Vol. 84, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 14, 1978 Pages 184-194

# IDENTIFICATION AND SYNTHESIS OF A HEPTAPEPTIDE IN UREMIC FLUID*

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Received July 19,1978

## SUMMARY

A heptapeptide isolated from uremic fluid was synthesized by conventional method. The total amino acid sequence of this peptide was deduced as follows: H-His-Pro-Ala-Glu-Asn-Gly-Lys-OH. Structural similarity was soon realized between this peptide and heptapeptde moiety corresponding to position 13 through 19 of $\beta_2$-microglobulin.

## INTRODUCTION

The sequare meter-hour hypothesis (1) has prompted research into role of middle molecular substances (MMS) in inducing clinical symptoms of uremic toxicity (2-4). According to many clinical reports MMS (Mw 300-5000) appear to play an important role in uremic neuropathy, immune deficiency, anemia and probably other sighns of uremic toxaemia (5-9). It has been shown that they are ninhydrin positive and that their concentration in the blood of uremic patients is much higher than in the blood of patients with normal kidney (3).
It has also been found that these substances pass through natural and artifical dialysis membranes because after peritoneal dialysis and hemodialysis their concentration in the blood evidently decreases (2,5,6). So far these substances have not been identified precisely but some authors suggest their peptide nature (3).

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* Amino acids, peptides and their derivatives in this paper are of the L-configuration. The following abbreviations are used: Z= benzyloxycarbonyl, Boc= tert-butoxycarbonyl, OBzl= benzyl ester, ONp= p-nitrophenyl ester, OMe= methyl ester, PTH= phenylthiohydantoin

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0006-291X/78/0841-0184$01.00/0

A peptide was isolated from uremic fluid and its entire amino acid sequence was disclosed as the linear heptapeptide. We have identified the heptapeptide as
1 2 3 4 5 6 7
H-His-Pro-Ala-Glu-Asn-Gly-Lys-OH
by amino acid composition and characteristics of its PTH-derivatives. Residue 5 is probably asparagine rather than aspartic acid in view of the Rf of the PTH-derivative. Structural similarity was soon realized between this peptide (H-His-Pro-Ala-Glu-Asn-Gly-Lys-OH) and the heptapeptide fragment corresponding to position 13 through 19 of $\beta_2$-microglobulin. The heptapeptide obtained from uremic fluid was found to be homogeneous to synthetic peptide by paper chromatography, paper electrophoresis and optical rotation. Further precise biological evaluation of the heptapeptide will be required in further.

## PATIENT SELECTION

An uremic patient with severe neuropathy characterized by motor paralysis, areflexia, muscular atrophy were selected for study.

## EXPERIMENTAL

Melting points were uncorrected. Rotation was determined with an Atago Polax. Amino acid analysis was performed with a JEOL JLC-8AH amino acid analyzer. Evaporations were carried out in a rotary evaporator under reduce pressure at a temperature of 35°. The purity of the separated products were by paper chromatography using Toyo Roshi No. 51, at room temperature. $Rf^1$ value refer to Partridge system (10) and $Rf^2$ values refer to the system of nBuOH-pyridine-AcOH-$H_2O$ (30:20:6:24) (11). The brief details of the separation of extracorporeal ultrafiltration method (ECUM) fluid was summerized in Chart I.

### 1 Ultrafiltration

ECUM fluid (1.5l) was ultrafiltered using an Amicon Centriflo membrane DM-5 which has a molecular cut off at approximately 5000 dalton.

### 2 Ultrafiltration

The filtrate of 1 was ultrafiltered using an Amicon Centriflo membrane UM-05 which has a molecular cut off at approximately 500 dalton.

### 3 Sephadex G-25 gel filtration

This concentrated fluid (10ml) of 2 was fractioned on a column (2.6X94.0cm) of Sephadex G-25 with 1% AcOH at a flow rate of 1.3ml/min. 4ml of each samples were collected and their absorptions at 230nm

ECUM fluid (1.5l containing 0.02% $NaN_3$)
↓
1. Amicon Centriflo membrane DM-5
↓ cut-off at approximately 5000 dalton
2. Amicon Centriflo membrane UM-05
↓ cut-off at approximately 500 dalton
3. Sephadex G-25
column size: 2.6X94.0cm
eluate: 1% AcOH
↓
4. Sephadex G-15
column size: 2.6X96.0cm
eluate: 1% AcOH
↓
5. CM-Sephadex C-25
column size: 2.6X96.0cm
eluate: $NH_4OAc$ buffer (PH 6.50)
↓
H-His-Pro-Ala-Glu-Asn-Gly-Lys-OH

Chart 1. Purification of H-His-Pro-Ala-Glu-Asn-Gly-Lys-OH from uremic fluid

were measured (Fig. 1). Corresponding to the Pauly positive peak (tube No. 80-120) were collected, evaporated to dryness in vacuum, and lyophilized (Fig. 2). This peak is present in ECUM fluid of a uremic patient but not detected in normal plasma (Fig. 1).Yield 600mg.

4 Sephadex G-15 gel filtration

The crude material (500mg) of 3 in $H_2O$ (10ml) was added to a Sephadex G-15 column (2.6X96.0cm) which was eluted with 1% AcOH. Fractions of 4ml each were collected at a flow rate of 1.0ml/min and absorbance of each fraction was determined at 230nm (Fig. 3). The each fraction was determined at 230nm (Fig. 3). The each fractions were located by Pauly reaction. Fractions corresponding to the Pauly positive peak (tube No. 40-75) were pooled, evaporated to dryness in vacuum, and lyophilized (Fig. 4). Yield 200mg.

5 CM-Sephadex C-25

The crude material (130mg) of 4 in $H_2O$ (10ml) was applied to a column of CM-Sephadex C-25 (1.8X46.0cm), which was eluted with a linear gradient elution from $H_2O$ (300ml) in mixing chamber to 0.06 M $NH_4OAc$ buffer (PH 6.50, 300ml) in reservoir. Fractions of 4ml each were collected at a flow rate of 2ml/min with an automatic fraction collector and the absorbancy of each fraction was determined at 230nm (Fig. 5). The eluates in tubes No. 84 to 108 containing the heptapeptide were pooled, evaporated in

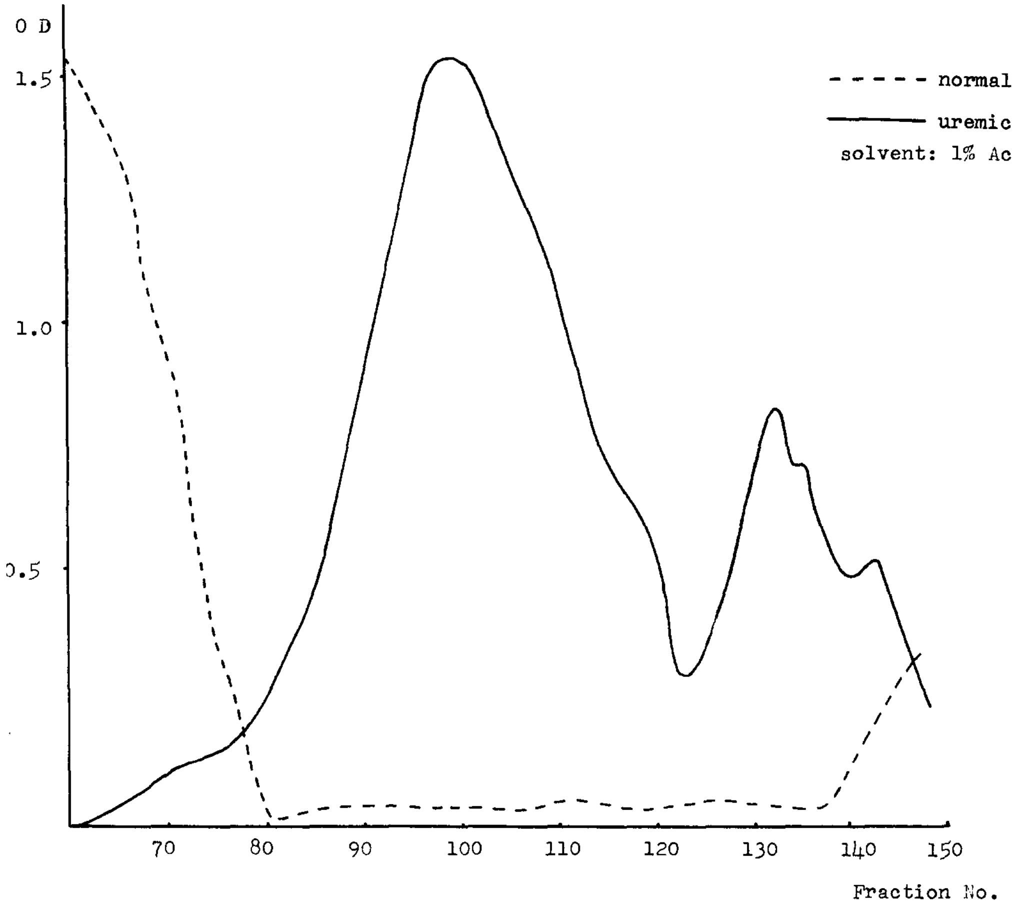


Fig. 1 Elution pattern of Sephadex G-25 (230nm)

vacuum and lyophilized. Yield 40mg, mp 146-151°, (α)$^{26}_{D}$ -38° (c= 0.6, $H_2O$), Rf$^1$ 0.26, Rf$^2$ 0.31 single ninhydrin and Pauly positive spot. Amino acid ratios in the acid hydrolysate by 5.5N HCl; Pro 0.92, His 0.93, Ala 1.01, Glu 0.89, Asp 0.93, Gly 1.02, Lys 0.82.

6 Edman sequence analysis

For the sequence analysis of the heptapeptide manual Edman procedure (12) was used. The peptide sample (1.5 µmoles) was dissolved in 0.3ml of pyridine-water (3:2 V/V) containing dimethylallylamine and adjusted to PH 9.5 with trifluoroacetic acid (TFA). After addition of 15 µl phenylisothiocyanate, the tube was flushed with a gentle stream of $N_2$ for 5-10 sec, stoppered and left at 40° for 1hr. The solution was then extracted 3 times with 1.2ml of benzene. The aqueous phase was subsequently freeze-dried and remaining reagents removed by sublimation in vacuum at 50° for 15min with solid $CO_2$-ethanol as cold trap. Cleavage was performed in 1.5 µl of TFA for 20min at 40°. The residual peptide was precipitated with 0.6ml of ethylenedichloride and washed with 0.6ml of ethylenedichloride. The precipitate was dried over night in vacuum over $P_2O_5$ and KOH and used for the next degradation cycle. The intermediate

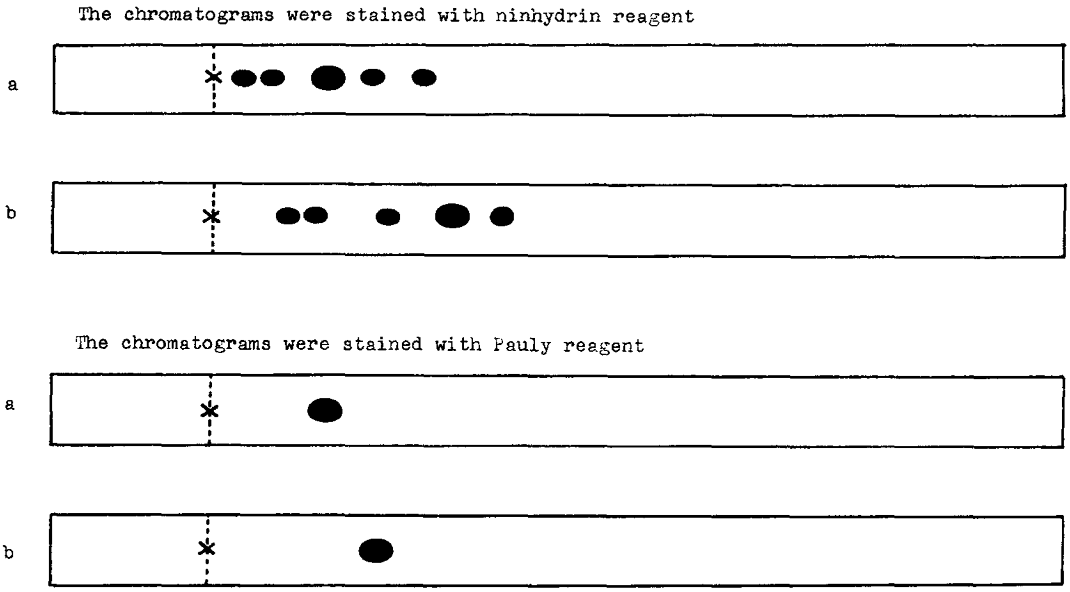


Fig. 2 Paper chromatograms of Fraction No. 80-120; Toyo Roshi No. 51, solvent: a= Partridge system, b= Waley system.

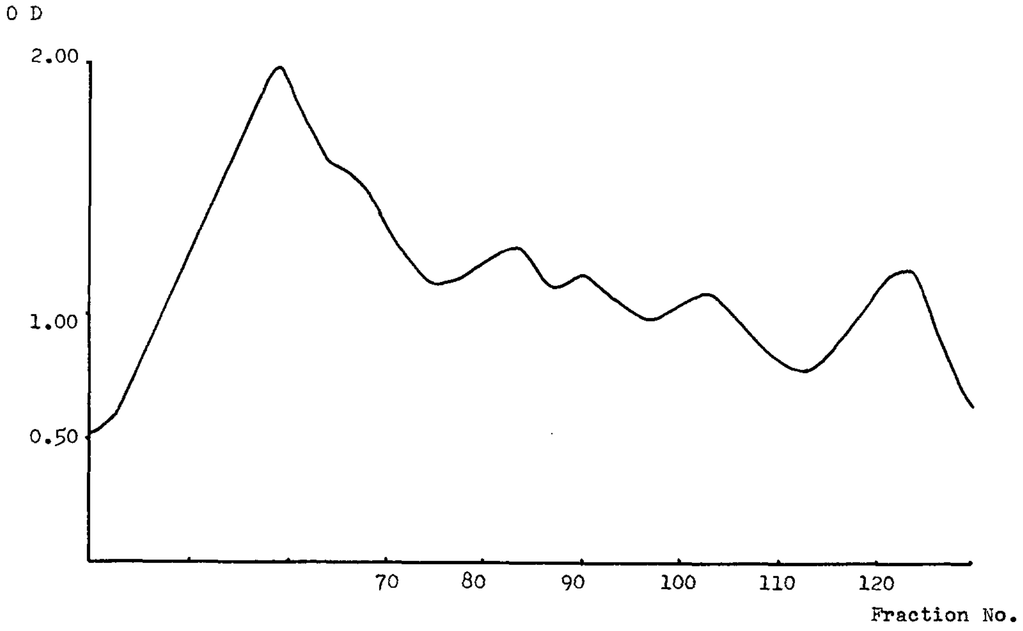
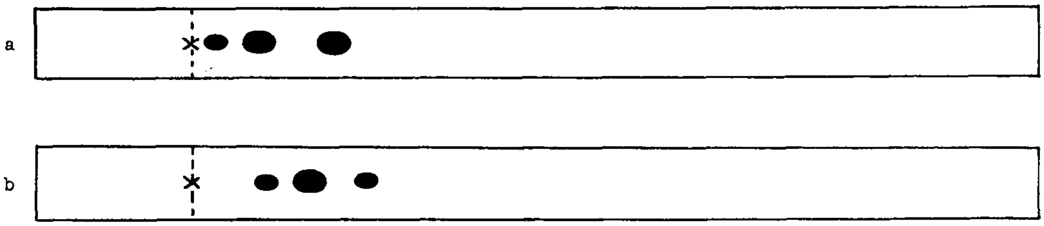


Fig. 3 Elution pattern of Sephadex G-15 (230nm)

The chromatograms were stained with ninhydrin reagent



The chromatograms were stained with Pauly reagent

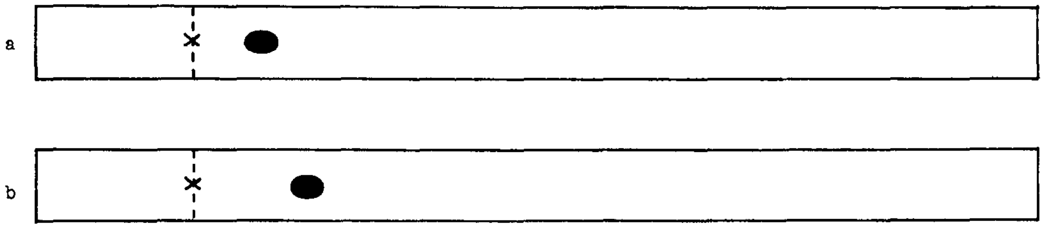


Fig. 4 Paper chromatograms of Fraction No. 40-75; Toyo Roshi No. 51, solvent: a= Partridge system, b= Waley system.

thiazolinone contained in the ethylenedichloride phase was without delay brought to dryness and converted to the corresponding thiohydantoin by treatment with 0.3ml of 1N HCl at 80° for 10min. The thiohydantoin derivative was extracted two times with 0.5ml of EtOAc. The extract was brought to dryness and dissolved in EtOAc or 90% AcOH. The amino acid phenylthiohydantoins contained in the organic phase were routinely identified by thin-layer chromatography in silica gel (containing 1% starch, 0.1% EDTA and fluorescence indicator) in $CH_3Cl$:MeOH (90:10) solvent system.
The results of this degradation study indicated the following sequence:

H-His-Pro-Ala-Glu-Asn-Gly-Lys-OH

## Synthesis of H-His-Pro-Ala-Glu-Asn-Gly-Lys-OH

The heptapeptide was synthesized as authentic specimen for identification of the isolated peptide (Fig. 6).
Unless otherwise mentioned, Z-group of the protected peptides were deblocked with catalytic hydrogenation in the presence of 10% Pd-C and Boc-group with TFA and the resulting amino components were chromatographed on filter paper.

Boc-Asn-Gly-Lys(Z)-OBzl (I)----- Boc-Gly-Lys(Z)-OBzl (13) (3.8g) was treated with TFA (5.4ml) and anisole at room temperature for 30min and dry $Et_2O$ was added. The resulting powder was dried over KOH pellets in vacuo for 1hr and then dissolved in dimethylformamide (DMF) (30.0ml) together with $Et_3N$ (1.1ml). To this solution were added Boc-Asn-ONp (14)

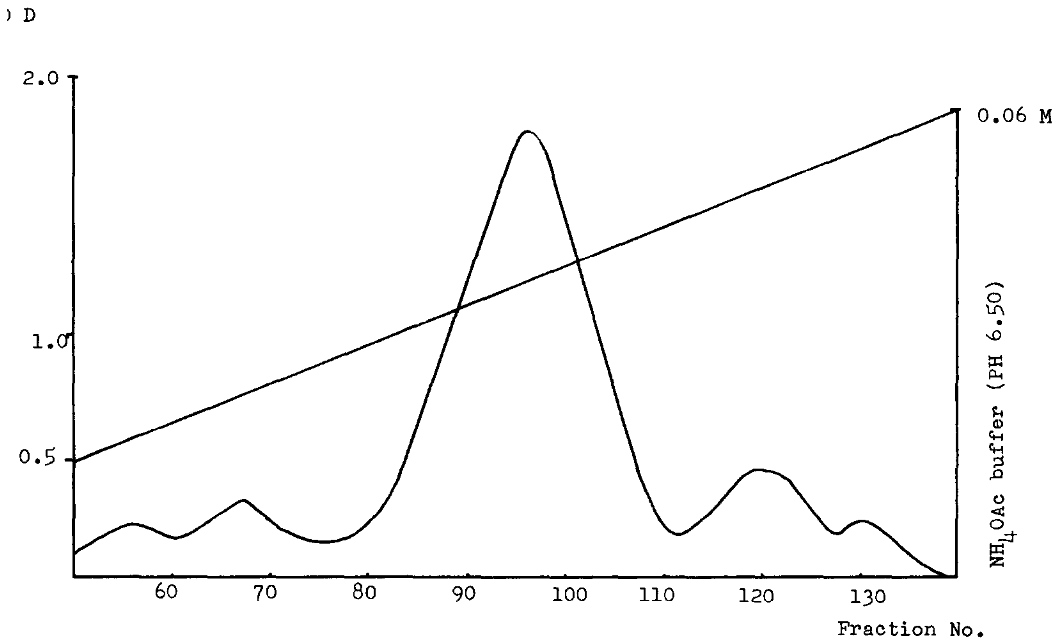


Fig. 5 Elution pattern of CM-Sephadex C-25

(2.8g) with stirring at room temperature, the reaction mixture was diluted with 1N $NH_4OH$ (3.0ml) with stirring. After 1hr, the mixture was extracted with EtOAc, washed with 1N $NH_4OH$, $H_2O$, 1N citric acid and $H_2O$, dried over $MgSO_4$ and then the solvent was evaporated. The residue was precipitated from EtOAc and $Et_2O$. Yield 3.0g (65%), mp 55-59°, $(\alpha)_D^{22}$ -15. (c=1.0, DMF), Anal. Calcd. for $C_{32}H_{43}O_9N_5$: C, 59.89; H, 6.76; N, 10.92. Found: C, 59.52; H, 7.01; N, 10.89. De-Boc peptide ester, $Rf^1$ 0.76, $Rf^2$ 0.84 single ninhydrin positive spot.

Boc-Glu(OBzl)-Asn-Gly-Lys(Z)-OBzl (II)----- I (2.000g) was treated with TFA (3.0ml) and anisole in the same manner as described I. To an ice-cold solution of the resulting H-Glu(OBzl)-Asn-Gly-Lys(Z)-OBzl, Boc-Glu(OBzl)-OH (15) (1.200g) and $Et_3N$ (0.5ml) in DMF (15.0ml) were added N-hydroxy 5-norbornene-2,3-dicarboximide (HONB) (16) (0.600g) and dicyclohexylcarbodiimide (DCC) (0.700g) with stirring. The mixture was stirred at 0° for 2hr and additional 12hr at 4°. The reaction mixture was filtered

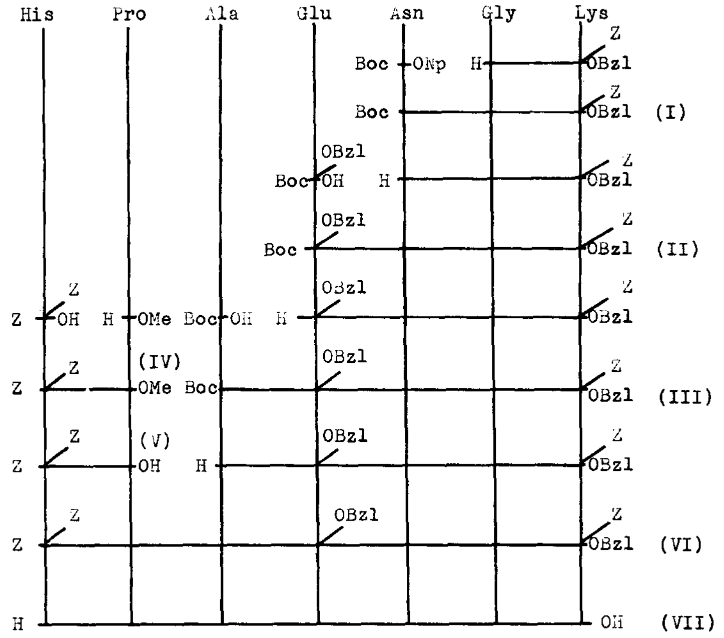


Fig. 6 Synthetic route for H-His-Pro-Ala-Glu-Asn-Gly-Lys-OH

off to remove the formed DCU and the filtrate was diluted with EtOAc. The EtOAc-extracts were washed with 1N $NaHCO_3$, $H_2O$, 1N citric acid and $H_2O$, dried over $MgSO_4$ and evaporated to dryness in vacuum. The residue was precipitated from EtOAc and $Et_2O$. Yield 1.4g (52%), mp 106-111°, $(\alpha)_D^{26}$ -12.0° (c= 1.0, DMF), Anal. Calcd. for $C_{44}H_{56}O_{12}N_6$: C, 61.14; H, 6.56; N, 9.76. Found: C, 61.01; H, 6.96; N, 9.35. De-Boc peptide ester $Rf^1$ 0.60, $Rf^2$ 0.80 single ninhydrin positive spot.

Boc-Ala-Glu(OBzl)-Asn-Gly-Lys(Z)-OBzl (III)----- The compound was prepared from II (1.000g), DCC (0.240g), HONB (0.220g) and Boc-Ala-OH (0.230g) essentially in the same manner as described in the preparation of II. The product was recrystallized from EtOAc. Yield 0.638g (58%), mp 125-130°, $(\alpha)_D^{25}$ -9.0° (c= 1.0, DMF), Anal. Calcd. for $C_{47}H_{61}O_{13}N_7$: C, 60.57; H, 6.60; N, 10.52. Found: C, 60.91; H, 7.01; N, 10.13. De-Boc peptide ester $Rf^1$ 0.73, $Rf^2$ 0.89 single ninhydrin positive spot.

Z-His(Z)-Pro-OMe (IV)----- The compound was prepared from H-Pro-OMeHCl

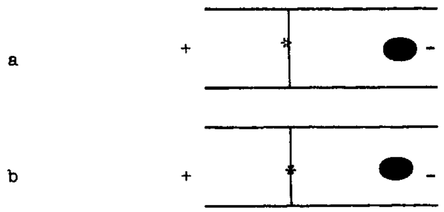


Fig. 7 Paper electrophoresis of heptapiptide (a) and synthetic heptapeptide (b). Electrophoresis was carried out on Toyo Roshi No. 51 (2.0X40.0cm) using acetate buffer PH 2.8, at a potential gradient of 60V/cm for 120min, Ninhydrin and Pauly positive spot.

(414mg) and Z-His(Z)-OH (17) (1.100mg), DCC (566mg) and HONB (494mg) essentially in the same manner as described in the preparation of II. The product was precipitated from EtOAc and petroleum ether. Yield 711mg (55%), mp 82-87°, $(\alpha)_D^{22}$ -40.0° (c= 1.0, DMF), Anal. Calcd. for $C_{28}H_{28}O_7N_4$ C, 63.15; H, 5.30; N, 10.52. Found: C, 62.89; H, 5.78; N, 10.89. De-Z peptide ester $Rf^1$ 0.18, $Rf^2$ 0.38 single ninhydrin and Pauly positive spot.

Z-His(Z)-Pro-OH (V)----- The protected dipeptide ester IV (200mg) in dioxane (2.0ml) was saponified with 1N NaOH (0.4ml) for 1hr at room temperature and diluted with $H_2O$ (5ml). The solution was washed with EtOAc twice and aqueous layer was acidified with 4N HCl to Congo red and saturated with NaCl. The precipitate was extracted with EtOAc and the solution was washed with $H_2O$. The EtOAc layer was dried over $MgSO_4$ and the solvent was precipitated from EtOAc and $Et_2O$. Yield 100mg (56%), mp 72-76°, $(\alpha)_D^{22}$ -48.0° (c= 0.8, DMF), Anal. Calcd. for $C_{27}H_{26}O_7N_4$: C, 62.54; H, 5.06; N, 10.81. Found: C, 62.09; H, 5.42; N, 10.92. De-Z peptid ester $Rf^1$ 0.09, $Rf^2$ 0.19 single ninhydrin and Pauly positive spot.

Z-His(Z)-Pro-Ala-Glu(OBzl)-Asn-Gly-Lys(Z)-OBzl (VI)----- The compound was prepared from III (233mg), DCC (50mg), HONB (50mg) and V (140mg) essentially in the same manner as described in the preparation of II. The product was recrystallized from EtOAc. Yield 175mg (52%), mp 130-134°, $(\alpha)_D^{25}$-8.0° (c= 1.0, DMF), Anal. Calcd. for $C_{69}H_{79}O_{17}N_{11}$: C, 62.10; H, 5.60; N, 11.55. Found: C, 61.71; H, 6.01; N, 11.09.

H-His-Pro-Ala-Glu-Asn-Gly-Lys-OH (VII)----- The fully protected heptapeptide VI (100mg) was hydrogenated in 50% AcOH (12ml) over 10% Pd-C for 18hr. The catalyst was removed by the aid of Cellite. The solution was evaporated to dryness and the residue was dried over KOH pellets in vacuum. The solution of the crude product in $H_2O$ (10ml) was added to a Sephadex G-15 column (1.8X42.0cm) which was eluted with 1% AcOH. Fractions of 5ml each were collected at a flow rate of 1ml/min with an automatic fraction collector and the absorbancy of each fraction was determined at 230nm. The eluates in tubes No. 21 to 31 were pooled, evaporated to dryness in vacuum and lyophilized. $NH_4OAc$ was removed by repeated lyophilization to constant weight. Yield 51mg (91%), mp 149-158°, $(\alpha)_D^{25}$ -40° (c= 0.8, $H_2O$), $Rf^1$ 0.23, $Rf^2$ 0.33 single ninhydrin and Pauly positive spot; amino acid ratios in the acid hydrolysate: His 0.89, Pro 0.91, Ala 0.91, Glu 0.86, Asp 0.99, Gly 1.01, Lys 1.02.

### High voltage paper electrophoresis

The heptapeptide obtained by gel filtration on CM-Sephadex C-25 was subjected to paper electrophoresis. The electrogram of heptapeptide was compared with that of synthetic heptapeptide (Fig. 7 ). The isolated peptide and the synthetic peptide had identical movement values in the electrograms used (Fig. 7 ).

## ACKNOWLEDGEMENT

We wish to thank Miss H. Asano for performing amino acid analysis. Thanks are also due to the Central analysis, Room of Pharmaceutical Institute, Tohoku University for elemental analysis.

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