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Studies on Separation of Amino Acids and Related Compounds. VII. Separation of L-Aspartyl-(α,β)-L-histidine and of L-Glutamyl-(α,γ)-L-histidine[1,2]

Mitsuteru HIRATA,* Kosaku NODA,** and Nobuo IZUMIYA***

Laboratory of Biochemistry, Faculty of Science, Kyushu University, Hakozaki, Fukuoka

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As control compounds, the structural α- and ω-isomers of L-aspartyl- and L-glutamyl-L-histidine were synthesized by a conventional manner. The experiments on a column chromatography of Dowex 50 were carried out with a model mixture containing α- and ω-isomer, and the complete separation of the isomers was observed at acidic condition. A mixture of α- and ω-isomers of L-aspartyl- or L-glutamyl-L-histidine prepared through the coupling of benzyloxycarbonyl-L-aspartic or glutamic acid anhydride with L-histidine were separated into each isomer with a large column in moderate yields.

An α-aspartyl or α-glutamyl dipeptide is synthesized without any special difficulty by the coupling of benzyloxycarbonyl-ω-benzyl-aspartic or -glutamic acid with an amino acid benzyl ester and subsequent deprotection from a coupling product.[3] Similarly, benzyloxycarbonyl-α-benzyl-aspartic or -glutamic acid is used for synthesis of a β-aspartyl or γ-glutamyl peptide.[3] An intramolecular anhydride[4] (XIV) is derived from benzyloxycarbonyl-L-aspartic acid by the action of acetic anhydride. Coupling reaction of the anhydride (XIV) with an amino acid ester produces a mixture of α- and β-dipeptide derivative, and the latter can be removed from the reaction mixture by extraction with sodium bicarbonate solution.[3] Similarly, benzyloxycarbonyl-L-glutamic acid anhydride[5] (XVI) is used for synthesis of α- and γ-glutamyl peptide.[3] Although the preparation of the anhydride (XIV or XVI) is much easier than that of benzyloxycarbonyl-α or ω-benzyl-aspartic or glutamic acid, the application of the anhydride for synthesis of α- or ω-peptide is restricted by the low yields and the elaborate preparative efforts.

We attempted to develop a convenient procedure separating α- and ω-structural isomers of L-aspartyl or L-glutamyl dipeptide by a column chromatography. We selected L-aspartyl- and L-glutamyl-L-histidine in this study because some of these dipeptides seemed interesting from the stand point of biochemical materials; β-L-aspartyl-L-histidine (VII) is a normal constituent of human urine,[6] and the VII and α-L-glutamyl-L-histidine (VI) possess tetanus toxic activities.[7]

* Present address: Tokyo Research Laboratories, Kowa Co. Ltd., Higashimurayama, Tokyo.

** Present address: LBS-NICHD, National Institutes of Health, Bethesda, Md., U. S. A.

*** To whom the reprint request should be addressed.

1) Part VI of this series: M. Muraoka and N. Izumiya, *Memoirs Fac. Sci., Kyushu Univ., Series C*, **7**, 133 (1970).

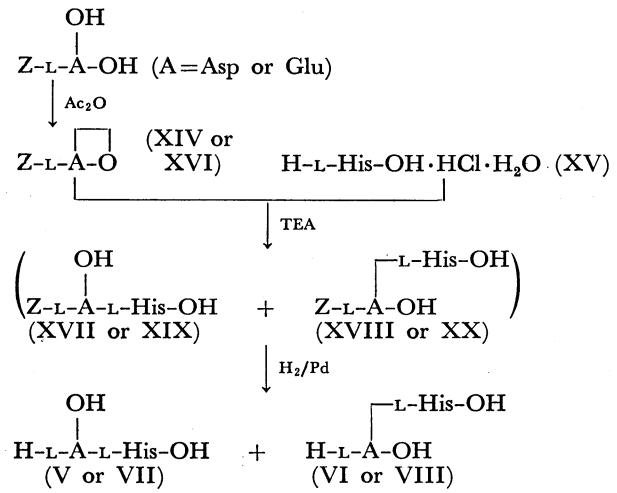
2) Presented at the 24th Annual Meeting of the Chemical Society of Japan, Osaka, April, 1971.

3) J. P. Greenstein and M. Winitz, "Chemistry on the Amino Acids," Vol. 2, John Wiley & Sons New York (1961), p. 1092; E. Schröder and K. Lübke, "The Peptides," Vol. 1, Academic Press, New York (1965), p. 181.

4) W. J. Le Quesne and G. T. Young, *J. Chem. Soc.*, **1952**, 24.

5) W. J. Le Quesne and G. T. Young, *ibid.*, **1950**, 1954.

6) Y. Kakimoto and M. D. Armstrong, *J. Biol. Chem.*, **236** 3280 (1961).

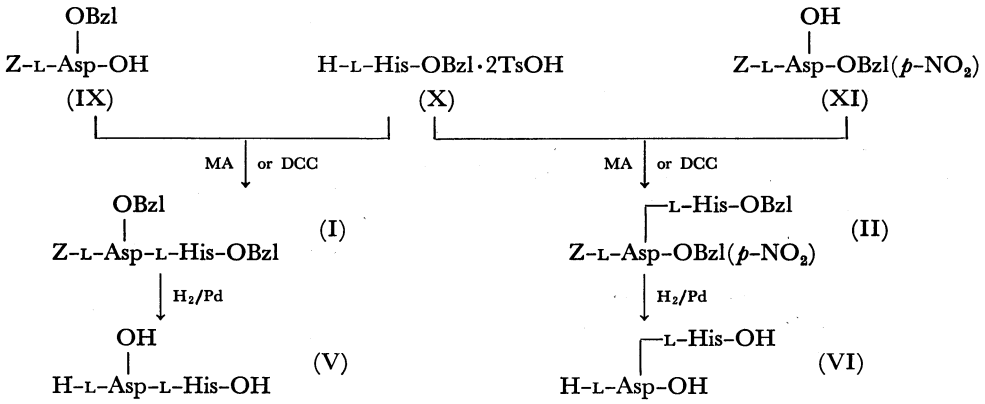


Scheme 1.

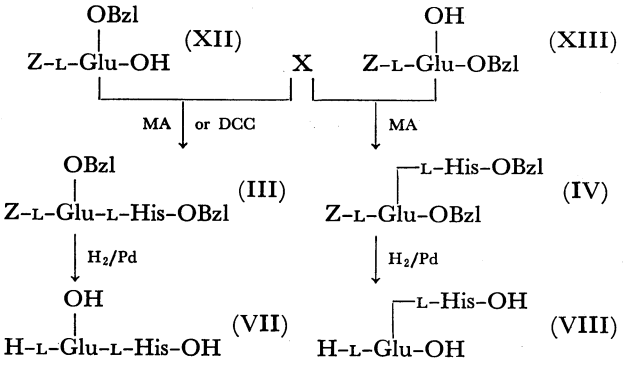
The reaction route in this study is shown in Scheme 1.[8] A coupling reaction of the anhydride (XIV or XVI) and L-histidine will produce a mixture of XVII and XVIII or XIX and XX. A whole reaction mixture is subjected to hydrogenolysis and each isomer in a hydrogenated mixture is attempted to separate by the application of a Dowex 50 column.

It should be noted that Kornguth *et al.* reported the separation of a mixture of ε-(α-L-glutamyl)-L-lysine and ε-(γ-L-glutamyl)-L-lysine by an amino acid analyzer.[9] We reported the experimental results to separate the leucyl dipeptide diastereomers by a Dowex column in the analytical and preparative scale,[10] and to separate glycyl tripeptide diastereomers by an amino acid analyzer.[11]

Each isomer of L-aspartyl- and L-glutamyl-L-histidine were prepared in the conventional manner



Scheme 2.



Scheme 3.

shown in Schems 2 and 3. The acid (XI) was used instead of benzyloxycarbonyl-α-benzyl-L-aspartic acid because the preparation[12] of the latter is considerably troublesome compared with that of XI or XIII. It was indicated that a synthetic isomer gave single spot on a paper chromatogram, but the R_f value was different from that of the corresponding isomer (Table 1).

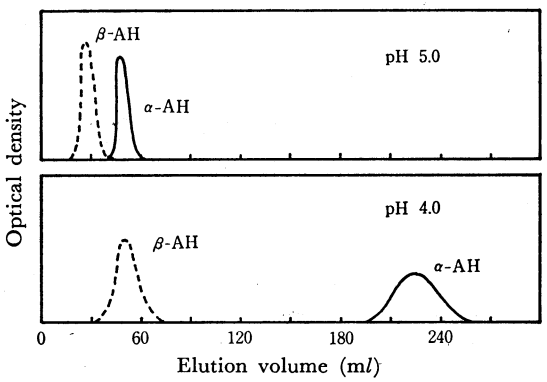


Fig. 1. Effect of pH in buffer system on separation of Asp-His.

7) J. H. Mueller and P. A. Miller, *ibid.*, **223**, 185 (1956).

8) Abbreviations: Z, benzyloxycarbonyl; OBzl, benzyl ester; OBzl(*p*-NO_2), *p*-nitrobenzyl ester; DCC, dicyclohexylcarbodiimide; MA, mixed anhydride; TEA, triethylamine; DMF, dimethylformamide; THF, tetrahydrofuran.

9) M. L. Kornguth, A. Neidle, and H. Waelsch, *Biochemistry*, **2**, 740 (1963).

10) K. Noda, H. Okai, T. Kato, and N. Izumiya, This Bulletin, **41**, 401 (1968).

11) M. Muraoka, N. Yoshida, K. Noda, and N. Izumiya, *ibid.*, **41**, 2134 (1968); N. Izumiya and M. Muraoka, *J. Amer. Chem. Soc.*, **91**, 2391 (1969).

12) M. Bergmann, L. Zervas, and L. Salzmann, *Ber.*, **66**, 1288 (1933); G. L. Miller, O. Behrens, and V. du Vigneaud, *J. Biol. Chem.*, **140**, 411 (1941); P. M. Bryant, R. H. Moore, P. J. Pimlott, and G. T. Young, *J. Chem. Soc.*, **1959**, 3868.

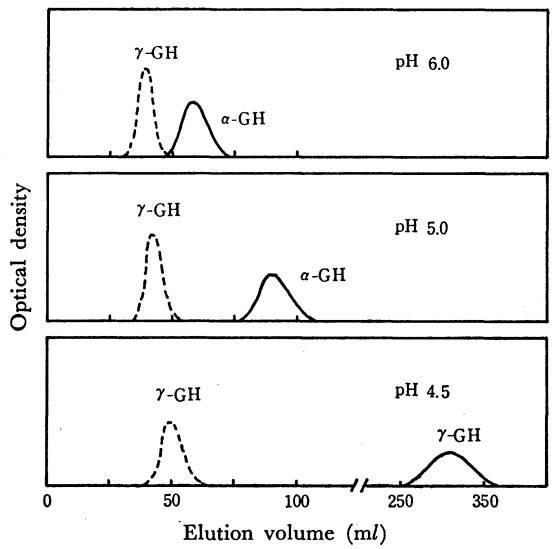


Fig. 2. Effect of pH in buffer system on separation of Glu-His.

Preliminary experiments on a model mixture were performed with a small column (0.9×50 cm) of Dowex 50 using 0.2 N ammonium acetate buffers at different pH, in order to find an optimal condition for the separation. The elution patterns of a model mixture composed of two isomers in the same amount were indicated in Figs. 1 and 2. The α- and γ-isomer of glutamyl-histidine in the mixture were separated each other more efficiently in general than the α- and β-aspartyl-histidine. It was observed that peaks of the free amino acids, aspartic acid and histidine, did not overlap with those of α- and β-aspartyl-histidine when a buffer of pH 4.0 or 5.0 was used. Similarly, peaks of glutamic acid and histidine did not overlap with those of the α- and γ-dipeptide when a buffer of pH 4.5, 5.0 or 6.0 was used.

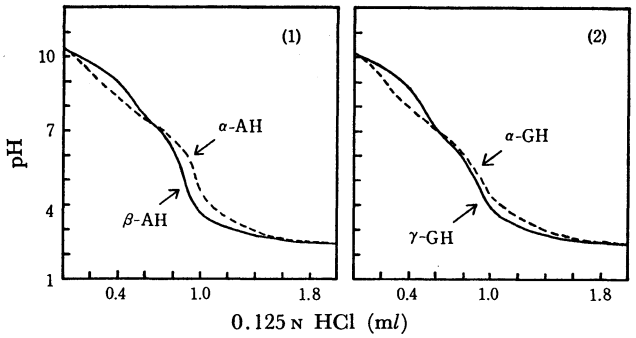


Fig. 3. pH Titration curve of Asp-His (1) and Glu-His (2).

In a previous paper,[10] it was observed that an L-L diastereomer of leucyl-alanine with a solvent of pH 4.5 in a Dowex 50 column was eluted more slowly than the corresponding L-D isomer, whereas the L-L isomer with a solvent of pH 5.5 was eluted rapidly than the L-D. It was also observed that the titration curves for the diastereomers crossed each other around pH 5.0. Then, it was suggested that the positions of peaks were correlated with the pH titration curves. In this study, the titration of α- and ω-isomer or L-aspartyl- or L-glutamyl-L-histidine indicated that the titration curves crossed each other around pH 7 in both cases (Fig. 3). Therefore, separation studies of the model mixture with the small column were performed with solvents of pH 7.0, 8.0 and 9.0; it was found that each of the peptides was eluted very rapidly near to a void volume and not separated each other. Thus, the correlation between the peak positions and the pH titration curves by α- and ω-isomer in this study could not be established.

As the experiments on a preparative scale, two materials which prepared following Scheme 1 were tried to separate into α- and ω-isomer with a large column (1.8×110 cm) of Dowex 50. Each material was composed of four ninhydrin positive components, namely α- and ω-isomer of L-aspartyl or L-glutamyl-L-histidine as majors and aspartic or glutamic acid and histidine as minors. Although a buffer of pH 4.0 or 4.5 might be an efficient solvent for complete separation (see, Figs. 1 and 2), we used a pH 5.0 buffer for an economy of running time. When the material derived from 5 mmol of benzyloxycarbonyl-L-aspartic acid anhydride (XIV) was applied for the column with the pH 5.0 buffer, pure α- and β-L-aspartyl-L-histidine were isolated in 31% and 22% yield respectively. In the similar manner, pure α- and γ-L-glutamyl-L-histidine were isolated in 21% and 28%. Thus, it was indicated that the structural isomers of L-aspartyl- and L-glutamyl-L-histidine can be prepared conveniently by the use of the anhydride and following application of a column chromatography.

Experimental

The R_f(tlc) refers to thin layer chromatography with a solvent of chloroform–methanol (5 : 1, v/v).

α-(Benzyloxycarbonyl-β-benzyl)-L-aspartyl-L-histidine Benzyl Ester (I). (a) By MA Method: To a solution of benzyloxycarbonyl-β-benzyl-L-aspartic acid[13] (IX) (3.57 g, 10 mmol) and TEA (1.4 ml, 10 mmol) in THF (20 ml) at −5°C was added isobutyl chloroformate (1.31 ml, 10 mmol). After 15 min, a mixture of L-histidine benzyl ester di-*p*-toluenesulfonate[14] (X) (5.90 g, 10 mmol) and TEA (2.8 ml, 20 mmol) in DMF (20 ml) was added to the solution. The mixture was allowed to stand overnight at room temperature, and then evaporated to dryness *in vacuo*. After the residue was dissolved in ethyl acetate (30 ml), the solution was washed successively with 4% sodium bicarbonate and water, dried over sodium sulfate, and then evaporated. The product was obtained as a semi-solid; yield, 3.1 g (53%); R_f (tlc) 0.59.

(b) By DCC Method: To a solution of IX (0.71 g, 2 mmol) and X (1.18 g, 2 mmol) in DMF (10 ml) were added TEA (0.56 ml, 4 mmol) and DCC (0.41 g, 2 mmol) at 0°C. After the mixture had been stirred overnight at room temperature, it was evaporated and ethyl acetate was added to residue. After dicyclohexylurea was filtered off, the filtrate was washed with 4% sodium bicarbonate and water, dried, and evaporated to give a semi-solid; 0.78 g (67%); R_f(tlc) 0.59.

13) N. Izumiya, H. Uchio, and T. Yamashita, *Nippon Kagaku Zasshi*, **79**, 420 (1958).

14) S. Akabori, S. Sakakibara, and S. Shina, This Bulletin, **31**, 784 (1958).

β-(Benzyloxycarbonyl-α-p-nitrobenzyl)-L-aspartyl-L-histidine Benzyl Ester (II). Benzyloxycarbonyl-α-*p*-nitrobenzyl-L-aspartic acid[15] (XI) (4.02 g, 10 mmol) and X (5.9 g, 10 mmol) were coupled by the MA method as described for the preparation (method a) of I; yield of an oily product, 3.28 g (52%); R_f(tlc) 0.61. The same compound (II) was prepared by the DCC method from XI and X as described for the preparation (method b) of I; yield of an oil, 57%; R_f (tlc) 0.61.

α-(Benzyloxycarbonyl-γ-benzyl)-L-glutamyl-L-histidine Benzyl Ester (III). (a) By MA Method: Benzyloxycarbonyl-γ-benzyl-L-glutamic acid[16] (XII) (3.71 g, 10 mmol) and X (5.9 g, 10 mmol) was coupled as described for the preparation (method a) of I, and the resulting crude product was recrystallized from ethyl acetate–ether–petroleum ether; yield, 2.7 g (45%); mp 92—93°C; $[\alpha]_D^{25}$ −11.0° (*c* 2, ethanol); R_f (tlc) 0.54.

Found: C, 65.70; H, 5.95; N, 9.36%. Calcd for $C_{33}H_{34}O_7N_4$: C, 65.51; H, 5.84; N, 9.55%.

(b) By DCC Method: III was prepared from XII and X; yield, 39%; mp 93—94°C; $[\alpha]_D^{25}$−11.5° (*c* 2, ethanol); R_f (tlc) 0.54 (Found: C, 65.55; H, 5.91; N, 9.32%).

γ-(Benzyloxycarbonyl-α-benzyl)-L-glutamyl-L-histidine Benzyl Ester (IV). This was obtained from benzyloxycarbonyl-α-benzyl-L-glutamic acid[17] (XIII) (3.71 g, 10 mmol) and X (10 mmol) by the MA method; yield, 2.04 g (34%); mp 130—132°C; $[\alpha]_D^{25}$−13.2° (*c* 2, ethanol); R_f (tlc) 0.52.

Found: C, 65.82; H, 6.02; N, 9.27%. Calcd for $C_{33}H_{34}O_7N_4$: C, 65.51; H, 5.84; N, 9.55%.

α-L-Aspartyl-L-histidine (α-Asp-His or α-AH) (V). A solution of I (2.92 g, 5 mmol) in 90% acetic acid (25 m*l*) was treated with hydrogen in the presence of palladium black. The filtrate from the catalyst was evaporated to dryness. The residual crystals were recrystallized from water-ethanol; yield, 1.15 g (80%); mp 205—207°C (decomp.); $[\alpha]_D^{25}$ +12.0° (*c* 2, water) (Found as monohydrate: C, 41.45; H, 5.81; N, 19.66%). (lit,[6]; mp 190—194°C; $[\alpha]_D$ +9.7° (water).)

β-L-Aspartyl-L-histidine (β-Asp-His or β-AH) (VI). This was obtained by hydrogenolysis of II (1.89 g, 3 mmol) as described above; yield, 0.354 g (41%); mp 234—238°C (decomp.); $[\alpha]_D^{25}$ +36.8° (*c* 2, water) (Found as monohydrate: C, 41.37; H, 5.66; N, 19.63%). (lit.[18]; mp 235—240°C; $[\alpha]_D$ +38° (water).)

α-L-Glutamyl-L-histidine (α-Glu-His or α-GH) (VII). This was obtained from III (1.8 g, 3 mmol); yield, 0.707 g (78%); mp 199—204°C (decomp.); $[\alpha]_D^{25}$ +18.5° (*c* 2, water) (Found as monohydrate: C, 43.84; H, 6.08; N, 18.26%). (lit,[19]; mp 177—178°C.)

γ-L-Glutamyl-L-histidine (γ-Glu-His or γ-GH) (VIII). This was obtained from IV (1.8 g, 3 mmol); yield, 0.435 g (48%); mp 203—205°C (decomp.); $[\alpha]_D^{25}$ +12.3° (*c* 2, water).

Found: C, 44.01; H, 6.13; N, 18.37%. Calcd for $C_{11}H_{16}O_5N_4 \cdot H_2O$: C, 43.70; H, 6.00; N, 18.54%.

Paper Chromatography of the Dipeptides. The experiments were carried out with ascending technique with Toyo Roshi No. 52 paper, and a peptide on the paper was developed with ninhydrin. It was observed that each (V, VI, VII or VIII) of synthetic dipeptides showed single spot with three different solvents. As recognized from Table 1, the isomers of Asp-His or Glu-His were clearly separated on a chromatogram.

Table 1. R_f values of dipeptides on paper chromatography

Dipeptide	R_f		
	BAPW[a]	BPA[b]	PW[c]
α-Asp-His	0.13	0.17	0.16
β-Asp-His	0.09	0.12	0.11
α-Glu-His	0.19	0.19	0.18
γ-Glu-His	0.12	0.13	0.14

a) *n*-butanol - acetic acid - pyridine - water; 4 : 1 : 1 : 2, v/v.
b) *n*-butanol - acetic acid - water; 3 : 1 : 1, v/v.
c) pyridine - water; 4 : 1, v/v.

Column Chromatography of Model Mixture Composed of Isomeric Dipeptides. A model mixture of Asp-His was made by dissolving each 0.05 mmol of V and VI in water (0.5 m*l*). Similarly, a model mixture of Glu-His (VII and VIII) was prepared. A column (0.9×50 cm) with Dowex 50×8 (200—400 mesh), NH_4^+ form, was equilibrated by washing with 0.2 N ammonium acetate buffer of a definite pH (4.0, 4.5, 5.0 or 6.0), and a model mixture was applied to a column. The column was eluted with the same buffer at room temperature, and two m*l* fractions were collected at a flow rate of about 20 m*l*/hr. Approximately 0.01 m*l* from each tube was spotted on a strip of filter paper. The strip was dried at 100°C for 5 min, immersed in a 0.2% ninhydrin-acetone solution, and again heated. The degrees of coloration developed were determined by a densitometer as described previously.[20] The resulting patterns are shown in Figs. 1 and 2. The nature of a peptide in each peak in Figs. 1 and 2 was identified by means of paper chromatography after the corresponding fractions were concentrated by evaporation.

pH Titration Curve of the Dipeptides. Each isomer (0.05 mmol) of Asp-His and Glu-His was dissolved in 0.1 N sodium hydroxide (1 m*l*), and an automatic titration was carried out with 0.125 N hydrochloric acid with Radiomer pH-stat, model TTT-1, at room temperature. The resulting patterns are shown in Fig. 3.

Preparative Separation of α-Asp-His and β-Asp-His from a Mixture. A solution of benzyloxycarbonyl-L-aspartic acid anhydride[4] (XIV) (1.25 g, 5 mmol) in THF (5 m*l*) was added to a solution of L-histidine monohydrochloride monohydrate (XV) (1.05 g, 5 mmol) and TEA (2.8 m*l*, 20 mmol) in water (10 m*l*) under vigorous stirring at room temperature. After 15 min, the reaction mixture was evaporated to dryness and the residue was dissolved in 90% acetic acid (30 m*l*). The solution was treated with hydrogen in the presence of palladium black. Paper chromatography on the hydrogenated solution showed the presence of α-Asp-His, β-Asp-His, aspartic acid and histidine. The filtrate from the catalyst was evaporated to dryness, and the residue was dissolved in 0.2 N ammonium acetate buffer (1 m*l*) of pH 5.0. The solution was put on a column (1.8×110 cm) of Dowex 50×8 treated previously with the same buffer. The column was eluted with the same buffer (650 m*l*) at a flow rate of 60 m*l*/hr, and each 10 m*l* was fractionated (No. 1—No. 65). Then, the eluting solvent was changed to 2 N ammonium hydroxide. The material in each fraction was identified by means of paper chromatography; it was found

15) E. Schröder and E. Klieger, *Ann. Chem.*, **673**, 208 (1964).
16) W. E. Hanby, S. G. Waley, and J. Watson, *J. Chem. Soc.*, **1950**, 3239.
17) E. Klieger and H. Gibian, *Ann. Chem.*, **655**, 195 (1962).
18) V. du Vigneaud and M. Hunt, *J. Biol. Chem.*, **125**, 269 (1938).
19) F. Schneider, *Z. Physiol. Chem.*, **320**, 82 (1960).
20) H. Aoyagi, H. Okai, M. Ohno, and N. Izumiya, *Nippon Kagaku Zasshi*, **85**, 656 (1964); H. Aoyagi, M. Ohno, N. Izumiya, and B. Witkop, *J. Org. Chem.*, **29**, 1382 (1964).

that the fractions 13—15 contained aspartic acid, the 28—40 β-Asp-His, the 48—61 α-Asp-His and the 70—73 histidine. The fractions from tube number 48 to 61 were evaporated *in vacuo* to dryness. To remove the ammonium acetate contaminated, the residue dissolved in water was treated with a column of Dowex 50×8, H^+ form, and the column was washed with water and eluted with aqueous ammonium hydroxide. The eluate was evaporated, and the residual crystals were recrystallized from water–ethanol; yield of α-Asp-His monohydrate, 440 mg (31% from XIV); mp 205—208°C (decomp.); $[\alpha]_D^{25}$ +12.5° (*c* 2, water) (Found: C, 41.37; H, 5.64; N, 19.70%). A mixture of this product and an authentic sample obtained from I showed a single spot in paper chromatography with different solvent systems.

The fractions from 28 to 40 were treated in the same manner described above to give pure β-Asp-His monohydrate, 308 mg (22% from XIV); mp 234—237°C (decomp.); $[\alpha]_D^{25}$ +37.2° (*c* 2, water) (Found: C, 41.42; H, 5.51; N, 19.66%).

Preparative Separation of α-Glu-His and γ-Glu-His from a Mixture. Benzyloxycarbonyl-L-glutamic acid anhydride[5] (XVI) (1.32 g, 5 mmol) was coupled with XV (5 mmol), and the reaction mixture was treated exactly in the same manner as described above. As only one exception, the eluting solvent was changed from 0.2 N ammonium acetate buffer (pH 5.0) to 2 N ammonium hydroxide at the fraction number 85. It was found that the fractions 10—12 contained glutamic acid, the 16—24 γ-Glu-His, the 59—81 α-Glu-His and the 90—94 histidine. The fractions from 59 to 81 were treated in the same manner as described above to give pure α-Glu-His monohydrate, 314 mg (21% from XVI); mp 202—205°C (decomp.); $[\alpha]_D^{25}$ +18.2° (*c* 2, water) (Found: C, 43.60; H, 6.19; N, 18.75%).

The fractions from 16 to 24 yielded 409 mg (28%) of pure γ-Glu-His monohydrate; mp 205—207°C (decomp.); $[\alpha]_D^{25}$ +11.6° (*c* 2, water) (Found: C, 43.52; H, 6.12; N, 18.66%).

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