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Synthesis of [Gln⁴]-Neurotensin Using a New Solid Support, the 4-(Hydroxymethyl)phenylacetamidomethyl-resin

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For biomedical studies, [Gln⁴]-neurotensin, which appears to be the naturally occurring form of neurotensin, was synthesized using a recently designed new resin for the Merrifield solid-phase synthesis of peptides, 4-(hydroxymethyl)phenylacetamidomethyl resin (PAM-resin). This synthesis was compared to the synthesis of [Gln⁴]-neurotensin by the use of oxymethyl-copoly-(styrene-divinylbenzene) as the solid support. The PAM-resin was superior, since the yield of [Gln⁴]-neurotensin was doubled and fewer purification steps were necessary.

INTRODUCTION

In 1976, Mitchell *et al.* (*1*) designed a new resin for solid-phase peptide synthesis, the 4-(hydroxymethyl)phenylacetamidomethyl resin (PAM-resin). This new resin was also synthesized by Yamaguchi *et al.* (*2*) by an improved procedure. This resin provides, under acidic conditions (50% TFA in CH_2Cl_2), a linkage which is 100-fold more stable during the growth of the peptide chain than that provided by the commonly used oxymethyl-copoly(styrene-divinylbenzene). The higher stability of the resin–peptide bond resulted at the end of the synthesis, in a higher yield of the crude peptide which was also of a higher purity, since late initiation of peptide chains and formation of deletion peptides lacking one or more residues at the carboxyl terminal were minimized.

Carraway and Leeman reported (*3*, *4*) the isolation of a new peptide from bovine hypothalami during 1971–73. This new peptide, neurotensin, induced hypotension in rats, stimulated the contraction of the guinea pig ileum and the rat uterus, and produced relaxation of the rat duodenum. Amino acid sequence analysis (*5*) proved that neurotensin is the tridecapeptide: <Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH.

A priori, it was considered quite possible by Folkers *et al.* (*6*), that the tedious and time-consuming isolation of neurotensin could have inadvertently allowed the hydrolysis of a [Gly⁴]-moiety, if this moiety were actually present in the native neurotensin. Guided by this consideration, neurotensin and three of its analogs were synthesized. [Gln⁴]-neurotensin showed the same biological activities as neurotensin, and it was found to be hydrolyzed to neurotensin under conditions which simulated certain conditions of the isolation.

To facilitate the biomedical evaluation of [Glu⁴]-neurotensin, it was necessary to synthesize a larger amount. To simplify the purification of the crude peptide, and to obtain the pure peptide in a higher yield, the PAM-resin was used as solid support for

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the synthesis. Based on the synthesis of Folkers *et al.* (*6*) the PAM-resin yielded a crude peptide which required only two purification steps to obtain a product which was homogeneous in six tlc systems and by electrophoresis. After lyophilization of the fractions containing the desired [Gln^4]-neurotensin, a yield of 21% (170 mg) of the tridecapeptide was obtained as in white powder. In the first synthesis (*6*), the yield of pure peptide was only about 10%. Electrophoresis and tlc showed that the peptides synthesized from both resins were identical in properties.

These findings underscore the data of Mitchell *et al.* (*1*) on the greater stability of the PAM-resin–peptide bond under the conditions of the solid-phase peptide synthesis and offer a better approach to the synthesis of long peptides, since the formation of failure sequences is minimized.

EXPERIMENTAL

Pure L-amino acid derivatives, purchased from Beckman, Inc., Palo Alto, Calif.; Bachem, Inc., Marina del Rey, Calif.; and Peninsula Laboratories, San Carlos, Calif., were used as starting materials. The amino acid analyses were carried out on a Beckman amino acid analyzer, Model 119, after hydrolysis of the samples in 6 *N* HCl for 24 hr in evacuated sealed tubes at 110°C. The synthesis was carried out by the Merrifield solid-phase procedure on a Beckman Model 990 automatic peptide synthesizer. The α-amino groups of the used L-amino acid derivatives were protected by the t-amyloxycarbonyl group for Arg. The side chain protecting groups were (2-Br-Z) for Tyr, (2-CL-Z) for Lys, and (Tos-) for Arg. <Glu was incorporated into the peptide chain by the Z-<Glu derivative. The following solvent systems were used for tlc analysis: (1) *n*-BuOH/EtOAc/AcOH/H_2O (1:1:1:1), (2) EtOAc/Py/AcOH/H_2O (5:5:1:3), (3) *n*-BuOH/Py/AcOH/H_2O (30:30:6:24), (4) 2-propanol/1 *N* AcOH (2:1), (5) $CHCl_3$/conc. NH_4OH/MeOH (60:20:45), and (6) Py/AcOH/H_2O (50:30:15). The 4-(hydroxymethyl)phenylacetamidomethyl-resin (PAM-resin) was synthesized by using an improved method (*2*), which was based upon the method originally published by Mitchell *et al.* (*1*).

Boc-Leucyl-4-(oxymethyl)phenylacetamidomethyl-resin. A solution of Boc-leucine (0.5 g, 2.16 mmol) and carbonyldiimidazole (0.3 g, 1.85 mmol) in CH_2Cl_2 (15 ml) was kept at −20°C for 30 min and at −5°C for another 30 min and then aded to a 50-ml round-bottomed flask, containing 2 g of 4-(hydroxymethyl)phenylacetamidomethyl-resin. The suspension was stirred at room temperature for 8 hr. The resin was collected by filtration, washed with CH_2Cl_2, and acetylated by being stirred with acetic anhydride–pyridine (1:1, 20 ml) for 40 min. Subsequent filtration, washing with DMF, CH_2Cl_2, isopropanol, and CH_2Cl_2, and drying under vacuum afforded the desired Boc-Leu-PAM-resin, which contained 0.89 mmol of leucine/g of substituted resin, according to the amino acid analysis after hydrolysis of the resin with 6 *N* HCl–propionic acid (1:1) at 120°C overnight.

[Gln^4]-Neurotensin (<Glu-Leu-Tyr-Gln-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH). Boc-Leu-PAM-resin (2.25 g, 2.025 mmol) was introduced into the reaction vessel and washed with CH_2Cl_2 (3 × 2 min), then the Boc-group was removed by prewashing with 30% (v/v) TFA in CH_2Cl_2 (2 min) and two successive deblocking steps with 30% TFA

(2 × 30 min). The resin was washed free of TFA with CH_2Cl_2 (3 × 2 min), isopropanol (2 × 2 min), and CH_2Cl_2 (4 × 2 min). The amino group was liberated from the TFA-salt by prewashing the resin with 10% (v/v) triethylamine in CH_2Cl_2 (2 × 5 min) and neutralization with 10% triethylamine in CH_2Cl_2 (30 min). The resin was washed neutral with CH_2Cl_2 (4 × 2 min). Except for the incorporation of [Asn^5] and [Gln^4], the coupling was performed in the following way: A twofold excess (4.05 mmol) of the appropriate Boc-amino acid dissolved in CH_2Cl_2 (25 ml) was added to the resin. After stirring for 5 min, a twofold excess of DCC (4.05 mmol) in CH_2Cl_2 (8.5 ml) was added, and the mixture was allowed to react for 4 hr. To get more complete coupling reactions and a peptide of higher purity, double coupling was performed for each amino acid in the following way (except for [Asn^5] and [Gln^4]): The resin was rewashed with 10% triethylamine in CH_2Cl_2 (4 × 2 min). Then, an equimolar amount of the appropriate Boc-amino acid (2.025 mmol) in CH_2Cl_2 (25 ml), and after 5 min of stirring, an equimolar amount of DCC (2.025 mmol) in CH_2Cl_2 (4.25 ml), were added. The mixture was allowed to react for another 4 hr. In the case of [Tyr^{11}], the order of addition of the Boc-amino acid and the DCC was reversed to avoid formation of undesired diketopiperazine derivatives as much as possible (*7*). In case of [Asn^5] and [Gln^4], the active ester method was used for incorporation; i.e., the resin, after deprotection and neutralization, was allowed to react with a fivefold excess (10.125 mmol) of the *p*-nitrophenyl ester derivative of either Boc-Asn or Boc-Gln, dissolved in purified DMF/CH_2Cl_2 (1 : 1, 30 ml), for 24 hr. Double coupling was done with a twofold excess (2.025 mmol) of the *p*-nitrophenyl ester derivatives in DMF/CH_2Cl_2. The completeness of the coupling reactions was monitored by the ninhydrin color test procedure of Kaiser *et al.* (*8*). When the color test was still positive after double coupling, the remaining free amino groups were acetylated with acetic anhydride in CH_2Cl_2 (10% by volume) for 30 min. After the double-coupling steps, the resin was washed with CH_2Cl_2 (3 × 2 min), isopropanol (3 × 2 min), DMF (3 × 2 min), and CH_2Cl_2 (3 × 2 min). Twelve successive cycles of deprotection, neutralization, coupling, and double coupling were carried out in this manner with the following amino acid derivatives: Boc-Ile; Boc-Tyr (2-Br-Z); Boc-Pro; Boc-Arg (Tos); Boc-Arg (Tos); Boc-Pro; Boc-Lys (2-Cl-Z); Boc-Asn-ONP; Boc-Gln-ONP; Boc-Gln-ONP; Boc-Tyr (2-Br-Z); Boc-Leu; and Z-<Glu.

At the conclusion of the synthesis, the protected peptide resin was washed with CH_2Cl_2 (3 × 2 min), 30% TFA in CH_2Cl_2 (3 × 2 min), CH_2Cl_2 (3 × 2 min), isopropanol (3 × 2 min), CH_2Cl_2 (3 × 2min), DMF (3 × 2 min), and CH_2Cl_2 (3 × 2 min) and dried *in vacuo* to give 5.2 g of the protected [Gln^4]-neurotensin–PAM-resin; 2 g of this peptide–resin was treated with 25 ml of dried (CoF_3) HF to cleave the peptide–resin bond and deprotect the side chains. The cleavage reaction was run at 0°C for 60 min in the presence of 3 ml of anisole in a reaction apparatus for anhydrous hydrogen fluoride, manufactured by the Toho Kasei Co., Ltd., Osaka, Japan. After evaporation of the excess HF under reduced pressure, the resin was washed with ethylacetate to remove anisole and anisole breakdown products, followed by 10% aqueous acetic acid to extract the peptide. Lyophilization of this extract gave 890 mg of the crude [Gln^4]-neurotensin.

This crude peptide (800 mg) was purified by gel filtration on Bio-Gel P-2 (200–400 mesh, 60 × 5-cm column) with 1.3%aqueous acetic acid as eluant. The fractions containing the main product in reasonably pure form were collected and lyophilized to

give about 600 mg of a white powder. This material was further purified by partition chromatography on Sephadex G 25 (60 × 5-cm column) with the system *n*-butanol/AcOH/H_2O (4 : 1 : 5) as eluting solvent. The flow rate was 5 ml/30 min, and the temperature during chromatography was about 4°C. The fractions which contained the pure [Gln^4]-neurotensin (tlc-control) were lyophilized to give 170 mg of a white powder which was identical in properties with a sample of [Gln^4]-neurotensin synthesized previously by Folkers *et al.* (*6*) using the conventional Merrifield resin.

The tlc values were: R_f^1 0.59; R_f^2 0.87; R_f^3 0.59; R_f^4 0.50; R_f^5 0.32; and R_f^6 0.98. Each system revealed only one spot with Pauly, I_2, and chlorine-*o*-toluidin reagents. Only one compound, moving toward the cathode, was observed on paper electrophoresis (*6*).

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