Solid-Phase Synthesis of Peptide Amides on a Polystyrene Support Using Fluorenylmethoxycarbonyl Protecting Groups

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Received September 23, 1986

We have investigated new routes for the synthesis of acid-sensitive amino acids using *N*-fluorenylmethoxycarbonyl amino acids and mild acid labile side chain protection in order to avoid HF cleavage as final step. The 4-(benzyloxy)benzylamine resin, applied successfully by Pietta and Brenna, proved to be unsuitable for the synthesis of peptide amides since we have found that trifluoroacetic acid treatment of peptide–resins yielded the peptide still bearing a *p*-hydroxybenzyl group on the carboxamide nitrogen. We have successfully used the 2,4-dimethoxybenzhydrylamine support for the synthesis of peptide amides. Cholecystokinin octapeptide and GnRH were synthesized on the 2,4-dimethoxybenzhydrylamine resin and cleaved from the polymer with trifluoroacetic acid–thioanisole (4:1), and 95% trifluoroacetic acid containing phenol (2.5%) and dimethyl sulfide (2.5%), respectively. The final cleavage conditions had to be optimized in order to obtain good yields, suggesting that side chain protections of certain amino acids, possibly arginine and tryptophan, may have to be further refined.

Introduction

A strategy for the solid-phase synthesis of peptide amides containing acid-sensitive amino acids (e.g., tyrosine *O*-sulfate and γ-carboxyglutamic acid) has not yet been perfected. Benzhydrylamine-type polymers[1-3] and protection of the α-amino function using the *tert*-butyloxycarbonyl group have been successfully used for the synthesis of numerous peptide amides. However, the very strongly acidic conditions (HF, CF_3SO_3H, and others) necessary for deprotecting and cleaving the peptide from the polystyrene-type resin have made impossible the one-step solid-phase synthesis of important, acid-labile natural polypeptide amides such as gastrin-II, caerulein, cholecystokinin-8 (CCK-8), cholecystokinin-33, and γ-carboxyglutamic acid containing peptide amides, etc.

Such peptide amides could hypothetically be synthesized on a polar poly(dimethylacrylamide) support[4,5] with protection of the α-amino function by using the base labile fluorenylmethoxycarbonyl (Fmoc) group. However, the ammonolytic cleavage of the protected peptides from the resin is not without limitations as transesterification can occur as a side reaction at the C-terminus[6,7] or at the ω-carboxyl groups. This is not to mention the possible limited racemization of the C-terminus amino acid, or such reactions of β-*tert*-butylaspartyl peptides as those mediated by amminolysis and reported by Schon and Rill.[8] Furthermore, the disadvantageous swelling properties of poly(dimethylacrylamide) matrices and the rigidity of the polyamide–kieselguhr supports[9] are not attractive for practical purposes (generally low substitution of the polymeric matrix). Because of our extensive experience with the chemically and mechanically favorable polystyrene-type supports, we wanted to use it for the synthesis of polypeptide amides in combination with Fmoc–amino acids bearing mild acid labile side chain protection such as the *tert*-butyl ester, ether, or urethane. Pietta and Brenna[10] used the 4-(benzyloxy)benzylamine support in combination with the very acid labile 2-phenylisopropyloxycarboxyl (Ppoc) group for the synthesis of some simple amino acid and peptide amides on a polystyrene-type resin. According to these authors, a mild acidic treatment (50% trifluoracetic acid in methylene chloride) selectively cleaved the N–C bond between the C-terminal carboxamide group and the polymer support, leaving the amide function intact. The same polymer was used by Colombo for the synthesis of chicken vasoactive intestinal peptide using Fmoc–amino acids.[11]

Pietta's results, however, with the 4-methoxybenzyl (Pmb) as a rather stable amido-protecting group (see below) does not support his claim of the acid lability of the pseudo-4-methoxybenzyl group when attached to a polymer (see above). In his first experiments[1] he found that the 4-methoxybenzyl group was stable in CF_3COOH, 1 N HCl in acetic acid and 4 N HCl in dioxane, and only anhydrous HF cleaved this protecting group. He has even synthesized tetragastrin (H-Trp-Met-Asp-Phe-NH_2) using Boc–amino acids and Pmb as the C-terminal amide protecting group. (The Pmb group proved to be stable during the standard Boc-cleavage methods and was cleaved with HF/anisole as the final step.) In another report[12] he published that "prolonged reaction times (12 h) were found necessary to complete removal" of the 4-methoxybenzyl residue from the amide group of glutamine using liquid HF. These experiments are in agreement with the results of Weygand et al.[13] who found that the 4-methoxybenzyl residue is ill-suited for amide protection because of the strong acid conditions required for its cleavage. Pietta et al. found even the 2,4-dimethoxybenzyl (2,4-DMB) group quite stable under acidic cleavage conditions.[13,14]

In general, sensitivity toward cleaving agents of protecting groups whether free or polymer-bound has been

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0022-3263/87/1952-1197$01.50/0

shown to be mostly the same. We have synthesized a series of polymers on the basis of this principle; these included amide-protecting polymers such as benzhydrylamine and 4-methylbenzhydrylamine resins.[15] Therefore, it was incongruous that the Pmb group would be stable in TFA or even in HF[1,12,13] and when attached to a polystyrene polymer, would cleave in 50% TFA in CH_2Cl_2 to yield the amide function.[10,11] To clarify this issue, we synthesized and characterized the 4-(benzyloxy)benzylamine (BBA) as well as the 2,4-dimethoxybenzhydrylamine (2,4-DMBHA)[1] supports and tried to apply them to the synthesis of peptide amides using Fmoc-amino acids.

Results and Discussion

In a first experiment, we repeated the experimental protocol of Pietta and Brenna[10] and prepared the 4-(benzyloxy)benzylamine support ("Pietta-BBA-resin", 1) by reacting Merrifield's chloromethylated resin with 4-cyanophenol. The resin was then reduced with $LiAlH_4$ in the presence of NH_3. Fmoc-phenylalanine was coupled to this polymer and cholecystokinin-octapeptide [H-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-OH, that is CCK-8 when amidated at the C-terminus] was built up on the support by using *N*-Fmoc amino acids bearing *tert*-butyl side chain protection where needed. The octapeptide was cleaved from the resin with 50% TFA in CH_2Cl_2 (1 h) and compared to an authentic sample of CCK-8 synthesized by the liquid-phase method.[16] Although the amino acid composition of the CCK-8 cleaved from the Pietta-resin gave the expected amino acid ratios, this peptide showed very different chromatographic behavior when compared to an authentic of CCK-8 sample both on HPLC column and on TLC plates. We therefore attempted to synthesize H-Phe-NH_2 on the Pietta-BBA-resin and after cleavage with 50% TFA in CH_2Cl_2 obtained several ninhydrin positive materials, none of which had the chromatographic properties of the target compound. We presumed that the $LiAlH_4$ reduction step applied for conversion of the cyano function to the amino group generated a series of side products which disturbed the synthetic and cleavage steps.

In the next experiment we prepared the 4-(benzyloxy)benzylamine support through a new and unequivocal synthetic route. *N*-Fmoc-4-hydroxybenzylamine was synthesized, characterized and coupled to chloromethylated Merrifield resin in the presence of KF.[17] Cleavage of the *N*-Fmoc group (20% piperidine in DMF, 10 min) resulted in the 4-(benzyloxy)benzylamine support with a better defined chemical structure. Nevertheless, we failed to synthesize CCK-8 or even H-Phe-NH_2 on this new polymer; indeed, cleavage of the C-terminal phenylalanine amino acid or peptide with 50% TFA in CH_2Cl_2 resulted again in materials of expected amino acid composition, with chromatographic properties similar to those obtained in our first experiment but which did not match those of authentic samples. We hypothesized that the 4-methoxybenzyl residue (Pmb) as the amide-protecting group was too stable in 50% TFA/CH_2Cl_2 as expected from the literature data[1,12,13,18] and that both phenylalanine and CCK-8 still contained this group at their C-terminus. Mass spectrometric analysis of the peptides that were synthesized on the 4-(benzyloxy)benzylamine resin confirmed this hypothesis. The ether linkage between the Merrifield polymer and the 4-hydroxybenzylamine (Phb-amine) spacer seems to be unexpectedly labile toward acids and was at least partially cleaved with 50% TFA/CH_2Cl_2, to yield CCK-8-Phb and nonsulfated CCK-8 Phb (see Experimental Section).

Table I. Percent Cleavage of Amino Acid Amides from the 2,4-DMBHA Resin

cleavage agent	time	H-Gly-NH_2	H-Phe-NH_2
TFA–CH_2Cl_2 (1:1)	10 min	90	6
	30 min	98	10
	1 h	100	14
	2 h	100	18
	3 h	100	20
TFA–thioanisole (4:1)	10 min	97	60
	30 min	100	72
	1 h	100	80
	2 h	100	92
	3 h	100	100

The fact that Pietta and Brenna have synthesized only very simple amino acid and peptide amides[10] on this support and never used chromatographic data and reference standard substances for the identification of their materials, and their relying on a correct amino acid composition of the products for structural proof led them to unfortunate conclusions. Later, Colombo synthesized chicken VIP[11] on the Pietta-BBA-resin and proved the structure of the final product again by amino acid analysis, elemental analysis, and optical rotation, methods by which one cannot detect the presence of a C-terminal amide protecting group. Neither can we accept the only biological experiment made with this synthetic chicken VIP as a proof of structure. Besides Pietta and Brenna's[10] and Colombo's[11] works, we have not found any successful application of the 4-(benzyloxy)benzylamine resin in the literature.

The 4-(benzyloxy)benzylamine polymer is therefore unsuitable for the synthesis of peptide amides using the Fmoc strategy. From the known acid lability of some amide protecting groups previously described, we have designed a new polymer which should allow the synthesis of peptide amides using N^{α}-Fmoc amino acids during the synthesis and TFA in the final cleavage step. Indeed, one could conclude that the presence of an increased number of methoxy groups on the aromatic rings of diphenylmethane would concomitantly result in the graded weakening of the peptide amide to the methine group of the diphenylmethane. For example, the bond between the peptide amide and the 4-methoxybenzhydryl resin proved to be too labile for conventional synthesis using the Boc strategy and TFA deblocking.[19] Even more drastic is the observation by Juhasz and Bajusz,[20] that the 2,2′,4,4′-tetramethoxybenzhydryl residue could be cleaved from peptide amides in 5–15 min with 80% TFA in $CHCl_3$ or H_2O.

Whereas Pietta and Marshall[1] had reported on the 2,4-dimethoxybenzhydrylamine resin (2,4-DMBHA), they never used nor characterized it. We expected that the presence of the two methoxy groups in 2- and 4-positions should increase the acid lability of the polymer–peptide amide link. According to a previously published series of reactions,[15] the preparation of 2,4-DMBHA was straightforward, giving 0.33 mmol of NH_2 group per gram of resin. Coupling of Fmoc-glycine or Fmoc-phenylalanine with DCC in CH_2Cl_2 was complete within an hour. After Fmoc cleavage, amino acid amides were cleaved from the resin

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with TFA. Glycine amide cleaves with 50% TFA/CH_2Cl_2 in 10 min from the 2,4-DMBHA support, the phenylalanine–amide cleavage was only partially successful under these conditions. Therefore, we used a TFA–thioanisole (8:2) system[21] for the efficient removal of the 2,4-dimethoxybenzhydryl-protecting group from the Phe-amide by a "push–pull" mechanism: Phe-amide was cleaved in a short time (Table I). The use of 7% 4-(methylthio)phenol in TFA[22] led to the same result. However, further experiments showed that Tyr(*O*-sulfate) was unstable under these conditions. In order to prove the suitability of this resin for the synthesis of peptide amides, CCK-octapeptide was built up on the 2,4-DMBHA resin, using N^{α}-Fmoc amino acids and *tert*-butyl side chain protection where needed. Fmoc-Tyr(*O*-sulfate) was coupled as the pentafluorophenyl ester, other couplings were performed with DCC. After the last synthesis step, the protecting groups (including the bond to polymer support) were cleaved from the resin with a TFA–thioanisole (8:2, (v/v) mixture for 15 min. The crude peptide containing mostly CCK-8 and nonsulfated CCK-8 was purified on a Vydac C_{18} preparative HPLC column, yielding pure CCK-8, which coeluted with the reference standard in different HPLC systems. Amino acid analysis and optical rotation gave the expected values.

As another example, GnRH decapeptide was also synthesized on the polymer with the Fmoc strategy using *tert*-butyl-based side chain protecting groups for serine and tyrosine. The indole ring was unprotected, whereas N^{α},N^{im}-bis(Fmoc)histidine was used. In an experiment we had some difficulties coupling N^{α}-Fmoc-N^{g}-Boc-arginine on solid phase as mentioned by Atherton et al.;[23] therefore we used N^{α}-Fmoc-N^{g}-Mtr-arginine in the synthesis. GnRH was cleaved from the support and deprotected after a 45-min treatment of the peptide–resin with 95% TFA containing phenol and dimethyl sulfide.[23] The crude GnRH was purified by using HPLC. The pure GnRH coeluted with the peptide standard in two different HPLC systems that would discriminate between the C-terminal free acid and amide and had the expected amino acid composition.

Conclusion

While investigating new routes for the synthesis of peptide amides using the Fmoc strategy, we have found that the 4-(benzyloxy)benzylamine resin[10] was not suitable for the synthesis of peptide amides whereas the 2,4-dimethoxybenzhydrylamine support appears promising. Gly-amide could be cleaved from the polymer with a short treatment of 50% TFA in CH_2Cl_2, while Phe-amide could only be obtained in high yields by using more rigorous acid treatment (TFA-thioanisole). Cholecystokinin-octapeptide and GnRH were synthesized on the 2,4-DMBHA resin and cleaved from the polymer with TFA-thioanisole and 95% TFA, respectively, by using acid-labile side chain protection. N^{α}-Fmoc amino acids and the 2,4-DMBHA resin are suitable for the synthesis of peptide amides, avoiding the HF cleavage as final step. Certain limitations are to be noted: (a) all amino acids do not cleave at the same rate from the polymer, hence the need for different cleavage conditions, taking advantage of different reaction mechanisms, and (b) in view of the relatively slow cleavage of the N^{g}-Mtr group of arginine with TFA, side chain protection for arginine may have to be further refined.

Experimental Section

Protected amino acids were bought from Bachem (Torrance, CA). All solvents were reagent or HPLC grade. DMF and piperidine were redistilled.

Peptide retention times (t_r) were measured by RP-HPLC under isocratic conditions (Vydac C_{18} column, 25 × 0.46 cm, 5 μm; pore size 300A; flow rate, 2 mL/min; UV detection at 210 nm). Preparative HPLC purification was carried out on a Waters Prep LC500 liquid chromatograph using custom-made cartridges. (5.7 × 30 cm, Vydac C_{18} 15–20 μm; pore size 300A). Optical rotations were measured on a Perkin-Elmer 141 spectropolarimeter. Peptide hydrolyses were performed in 4 M methanesulfonic acid containing 0.2% tryptamine (24 h 110 °C, in a sealed ampule under high vacuum). Analyses were carried out on a Perkin-Elmer automatic amino acid analyzer using *o*-phthalaldehyde (OPA) post column derivatization. TLC was performed on precoated silica gel plates (60F-254, 0.2 mm thick, Merck) with the following solvent systems: (a) 1-butanol–acetic acid–water (upper phase), 4:1:1; (b) $CHCl_3$–acetone, 7:3.

A. 4-(Benzyloxybenzyl)amine Resin. *N*-(Fluorenylmethoxycarbonyl)-4-hydroxybenzylamine. To a stirred solution of 4-hydroxybenzylamine (3.1 g, 15 mmol) in H_2O–THF (1:1, v/v) (50 mL) containing $NaHCO_3$ (1.68 g, 20 mmol) was added Fmoc-OSu (5 g, 15 mmol) over 1 h; the reaction was completed overnight. After dilution with water (150 mL) and acidification with $NaHSO_4$, the product was extracted with ethyl acetate (3 × 50 mL), and the organic phase was washed with H_2O, dried over $MgSO_4$, and evaporated. The resulting white solid was triturated with petroleum ether and filtered off, yielding 4.15 g (12 mmol, 80%) of pure product, mp 128–130 °C, R_f(b) 0.75.

To a stirred suspension of 10 g of chloromethylated polystyrene resin (0.7 g equiv of Cl per gram) cross-linked with 1% divinylbenzene (Bio-Beads, SX-1, 200–400 mesh) in dimethylacetamide (DMA, 80 mL) were added *N*-(fluorenylmethoxycarbonyl)-4-hydroxybenzylamine (3.45 g, 10 mmol) and KF (1.2 g, 20 mmol). The mixture was reacted at 80 °C overnight. After cooling, the resin was extensively washed with DMA, DMA–H_2O (1:1), H_2O, H_2O–CH_3OH (1:1), CH_3OH–CH_2Cl_2 (1:1), CH_2Cl_2, and CH_3OH and dried. The product (10.2 g) exhibited a strong absorption at 1700 cm^{-1} (KBr pellet). The resin was successively treated with 20% piperidine in DMF (v/v) for 3 and 7 min (standard deblocking), washed with DMF, CH_2Cl_2, and CH_3OH, and dried (10 g). The IR showed a broad absorption at 3500–3100 cm^{-1}. The substitution, determined by the Gisin[25] procedure, was ca. 0.25 mequiv of NH_2/g of polymer.

This resin was also obtained with the method of Pietta and Brenna.[10]

Attempted Syntheses of Cholecystokinin-octapeptide on 4-(Benzyloxy)benzylamine Resin. (1) 4-(Benzyloxy)benzylamine resin (2 g, 1.3 mmol) prepared according to Pietta and Brenna[10] was used for the manual synthesis of CCK-8. Three-fold excess (4 mmol) of the following amino acid derivatives and diisopropylcarbodiimide were used in each successive cycle: Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Met-OH, Fmoc-Trp-OH, Fmoc-Gly-OH, Fmoc-Met-OH, Fmoc-Tyr(SO_3Na)OH, and Fmoc-Asp(O-*t*-Bu)OH. The Tyr derivative was coupled in the presence of 1 equiv of pentafluorophenol (Pfp) until a negative Kaiser test[26] was obtained. The following synthesis cycle was used.

(a) Fmoc cleavage: repeated treatment (3 + 7 min) with 20% piperidine in DMF (20 mL).

(b) Washing: DMF (20 mL), CH_2Cl_2, and CH_3OH (six times alternatively, each 20 mL), three times with CH_2Cl_2 (20 mL).

(c) Coupling: Fmoc amino acids were dissolved in 15 mL of CH_2Cl_2–DMF (8:2, v/v) mixture, and 1 M carbodiimide solution (4 mL in CH_2Cl_2) was added.

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(d) Washing: CH_3OH and CH_2Cl_2 (six times alternatively), followed by three washings with CH_2Cl_2; cleavage was monitored with the Kaiser test.

After the last coupling step, the Fmoc group was cleaved; the resin was washed and dried, yielding 3.2 g of octapeptide polymer. The peptide–polymer (3 g) was treated with CH_2Cl_2-TFA-$(CH_3)_2S$-ethanedithiol (45:50:2.5:2.5, v/v; 40 mL) for 30 min. After filtration and rapid evaporation (5 min), the peptide was precipitated with ether, filtered off, and dried, yielding 1 g of crude product. No traces of CCK-8 or unsulfated CCK-8 (NS) could be found by HPLC (main peak t_r, 11.2 min; t_r of CCK-8, 12.5 min). Similarly, on TLC, a major spot was seen at R_f(a) = 0.70; R_f(a) of CCK-8 = 0.55. Upon acid hydrolysis, the compound gave the correct amino acid analysis: Gly 1.00, Asp 2.05, Met 1.86, Tyr 1.06, Phe 1.02, Trp 0.89. Ir: sharp band at 1050 cm^{-1} (*O*-sulfate group). The product was purified on a preparative Vydac C_{18} column by using 0.05 M (pH 6.5) CH_3COONH_4 buffer. CCK-8 (Phb)NS Mass spectrum: protonated isotopic molecular ion at 1169.46 (calcd 1169.44); CCK-8 (Phb) protonated isotopic molecular ion at 1249.46 (calcd 1249.49). Amino acid analysis gave the expected ratios.

(2) CCK-8 was also assembled by using the same protocol as above on the 4-(benzyloxy)benzylamine resin (4 g, 1 mmol), synthesized by our method. Cleavage of the peptide from the resin resulted in 0.6 g of crude product. The materials contained no trace of cholecystokinin-octapeptide when monitoring by TLC or HPLC and was identical with the main product of the former synthesis and demonstrated to coelute with it.

Attempted Synthesis of Phenylalanine Amide on 4-(Benzyloxy)benzylamine Resins. (1) Fmoc-Phe-OH (1.95 g, 5 mmol), dissolved in 15 mL of CH_2Cl_2, was coupled to the 4-(benzyloxy)benzylamine resin [prepared according to the method of Pietta and Brenna[10]] with 1 M diisopropylcarbodiimide in CH_2Cl_2 (5 mL). After cleavage of the Fmoc-protecting group (as above), the phenylalanyl-resin was treated with TFA–CH_2Cl_2 (1:1, v/v) for 30 min. The resin was filtered off, the solvents were removed in vacuo, and the product was precipitated with ether. The white solid material (100 mg) contained no H-Phe-NH_2 according to analytical HPLC (t_r = 5.5 min; t_r of the standard H-Phe-NH_2 was 4 min, in 0.1% TFA containing 6% acetonitrile).

(2) Fmoc-Phe-OH (390 mg, 1 mmol), dissolved in CH_2Cl_2 (10 mL), was coupled to the 4-(benzyloxy)benzylamine resin prepared by using the characterized *N*-Fmoc-4-hydroxybenzylamine. Treatment of this Phe-resin with TFA–CH_2Cl_2 (1:1) resulted in the same product as above.

B. 2,4-Dimethoxybenzhydrylamine Resin. To a stirred suspension of 100 g of polystyrene resin cross-linked with 1% divinylbenzene (Bio-Beads SX-1, 200–400 mesh) in nitrobenzene (1000 mL) were added 2,4-dimethoxybenzoyl chloride (70 g)[26] and anhydrous aluminum chloride (80 g). After 20 min, the reaction mixture had thickened and had warmed up to ca. 35 °C, at which time the resin was filtered off, thoroughly washed with nitrobenzene, isopropyl alcohol, H_2O, H_2O–CH_3OH (1:1), CH_3OH, CH_2Cl_2, and CH_3OH, and dried, resulting in 119 g of keto resin. The product exhibited a strong carbonyl absorption band at 1650 cm^{-1}.

The keto resin (100 g) was added to melted ammonium formate (2 kg) under mechanical stirring and was allowed to react at 165–168 °C.[15] After 20 h the mixture was cooled, and the resin was filtered off, extensively washed with water, CH_3OH, CH_2Cl_2, and CH_3OH, and dried, yielding the formylamino resin (100 g). (IR: strong absorption band at 1700 cm^{-1}). The resin was added to a mixture of EtOH (700 mL) and concentrated HCl (300 mL) and then stirred under reflux for 2 h. After cooling to room temperature, the resin was collected by filtration, washed with EtOH, H_2O, CH_3OH, and CH_2Cl_2, suspended in trifluoroacetic acid, containing 5% ethanedithiol for 40 min, washed with IPA (2% ethanedithiol), neutralized with 5% diisopropylethylamine in CH_2Cl_2, and finally washed with CH_2Cl_2 and CH_3OH and dried to a constant weight (98 g). Substitution was ca. 0.33 mequiv of NH_2/g of resin.

C. Cleavage Experiments. Fmoc-Gly-OH (0.3 g, 1 mmol) or Fmoc-Phe-OH (0.39 g, 1 mmol) was dissolved in CH_2Cl_2 and coupled to 2,4-DMBHA resin (1 g of each) by using DCC (1 mmol). Couplings were complete in 1 h. After washing, the Fmoc group was cleaved with 20% piperidine in DMF, and the amino acid resins were washed and dried. Cleavages were performed in TFA–thioanisole (4:1, v/v) and TFA–CH_2Cl_2 (1:1, v/v). Amino acid–resin samples (500 mg each) were treated with these reagents (5 mL), and aliquots (0.8 mL) were taken out at regular intervals. After evaporation to dryness (N_2 stream), the products were distributed in water (2 mL) and ether (2 mL), and the amino acid amide content in the aqueous phase was measured by analytical HPLC (solvent system for H-Gly-NH_2: 0.1% TFA, containing 1.2% acetonitrile; t_r 3.2 min. For H-Phe-NH_2: 0.1% TFA, containing 12% acetonitrile; t_r 4.5 min). Results are summarized in Table I.

L-Aspartyl-L-(*O*-sulfato)tyrosyl-L-methionyl-glycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalaninamide (CCK-8). The peptide was assembled on a 2,4-DMBHA (1 g; 0.33 mmol of NH_2/g) by using three-fold excess of *N*-Fmoc–amino acids (1 mmol) dissolved in CH_2Cl_2–DMF (4:1) (5 mL) and diisopropylcarbodiimide in CH_2Cl_2 (1 mmol) at each coupling step. Tyrosine was coupled twice as Fmoc-Tyr(SO_3Na)-OPfp. (See above for coupling schedule.) After the last coupling step, the Fmoc group was cleaved, and the resin was washed and dried (1.3 g). The octapeptide resin (1 g) was treated with a mixture of trifluoroacetic acid–thioanisole (4:1) (5 mL) for 10 min. After evaporation to dryness the peptide was precipitated with ether and extracted with 2% $NaHCO_3$ solution (100 mL). The solution of the crude peptide was loaded onto a preparative HPLC column (Vydac C_{18} cartridge, 15–20 μm) and purified by using a 0.05 M (pH 6.5) CH_3COONH_4 buffer solution and a linear gradient of acetonitrile (from 12% to 25% in 60 min). The fractions were checked on analytical HPLC. Pure fractions were collected, pooled, and lyophilized, yielding 23 mg of highly purified CCK-8. Amino acid composition gave the expected ratios: $[\alpha]^{20}_D$ −21.2° (*c* 1, DMF); analytical HPLC, t_r = 12.5 (0.05 M CH_3COONH_4 [pH 6.5] as a buffer, 60% CH_3CN in A as B buffer; gradient runs from 30% to 50% B in 20 min). The compound was shown to coelute with authentic CCK-8.

L-Pyroglutamyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosylglycyl-L-leucyl-L-arginyl-L-prolylglycinamide (GnRH). The peptide was built up on the 2,4-DMBHA resin (1 g; 0.33 mmol). Three-fold excess of N^{α}-Fmoc amino acid derivatives (1 mmol) and diisopropylcarbodiimide (1 mL of 1 M solution in CH_2Cl_2) were used in each cycle. Serine and tyrosine were coupled as *O*-*tert*-butyl derivatives, the arginine guanidino group was protected with Mtr and the histidine imidazole N with Fmoc. The same synthetic cycle was used as for CCK-8. Pyroglutamic acid was coupled unprotected in DMF. After the last coupling step, the peptide–resin was washed, dried (1 g), and treated (45 min) with a mixture of phenol (0.125 mL), dimethyl sulfide (0.125 mL), and trifluoracetic acid (4.75 mL) for 45 min at room temperature. The solvents were evaporated in vacuo; the peptide was precipitated with ether and extracted from the resin by washing with water (15 mL). The aqueous solution of the crude peptide was loaded onto a preparative HPLC column (Vydac C_{18}, 5 μm) and purified by using both TEAP and 1% TFA buffers and acetonitrile gradient (15% B to 35% B in 60 min, respectively). Pure fractions were pooled and lyophilized. Amino acid composition gave the expected ratios. FAB mass spectrometric analysis gave a protonated isotopic molecular ion at *m/z* 1182.58 for a calculated value of *m/z* 1182.58. Coelution experiment on HPLC with an authentic sample (Vydac C_{18}, 5 μm) gave one single, sharp symmetrical peak.

Acknowledgment. Research was supported by NIH Grants HD13527 and AM26741. We are indebted to C. Hoeger for extensive discussion and contribution to the manuscript. We thank J. Dykert and C. Miller for technical assistance and R. Hensley for manuscript preparation. FAB mass spectrometric analysis was performed by T. Lee at the Beckman Institute, Duarte, CA.

Registry No. CCK-8, 25126-32-3; GnRH, 33515-09-2; FMOC-OSu, 82911-69-1; FMOC-Phe-OH, 35661-40-6; FMOC-Gly-OH, 29022-11-5; FMOC-Met-OH, 71989-28-1; FMOC-Trp-OH, 35737-15-6; FMOC-Tyr(SO_3Na)OH, 106864-37-3; FMOC-Asp(OBu-*t*)OH, 71989-14-5; 4-hydroxybenzylamine, 696-60-6; *N*-(fluorenylmethoxycarbonyl)-4-hydroxybenzylamine, 106864-36-2; 2,4-dimethoxybenzoyl chloride, 39828-35-8.