BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 41 2134—2140 (1968)

Studies on Separation of Amino Acids and Related Compounds. IV. Separation of Diastereomeric Tripeptides Containing Basic and Acidic Amino Acid Residue[1]

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(Received March 4, 1968)

Twelve pure tripeptides have been synthesized and the separation of each mixture composed of two diastereomeric tripeptides with an amino acid analyzer has been studied. Among six model systems of diastereomeric mixture studied, a mixture of the LL and LD isomers of glycyl-ornithyl-glutamic acid was separated most efficiently.

The several methods of measuring the extent of racemization of the step of the peptide bond formation have been reported in the literature.[2] Anderson *et al.*[3] found that benzyloxycarbonyl-glycyl-L-phenylalanyl-glycine ethyl ester can be separated from the racemate by fractional crystallization in which the less soluble racemate separates in the first crops; many investigators have used this Anderson's test for the study of racemization as a model system in different coupling procedures.[2] Williams *et al.* suggested the fractional crystallization of benzoyl-leucyl-glycine ethyl ester which is synthesized from benzoyl-L-leucine and glycine ethyl ester with a coupling procedure.[4] Clayton *et al.* indicated that a diastereomeric mixture of benzyloxycarbonyl-glycyl-alanyl-L-phenylalanyl-glycine can be separated by countercurrent distribution.[5] Weygand *et al.* have taken advantage of separating diastereomeric trifluoroacetyl-dipeptide methyl esters by gas chromatography for the study of racemization.[6] Taschner *et al.* were able to detect a small amount of the LD diastereomer in an LL dipeptide by paper chromatography; they have applied this technique in order to examine the degree of racemization caused by the activation of an acyl-dipeptide acid.[7] Halpern *et al.* have described the use of NMR procedure for detection of racemization.[8]

We have been attempting to find a convenient procedure of racemization-test with the application an of amino acid analyzer. A procedure which we have designed is as follows. When an acyl-dipeptide acid is condensed with an amino acid benzyl ester by an usual coupling method, an acyl-tripeptide benzyl ester will be obtained. The crude product is subjected directly to hydrogenolysis, and the hydrogenated material is submitted to an amino acid

$$\text{Z-Gly-L-A-OH} + \text{H-L-B-OBzl} \xrightarrow[\text{racemization of A residue)}]{\text{(with partial or complete}} \text{Z-Gly-A-B-OBzl (LL isomer + DL isomer)} \xrightarrow[H_2]{} \text{H-Gly-A-B-OH (LL isomer + DL isomer)}$$

Fig. 1. Supposed sequence of synthesis of tripeptide diastereomers.
Z-, benzyloxycarbonyl; -OBzl, benzyl ester; A or B, certatin amino acid residue bearing an asymmetrical carbon.

1) Part III of this series: K. Noda, H. Okai, T. Kato and N. Izumiya, This Bulletin, **41**, 401 (1968).
2) E. Schröder and K. Lübke, "The Peptides," Vol. I, Academic Press, New York (1966), p. 319; M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," Interscience Publishers, New York (1966), p. 137.
3) G. W. Anderson, J. Blodinger and A. D. Welcher, *J. Am. Chem. Soc.*, **74**, 5309 (1952).
4) M. W. Williams and G. T. Young, *J. Chem. Soc.*, **1964**, 881.
5) D. W. Clayton, J. A. Farrington, G. W. Kenner and J. M. Turner, *ibid.*, **1957**, 1398.
6) F. Weygand, A. Prox and W. König, *Chem. Ber.*, **99**, 1451 (1966).
7) E. Taschner, A. Chimiak, J. F. Biernat, T. Sokolowska, C. Wasielewski and B. Rzeszotarska, "Peptide Symposium," Pergamon, Oxford (1963), p. 109.
8) B. Halpern, L. F. Chew and B. Weinstein, *Tetrahedron Letters*, **1967**, 3075; *J. Am. Chem. Soc.*, **89**, 5051 (1967).

analyzer (Fig. 1). In the present paper we describe the syntheses of several model peptides and the separation experiments of each mixture of diastereomeric peptides by the analyzer.

The literature survey on the separation of dipeptide diastereomers by an amino acid analyzer, has revealed that two groups reported the separation of a mixture of alanyl-alanines.[9] We also described in the previous paper the separation of leucyl-valine diastereomers.[1] Though the model dipeptides are not true diastereomers it would be of interest to note that Kornguth *et al.* have reported the complete separation of a mixture of ε-(α-L-glutamyl)-L-lysine and ε-(γ-L-glutamyl)-L-lysine.[10] A separation study of tripeptide diastereomers by an amino acid analyzer has not been performed to date as far as we recognize.

For model peptide, we chose tripeptide, which is composed of glycine, a basic and an acidic amino acid, with the surmise that a diastereomeric mixture of this polyfunctional neutral tripeptide might be efficiently separated under an appropriate condition. Each pure diastereomer of glycyl-lysyl-glutamic acid, glycyl-lysyl-aspartic acid, glycyl-ornithyl-glutamic acid and glycyl-ornithyl-aspartic acid were prepared in the conventional manner as the first step of the study. For example, glycyl-L-lysyl-L-glutamic acid was synthesized as shown in Fig. 2. Thus eight tripeptides (XXIII—XXX) of Gly-Lys-Glu type were obtained as hygroscopic crystals and their homogeneity was ascertained by paper and thin-layer chromatographies and electrophoresis.

As the control material for the experiment with the analyzer, several dipeptides (VII—X) of Lys-Glu type were also synthesized by the usual way.

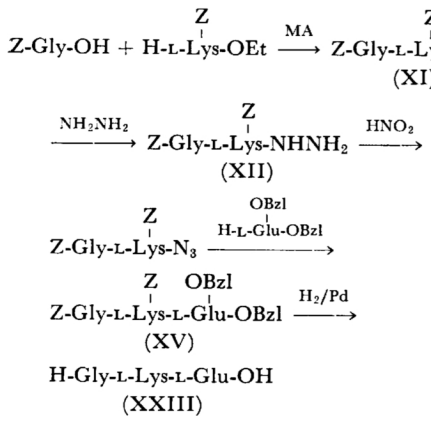

Fig. 2. Sequence of synthesis of tripeptide (XXIII).
MA, the mixed anhydride method.

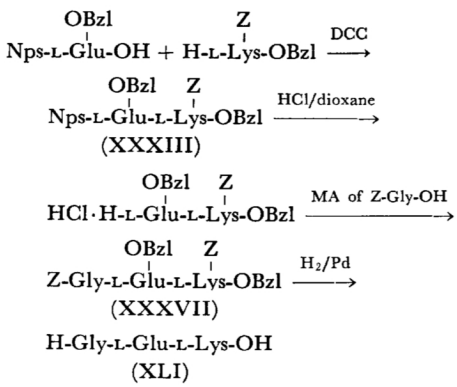

Fig. 3. Sequence of synthesis of tripeptide (XLI).
Nps-, *o*-nitrophenylsulfenyl; DCC, dicyclohexylcarbodiimide.

Since any mixture of diastereomeric tripeptides of Gly-Lys-Glu type was not separated completely by the analyzer as described later, syntheses of model tripeptides of Gly-Glu-Lys type were further attempted. Each pure diastereomer of glycyl-glutamyl-lysine and glycyl-aspartyl-lysine was prepared in the manner of the stepwise elongation to preclude racemization. For example, synthetic sequence of glycyl-L-glutamyl-L-lysine was noted in Fig. 3.

As a preliminary experiment, separation studies of mixture of diastereomeric peptides with paper chromatography and electrophoresis were performed. As noted in Table 1, it was observed that the mixture of dipeptide diastereomers was separated each other more efficiently in general than a mixture of tripeptides.

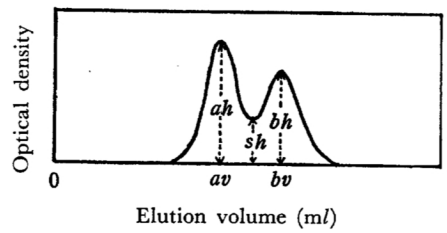

Fig. 4. Model elution pattern of a diastereomeric mixture.

A model elution pattern of a mixture composed of two diastereomers in the same amounts by an amino acid analyzer was indicated in Fig. 4. For the approximate expression of the efficiency of separation, we introduced the term rs, apparent ratio of separation; rs is calculated by the equation of $[sh/(ah+bh)\times 2]\times 100$.

Each mixture of the diastereomeric dipeptides or tripeptides was subjected to the analyzer. For the elution of columns three procedures were employed and the results obtained were summarized in Table 2. In procedure (i) the elution was carried out with a medium column (0.9×50 cm) and pH 5.28 buffer; any peptide mixture was found not to be

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TABLE 1. R_f AND R_{Gly} VALUES OF PEPTIDES ON PAPER CHROMATOGRAPHY AND ELECTROPHORESIS

Compound		Paper chromatography R_f[b]	Paper electrophoresis R_{Gly}[a]		
			pH 1.8[c]	pH 3.25[d]	pH 4.25[d]
Lys-Glu,	L-L	0.23	1.13	1.14	1.10
Lys-Glu,	L-D	0.18	1.13	1.09	1.10
Lys-Asp,	L-L	0.18	1.09	1.14	1.12
Lys-Asp,	L-D	0.17	1.09	1.10	1.12
Gly-Lys-Glu,	L-L	0.15	1.03	1.21	1.08
Gly-Lys-Glu,	L-D	0.15	1.03	1.21	1.08
Gly-Lys-Asp,	L-L	0.12	1.11	1.18	1.14
Gly-Lys-Asp,	L-D	0.12	1.11	1.18	1.14
Gly-Orn-Glu,	L-L	0.15	1.10	1.23	1.12
Gly-Orn-Glu,	L-D	0.15	1.10	1.23	1.12
Gly-Orn-Asp,	L-L	0.13	1.44	1.22	1.11
Gly-Orn-Asp,	L-D	0.13	1.44	1.22	1.11
Gly-Glu-Lys,	L-L	0.16	1.12	1.14	1.03
Gly-Glu-Lys,	D-L	0.14	1.12	1.14	1.03
Gly-Asp-Lys,	L-L	0.13	1.12	1.13	1.01
Gly-Asp-Lys,	D-L	0.12	1.12	1.13	1.01

a) Calculated relatively from a distance travelled of glycine as a control compound.
b) Solvent system: *n*-butanol - acetic acid - pyridine - water (4 : 1 : 1 : 2, v/v).
c) Solvent system: formic acid - acetic acid - methanol - water (1 : 3 : 6 : 10, v/v).
d) Solvent system: 0.2 M sodium citrate buffer.

TABLE 2. VALUES RELATING TO CHROMATOGRAPHY OF PEPTIDES WITH AMINO ACID ANALYZER

		Effluent volume (av or bv, m*l*) and apparent ratio of separation (rs, %)					
		(i)[a]		(ii)[b]		(iii)[c]	
		av or bv	rs	av or bv	rs	av or bv	rs
Lys-Glu,	L-L	46	0	62	77	411	91
Lys-Glu,	L-D	46		51		399	
Lys-Asp,	L-L	43	0	44	77	288	100
Lys-Asp,	L-D	43		36		236	
Gly-Lys-Glu,	L-L	47	0	88	0	409	50
Gly-Lys-Glu,	L-D	47		88		418	
Gly-Lys-Asp,	L-L	59	0	61	0	332	0
Gly-Lys-Asp,	L-D	59		61		332	
Gly-Orn-Glu,	L-L	41	0	74	0	397	64
Gly-Orn-Glu,	L-D	41		74		403	
Gly-Orn-Asp,	L-L	45	0	49	0	274	0
Gly-Orn-Asp,	L-D	45		49		274	
Gly-Glu-Lys,	L-L	42	0	51	0	400	0
Gly-Glu-Lys,	D-L	42		51		400	
Gly-Asp-Lys,	L-L	33	0	31	0	396	10
Gly-Asp-Lys,	D-L	33		31		393	

a) With medium column (0.9×50 cm) and 0.2 M sodium citrate buffer (pH 5.28).
b) With small column (0.6 ×10 cm) and 0.2 M sodium citrate buffer (pH 4.25).
c) With the small column, 0.2 M sodium citrate at pH 3.25 was applied until 360 m*l*, and then the system was changed to the pH 4.25 buffer.

separated. In scheme (ii) a smaller column (0.6 ×10 cm) was used and eluted with pH 4.25 buffer; any tripeptide system of Gly-Glu-Lys or Gly-Lys-Glu type was no separated though both dipeptide systems separated considerably. In scheme (iii) the elution was initiated with pH 3.25, and at fraction 360 m*l* the eluting solution was changed to pH 4.25 buffer. At first four systems of Gly-Lys-Glu type were studied; although separation of glycyl-ornithyl-glutamic acid diastereomers occurred most efficiently, the separation was not still completed (Fig. 7). Therefore, the analysis was further extended to two model systems of glycyl-glutamyl-lysine and glycyl-aspartyl-lysine; the results were

worse than that of the system of glycyl-ornithyl-glutamic acid. Several chromatographic patterns obtained were indicated in Figs. 5—7 for examples.

Though it appears that a reaction sequence to afford glycyl-ornithyl-glutamic acid from benzyloxycarbonyl-glycyl-δ-benzyloxycarbonyl-L-ornithine and L-glutamic acid dibenzyl ester can be used for the detection of racemization, the separation study of several dipeptide diastereomers, such as α-acetyl-lysyl-alanine, and tripeptide diastereomers is being continued in this laboratory in order to find a model system in better separation.

Experimental

The melting points were uncorrected. Optical rotations were determined with a Yanagimoto Photometric Polarimeter, OR-20 type.

D-Glutamic Acid Dibenzyl Ester *p*-Toluenesulfonate (I). This compound was obtained according to the procedure for the L-isomer.[11] Yield, 72%; mp 140—141°C; $[\alpha]_D^{20}$ −6.9° (*c* 2, methanol). Reported value for the L-isomer; mp 142°C; $[\alpha]_D$ +7.6° (methanol).[11]

D-Aspartic Acid Dibenzyl Ester *p*-Toluenesulfonate (II). This compound was obtained according to the procedure for the L-isomer.[11] Yield, 81%; mp 155—156°C; $[\alpha]_D^{20}$ +1.0° (*c* 2, methanol). Reported value for the L-isomer; mp 151—152°C; $[\alpha]_D^{20}$ +1.0° (methanol).[11,12]

Dibenzyloxycarbonyl-lysyl-glutamic Acid Dibenzyl Ester. *The LL Isomer (III).* A suspension of dibenzyloxycarbonyl-L-lysine dicyclohexylammonium salt[13] (1.78 g, 3 mmol) in ethyl acetate was stirred with 0.5 M citric acid (12 m*l*) during 1 hr. The ethyl acetate layer was separated and washed with water, and dried over sodium sulfate. The filtrate was evaporated *in vacuo* and the residual oil (dibenzyloxycarbonyl-L-lysine) was dissolved in tetrahydrofuran (6 m*l*). The solution was treated with triethylamine (0.42 m*l*, 3 mmol) and isobutyl chloroformate (0.39 m*l*, 3 mmol) at −5°C.[14] After 15 min, a mixture of L-glutamic acid dibenzyl ester *p*-toluenesulfonate (1.50 g, 3 mmol) in chloroform (6 m*l*) and triethylamine (0.42 m*l*, 3 mmol) was added. The mixture was left to stand overnight at room temperature, and then evaporated *in vacuo*. The residue was dissolved in ethyl acetate (20 m*l*), then the solution was washed successively with 4% sodium bicarbonate, 2% hydrochloric acid and water, and dried over sodium sulfate. The filtrate was evaporated *in vacuo*, and the oily residue was solidified by the addition of ether and petroleum ether. The product was recrystallized from ethyl acetate - ether - petroleum ether. Yield, 1.51 g (70%); mp 113°C; $[\alpha]_D^{25}$ −14.2° (*c* 1, dimethylformamide).

Found: C, 67.85; H, 6.42; N, 5.98%. Calcd for $C_{41}H_{45}N_3O_9$: C, 68.03; H, 6.27; N, 5.81%.

The LD Isomer (IV). This compound was obtained from dibenzyloxycarbonyl-L-lysine and I by the mixed anhydride method described above. Yield, 55%; mp 95°C; $[\alpha]_D^{20}$ +6.4° (*c* 1, dimethylformamide).

Found: C, 68.03; H, 6.32; N, 5.98%.

Dibenzyloxycarbonyl-lysyl-aspartic Acid Dibenzyl Ester. *The LL Isomer (V).* This compound was obtained from dibenzyloxycarbonyl-L-lysine and L-aspartic acid dibenzyl ester by the mixed anhydride method. Yield, 74%; mp 139—140°C; $[\alpha]_D^{25}$ −9.4° (*c* 1, dimethylformamide).

Found: C, 67.68; H, 6.11; N, 5.92%. Calcd for $C_{40}H_{43}N_3O_9$: C, 67.44; H, 6.52; N, 5.92%.

The LD Isomer (VI). This compound was obtained from dibenzyloxycarbonyl-L-lysine and II. Yield, 66%; mp 97—98°C; $[\alpha]_D^{20}$ +6.2° (*c* 1, dimethylformamide).

Found: C, 67.65; H, 6.19; N, 5.98%.

Lysyl-glutamic Acid (Lys-Glu). *The LL Isomer (VII).* A solution of III (0.72 g, 1 mmol) in a mixture of acetic acid (3 m*l*), methanol (1.2 m*l*) and water (0.6 m*l*) was treated with hydrogen in the presence of palladium black. The filtrate from the catalyst was evaporated *in vacuo* to dryness. The residual crystals were recrystallized from water - ethanol. Yield, 0.23 g (85%); mp 194—196°C (decomp.); $[\alpha]_D^{20}$ +20.1° (*c* 3.1, water) (Found as a hemihydrate: C, 46.34; H, 7.91; N, 14.49%). Bergmann *et al.*[15] reported the synthesis of this compound by hydrogenolysis of Z-L-Lys(ε-Z)-L-Glu-OH which was obtained through the saponification of Z-L-Lys(ε-Z)-L-Glu(γ-OEt)-OEt; mp 197°C; $[\alpha]_D^{19}$ +22.9° (water).

The LD Isomer (VIII). This compound was obtained by hydrogenolysis of IV as described above; yield, 96%; $[\alpha]_D^{20}$ +33.4° (*c* 1, water). The elemental analysis could not be performed because of its hygroscopic character compared with the LL isomer (VII), however the homogeneity of this compound VIII was confirmed by paper chromatography and electrophoresis.

Lysyl-aspartic Acid (Lys-Asp). *The LL Isomer (IX).* This compound was obtained by hydrogenolysis of V. Yield, 86%; $[\alpha]_D^{25}$ +25.3° (*c* 1.3, water) (Found as a monohydrate: C, 42.81; H, 7.73; N, 14.68%). Bergmann *et al.*[16] reported the synthesis of this compound by hydrogenolysis of Z-L-Lys(ε-Z)-L-Asp-OH; $[\alpha]_D^{25}$ +23° (water).

The LD Isomer (X). The compound VI was hydrogenated and the filtrate from the catalyst was evaporated to dryness. The residual crystals were collected with the aid of ethanol. Yield, 86%; mp 180—182°C (decomp.); $[\alpha]_D^{25}$ +30.6° (*c* 1, water).

Found: C, 42.66; H, 7.86; N, 14.68%. Calcd for $C_{10}H_{19}O_5N_3 \cdot H_2O$: C, 43.00; H, 7.58; N, 15.03%.

Benzyloxycarbonyl-glycyl-ε-benzyloxycarbonyl-L-lysine Ethyl Ester (XI). This compound was obtained from benzyloxycarbonyl-glycine and ε-benzyloxycarbonyl-L-lysine ethyl ester *p*-toluenesulfonate[17] by the mixed anhydride method as described for the preparation of III. Yield, 76%; mp 77—78°C; $[\alpha]_D^{21}$ +3.2° (*c* 1, ethyl acetate).

11) N. Izumiya and S. Makisumi, *Nippon Kagaku Zassi* (*J. Chem. Soc. Japan, Pure. Chem. Sect.*), **78**, 662, 1768 (1957).

12) We wish to correct the value of the L-isomer as $[\alpha]_D^{20}$ −1.0° (*c* 2, methanol).

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14) J. R. Vaughan, Jr., and R. T. Osato, *J. Am. Chem. Soc.*, **73**, 5553 (1951).

15) M. Bergmann, L. Zervas and J. P. Greenstein, *Ber.*, **65**, 1692 (1932).

16) M. Bergmann, L. Zervas, H. Rinke and H. Schleich, *Z. Physiol. Chem.*, **224**, 26 (1934).

17) T. Kato, S. Makisumi, M. Ohno and N. Izumiya, *Nippon Kagaku Zasshi* (*J. Chem. Soc. Japan, Pure Chem. Sect.*), **83**, 1151 (1962).

Found: C, 62.50; H, 6.66; N, 8.42%. Calcd for $C_{26}H_{33}O_7N_3$: C, 62.49; H, 6.87; N, 8.43%.

Benzyloxycarbonyl-glycyl-ε-benzyloxycarbonyl-L-lysine Hydrazide (XII). A solution of XI (4.99 g, 10 mmol) and hydrazine hydrate (1.21 m*l*, 25 mmol) in methanol was allowed to stand at 30°C for 48 hr. The excess hydrazine was evaporated *in vacuo*, and then water (50 m*l*) was added to the residue. The resulting crystals were collected by filtration and washed with water. Yield, 4.3 g (91%); mp 163—165°C; $[\alpha]_D^{25}$ −1.2° (*c* 1, dimethylformamide) (Found: C, 59.25; H, 6.31; N, 14.41%). Erlanger and Brand[18] reported the synthesis of this compound from Z-Gly-L-Lys(ε-Z)-OMe with hydrazine; mp 167°C.

Benzyloxycarbonyl-glycyl-δ-benzyloxycarbonyl-L-ornithine Ethyl Ester (XIII). This compound was obtained from benzyloxycarbonyl-glycine and δ-benzyloxycarbonyl-L-ornithine ethyl ester *p*-toluenesulfonate[19] by the mixed anhydride method as described for the preparation of III. Yield, 71%; mp 79—80°C; $[\alpha]_D^{20}$ −7.6° (*c* 1, dimethylformamide).

Found: C, 61.84; H, 6.44; N, 8.66%. Calcd for $C_{25}H_{31}O_7N_3$: C, 61.58; H, 6.32; N, 8.56%.

Benzyloxycarbonyl-glycyl-δ-benzyloxycarbonyl-L-ornithine Hydrazide (XIV). The compound XIII was converted to the hydrazide as described for the preparation of XII. Yield, 87%; mp 141—143°C; $[\alpha]_D^{20}$ +0.8° (*c* 1, dimethylformamide).

Found: C, 58.10; H, 6.25; N, 14.75%. Calcd for $C_{23}H_{29}O_6N_5$: C, 58.18; H, 6.24; N, 14.62%.

Benzyloxycarbonyl-glycyl-ε-benzyloxycarbonyl-lysyl-glutamic Acid Dibenzyl Ester. *The* LL *Isomer* (*XV*). To a chilled solution of XII (1.94 g, 4 mmol) in glacial acetic acid (40 m*l*) and 2 N hydrochloric acid (2.4 m*l*) was added sodium nitrite (0.32 g) with stirring. After the solution had been allowed to stand for 5 min at −5°C, it was diluted with cold water (100 m*l*), and the protected dipeptide azide was extracted three times with 50 m*l* portions of ethyl acetate. The combined ethyl acetate solution was washed with 4% sodium bicarbonate and water, dried over sodium sulfate, and then added to a cold solution of L-glutamic acid dibenzyl ester *p*-toluenesulfonate (2.00 g, 4 mmol) in triethylamine (0.56 m*l*, 4 mmol) and dimethylformamide (10 m*l*). After the mixture had been stirred for 3 days at 0°C and evaporated *in vacuo*, the residue was dissolved in ethyl acetate and washed successively with 4% sodium bicarbonate, 2% hydrochloric acid and water, and dried over sodium sulfate. The filtrate was evaporated *in vacuo* and the oily residue was solidified by the addition of ether and petroleum ether. The product was recrystallized from ethyl acetate - ether - petroleum ether. Yield, 2.17 g (69%); mp 117—118°C; $[\alpha]_D^{20}$ −10.8° (*c* 1, dimethylformamide). For the elemental analysis, a part of the crystals was purified further by column chromatography of Sephadex LH-20 (column size; 0.9×50 cm) using dioxane as a solvent.

Found: C, 65.82; H, 6.20; N, 7.28%. Calcd for $C_{43}H_{48}O_{10}N_4$: C, 66.14; H, 6.20; N, 7.18%.

The LD *Isomer* (*XVI*). This compound was obtained from XII and I by the azide method. Yield, 63%; mp 122°C; $[\alpha]_D^{20}$ +3.6° (*c* 1, dimethylformamide).

Found: C, 65.87; H, 6.26; N 7.38%.,

Benzyloxycarbonyl-glycyl-ε-benzyloxycarbonyl-lysyl-aspartic Acid Dibenzyl Ester. *The* LL *Isomer* (*XVII*). The protected tripeptide ester (XVII—XXII) were obtained in the same manner from the corresponding protected dipeptide hydrazide (XII or XIV) and the corresponding amino acid dibenzyl ester as described for the preparation of XV. Yield, 64%; mp 122—123°C; $[\alpha]_D^{20}$ −8.6° (*c* 1, dimethylformamide).

Found: C, 65.72; H, 6.10; N, 7.44%. Calcd for $C_{42}H_{46}O_{10}N_4$: C, 65.76; H, 6.05; N, 7.31%.

The LD Isomer (*XVIII*). Yield, 60%; mp 105—106°C; $[\alpha]_D^{20}$ +6.2° (*c* 1, dimethylformamide).

Found: C, 65.31; H, 6.15; N, 7.76%.

Benzyloxycarbonyl-glycyl-δ-benzyloxycarbonyl-ornithyl-glutamic Acid Dibenzyl Ester. *The* LL *Isomer* (*XIX*). Yield, 61%; mp 84°C; $[\alpha]_D^{20}$ −10.0° (*c* 1, dimethylformamide).

Found: C, 65.46; H, 6.05; N, 7.18%. Calcd for $C_{42}H_{46}O_{10}N_4$: C, 65.76; H, 6.05; N, 7.31%.

The LD Isomer (*XX*). Yield, 76%; mp 96°C; $[\alpha]_D^{20}$ +5.2° (*c* 1, dimethylformamaide).

Found: C, 65.37; H, 6.00; N, 7.33%.

Benzyloxycarbonyl-glycyl-δ-benzyloxycarbonyl-ornithyl-aspartic Acid Dibenzyl Ester. *The* LL *Isomer* (*XXI*). Yield, 64%; mp 127—129°C; $[\alpha]_D^{20}$ −8.0° (*c* 1, dimethylformamide).

Found: C, 65.03; H, 5.83; N, 7.84%. Calcd for $C_{41}H_{44}O_{10}N_4$: C, 65.41; H, 5.89; N, 7.44%.

The LD *Isomer* (*XXII*). Yield, 61%; mp 103—104°C; $[\alpha]_D^{20}$ +6.4° (*c* 1, dimethylformamide).

Found: C, 64.98; H, 5.83; N, 7.77%.

Glycyl-lysyl-glutamic Acid (Gly-Lys-Glu), LL Isomer (XXIII) and LD Isomer (XXIV); Gly-Lys-Asp, LL Isomer (XXV) and LD Isomer (XXVI); Gly-Orn-Glu, LL Isomer (XXVII) and LD Isomer (XXVIII); Gly-Orn-Asp, LL Isomer (XXIX) and DL Isomer (XXX). Each of benzyloxycarbonyl-tripeptide benzyl ester (XV—XXII) (2 mmol) obtained above was suspended in a solution (20 m*l*) of acetic acid, methanol and water (6:1:3), and the mixture was treated with hydrogen in the presence of palladium black. As the hydrogenolysis had proceeded the suspended material went into the solution. The catalyst was removed by filtration, and the filtrate was evaporated *in vacuo* to dryness. The residual oil become hygroscopic crystals upon treatment with ethanol. The yields were almost quantitative. The homogeneity of these peptides was established by paper chromatography and electrophoresis. Elemental analysis was not performed because of its highly hygroscopic character. Amiard and Goffinet[20] reported the synthesis of XXIII by saponification and detritylation of Tri-Gly-L-Lys(ε-Tri)-L-Glu(γ-OEt)-OEt.

***o*-Nitrophenylsulfenyl-γ-benzyl-D-glutamic Acid Dicyclohexylammonium Salt (XXXI).** This compound was obtained according to the procedure for the L isomer.[21] Yield, 66%; mp 167—168°C; $[\alpha]_D^{20}$ +33.7° (*c* 3.5, chloroform). Reported value for the L isomer,[21] mp 168°C; $[\alpha]_D^{20}$ −34.0° (chloroform).

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20) G. Amiard and B. Goffinet, *Bull. Soc. Chim. France*, **1957**, 1133.

21) L. Zervas, D. Borovas and E. Gazis, *J. Am. Chem. Soc.*, **85**, 3660 (1963).

o-Nitrophenylsulfenyl-β-benzyl-D-aspartic Acid Dicyclohexylammonium Salt (XXXII). This compound was obtained according to the procedure for the L isomer.[22] Yield, 59%; mp 165°C; $[\alpha]_D^{20}$ +41.0° (*c* 4, chloroform). Reported value for the L isomer;[22] mp 165—166°C; $[\alpha]_D^{20}$ −38.0° (chloroform).

o-Nitrophenylsulfenyl-γ-benzyl-glutamyl-ε-benzyloxycarbonyl-lysine Benzyl Ester. *The LL Isomer (XXXIII).* To a solution of *o*-nitrophenylsulfenyl-γ-benzyl-L-glutamic acid dicyclohexylammonium salt[21] (1.72 g, 3 mmol) and ε-benzyloxycarbonyl-L-lysine benzyl ester *p*-toluenesulfonate[23] (1.63 g, 3 mmol) in chloroform (12 m*l*) was added dicyclohexylcarbodiimide (0.62 g, 3 mmol) with stirring at −5°C. After 2 hr at −5°C, the stirring was continued at room temperature overnight. After the reaction mixture was evaporated *in vacuo*, the residue was treated with ethyl acetate (20 m*l*), and insoluble precipitate of dicyclohexylurea was removed by filtration. The filtrate was washed successively with 4% sodium bicarbonate, 0.5 M citric acid and water, dried over sodium sulfate and evaporated *in vacuo* yielding an oily residue. Yield of the oil, 2.01 g (90%). This oil did not crystallize on prolonged standing in a refrigerator.

The DL Isomer (XXXIV). This compound was obtained from XXXI and ε-benzyloxycarbonyl-L-lysine benzyl ester as described above. The product was recrystallized from ethyl acetate - ether - petroleum ether. Yield, 73%; mp 87—88°C; $[\alpha]_D^{20}$ +19.2° (*c* 1, chloroform).

Found: C, 63.07; H, 5.79; N, 7.59%. Calcd for $C_{39}H_{42}O_9N_4S$: C, 63.06; H, 5.70; N, 7.54%.

o-Nitrophenylsulfenyl-β-benzyl-aspartyl-ε-benzyloxycarbonyl-lysine Benzyl Ester. *The LL Isomer (XXXV).* This compound was obtained from *o*-nitrophenylsulfenyl-β-benzyl-L-aspartic acid dicyclohexylammonium salt[22] and ε-benzyloxycarbonyl-L-lysine benzyl ester as described above and recrystallized from ethyl acetate - ether - petroleum ether. Yield, 88%; mp 128—129°C; $[\alpha]_D^{20}$ −17.2° (*c* 1, dimethylformamide).

Found: C, 62.58; H, 5.51; N, 7.83%. Calcd for $C_{38}H_{40}O_9N_4S$: C, 62.62; H, 5.53; N, 7.69%.

The DL Isomer (XXXVI). This compound was obtained from XXXII and ε-benzyloxycarbonyl-L-lysine benzyl ester as described above. Yield, 82%; mp 118—119°C; $[\alpha]_D^{20}$ +11.8° (*c* 1, chloroform).

Found: C, 61.76; H, 5.40; N, 7.84%. Calcd for $C_{38}H_{40}O_9N_4S \cdot 1/2H_2O$: C, 61.86; H, 5.60; N, 7.60%.

Benzyloxycarbonyl-glycyl-γ-benzyl-glutamyl-ε-benzyloxycarbonyl-lysine Benzyl Ester. *The LL Isomer (XXXVII).* To a solution of the nitrophenylsulfenyl-dipeptide ester XXXIII (1.54 g 2 mmol) in dioxane (6 m*l*), 2 N hydrogen chloride in dioxane (4 m*l*) was added. After the solution was allowed to stand at room temperature for 30 min, it was evaporated *in vacuo*. The residual oil was washed several times with ether by the way of decantation; yield of the oily dipeptide ester monohydrochloride, 1.26 g (102%). Then the compound XXXVII was prepared from benzyloxycarbonyl-glycine (2 mmol) and the dipeptide ester monohydrochloride (1.26 g) obtained above by the mixed anhydride method as described for the preparation of III. The product was recrystallized from ethyl acetate - ether - petroleum ether. Yield, 1.11 g (71%); mp 97°C; $[\alpha]_D^{20}$ −9.0° (*c* 1, dimethylformamide).

Found: C, 66.23; H, 6.11; N, 7.21%. Calcd for $C_{43}H_{48}O_{10}N_4$: C, 66.14; H, 6.20; N, 7.18%.

The DL Isomer (XXXVIII). The crystalline dipeptide ester monohydrochloride (1.23 g) was obtained from the nitrophenylsulfenyl-dipeptide ester XXXIV (2 mmol) with the treatment of hydrogen chloride in dioxane as has been described above. Then, XXXVIII was prepared from benzyloxycarbonyl-glycine and the dipeptide ester monohydrochloride (1.23 g) by the mixed anhydride method. The product was recrystallized from ethyl acetate - ether - petroleum ether. Yield, 1.09 g (70%); mp 126°C; $[\alpha]_D^{20}$ −4.4° (*c* 1, dimethylformamide).

Found: C, 66.21; H, 6.13; N, 7.22%.

Benzyloxycarbonyl-glycyl-β-benzyl-aspartyl-ε-benzyloxycarbonyl-lysine Benzyl Ester. *The LL Isomer (XXXIX).* This compound was prepared from benzyloxycarbonyl-glycine and the crystalline dipeptide ester monohydrochloride which was obtained fromXXXV with hydrogen chloride in dioxane. The product was recrystallized from ethyl acetate - ether - petroleum ether. Yield, 61%; mp 117°C; $[\alpha]_D^{20}$ −18.6° (*c* 1, dimethylformamide).

Found: C, 65.76; H, 6.07; N, 7.27%. Calcd for $C_{42}H_{46}O_{10}N_4$: C, 65.76; H, 6.05; N, 7.31%.

The LD Isomer (XL). This compound was synthesized as described above from benzyloxycarbonyl-glycine and the crystalline dipeptide ester monohydrochloride which was obtained from XXXVI. Yield, 73%; mp 91°C; $[\alpha]_D^{20}$ +6.2° (*c* 1, dimethylformamide).

Found: C, 65.60; H, 6.08; N, 7.21%.

Glycyl-glutamyl-lysine (Gly-Glu-Lys). *The LL Isomer (XLI).* The compound XXXVII (0.78 g) was hydrogenated as has been described for the preparation of Lys-Glu (VII) and the filtrate from the catalyst was evaporated to dryness. The residual crystals were recrystallized from water-ethanol. Yield, 0.247 g (70%); $[\alpha]_D^{20}$ −22.4° (*c* 1, water).

Found: C, 44.28; H, 7.25; N, 15.66%. Calcd for $C_{13}H_{24}O_6N_4 \cdot H_2O$: C, 44.56; H, 7.48; N, 15.99%.

The DL Isomer (XLII). This was obtained by the

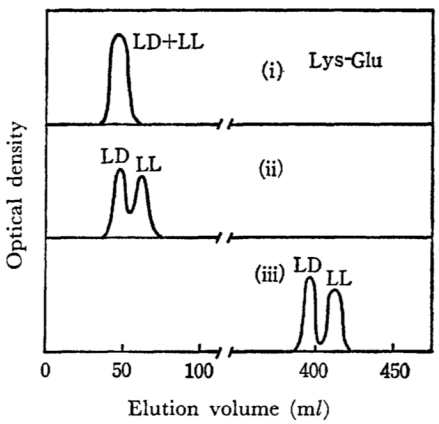

Fig. 5. Elution pattern of a diastereomeric mixture of Lys-Glu.
(i) 0.9×50 cm column and pH 5.28 buffer,
(ii) 0.6×10 cm column and pH 4.25 buffer,
(iii) 0.6×10 cm column, pH 3.25 buffer (360 m*l*) and then pH 4.25 buffer.

22) L. Zervas and C. Hamilidis, *ibid.*, **87**, 99 (1965).
23) O. Abe, H. Takiguchi, M. Ohno, S. Makisumi and N. Izumiya, This Bulletin, **40**, 1945 (1967).

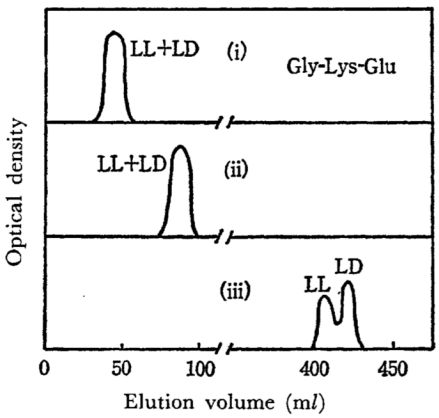

Fig. 6. Elution pattern of a diastereomeric mixture of Gly-Lys-Glu.
(i), (ii) and (iii); see Fig. 5.

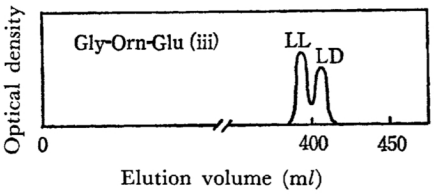

Fig. 7. Elution pattern of a diastereomeric mixture of Gly-Orn-Glu.
(iii); see Fig. 5.

hydrogenolysis of XXXVIII as described above. Yield, 82%; $[\alpha]_D^{20}$ +13.0° (*c* 1, water).

Found: C, 46.20; H, 7.28; N, 16.03%. Calcd for $C_{13}H_{24}O_6N_4 \cdot \frac{1}{2}H_2O$: C, 45.92; H, 7.35; N, 16.33%.

Glycyl-aspartyl-lysine (Gly-Asp-Lys). *The* LL *Isomer (XLIII).* This was obtained by hydrogenolysis of XXXIX. Yield, 71%; $[\alpha]_D^{20}$ −4.6° (*c* 1, water).

Found: C, 42.65; H, 7.10; N, 16.31%. Calcd for $C_{12}H_{22}O_6N_4 \cdot H_2O$: C, 42.85; H, 7.19; N, 16.66%.

The DL *Isomer (XLIV).* This was obtained by hydrogenolysis of XL. Yield, 85%; $[\alpha]_D^{20}$ +14.8° (*c* 1, water).

Found: C, 43.16; H, 7.42; N, 16.29%. Calcd for $C_{12}H_{22}O_6N_4 \cdot H_2O$: C, 42.85; H, 7.19; N, 16.66%.

Paper Chromatography and Paper Electrophoresis. A paper chromatography was carried out with ascending technique using Toyo Roshi No. 52, and a peptide on the paper was revealed with ninhydrin. As shown in Table 2, it was observed that the diastereomers of several peptides were separated on the chromatogram. The electrophoresis was carried out under the following condition: 500 V/30 cm with formic acid - acetic acid - methanol - water solution and 300 V/30 cm with 0.2 M sodium citrate buffer. As indicated in Table 2, it appeared that the ability to separate a diasteremeric mixture was less than that in the paper chromatography.

Separation Studies with Amino Acid Analyzer. A mixture of 0.6 μmol each of the LL form and the LD (or DL) form of di- or tripeptides was analyzed by Hitachi amino acid analyzer with spherical resin (No 3105), model KLA-3B, under the following conditions: flow rate, 60 ml/hr and jacket temperature, 55°C. Three different analyses were performed for each diastereomeric mixture as follows: (i) with 0.9×50 cm column, 0.2 M sodium citrate buffer at pH 5.28 was used; (ii) with 0.6×10 cm column, 0.2 M sodium citrate at pH 4.25 was used; (iii) with 0.6×10 cm column, 0.2 M sodium citrate at pH 3.25 was applied for 6 hr (toltal effluent volume, 360 ml), and then the buffer system was changed to pH 4.25. The results obtained were summarized in Table 2. The several chromatographic patterns with the amino acid analyzer were indicated in Figs. 5—7.

The authors wish to express their thanks to Dr. Haruhiko Aoyagi, in this laboratory for his valuable discussions.