Synthesis of 31- and 35-Amino Acid Carboxyl Terminal Fragments of the β Subunit of the Human Chorionic Gonadotropin

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The 31- and 35-amino acid carboxyl terminal fragments (hCG 117–147 and hCG 111–145) based on two sequences for the β subunit of human chorionic gonadotropin have been synthesized using the solid-phase technique. N^{α}-Boc, protected L amino acids were automatically coupled on the chloromethylated Merrifield resin by the DCC method, with the exception of Boc-Gln, which was incorporated by the ONP active ester method. The completed peptides were cleaved from the resins, deprotected with anhydrous liquid HF, and purified by a series of gel filtration, partition and ion-exchange chromatography columns, and preparative thin layer electrophoresis. The purity of the two hCG fragments was shown by thin layer chromatography, electrophoresis, and amino acid analyses.

Human chorionic gonadotropin (hCG) is a glycoprotein hormone of the placenta. It is composed of 2 subunits, α and β.[1] The β subunit is hormone specific, while the α subunit is common to other pituitary glycoprotein hormones such as luteinizing hormone, thyroid stimulating hormone, and follicle stimulating hormone (LH, TSH, and FSH).[2,3] The β subunit of hCG also has approximately 30 additional amino acids at its carboxyl terminus which are not present in the β subunits of other glycoprotein hormones. Consequently, this additional carboxyl terminal fragment is of interest from a biological standpoint as to its function in the activity and potency of hCG.

Two slightly differing structures for the β subunit of hCG have been proposed, one by Carlsen, Bahl, and Swaminathan[4] and the other by Morgan, Birken, and Canfield.[6] The structure of Morgan et al. contains 145 amino acid residues. The structure of Carlsen et al. has 147 residues, namely an additional Ser-Leu-Pro sequence at the carboxyl terminus and no Ser^{121} (Scheme I).

It is of interest to synthesize specifically selected fragments of the β subunit of hCG. Such synthetic peptides will then be coupled to one or more suitable protein carriers which will then be used to achieve antibodies in vivo. It is desirable to achieve antibodies against hCG, but not against LH, FSH, and TSH. Since there is no established basis to indicate which sequences of the β subunit of hCG of the additional carboxyl terminal fragment which might provide immunological differentiation, a priority of peptide sequences based on this β subunit are being synthesized to support the biological research to achieve such specific antibodies against hCG. We now describe the synthesis of a 31- and a 35-amino acid peptide fragment of this subunit. A total of five samples of the two synthetic peptides have been provided to Dr. Sheldon Segal and Dr. Harold A. Nash of the Population Council, the Rockefeller University, New York. Antibody titers produced in rabbits by the 31-unit peptide were lower than those produced by the β subunit of hCG. Both were injected as "tetramers" with Freund's Complete Adjuvant. The antibodies were assayed by measuring the binding of I^{125}hCG. Less than 1% binding of the labeled hCG was observed with a 1:250 dilution of antisera from rabbits injected with the "tetramer" of the 31-unit peptide. Approximately 50% binding with a 1:250000 dilution of antisera from rabbits injected with the tetramer of the β subunit was observed.[6]

Syntheses

The peptides were synthesized by the solid phase technique[7,8] using the chloromethylated Merrifield resin. The starting resins were prepared by esterification with the appropriate Boc-amino acid, namely Boc-Pro for hCG (117–147)C and Boc-Gln for hCG (111–145)M, and the resin in refluxing ethanol and triethylamine. Amino acid analyses gave a content of 0.68 mM Pro/g for the Boc-Pro resin and 0.39 mM Gln/g for the Boc-Gln resin.

Scheme I

(hCG 117–147)C (Carlsen et al.[4]): hentriacosapeptide Asp^{117}-Ser-Ser-Ser-Lys-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Pro-Asp-Thr-Pro-Ile-Leu-Pro-Gln-Ser-Leu-Pro-^{145}-OH

(hCG 111–145)M (Morgan et al.[6]): pentatriacosapeptide Asp^{111}-Asp-Pro-Arg-Phe-Gln-Asp-Ser-Ser-Ser-Ser-Lys-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Ser-Asp-Thr-Pro-Ile-Leu-Pro-Gln^{145}-OH

Since the Boc-Pro resin had a high amino acid content, Boc-Leu was coupled to the Pro resin in the limited ratio of 0.80 Lue to 1.0 Pro, in order to limit the resin content to allow sufficient space for the growing peptide chain. After acetylation of unreacted amino groups, the Leu content of the Boc-Leu-Pro resin was 0.32 mM/g. The couplings for the remainder of the syntheses of both peptides were done on a Beckman 990 automatic synthesizer.

α-Amino functions were protected by the *tert*-butyloxycarbonyl (Boc) group, except for arginine which was protected by the amyloxycarbonyl (Aoc) group which is more soluble in methylene chloride. Side chain protecting groups were benzyl (Bzl) for serine, threonine, and aspartic acid; tosyl (Tos) for aginine; and 2-chlorocarbobenzoxy (2-Cl-Z) for lysine. Deprotection of the amino-protected intermediates on the resin was accomplished with 30% trifluoroacetic acid (TFA) in methylene chloride, with neutralization on the resulting TFA salt by 10% triethylamine in methylene chloride to give the free amino group. Each amino acid was coupled by the dicyclohexylcarbodiimide (DCC) method[9] in methylene chloride or dimethylformamide, with the exception of Boc-Gln which was incorporated by the nitrophenyl active ester method[10] in dimethylformamide. Completeness of coupling was monitored by the ninhydrin color test procedure of Kaiser et al.[11] and when doubtful, the resin was double-coupled or acetylated with acetic anydride.

Double coupling was employed throughout the synthesis of hCG (117–147)C, the first coupling for 3–5 h, and the second coupling, with fresh Boc-amino acid, for 4–6 h, while the Boc-Gln^{144}-ONP active ester was incorporated once as a 5-fold excess for about 12 h. Ser^{145} and Pro^{143} through Pro^{140} were incorporated as 2.5-fold excesses for the first coupling and 1.5-fold excesses for the double coupling; the next ten amino acids, Thr^{139} through Pro^{128} as 4.0 and 2.5-fold excesses; and the last eleven amino acids, Leu^{127} through Asp^{117} as 5.0- and 3.0-fold excesses. The color tests were doubtful after coupling Ile^{141}, Pro^{140}, and Arg^{132}, and the resin was acetylated. From 3.0 g of Boc-Pro-CH_2-resin, there was obtained 5.145 g of the protected hentriacosa peptide resin.

Table I. Amino Acid Analyses[a] of hCG (117–147)C

Theory	Found: Sample 4B	Found: Sample 5B
2 Asp	2.13	1.89
1 Thr	0.73	0.79
7 Ser	7.22	7.65
1 Glu	0.85	0.95
11 Pro	11.56	11.78
1 Gly	0.91	0.80
1 Ala	0.95	0.86
1 Ile	0.67	0.52
4 Leu	4.06	3.82
1 Lys	0.95	1.06
1 Arg	1.02	0.91

[a] Amino acid analyses were done on a Beckman Model 119 amino acid analyzer set up for a single column methodology after hydrolysis of the peptide with 6 N HCl at 110 or 130 °C overnight.

Double couplings were not used in the synthesis of hCG (111–145)M. Instead the molar excess of each Boc-amino acid was increased with an average coupling time of 3–6 h. Pro^{144} through Gly^{136} were incorporated as 3.0-fold excesses; Pro^{135} and Leu^{134} as 3.22-fold excesses; Arg^{133} through Pro^{126} as 4.0-fold excesses; Pro^{125} through ASP^{117} as 5.0-fold excesses; Gln^{116} as a 12-fold excess for 12 h; and Phe^{115} through Asp^{111} as 6.0-fold excesses. Using these higher molar excesses, all the couplings appeared complete by the color tests, making double couplings unnecessary. However, Ile^{142} was double-coupled to Leu^{143} since this is known to be a difficult coupling; 3.18 g of Boc-Gln-CH_2-resin yielded 7.05 g of the protected pentatriacosa peptide resin.

Cleavage of the completed protected peptides from the resins, with simultaneous removal of the protecting groups and formation of the carboxyl terminal acid, was effected with anhydrous, liquid hydrogen fluoride in the presence of 10% anisole for 1 h at 0 °C.[12,13] The crude, deprotected peptides were extracted from the resin with diluted acetic acid, lyophilized, and purified as follows.

Experimental Section

Purification. hCG (117–147)C. The crude peptide (737 mg from 3.0 g of the hentriacosa resin) after HF was first desalted by gelfiltration on a 112 × 2.5 cm column of Bio-Gel P-4 eluted with 1 N HOAc in 15-mL fraction. Detection of the peptide by the Folin–Lowry procedure[14] at 660 nm showed a broad multishouldered peak. Lyophilization of the fractions corresponding to the top of the peptide peak (tubes 17–22) gave 465 mg of desalted peptide. This material showed two spots on TLC.

The desalted peptide was then subjected to partition chromatography on a 59 × 2.0 cm column of Sephadex G-25, eluted first with upper-phase and then lower-phase of the system *n*-BuOH–HOAc–H_2O (4:1:5) in 6-mL fractions. Detection of the peptide by the Folin–Lowry procedure showed one peak in the first portion of the upper-phase eluent at tubes 11–12 (2A), a smaller peak near the end of the upper-phase elution at tubes 53–59 (2D), and another peak at the beginning of the lower-phase elution at tube 60 (2E).

The upper-phase peak fractions (2A) upon lyophilization gave 80.6 mg of white solid, which was further purified by ion-exchange chromatography on a 28 × 1.5 cm column of carboxymethyl cellulose (CM-52) eluted in 6-mL fractions with 1 to 100 mM ammonium acetate buffer gradient at pH 3.5 giving one peak which yielded 52.3 mg (3B) of white solide. Upon amino acid analyses, this material proved to be a mixture of by-product peptides which were missing about seven or eight amino acids (namely 4.8 serines, 1 alanine, 1 lysine and 1 argine) and showed two high R_f spots on TLC.

The desired hentriacose peptide hCG (117–147)C was found in the trailing end of the upper-phase eluent (2D, 118.4 mg) and the first part of the lower-phase eluent (2E, 29.2 mg) of the 4:1:5 system partition column. Both fractions 2D and 2E had the same TLC pattern in *n*-BuOH–HOAc–EtOAc–H_2O (1:1:1:1) and *i*-PrOH–1 N HOAc (2:1) systems consisting of major low R_f spots due to by-product peptide contaminants.

Table II. Amino Acid Analyses[a] of hCG (111–145)M

Theory	Found: Sample 6B	Found: Sample 9A	Found: Sample 10A	Found: Sample 10B	Found: Sample 10C
4 Asp	3.59	4.32	3.77	3.77	4.32
1 Thr	0.87	0.53	0.59	0.65	0.45
8 Ser	8.84	9.79	6.44	7.48	8.16
2 Glu	1.86	1.52	1.77	1.84	1.64
10 Pro	10.58	10.32	11.95	11.68	11.9
1 Gly	1.16	0.88	1.59	1.15	0.86
1 Ala	0.80	0.92	1.10	1.06	0.88
1 Ile	0.69	0.31	0.49	0.45	0.45
3 Leu	2.73	2.20	2.28	2.23	1.89
1 Phe	1.10	1.00	1.79	1.52	0.97
1 Lys	0.81	1.18	1.59	1.03	0.85
2 Arg	1.92	2.00	1.92	2.02	1.64

[a] Amino acid analyses were done on a Beckman Model 119 amino acid analyzer set up for a single column methodology after hydrolysis of the peptide with 6 N HCl at 110 or 130 °C overnight.

Table III. Thin Layer Chromatography R_f Values[a] of β Subunit hCG (117–147)C

	R_f^1	R_f^2	R_f^3
hCG (117–147)C (5B)	0.15	0.17	0.98

[a] TLC systems on silica gel are: R_f^1, *n*-BuOH–HOAc–EtOAc–H_2O (1:1:1:1); R_f^2, *i*-PrOH–1 N HOAc (2:1); R_f^3, $CHCl_3$–MeOH–conc NH_4OH (60:45:20).

Fraction 2E from the lower-phase eluent of the partition column was then subjected to ion-exchange chromatography on a 28 × 1.5 CM-52 column eluted in 5-mL fractions with 1 to 100 mM ammonium acetate buffer gradient at pH 5.0. Detection of the peptide by the Folin–Lowry procedure showed only one peak at tubes 6 and 7, which gave 3.5 mg (4B) of white solid upon lyophilization. This material showed only one spot, R_f = 0.15, on TLC in the *n*-BuOH–HOAc–EtOAc–H_2O (1:1:1:1) system and was not contaminated by the higher R_f by-product peptides which eluted later, mixed with some more of the hCG (117–147)C.

Since the desired hentriacose peptide was also found in the trailing end of the upper-phase eluent of the partition column (fraction 2D), it seemed appropriate to purify further this material by some technique employing an organic solvent rather than by ion exchange. Thus, fraction 2D was subjected to chromatography on a 39 × 1.0 cm column of Sephadex LH-20 eluted with *n*-BuOH–H_2O (6:100) in 4-mL fractions. Detection of the peptide by Folin–Lowry showed one sharp peak of tube 6 which upon lyophilization gave 57.0 mg (5B) of white material identical by TLC to fraction 4B. Material which eluted in the shoulder and beyond the peptide peak (5C, 36.3 mg) contained primarily the desired hCG (117–147)C contaminated by some of the higher R_f by-product peptides.

The data on amino acid analyses of fractions 4B and 5B, Table I, are consistent with the 31 amino acids of the hCG (117–147)C fragments. The low values for Thr are to be expected due to some oxidation during hydrolysis. The low values for Ile are due to incomplete hydrolysis of the Ile^{141}–Leu^{142} bond. Both samples 4B and 5B were identical by thin layer chromatography, single spot R_f's in 3 systems, Table III, and each showed the same one spot moving toward the cathode on electrophoresis.

Consequently, both ion-exchange chromatography on CM-52 and chromatography on LH-20 were successful in separating the desired hentriacosa peptide hCG (117–147)C from the failure sequence peptides.

hCG (111–145)M. The crude peptide from HF (1.56 g from 4.0 g of the protected peptide resin) was first desalted on a 118.5 × 2.5 cm column of Bio-Gel P-4, eluted with 1 N HOAc in 18-mL fractions. Detection of the peptide by Folin–Lowry showed one broad, multishouldered peak. Lyophilization of the fractions corresponding to the top of the peak (tubes 2–21) gave 367.8 mg (6B) of white solid which showed two spots on TLC in the *n*-BuOH–HOAc–EtOAc–H_2O

(1:1:1:1) system, a major low R_f spot for the desired pentatriacose hCG (111–145)M peptide and a minor mid R_f streak for impurities. Amino acid analyses of the desalted peptide (6B), listed in Table III, were almost acceptable for the pentatriacosa peptide.

The material was then chromatographed on a 58 × 2.0 cm column of Sephadex LH-20, eluted with *n*-BuOH-H_2O (6:100) in 7-mL fractions with detection of the peptide by a UV monitor at 256 nm (Phe), to give a single symmetrical peak. Lyophilization of tubes 14–16, corresponding to the top of the peptide peak, gave 225.5 mg (7B) of white solid which on TLC showed primarily the low R_f spot for the pentatriacosa peptide contaminated by only a small amount of the higher R_f impurity.

Since the hentriacosa peptide hCG (117–147)C eluted in the lower phase of the 4:1:5 system partition column, it was decided to use a different partition system for the pentatriacosa peptide hCG (111–145)M. Fraction 7B was partitioned on a 57 × 2.0 cm column of Sephadex G-25 eluted with upper and then lower phase of the system 0.1 N HOAc–*n*–BuOH–Pyr (11:5:3), in 5.5-mL fractions with detection of the peptide by Folin–Lowry. No peak appeared in the upper-phase eluent, but there was a sharp, symmetrical peak at the beginning of the lower-phase elution. Lyophilization of tubes 43 and 44, corresponding to the top of this peak, gave 74.7 mg (8B) of white solid which now showed only the one low R_f spot on TLC for the desired pentatriacosa peptide.

A further purification of 50 mg of fraction 8B was carried out by ion-exchange chromatography on a 28 × 1.5 cm -52 column eluted in 25-mL fractions with 1 to 500 mM ammonium acetate buffer gradient, pH 6.4. A single, symmetrical peak was detected by the UV monitor at 256 nm and by Folin–Lowry at 660 nm. Lyophilization of tube 2, corresponding to the top of this peak, gave 27.6 mg (9A) of purified hCG (111–145)M, which gave the amino acid analysis of ratios listed in Table II.

9A (5.76 mg) was further purified by preparative thin layer electrophoresis on cellulose plates (160 μ thick, Eastman chromagram sheet) at 500 V in pyridine acetate buffer of pH 6.5 to give 2.0 mg of pentatricosa hCG (111–145)M (10A; IBR 12755). The amino acid analytical ratios are in Table II. Two separate purifications in the same manner of 8.97 mg of (9A) and 12.87 mg of (9A) gave 3.6 mg (10B; IBR 13202) and 5.5 mg (10C, IBR 13669) of pentatriacosa hCG (111–145)M. The amino acid analytical results are in Table II.

The purified hCG (111–145)M showed one spot by electrophoresis moving toward the cathode in pyridine acetate buffers of pH 3.6 and pH 6.5.

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Registry No.—Boc-Pro, 15761-39-4; Boc-Gln, 4530-20-5; (hCG 117–147)C, 63215-95-2; (hCG 111–145)M, 63301-41-7.

References and Notes

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Metabolites of the Red Alga *Laurencia subopposita*

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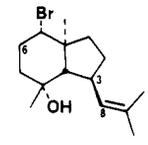
The red alga *Laurencia subopposita* contains a variety of secondary metabolites, some of which have previously been described from other sources. We have previously reported that the major metabolite was oppositol (1). The antibiotic activity of the alga was due to 7-hydroxylaurene (2) and 10-bromo-7-hydroxylaurene (3). Laurene (4), isoprelaurefucin (6), laurefucin (7), acetyllaurefucin (8), and oplopanone (19) have all been described previously. Two epimeric diols 13 and 14 were shown to be related to oppositol (1). A diol 21 having the germacrane skeleton rearranged on dehydration to give 8(15)-dehydrooplopanone (26). Two aromadendrene alcohols 27 and 29 were isolated. The optical enantiomer of 1-hydroxylalloaromadendrene (27) was synthesized by oxidation of alloaromadendrene (28) with selenium dioxide.

Red algae of the genus *Laurencia* have proved to be a most prolific source of halogenated metabolites of three major classes, sesquiterpenes, diterpenes, and acetylenes.[1] The halogenated sesquiterpenes may be further subdivided into three groups: the aromatic compounds, the chamigrenes and their precursors and rearrangement products, and the oppositol–eudesmane group. Although it is not uncommon to find representatives of more than one group in a *Laurenica* species, the structural elucidations have generally been described separately. In this paper we wish to present an account of the diversity of chemical structures which may be found in *Laurencia subopposita*.

We have previously reported that the major metabolite of *Laurencia subopposita* (J. G. Agardh) Setchell was oppositol (1), a sesquiterpene having a previously undescribed carbon skeleton.[2] On reinvestigation of the metabolites of *L. subopposita*, we found that oppositol (1) was not an antibiotic, as had been previously stated. The mild antibiotic activity of oppositol (1) had been due to the presence of traces of 7-hydroxylaurene (2) and/or 10-bromo-7-hydroxylaurene (3).

Hexane, ether, and acetone extracts of powdered, air-dried



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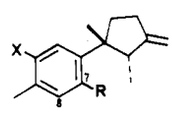
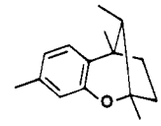


2 R = OH X = H
3 R = OH X = Br
4 R = H X = H



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