Solid Phase Synthesis of a Nonapeptide of Melittin

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Synopsis

The venom of the honey bee, *Apris mellifica*, is a strongly hemolytic polypeptide termed Melittin. It contains 26 amino acids, and the complete amino acid sequence of Melittin has recently been published[1-2]. This paper reports the synthesis of a nonapeptide, Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu, of Melittin. An instrument, which can perform both automatically and manually all of the operation in the stepwise synthesis of peptide by the solid phase method, was constructed[3]. The peptide was synthesized using manually the instrument. The peptide was purified by recrystallization and characterized by paper electrophoresis and amino acid analysis, and obtained in over all yield of 90%.

I. Introduction

The synthesis of several biologically active peptides has been performed by the classical methods[4-8]. However, many months and much trouble and labour were required with these methods. In 1962, R.B. Merrifield reported the new synthetic method of peptide, so-called, the solid phase peptide synthesis[9]. But, as this method had a few defects, it was improved by him[10], and bradykinin was synthesized for 8 days by the automatic instrument[11]. Now, the general scheme of the solid phase peptide synthesis is shown in Fig. 1. The copolystyrene-2%-

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* Yôshirô MIURA, Michihiro TOYAMA and Shûichi SETO, Presented at the 21th Annual Meeting of The Chemical Society of Japan, Osaka, Apr. 1～4, 1968.

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divinylbenzene, 200-400 mesh beads, which is insoluble in usual organic solvents (for example, acetic acid, dimethylformamide, ethanol, methylene chloride) and anhydrous mineral acid, is chloromethylated with chloromethyl methyl ether and anhydrous $SnCl_4$. Then, the protected amino acid is esterified on the copolymer with triethylamine. After the deprotection step, excess next protected amino acid is added into the copolymer with dicyclohexylcarbodiimide, and after the coupling reaction, unreacted amino acid and by-products of the reagent are removed by filtration.

As the same procedures are performed, a peptide chain is synthesized in a stepwise manner, and after the synthesis of the entire peptide, the peptide chain can be cleaved from the copolymer with HBr-CF_3COOH.

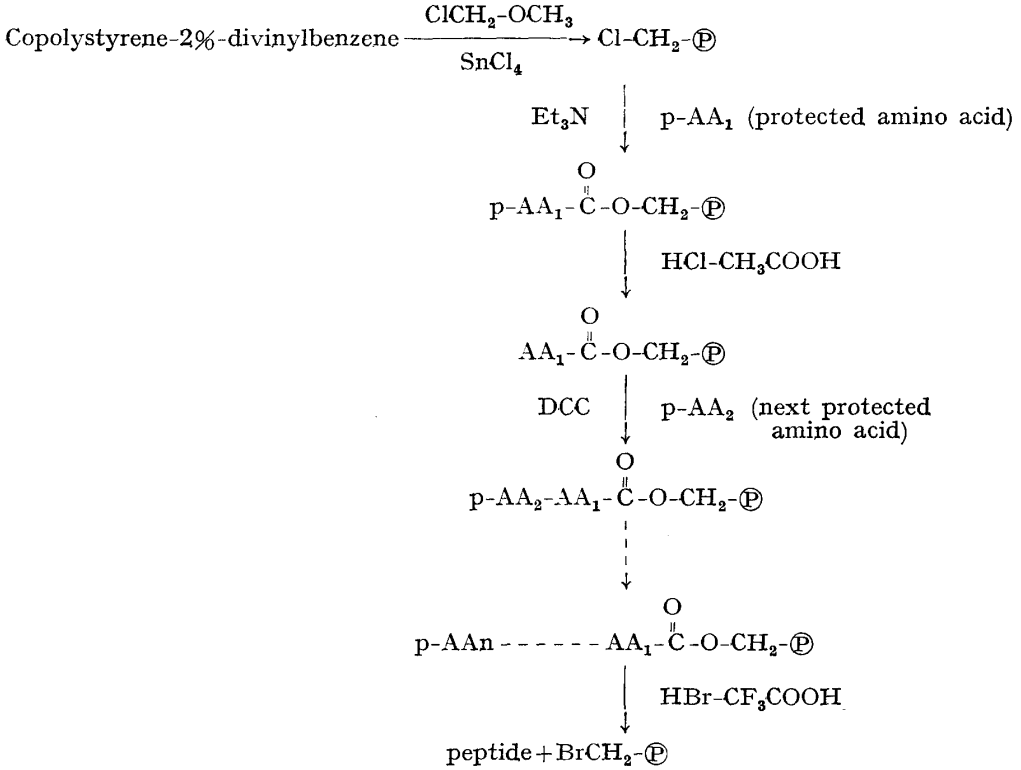

Fig. 1. The scheme of the solid phase peptide synthesis. Abbreviation used: Ⓟ Copolystyrene–2%–divinylbenzene, DCC. dicyclohexylcarbodiimide.

The characteristics of this method are shown below.

(1) Because of using the insoluble resin, an unreacted amino acid and by-products of the reagent are removed by filtration, therefore the preparation of intermediate of peptide is simplified.

(2) The synthesis of peptides is done very rapidly by the automatic instrument.

(3) As excess protected amino acid is used, the reaction is proceeded quantitatively.

II. Results

The complete amino acid sequence of Melittin is shown as following. H-Gly-

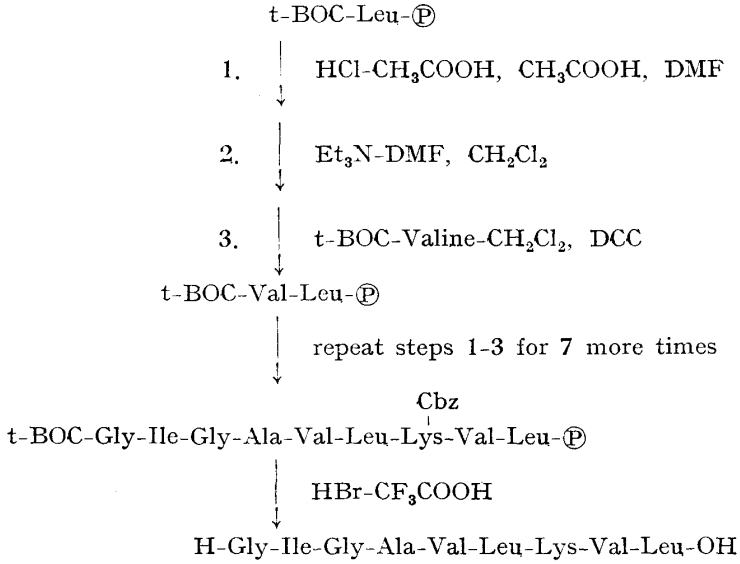

Fig. 2. The synthesis of a nonapeptide of Melittin. Amino acids are the L-isomer. Abbreviation used; Ⓟ, Copolystyrene–2% divinyl-benzene; t-BOC. tertiary-Butyloxycarbonyl group;Cbz. Benzyloxycarbonyl group; DCC, Dicyclohexyl-carbodiimide; DMF, Dimethylformamide.

Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2

The synthesis of a nonapeptide (1–9) of Melittin is shown in Fig. 2.

The chloromethylated copolymer of styrene and 2% divinylbenzene (Infrared spectrum in Fig. 3–1) was used, and the C-terminal amino acid was covalently attached by esterification with t-BOC*-Leucine triethylammonium salt. The infrared spectrum of t-BOC-Leucyl-Resin is shown in Fig. 3-2. Then, the t-BOC-group was removed with excess hydrogen chloride in acetic acid for 30 min. The neutralization of the hydrogen chloride was carried out with triethylamine in dimethylformamide. In the coupling step, the excess t-BOC-Amino acids and dicyclohexylcarbodiimide in methylene chloride were used to complete the reaction. When the successive sequential coupling reaction of t-BOC-amino acids in methylene chloride proceeded, a portion of the peptide-containing resin was removed from the reaction vessel, and its infrared spectrum was measured. The νC=O absorption of amide (secondary) increased gradually. It seemed that the coupling reaction was carried out almost completely. Fig. 3-3 shows the infrared spectrum of t-BOC-Glycyl-isoleucyl-glycyl-alanyl-valyl-leucyl-ε-benzyloxycarbonyl-lysyl-valyl-leucyl-Resin.

The peptide was cleaved from resin by passage of a slow stream of hydrogen bromide in trifluoroacetic acid. In this time, ε-benzyloxycarbonyl group was removed. The crude peptide was isolated after filtration and drying in a desiccator over potassium hydroxide.

* Abbreviation used in this work; t-BOC, tertiary-Butyloxycarbonyl.

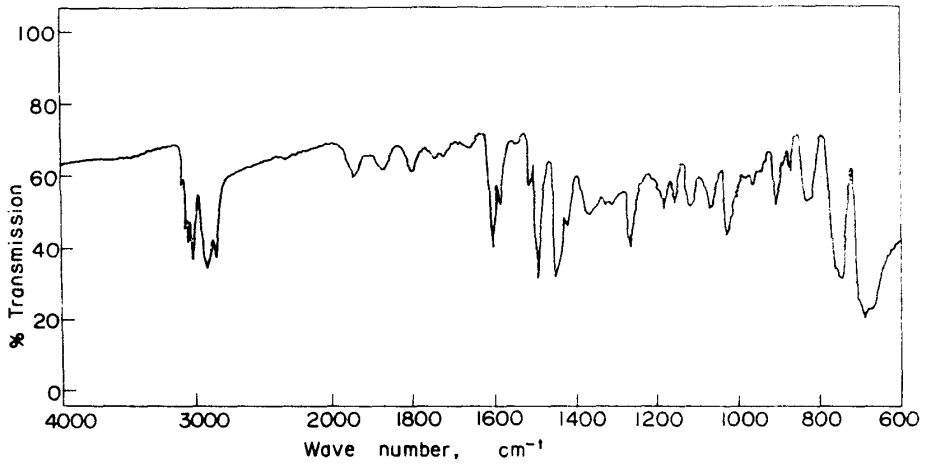

Fig. 3-1. Infrared absorption spectrum of the chloromethylated copolymer of styrene and 2% divinylbenzene inKBr disk.

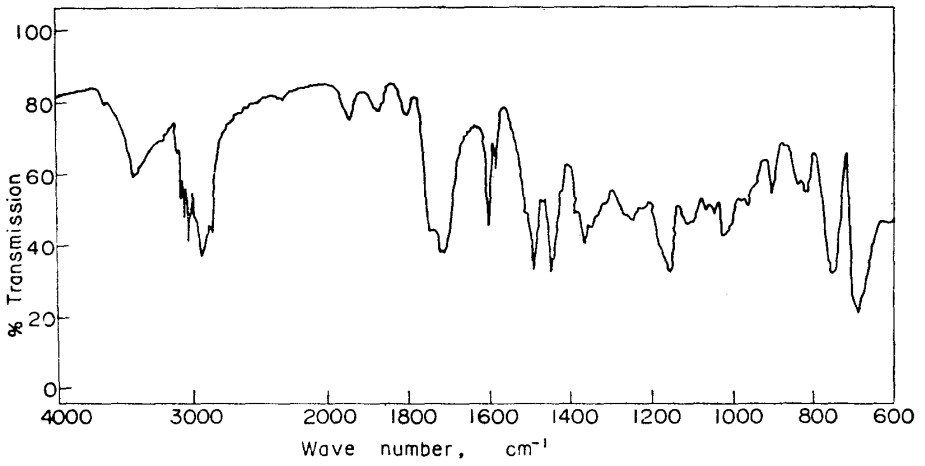

Fig. 3-2. Infrared absorption spectrum of t-BOC-Leucyl-Resin in KBr disk.

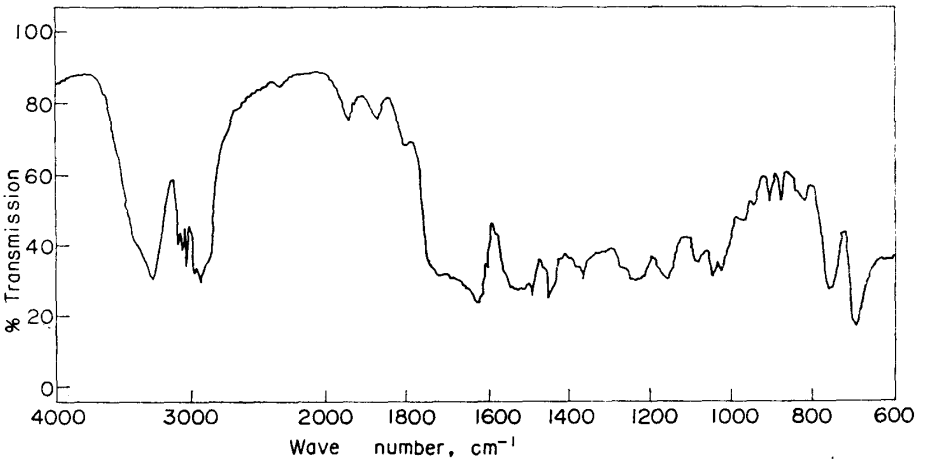

Fig. 3-3. Infrared absorption spectrum of t-BOC-Glycyl-isoleucyl-glycyl-alanyl-valyl-leucyl-ε-benzyloxycarbonyl-lysyl-valyl-leucyl-Resin in KBr disk.

Based on a quantitative amino acid analysis, the yield was 90.4%, the amino acid ratio, Gly, 2.00; Ile, 1.11; Ala, 0.92; Val, 2.35; Lys, 0.88; Leu, 2.16. Paper chromatography of the crude peptide showed a few ninhydrin spots. The nonapeptide was purified with n-butyl alchol and H_2O. A pure nonapeptide, Glycyl-isoleucyl-glycyl-alanyl-valyl-leucyl-lysyl-valyl-leucine was obtained from an aqueous layer. Infrared spectrum of a nonapeptide is shown in Fig. 4: The amino acid analysis ratios, Gly, 2.07; Ile, 0.96; Ala, 1.01, Val, 2.01; Lys, 0.93; Leu, 2.00.

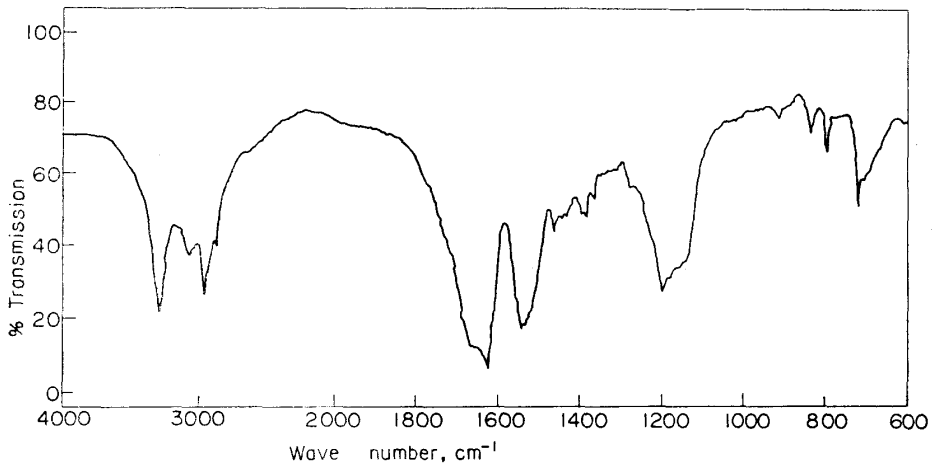

Fig. 4. Infrared absorption spectrum of a nonapeptide, Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu·CF_3CO_2H in KBr disk.

Also, from a n-butyl alcohol layer, amorphous powder which behaved exactly like a nonapeptide when compared by paper electrophoresis and paper chromatography, was obtained. As a nonapeptide contains basic amino acid, lysine, negative electrophoretic mobility at pH 8.6, was expected. In the fact, it showed negative mobility. Therefore, a nonapeptide of Melittin was synthesized with simple procedures by the solid phase method. In this way, the peptide was synthesized more easily with a simple manner than the classical methods. Several biologically active peptides will be synthesized by the solid phase method using an automatic peptide synthesizer.

III. Experimental Section

All of the t-BOC-amino acids were synthesized according to the modification of the procedure of Schwyzer et al.[12]. Paper electrophoresis was performed at 3 mA on a 5X6 cm cellulose acetate membrane using a pH 8.6 Veronal buffer solution. The peptide was detected by ponceau 3R 0.4% trichloroacetic acid aqueous solution. Solvent used for ascending paper chromatography on No. 50 Toyoroshi filter paper was n-butyl-alchol-pyridine-acetic acid-water (BPAW) (15:10:3:12). Quantitative amino acid analyses of peptide hydrolysate were done using a Hitachi

(12) R. Schwyzer, P. Sieber, and H. Kapperer, Helv. Chim. Acta **42** (1959), 2622.

KLA-3B amino acid analyzer. For amino acid analysis, the samples were hydrolyzed in 6N HCl in a sealed tube (vacuum) at 110° for 24 hr. The polystyrene-2%-divinyl-benzene copolymer resin was purchased from Bio-Rad Laboratories Richmond, Claif. and chloromethylated according to the procedure of Merrifield[11]. Infrared spectra of resin were measured using a Hitachi EPI-G21 spectrophotometer.

t-BOC-Leucyl-Resin. A solution of 2.55 g (11.0 mmoles) of t-BOC-Leucine and 1.10 g (11.0 mmoles) of triethylamine in 20 ml absolute alcohol was added to 10.00 g of the chloromethylated copolystryene-2%-divinylbenzene which contained 5.48% Chlorine (1.55 mmoles Cl/g), and the mixture was stirred at 80°C for 48 hr[11]. As the chloromethylation of copolymer and the esterification of t-BOC-Leucine on it were done at random, the hydrolysis of the t-BOC-Leucyl-resin had to be performed several times to obtain the accurate content of Leucine.

An aliquot (51.1 mg) of t-BOC-Leucyl-resin was hydrolyzed by refluxing a suspension of resin for 18 hr in a mixture of equal volumes of dioxane and concentrated hydrochloric acid[13]. Amino acid analysis showed that the substituted polymer contained 0.219 m mole of Leucine/g.

In the hydrolysis of 50.3 mg of t-BOC-Leucyl-resin, the amino acid analysis showed 0.256 mmole of Leucine/g., in a 52.1 mg portion of t-BOC-Leucyl- resin, it showed 0.477 mmole of Leucine/g. The average value is 0.341 mmole Leucine/g of t-BOC-Leucyl-resin.

t-BOC-Glycyl-isoleucyl-glycyl-alanyl-valyl-leucyl-ε-Benzyloxycarbonyl-lysyl-valyl-leucyl-Resin. A 10.7 g sample of t-BOC-Leucine resin (3.65 mmoles leucine) was loaded into the reaction vessel of the manual peptide synthesis instrument. The following cycle of reaction was used to introduce each residue[13]: (1) washed with three 60 ml portions of glacial acetic acid; (2) t-BOC-group cleaved by 60 ml of 1 N HCl in glacial acetic acid for 30 min; (3) washed with three 60 ml portions of glacial acetic acid; (4) washed with three 60 ml portions of absolute ethanol; (5) washed with three 60 ml portions of dimethylformamide; (6) neutralized the hydrochloride with 6 ml of triethylamine in 60 ml of dimethylformamide; (8) washed with three 60 ml portions of methylene chloride (9) introduced 14.6 mmoles of the appropriate t-BOC amino acid in 40 ml of methylene chroride and allowed to mix for 10 min; (10) introduced 14.6 mmoles of dicyclohexylcarbodiimide in 5 ml of methylene chloride and allowed to react for 2 hr: (11) washed with three 60 ml portions of methylene chloride: (12) washed with three 60 ml portions of absolute ethanol. The washing time of each step (except (2), (6), (9), (10)) was 3-4 min. At the end of synthesis, the resin was transferred into a 200 ml beaker and dried overnight in a vacuum desiccator over KOH. The protected peptide-resin (12.2 g) was obtained.

Glycyl-isoleucyl-glycyl-alanyl-valyl-leucyl-lysyl-valyl-leucine. A5.0 g sample of

(13) G.R. Marshall and R.B. Merrifield, Biochemistry, **4** (1965), 2394.

the protected nonapeptide resin was suspended in 30 ml of trifluoroacetic acid, and a slow stream of hydrogen bromide was passed through with occasional shaking for 90 min under anhydrous conditions[13]. The mixture was filtered and the polymer was washed with two 5 ml portions of trifluoroacetic acid. The filtrate was evaporated at 25° on the rotary evaporator and then dried in a desiccator over KOH. The syrupy product was dissolved in about 1 ml of H_2O and dried in a desiccator over KOH. Crude peptide (1.4 g) was obtained. mp>280°. Amino acid analysis ratio; Gly, 2.00, Ile, 1.11, Ala, 0.92; Val, 2.35; Lys. 0.88; Leu. 2.08. The average value for each of the nine amino acid residues was 3.30 mmoles/g of peptide-resin. Therefore the yield for the cleavage step was 90%.

Purification of a nonapeptide (Glycyl-isoleucyl-glycl-alanyl-valyl-leucycl-lysyl-valyl-leucine) A 1.3 g sample of crude peptide was suspended in 50 ml of H_2O and 20 ml of n-butyl alcohol. The suspention was filtered and an aqueous layer was separated from a n-butyl alcohol layer. An isoluble product (159 mg) in both H_2O and n-butyl alcohol was obtained, mp>280°. The aqueous layer was concentrated at room temperature and a jelly material was precipitated. Amorphous powder (115 mg) was obtained; amino acid analysis ratio: Gly, 2.07; Ile, 0.96; Ala, 1.01; Val, 2.01; Lys, 0.93; Leu, 2.00, mp>280,° $[\alpha]_D^{23}$ -70.1 (C=1.7 in H_2O). By paper chromatography, Rf BPAW 0.71. Paper electrophoresis of a nonapeptide showed a single spot (Fig. 5). The n-butyl alcohol layer was concentrated at room temperature. Amorphous powder (204 mg) was obtained. It was dissolved in 25 ml of H_2O and 25 ml of acetone and purified with active charcoal. Paper electrophoresis of an aliquot of this solution showed a single spot which behaved exactly like a nonapeptide. (Fig. 5).

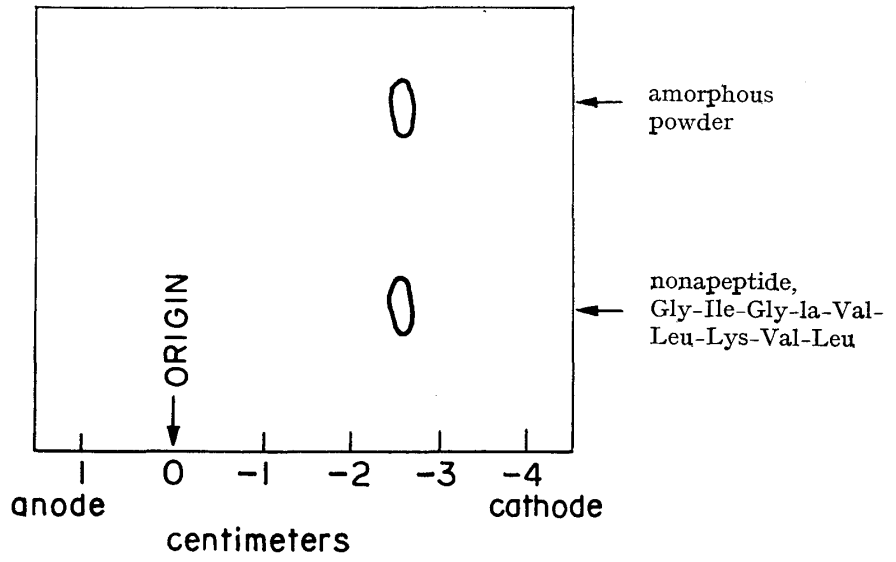

Fig. 5. Paper electrophoretic patterns of a nonapeptide and the amorphous powder. The electrophoresis was performed using a pH 8.6 Veronal buffer on cellulose acetate membrane at 3mA.

Then, the n-butyl alcohol layer was more concentrated and 267 mg of amorphous powder was obtained: amino acid analysis ratio: Gly, 2.00: Ile, 0.96; Ala, 0.99; Val, 2.07; Lys, 0.92; Leu, 2.06, mp>280°.

By paper chromatography, Rf BPAW 0.70. Infrared spectrum of this powder was identical with that of a nonapeptide. $[\alpha]_D^{23}$ -73.4 (C=0.74 in H_2O) Paper electrophoresis of this powder showed a single spot which behaved exactly like a nonapeptide.

Acknowledgements

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