absence of a single arginine residue (Table 2). In order both to confirm this and to identify the site of deletion, a portion of the synthetic peptide was digested with trypsin and the resulting fragments subjected to high-sensitivity microsequencing. The results (Figure 6a and Table 3) clearly showed that residue *25*—an arginine—was missing from the original peptide. This deletion can be traced back to the difficulties encountered in the synthesis following removal and washing of the peptide-resin immediately prior to the coupling of residue *25*.

Table 2. Amino acid analyses of crude (1) and purified (2) synthetic [Cys^{2}(Acm),Cys^{39}(Bu^{t})]-h-βCG(*1–74*)[a]

Amino Acid	Theory	1	2	1R	2R
Asp	8	7.80	7.86	7.20	7.70
Thr	4	3.57	3.73	3.80	3.79
Ser	16	16.72	16.40	16.74	16.39
Glu	4	4.12	4.20	4.28	4.05
Pro	20	19.81	19.77	19.73	19.85
Gly	2	2.24	2.46	2.27	2.32
Ala	2	2.21	2.36	2.10	2.11
Cys[b]	2				
Ile	2	1.80	1.94	2.49	1.91
Leu	6	6.06	6.09	6.10	6.19
Phe	2	1.99	1.94	1.82	2.07
Lys	2	2.04	2.14	1.97	2.20
Arg	4	2.64	3.12	3.51	4.05

R represents peptide from the second assembly. [a] Acm = acetamidomethyl. [b] Cys values not determined.

Following these observations, it was decided to attempt an improved assembly of the peptide using the aliquot of h-βCG-(*26–74*)-resin which had been removed from the previous synthesis.

The dried peptide-resin was first kept in freshly distilled DMF for 24 h in an effort to ensure maximum swelling of the poly-(dimethylacrylamide) support. It was then packed into the reaction column and washed well for 30 min with DMF. Deprotection of the N^{α}-Fmoc group from residue *26* (Leu) was

1 *5* *10* *15* *20* *25*
T-C-D-D-P-R-F-Q-D-S-S-S-S-K-A-P-P-P-S-L-P-S-P-S-R-L-P-G-P-

30 *35* *40* *45* *50* *55*
S-D-T-P-I-L-P-Q-T-C-D-D-P-R-F-Q-D-S-S-S-S-K-A-P-P-P-S-L-P-

60 *65* *70* *74*
S-P-S-R-L-P-G-P-S-D-T-P-I-L-P-Q

Table 3. Amino acid sequences of tryptic fragment of synthetic [Cys^{2}(Acm),Cys^{39}(Bu^{t})]-h-βCG(*1–74*)

H.p.l.c. fraction[a]	Sequence determined	Synthetic peptide (*1–74*) sequence
A	F–Q–D–S–S–S–S–K	*7–14, 44–51*
B	A–P–P–P–S–L–P–S–P–S–R	*15–25*,[b] *52–62*
C	L–P–G–P–S–D–T–P–I–L–P–Q	*63–74*
D	L–P–G–P–S–D–T–P–I–L–P–Q–T–C–D–D–P–R	*26–43*
E	T–C–D–D–P	*1–5*
F	A–P–P–P–S–L–P–S–P–S–L–P–G–P–S–D–T–P–I–L–P–Q–T–C–D–D–P–R	*15–43*[c]

[a] Fractions shown in Figure 6a and b. [b] Fragment *15–25* absent in des-Arg^{25}-[Cys^{2}(Acm),Cys^{39}(Bu^{t})]-h-βCG(*1–74*). [c] Corresponds to an Arg^{25} deletion.

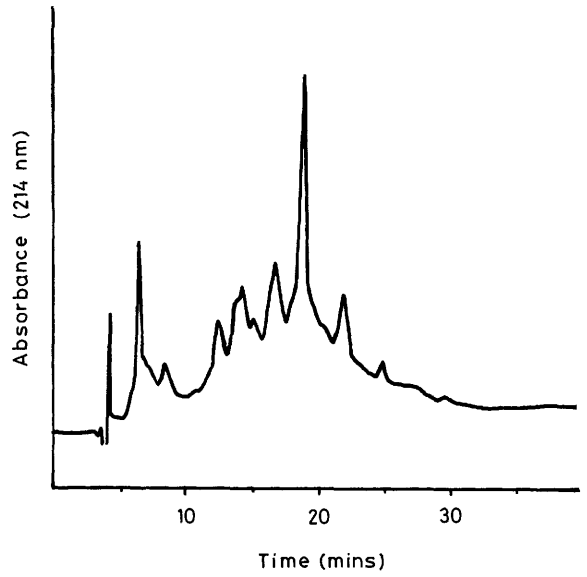


Figure 2. H.p.l.c. of total crude resin cleavage product from first assembly of h-βCG(*1–74*). Conditions: 40—54% B in 40 min. Flow rate 2.5 ml min^{-1}. Column: semi-preparative Vydac C4

with 20% piperidine in DMF for 30 min after which the synthesis continued using the same schedule as for the first assembly. The exception was the adoption of 50 min acylation times in order to compensate for potentially slower rates of coupling due to the resin support possibly being less swollen as a consequence of its previously dried form. Using a three-fold molar excess of acylating species, it was observed that two one-hour couplings were required of Fmoc-Arg(Mtr) anhydride in the presence of 1-hydroxybenzotriazole before a negative colour test was obtained. It is not known whether this sluggishness of acylation is due to a sequence-related effect or to the resin being incompletely reswelled from its previously dried state. Only a new assembly can determine this. Despite the difficulty with the

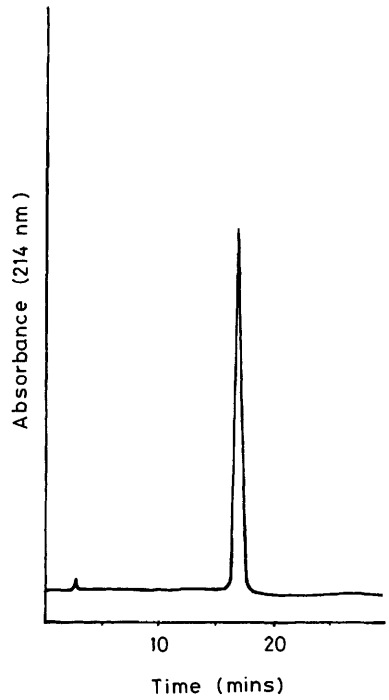


Figure 3. H.p.l.c. of synthetic [Cys^{2}(Acm),Cys^{39}(Bu^{t})]-h-βCG(*1–74*) from first assembly after purification by h.p.l.c. Conditions: 40—50% B in 35 min. Flow rate 1.0 ml min^{-1}. Column: analytical Vydac C4

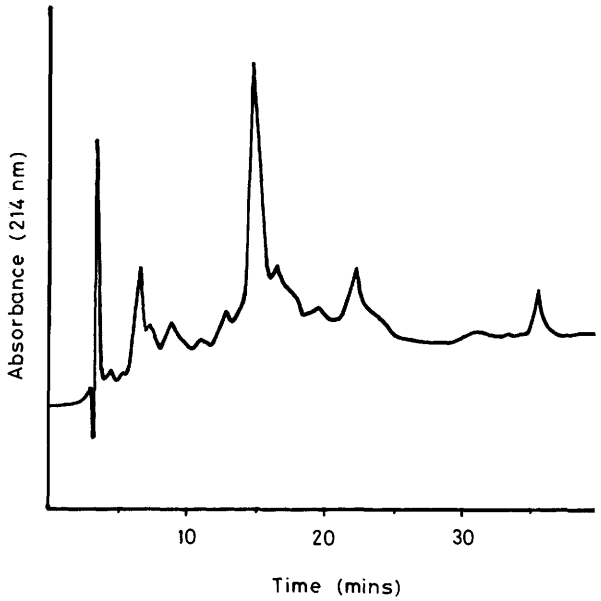


Figure 4. H.p.l.c. of total crude resin cleavage product from second assembly of [Cys^{2}(Acm),Cys^{39}(Bu^{t})]-h-βCG(*1–74*). Conditions: 40—50% B in 40 min. Flow rate 2.5 ml min^{-1}. Column: semi-preparative Vydac C4

coupling of this residue, no further complications were encountered during the remainder of the assembly. Cleavage and deprotection of the final peptide-resin was carried out as for the previous product as was desalting (Figure 4 for h.p.l.c.; Table 2 for amino acid analysis) and purification of the resulting crude di-*S*-protected peptide. Recovery from each step was similar to that for the first assembly and the overall yield of highly purified peptide was 8.7%. Amino acid analysis gave values close to theory (Table 2) and analytical h.p.l.c. using two different buffer

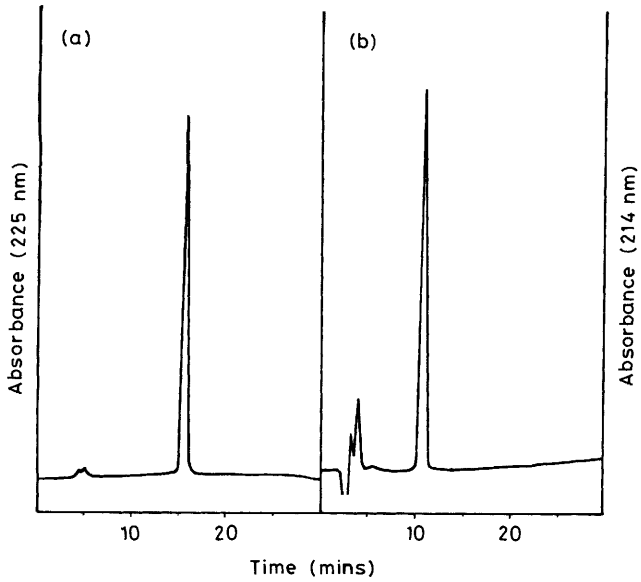


Figure 5. H.p.l.c. of synthetic [Cys^{2}(Acm),Cys^{39}(Bu^{t})]-h-βCG(*1–74*) from second assembly after purification by h.p.l.c. (a) Buffer A: 0.25M-triethylamine phosphate, pH 2.5. Buffer B: 40% A–60% acetonitrile. Conditions: 35—50% B in 35 min. Flow rate 2.0 ml min^{-1}. Column: semi-preparative Vydac C4. (b) Conditions as described in Figure 4

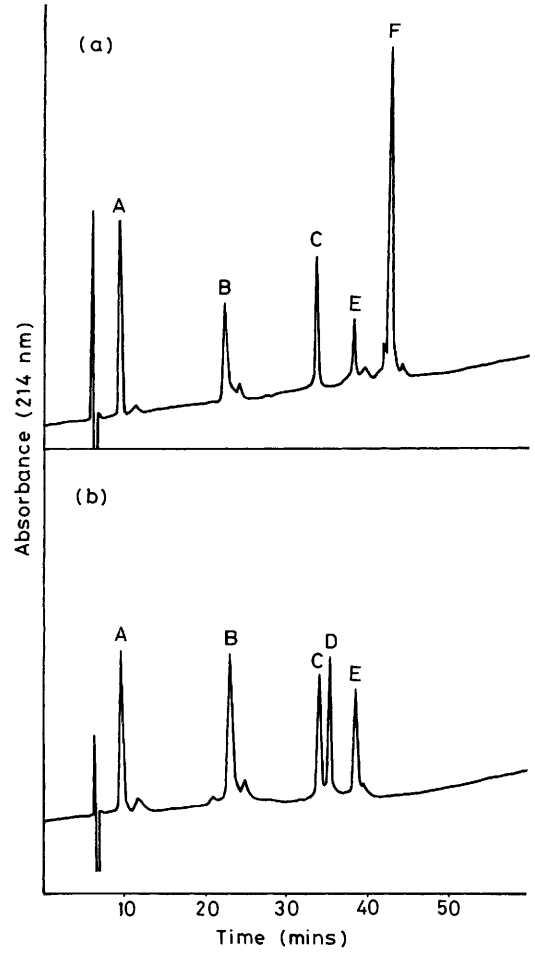


Figure 6. H.p.l.c. of tryptic digest of purified synthetic [(Cys^{2}(Acm), Cys^{39}(Bu^{t})]-h-βCG(*1–74*) from (a) first assembly, and (b) from second assembly. Conditions: 10—70% B in 60 min. Flow rate 2.5 ml min^{-1}. Column: semi-preparative Vydac C4

systems showed a high degree of purity (Figure 5). Tryptic digestion of an aliquot of the peptide and sequencing of the resulting fragments after their isolation was performed. The results (Figure 6b and Table 3) demonstrated the expected amino acid composition and sequence of the target product. This example highlights the usefulness of high-sensitivity micro-sequencing for assessing the integrity of composition of long and/or challenging synthetic peptide sequences.[23] It is clearly a technique which warrants more widespread adoption as a complementary analytical tool in the characterization of chemically synthesized peptides.

The result of this work augurs well for the assembly of large peptides using the continuous-flow method of Fmoc-polyamide solid-phase synthesis. On the basis of our limited experience, the following measures may prove useful for the general utility of this technique for the successful synthesis of peptides greater than, say, fifty amino acids. (i) Allowance for extra space in the reaction column (at least 10% of the total starting resin volume) in the event of unexpected resin swelling during the synthesis. (ii) Longer amine colour test development times are required with increasing length of synthesis. (iii) Likewise, longer N^{α}-deprotection and acylation times *may* be required towards the end of a long assembly. (iv) Solvent shock to the growing peptidyl-resin is to be avoided at all costs. At no stage of the synthesis should the peptide-resin be partially shrunk by washing with non-polar solvents or dried and then reswollen for continuing synthesis. It is possible that reswelling may not reach the same degree as prior to shrinkage. Resin 'fines' which may form during the assembly are best removed by repetitive decantation using DMF until decanted solution is totally clear.

The immunogenicity of the synthetic [Cys^{2}(Acm),Cys^{39}(Bu^{t})]-h-βCG-(*1–74*) peptide and its usefulness as a potential antipregnancy vaccine is currently being evaluated and will be reported elsewhere.

Experimental

Most N^{α}-fluorenylmethoxycarbonyl (Fmoc)-amino acid derivatives were obtained from Cambridge Research Biochemicals Ltd. (CRB), England. Fmoc-Cys(Acm)-OH, Fmoc-Gln(Mbh)-OH, and Fmoc-Lys(Boc)-ONp together with the 3,4,5-trichlorophenyl ester of 4-hydroxymethylphenoxyacetic acid (acid-labile handle) were each prepared in the laboratory using published procedures. All amino acid derivatives were of the L-form and were checked for purity by m.p. and on t.l.c. silica gel G plates with chloroform–methanol–acetic acid (85:10:5) as developer. Kieselguhr-supported poly(dimethylacrylamide) resin (Pepsyn K, ~0.11 mmol g^{-1}) was purchased from CRB. All solvents and reagents were purified as previously described.[14] DMF was freshly distilled daily during peptide synthesis.

Amino acid analyses were carried out on a Beckman system 6300 analyser. Peptides were quantitated by amino acid analysis after hydrolysis with 6M-hydrochloric acid, containing 0.1% phenol, in evacuated, sealed tubes for 24 h at 110 °C. High-performance liquid chromatography (h.p.l.c.) was carried out using a Shimadzu LC-4A system to which was connected a SPD-2AS variable-wavelength u.v.–visible spectrophotometer and a C-R2AX solvent programmer and recorder. Analytical h.p.l.c. was on a Vydac C4 column (250 × 4.6 mm id, 5 micron particle size). Semi-preparative h.p.l.c. was performed on a 250 × 7.0 mm i.d. column containing Vydac C4 packing material. The linear gradient for h.p.l.c. elution was established with solvents A and B. Solvent A consisted of 0.1% aqueous TFA and solvent B of 0.1% TFA in 60% aqueous acetonitrile.

Solid-phase Peptide Synthesis.—The assembly of [Cys^{2}(Acm), Cys^{39}(Bu^{t})]-h-βCG(*1–74*) was carried out semi-automatically

by the continuous-flow solid-phase procedure using a CRB Pepsynthesizer II. Each of the steps involved in the synthetic cycle was controlled by an Apple IIe microcomputer and was driven by CRB's version 2.3 software disc. The reaction protocol was as follows: Fmoc deprotection with 20% piperidine in DMF (10 min), washing with DMF (15 min), amino acid coupling (30 min), and washing with DMF (10 min). The synthesis was programmed to pause before each coupling (for manual addition of the activated amino acid) and again after each coupling (for removal of resin beads and subsequent testing of the presence of free α-amino groups with 2,4,6-trinitrobenzenesulphonic acid). The flow rate of the synthesizer's pump was set at 3.0 ml min^{-1}. The reaction column effluent was subjected to continuous u.v. monitoring at 307 nm by passage through a Varian model 635LC u.v. spectrophotometer.

Synthesis was carried out using Pepsyn K (1.5 g, 0.165 mmol). The solid phase was first functionalized, in the usual manner,[24] with a glycine internal reference amino acid and the acid-labile hydroxymethylphenoxyacetyl linkage agent. The *C*-terminal residue of the peptide was esterified onto the linkage using the symmetrical anhydride of Fmoc-Gln(Mbh)-OH in the presence of 0.1 mol equiv. of DMAP as catalyst. After 1 h of coupling (recirculation), the resin was washed well with DMF (10 min) and the coupling step was then repeated for a further 1 h using fresh reagents. For the above and all subsequent synthesis steps, three-fold molar excesses (0.495 mmol) of acylating species were employed. Preformed symmetrical anhydrides (prepared as described in ref. 13) of protected amino acids were used throughout the remainder of the synthesis. Suitably protected arginine symmetrical anhydride was coupled in the presence of an equivalent amount of catalyst, 1-hydroxybenzotriazole. Suitably protected glutamine and lysine were coupled as their *p*-nitrophenyl ester also in the presence of 1-hydroxybenzotriazole. The Fmoc group was used for N^{α}-protection throughout and sidechain protection was afforded by the following: 4-methoxy-2,3,6-trimethylbenzenesulphonyl (Mtr) for arginine, acetamidomethyl (Acm) and t-butyl (Bu^t) for cysteine, t-butyl for serine, threonine, and aspartic acid, and t-butoxycarbonyl (Boc) for lysine. No repeat couplings were required during the assembly. Peptide-resin aliquots were also removed at regular intervals for amino acid analysis (Table 1). At the completion of coupling residue *49* (Leu), all of the peptide-resin was removed from the reaction column and transferred to a glass beaker and gently washed well with DMF, methanol, dichloromethane, methanol, and DMF. After each wash, the solution and resin fines were removed by decantation. A portion of this peptide-resin was removed, separately washed with methanol, dichloromethane, and ether and dried *in vacuo* (0.23 g). The remainder was returned to the reaction column and synthesis continued as before. At the end of the assembly, the protected peptide-resin was removed from the column, washed thoroughly with DMF, t-amyl alcohol (2-methylbutan-2-ol), acetic acid, t-amyl alcohol, methanol, and ether, and dried *in vacuo* (1.50 g) (analysis, Table 1).

Second Assembly.—Dried [$Cys^2(Bu^t)$]h-βCG(*26–74*)-resin (0.224 g) was kept in freshly distilled DMF for 24 h, after which it was packed into the glass reaction column of the synthesizer. DMF was flushed through the resin for 30 min and then the synthesis mode commenced with the Fmoc-deprotection of residue *26* (Leu). The next amino acid (arginine *25*) required a double 1 h coupling in the presence of 1-hydroxybenzotriazole before a negative 2,4,6-trinitrobenzenesulphonic acid test was achieved. Coupling of the remaining protected amino acids (three-fold molar excesses) were of 50 min duration. No further recouples were required. Aliquots of peptide-resin were removed at regular intervals for analysis (Table 1). At the end of the synthesis, the peptide-resin was washed as before (0.240 g) (analysis, Table 1).

Cleavage, Deprotection, and Purification of First Assembly Peptide.—Dry synthetic protected peptide-resin (200 mg, 10.48 μmol peptide) was treated with a mixture of TFA, phenol, and anisole (95:2.5:2.5) for 6.5 h at room temperature. The filtered resin was washed well with neat TFA and the combined filtrate and washings were evaporated under reduced pressure. The resulting light orange oily residue was partitioned between water (50 ml) and ethyl acetate (50 ml); the aqueous layer was washed further with ethyl acetate (3 × 50 ml), the organic solvent layers were back-washed twice with water and the combined aqueous solution was freezer-dried. The residual resin was washed thoroughly with t-amyl alcohol, acetic acid, t-amyl alcohol, methanol, and ether, and dried *in vacuo*. A sample was subjected to analysis, the results of which indicated an extent of cleavage of 98.7%.

The crude peptide (10.33 μmol) was dissolved in 50% aqueous acetic acid (3 ml) and desalted on a 1.8 × 68.8 cm column of Sephadex G-25 with the same aqueous acid as eluant and a flow rate of 20.0 ml h^{-1}. Fractions were collected every 3 min and the absorbance of the eluate was monitored at 254 nm. Fractions 42—62, corresponding to the single major peak, were combined, diluted with water to 10% aqueous acetic acid, and freeze-dried to give the crude desalted peptide (9.20 μmol, 89.0% recovery); for h.p.l.c. see (Figure 2; analysis, Table 2). Final purification was achieved by semi-preparative h.p.l.c. in four portions. Highly purified *S*-protected synthetic peptide was obtained (0.91 μmol, 9.50% overall). H.p.l.c., Figure 3; amino acid analysis results are shown in Table 2.

Cleavage, Deprotection, and Purification of Second Assembly Peptide.—This was carried out exactly as described above. Peptide-resin (0.238 g, 10.32 μmol) was treated with aq. TFA (10 ml). Work-up and subsequent gel filtration yielded peptide (9.21 μmol; 89.2%). Analysis of the residual resin after further thorough washing indicated an extent of cleavage of 98.9%. The resulting peptide (amino acid analysis, Table 2; h.p.l.c., Figure 4) was purified by semi-preparative h.p.l.c. as described previously to give highly purified synthetic [Cys_2(Acm),$Cys^{39}(Bu^t)$]-h-βCG(*1–74*), (0.9 μmol, 8.7% overall yield; amino acid analysis results are shown in Table 2; h.p.l.c., Figure 5).

Tryptic Digestion and Peptide Mapping.—*S*-Protected peptide (30 nmol) was dissolved in 0.1M aqueous NH_4HCO_3 (200 μl). To this was added 48 μl of trypsin solution (48 μl; 0.9 mg enzyme/9 ml 0.1M aqueous NH_4HCO_3). The mixture was incubated at 37 °C for 2 h. After cooling, the sample was acidified with 0.1% aqueous TFA and subjected to semi-preparative h.p.l.c. on a Vydac C4. Each of the major peaks (Figure 6) were collected and peptide material isolated by freeze-drying. Each peptide fragment was characterized by both amino acid analysis and high sensitivity microsequencing (Table 3).

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References

1 W. R. Jones, 'Immunological Fertility Regulation,' Blackwell Scientific, Oxford, 1982.

2 G. L. Ada, A. Basten, and W. R. Jones, *Nature*, 1985, **317**, 288.
3 K. Talmadge, N. C. Vamvakopoulos, and J. C. Fiddes, *Nature*, 1984, **307**, 37.
4 V. C. Stevens, J. G. Powell, A. C. Lee, and D. Griffin, *Fertil. Steril.*, 1981, **36**, 98.
5 P. D. Griffin, in 'Immunological Approaches to Contraception and Promotion of Fertility,' ed. G. P. Talwar, Plenum Press, New York, 1986, p. 43.
6 G. P. Talwar, in 'Immunological Aspects of Reproduction and Fertility Control,' ed. J. P. Hearn, MTP Press, Lancaster, 1980, p. 217.
7 W. R. Jones, S. J. Judd, R. M. Y. Ing, J. Powell, J. Bradley, E. H. Denholm, U. W. Mueller, P. D. Griffin, and V. C. Stevens, *Lancet*, 1988, 1295.
8 R. C. Sheppard, *Chem. Bri.*, 1983, **19**, 402.
9 C. R. Wu, J. D. Wade, and G. W. Tregear, *Int. J. Pept. Protein Res.*, 1988, **31**, 47.
10 E. Atherton, E. Brown, R. C. Sheppard, and A. Rosevear, *J. Chem. Soc., Chem. Commun.*, 1981, 1151.
11 W. S. Hancock and J. E. Battersby, *Anal. Biochem.*, 1976, **21**, 261.
12 W. Konig and R. Geiger, *Chem. Ber.*, 1970, **103**, 2041.
13 E. Atherton, R. C. Sheppard, and P. Ward, *J. Chem. Soc., Perkin Trans. 1*, 1985, 2065.
14 R. Arshady, E. Atherton, D. L. J. Clive, and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1981, 529.
15 J. D. Wade, S. P. Fitzerald, M. R. McDonald, J. G. McDougall, and G. W. Tregear, *Biopolymers*, 1986, **25**, 521.
16 E. Brown, R. C. Sheppard, and B. J. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1983, 1161.
17 J. J. Pastuszak and A. Chimiak, *J. Org. Chem.*, 1981, **46**, 1868.
18 E. Atherton, M. Pinori, and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1985, 2057.
19 B. Kamber, A. Hartmann, K. Eisler, B. Riniker, H. Rink, P. Sieber, and W. Rittel, *Helv. Chim. Acta*, 1980, **63**, 899.
20 E. Atherton, A. Dryland, R. C. Sheppard, and J. D. Wade, in 'Peptides: Structure and Function,' eds. V. J. Hruby and D. H. Rich, Pierce Chemical Company, Illinois, 1983, p. 45.
21 A. Dryland and R. C. Sheppard, *Tetrahedron*, 1988, **44**, 859.
22 R. C. Sheppard, *Sci. Tools*, 1986, **33**, 9.
23 G. W. Tregear, J. van Rietschoten, R. Sauer, H. D. Niall, H. T. Keutman, and J. T. Potts Jr., *Biochemistry*, 1977, **16**, 2817.
24 E. Atherton, L. Cameron, and R. C. Sheppard, *Tetrahedron*, 1988, **44**, 843.

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