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Studies on Separation of Amino Acids and Related Compounds. V. A Racemization Test in Peptide Synthesis by the Use of an Amino Acid Analyzer[1,2]

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Several tripeptides, H-Gly-DL-Ala-B-OH, in which B residues are DL-alanine, L-valine, L-leucine, L-phenylalanine, L-proline, and L-serine have been synthesized and the separation of each glycyltripeptide diastereomers by an amino acid analyzer have been studied. Among six peptides studied, diastereomeric mixtures of H-Gly-DL-Ala-L-Val-OH and H-Gly-DL-Ala-L-Leu-OH were separated completely. A procedure to determine the amounts of LL and DL diastereomer of H-Gly-Ala-Leu-OH by the use of the analyzer was developed, and this procedure was applied to examine the influence of coupling reagents for racemization during coupling of benzyloxycarbonyl-glycyl-L-alanine and L-leucine benzyl ester by several reagents.

To determine the extent of racemization during peptide bond formation several methods have been reported in the literatures.[3] We have attempted to find a convenient racemization test with the application of an automatic amino acid analyzer though Bodanszky and Conklin already reported the use of the analyzer for such purpose.[4] They applied the analyzer for amino acid analysis of acid hydrolysate of a coupling product derived from Ac-L-Ile-OH[5] and H-Gly-OEt, and determined the amounts of L-isoleucine and D-alloisoleucine. Manning and Moore reported the use of the analyzer in the system involving the coupling of *N*-carboxyanhydride of L-leucine and a DL-amino acid.[6]

In this paper we report an accurate procedure to determine the degree of racemization by an amino acid analyzer and the results of the influence of several coupling reagents on the extent of racemization during peptide synthesis.

The proposed scheme for racemization test is shown in Fig. 1. The crude Z-tripeptide-OBz 1 is subjected to hydrogenolysis, the hydrogenated material is submitted to the analyzer, and the amounts of the LL and DL diastereomers are determined.

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

Fig. 1. Proposed sequence of synthesis of a diastereomeric mixture.

It was tried previously to discover a convenient system of glycyltripeptide diastereomers for separation by the analyzer.[1] We synthesized the LL and DL isomer of H-Gly-Lys-Glu-OH and similar several tripeptides, with the surmise that a diastereomeric mixture of a polyfunctional neutral tripeptide might be efficiently separated under appropriate conditions. It was observed, however, that all mixtures gave incomplete separation.[1] In the present investigation, we selected rather simple systems involving H-Gly-Ala-B-OH tripeptides in which B could be Ala, Val, Leu, Phe, Pro, and Ser residues.

1) Part IV of this series: M. Muraoka, N. Yoshida, K. Noda, and N. Izumiya, This Bulletin, **41**, 2134 (1968).

2) A part of this work has been briefly communicated: N. Izumiya and M. Muraoka, *J. Amer. Chem. Soc.*, **91**, 2391(1969).

3) For reviews, see: M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," Interscience Publishers, New York (1966), p. 137; T. Kato, H. Aoyagi, M. Waki, N. Mitsuyasu, and N. Izumiya, *Tampakushitsu-Kakusan-Koso*, **16**, 139 (1971).

4) M. Bodanszky and L. E. Conklin, *Chem. Commun.*, **1967**, 773.

5) Abbreviations used: Ac, acetyl; Z, benzyloxycarbonyl; OBzl, benzyl ester; TsOH, *p*-toluenesulfonic acid; HOSu, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; NEPIS, *N*-ethyl-5-phenylisoxazolium-3′-sulfonate; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; TEA, triethylamine; N-MM, *N*-methylmorpholine; THF, tetrahydrofuran; DMF, dimethylformamide; MA, mixed anhydride.

6) J. M. Manning and S. Moore, *J. Biol. Chem.*, **21**, 5591 (1968).

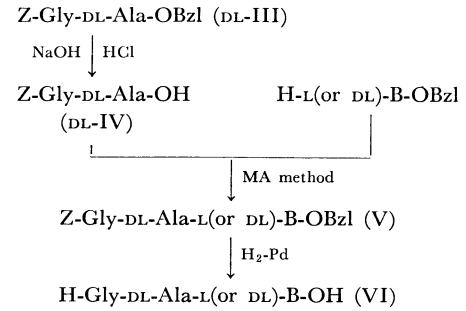

Fig. 2. Synthesis of a crude tripeptide consisting of diastereomers. B; DL-Ala, L-Val, L-Leu, L-Phe, L-Pro or L-Ser residue.

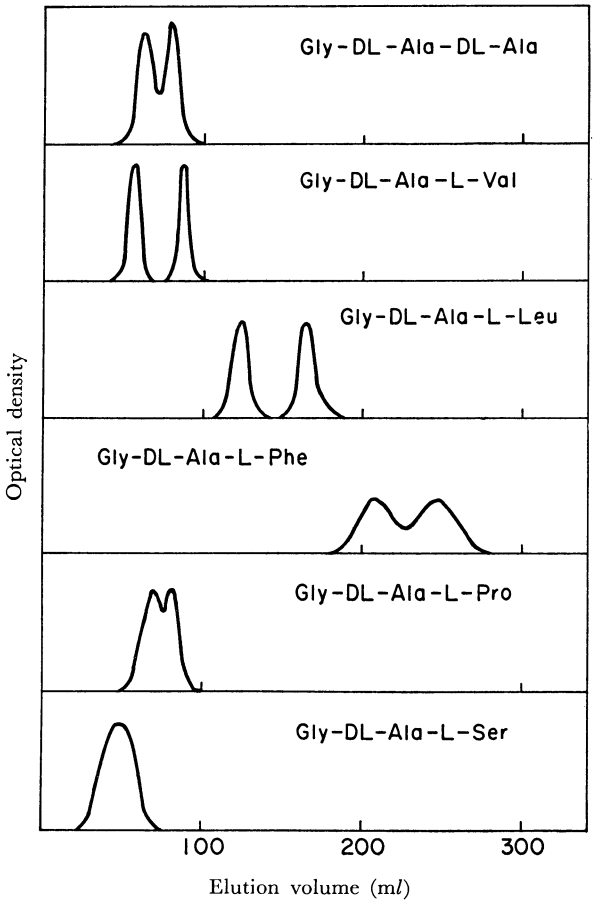

Fig. 3. Elution pattern of H-Gly-DL-Ala-B-OH (VI).

In the previous paper, each pure diastereomer of a tripeptide such as H-Gly-Lys-Glu-OH was prepared, and an artificial mixture of the diastereomers was subjected to separation experiment.[1] In this investigation, a crude tripeptide (VI) consisting of diastereomers was synthesized as shown in Fig. 2 instead of preparation of a pair of pure diastereomers. As shown in Fig. 3, the diastereomeric mixture containing Val or Leu residue as B component was found to be separated completely by the analyzer, and the other mixture containing Ala, Phe, Pro, or Ser separated incompletely.

A diastereomeric mixture of H-Gly-Ala-Leu-OH (IX) was the preferred system for the racemization test (see, Fig. 4) because two peaks of IX were not overlapped with either leucine or H-Gly-Ala-OH

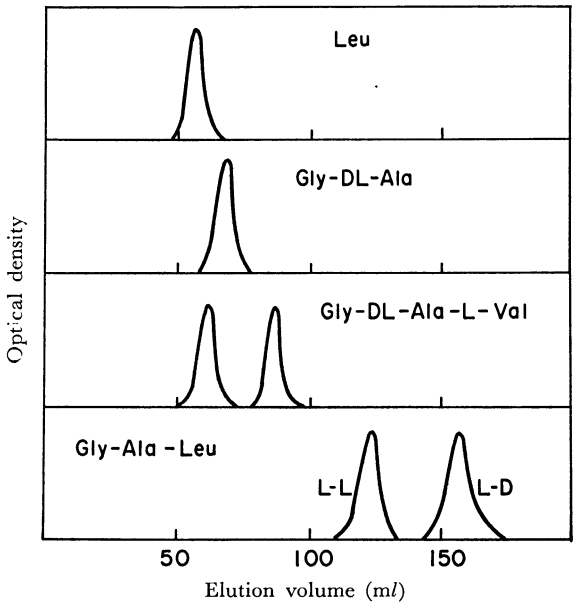

Fig. 4. Elution pattern of amino acid and peptides.

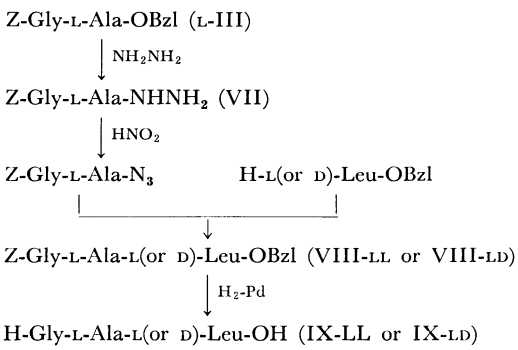

Fig. 5. Synthesis of a pure tripeptide.

which might be contaminated in a final crude tripeptide (see, Fig. 1).[7] A pair of pure diastereomers of IX was prepared as shown in Fig. 5 to be used for identification of two peaks in Fig. 3; the LL isomer was eluted faster than the corresponding LD isomer (Fig. 4).

The limit of detection of the LD contaminated in the LL isomer was studied with an artificial mixture of both isomers. Even when a mixture of the LL isomer (5 μmol) and DL (0.005 μmol) was analyzed, a small peak of the DL could still be recognized. It will be evident that the present method is more sensitive in detecting the slight occurrence of racemization than the Anderson test which has been used widely nowadays.[3]

The present method was applied in the detection of a possible racemization in azide procedure. A crude Z-Gly-Ala-Leu-OBzl obtained from pure Z-Gly-L-Ala-N_3 and H-L-Leu-OBzl was directly hydrogenated, and a part of the filtrate from a hydrogenated mixture was submitted to the analyzer. The material in the filtrate showed only single peak by the LL isomer,

7) Several related amino acids and peptides were subjected to the analyzer; glycine was eluted at 32 ml of effluent volume, alanine at 36 ml, LD isomer of H–Ala–Leu–OH at 126 ml, and its LL isomer at 138 ml.

Table 1. Extent of racemization during peptide bond formation[a)]

No.	Coupling reagent	Additional component	Solvent	Tertiary amine	Reaction time, hr	Reaction temp., °C	Yield of tripeptide (IX-LL plus IX-DL), %	Extent of racemization
1[b)]	Isobutyl chloroformate		THF	TEA	15	25	87	9.5
2	Isobutyl chloroformate		THF	NMM	15	25	91	2.4
3[c)]	Isobutyl chloroformate	HOSu	THF	TEA	2	25	55	1.1
4[c)]	Isobutyl chloroformate	HOSu	THF	NMM	2	25	63	0.2
5	DCC		THF	TEA	48	0	73	22
6	DCC		THF	NMM	48	0	77	21
7[d)]	DCC	HOSu	THF	TEA	48	0	98	0.0
8[c)]	NEPIS		CH_3CN	TEA	24	25	95	1.8
9	NEPIS		CH_3CN	NMM	24	25	72	1.7
10[c)]	EEDQ		THF	TEA	7	25	97	0.2
11	EEDQ		THF	NMM	7	25	91	0.2

a) All components (Z-Gly-L-Ala-OH, H-L-Leu-OBzl, coupling reagent, HOSu and tertiary amine) in the coupling were of equivalent weight.
b) The procedure following that in the literature[10] was noted in detail in the experimental part.
c) The procedure was similar to that described in the literatures: MA,[11] NEPIS,[15] and EEDQ.[16]
d) The procedure reported[12] was slightly modified; the components were of equivalent weight and the temperature was 0°C during the entire reaction.

using a load of up to 6 μmol. The result agreed with the fact that the azide procedure has been considered to be safe to avoid racemization.[3,8]

This procedure was employed to examine the influence of various coupling reagents to racemization, the experiments being summarized in Table 1. In a typical mixed anhydride (MA) method, the coupling yield of Z-Gly-L-Ala-OH and H-L-Leu-OBzl was approximately 87%[9] and the extent of racemization,[4] which is defined as {100[DL isomer]}/{[LL isomer]+[DL isomer]}, was calculated as 9.5. Anderson and his colleagues reported that the extent of racemization was 9 using fractional crystallization in case of the coupling of Z–Gly–L–Phe–OH and H–Gly–OEt.[10] The use of NMM instead of TEA decreased the recemization as 2.4 in this experiment, but Anderson *et al.* reported no isolation of a DL-tripeptide in their experiment.[10] The difference of these results may be due to the differences of the detection method for racemization and of the compounds investigated. The addition of HOSu to the MA system with TEA or NMM decreased the degree of racemization considerably; the results confirm the observation of Anderson and Callahan on the role of HOSu in the MA method.[11]

In the DCC method, the extents of racemization were calculated as 22 and 21 respectively when TEA and NMM were used (Table 1). It was reported previously that the extent of racemization was 9.4 in case of the coupling of Z–L–Leu–L–Phe–OH and H–L–Val–OBu^t,[12] and 9.1—10.6 in case of Z–Gly–L–Phe–OH and H–Gly–OEt.[13] No explanation for the higher degree of racemization observed in our experiment can be offered at the present time. On the contrary the coupling using HOSu and DCC gave no racemate; Weygand *et al.*[12] also reported the occurrence of less than 1% racemization under the similar conditions.

The extent of racemization were calculated as 1.7—1.8 and 0.2 in the applications of NEPIS[14,15] and EEDQ[16] respectively. These results showed similar levels of racemizations observed previously; 3.2 with NEPIS[17] and no detection of a recemate with EEDQ[16] were reported.

A simple and straightforward procedure described in this paper will be a useful test to detect possible

8) The occurrence of racemization have been reported recently even in the case of the azide procedure when unusual reaction condition was applied: G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **88**, 1338 (1966); H. Determann, "Peptides," ed. by H. C. Beyerman, A. Van de Linde, and W. Maassen van den Brink, North-Holland Pub. Co., Amsterdam (1967), p. 73.

9) Yield of 87% was derived from an analysis of H–Gly–Ala–Leu–OH (LL plus DL isomer) by an amino acid analyzer. We assume that the yield of free tripeptide corresponds to approximately that of Z–Gly–Ala–Leu–OBzl (VIII) because the hydrogenation of VIII will proceed quantitatively.

10) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **89**, 5012 (1967).

11) G. W. Anderson, F. M. Callahan, and J. E. Zimmerman, *ibid.*, **89**, 178 (1967).

12) F. Weygand, D. Hoffmann, and E. Wünsch, *Z. Naturforsch.*, **21b**, 426(1966).

13) G. W. Anderson and F. W. Callahan, *J. Amer. Chem. Soc.*, **80**, 2902(1958).

14) R. B. Woodward, R. A. Olofson, and H. Mayer, *Tetrahedron Suppl.*, **8**, 321(1966).

15) J. Ramachandran and C. H. Li, *J. Org. Chem.*, **27**, 4006 (1962).

16) B. Belleau and G. Malek, *J. Amer. Chem. Soc.*, **90**, 1651 (1968).

17) F. Weygand, A. Prox, and W. König, *Chem. Ber.*, **99**, 1451 (1966).

racemization when a new coupling method is developed in future.

Experimental

R_f of thin-layer chromatography with Merck silica gel G refers to *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2, vol) system. Benzyl ester toluenesulfonates of L-valine,[18] L-[18] and D-leucine,[19] L-phenylalanine,[18] and L-serine,[20] and H–Gly–DL–Ala–OH[21] were prepared following the literatures.

H–DL–Ala–OBzl·TsOH (I). This was prepared according to the procedure for the L isomer.[18] Yield, 82%, mp 109°C; R_f 0.74 (Found: C, 57.97; H, 6.13; N, 4.13%).

H–L–Pro–OBzl·TsOH (II). A mixture of L-proline, benzyl alcohol, toluenesulfonic acid, and benzene was treated following the general procedure.[18] Yield of II as an oil, 98%; R_f 0.69.

Z–Gly–DL–Ala–OBzl (DL–III). To a chilled solution (−10°C) of Z–Gly–OH (2.1 g) and TEA (1.4 m*l*) in THF (20 m*l*), isobutyl chloroformate (1.31 m*l*) was added. After 10 min, a mixture of I (3.52 g), TEA (1.4 m*l*) and chloroform (20 m*l*) was added to the solution. The reaction mixture was allowed to stand overnight and then evaporated to dryness. After the residual oil was dissolved in ethyl acetate, the solution was washed with 2% hydrochloric acid and 4% sodium bicarbonate, and dried over sodium sulfate. The filtrate was evaporated and the residue was crystallized by addition of petroleum ether. It was recrystallized from ethyl acetate-petroleum ether; yield, 2.54 g (69%); mp 91°C (Found: C, 64.94; H, 5.96; N, 7.52%). Wieland and Heinke prepared this compound by MA method with phosphorus oxychloride; mp 91—92°C.[22]

Z–Gly–L–Ala–OBzl (L–III). This was obtained from Z–Gly–OH and H–L–Ala–OBzl·TsOH as described above. Yield, 62%; mp 77°C; $[\alpha]_D^{25}$ −14.0° (*c* 1, ethyl acetate) (Found: C, 65.07; H, 6.01; N, 7.56%). Gante prepared this compound by a coupling of Z–Gly–OH and N-2,4-dinitrophenyloxycarbonyl–L–Ala–OBzl; mp 77—78°C.[23]

Z-Gly-DL-Ala-OH (DL-IV). To a solution of DL-III (3.7 g, 10 mmol) in methanol (30 m*l*), N sodium hydroxide (12 m*l*) was added. After 2 hr, N hydrochloric acid (12 m*l*) was added and the solution was evaporated. The resulting crystals were collected by aid of cold water. It was recrystallized from ethanol-ether; yield, 1.99 g (71%); mp 182°C (Found: C, 55.56; H, 5.84; N, 9.76%). Clayton *et al.* prepared this compound by a coupling of DL-alanine and a mixed anhydride derived from Z-Gly-OH; mp 183°C.[24]

Z-Gly-L-Ala-OH (L-IV). This was obtained from L-III as described above. Yield, 82%; mp 133°C; $[\alpha]_D^{20}$ −9.8° (*c* 4.4, ethanol) (Found: C, 55.58; H, 5.73; N, 9.81%). This was prepared previously by a coupling of L-alanine and Z-Gly-OH mixed anhydride,[24] and by saponification of Z-Gly-L-Ala-OEt[25]; mp 133°C,[24] 119.5°C;[25] $[\alpha]_D$ −9.5°,[24] −10.2° (ethanol).[25]

Z-Gly-DL-Ala-B-OBzl (B=DL-Ala, L-Val, L-Leu, L-Phe, L-Pro or L-Ser) (V). This compound was prepared from DL-IV (0.28 g, 1 mmol) and a benzyl ester (1 mmol) of an amino acid B as described for the preparation of DL-III. Ethyl acetate solution was dried, and evaporated to yield an oily product (0.3—0.4 g). It contained a compound V indicating R_f 0.94—0.96, and was used for the next step without purification.

H-Gly-DL-Ala-B-OH (VI). A solution of each V (0.3—0.4 g) in 90% acetic acid (5 m*l*) was treated with hydrogen in the presence of palladium black. After several hr, the material with R_f 0.94—0.96 disappeared and a material with R_f 0.21—0.46 appeared; a material in the solution showed R_f 0.21 (H-Gly-DL-Ala-B-OH in which B is L-Ser), 0.31 (DL-Ala), 0.34 (L-Pro), 0.43 (L-Val), 0.44 (L-Phe), and 0.46 (L-Leu). The filtrate from the catalyst was evaporated to dryness. The residual hygroscopic solid was used as such in an experiment with an amino acid analyzer as described later.

Z-Gly-L-Ala-NHNH$_2$ (VII). A solution of L-III (3.7 g) and hydrazine hydrate (2.4 m*l*) in methanol (10 m*l*) was allowed to stand at room temperature for 2 days. It was evaporated and the residual solid was recrystallized from acetone-ether; yield, 2.7 g (92%); mp 134°C; $[\alpha]_D^{25}$ −30.0° (*c* 1, methanol). This was prepared previously from Z-Gly-L-Ala-OEt; mp 133°C,[26] mp 158—160°C.[27]

Z-Gly-L-Ala-L-Leu-OBzl (VIII-LL). To a chilled solution of VII (2.36 g) in acetic acid (50 m*l*) and 2 N hydrochloric acid (10 m*l*), an aqueous solution (5 m*l*) containing sodium nitrite (0.64 g) was added. After 5 min at −5°C, the azide was extracted with ethyl acetate (50 m*l*×3). An organic layer was washed with 10% sodium bicarbonate and dried over sodium sulfate. The filtrate was added to a mixture of H-L-Leu-OBzl·TsOH (3.2 g) and TEA (1.12 m*l*) in DMF (40 m*l*). After 3 days at 0°C, the reaction mixture was evaporated and the residue was dissolved in ethyl acetate. Ethyl acetate solution was washed with 2% hydrochloric acid and 4% sodium bicarbonate, dried, and evaporated. The residual solid was recrystallized from ether-petroleum ether; yield, 2.72 g (70%); mp 102°C; $[\alpha]_D^{25}$ −31.6° (*c*, 1, ethyl acetate).

Found: C, 64.29; H, 7.00; N, 8.47%. Calcd for $C_{26}H_{33}O_6N_3$: C, 64.58; H, 6.88; N, 8.69%.

Z-Gly-L-Ala-D-Leu-OBzl (VIII-LD). Reaction of VII (2.36 g) and H-D-Leu-OBzl·TsOH (3.2 g) as described above yielded 2.48 g (64%) of VIII-LD; mp 125°C; $[\alpha]_D^{25}$ −1.0° (*c* 1, ethyl acetate).

Found: 64.80; H, 6.93; N, 8.49%. Calcd for $C_{26}H_{33}O_6N_3$: C, 64.58; H, 6.88; N, 8.69%.

H-Gly-L-Ala-L-Leu-OH (IX-LL). VIII-LL (1.93 g) was hydrogenated as described for the preparation of VI. The filtrate was evaporated and the resulting crystals were collected by aid of ethanol. It was recrystallized from water-methanol; yield of an air-dried material, 0.93 g (88%); mp 242—245°C (decomp); R_f 0.59; $[\alpha]_D^{25}$ −84.0° (*c* 1, water) (for $C_{11}H_{21}O_4N_3 \cdot 1/4\,H_2O$. Found: C, 50.31; H, 8.22; N, 15.80%; H_2O, 1.65%). The same compound without water of crystallization was prepared previously from Z-Gly-L-Ala-L-Leu-OMe; $[\alpha]_D^{23}$ −87° (water).[27]

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19) K. Noda, H. Okai, T. Kato, and N. Izumiya, This Bulletin, **41**, 401(1968).
20) H. Voss, *Z. Naturforsch.*, **20b**, 116(1965).
21) R. Shoenheimer, *Z. Physiol. Chem.*, **154**, 203(1926).
22) T. Wieland and B. Heinke, *Ann. Chem.*, **599**, 70(1956).
23) J. Gante, *Chem. Ber.*, **99**, 1576(1966).
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25) B. F. Erlanger and E. Brand, *J. Amer. Chem. Soc.*, **73**, 3508 (1951).
26) M. Bergmann and L. Zervas, *J. Biol. Chem.*, **113**, 341 (1936).
27) R. W. Holley and A. D. Holley, *J. Amer. Chem. Soc.*, **74**, 3069(1952).

H-Gly-L-Ala-D-Leu-OH (*IX*-LD). This was prepared from VIII-LD; yield of an air-dried material, 86%; mp 243—245°C (decomp); R_f 0.57; $[\alpha]_D^{25}$ −29.6° (*c* 0.4, water).

Found: C, 49.20; H, 8.29; N, 15.75%. Calcd for $C_{11}H_{21}O_4N_3 \cdot 1/2H_2O$: C, 49.24; H, 8.27; N, 15.66%. The air-dried sample lost 3.03% of its weight after being dried for 3 hr at 80°C *in vacuo*. Calcd for $1/2H_2O$: 3.35%.

Chromatography of Amino Acids and Peptides by Amino Acid Analyzer. A diastereomeric mixture (VI; approximately 1 μmol) of tripeptide was analyzed by a Hitachi amino acid analyzer, model KLA-3B, under the following conditions: length of column with spherical resin, 0.9×50 cm; solvent, a standard 0.2 M citrate buffer at pH 4.25; flow rate, 60 m*l*/hr; jacket temperature, 55°C. The patterns obtained are shown in Fig. 3. Similarly, amino acids (0.5 μmol), dipeptides (0.5 μmol), and an artificial mixture (1 μmol) of the LL and LD isomer of H-Gly-Ala-Leu-OH (IX) were analyzed; some of the patterns are shown in Fig. 4. It was observed that leucine was eluted at 58 m*l* of effluent volume, H-Gly-DL-Ala-OH at 73 m*l*, IX-LL at 129 m*l*, and IX-LD at 159 m*l*.

Detection of Racemization. *(a) Possible Racemization in Azide Method*: The azide derived from Z-Gly-L-Ala-$NHNH_2$ (VII) was condensed with H-L-Leu-OBzl as described in the preparation of VIII-LL. Ethyl acetate solution was evaporated, the crude Z-Gly-Ala-Leu-OBzl was hydrogenated, the filtrate from the catalyst was evaporated, and a part (6 μmol) of the residue was submitted to the analyzer. The product showed only single peak of IX-LL.

(b) Racemization in MA Method: Similar conditions (temperatures and reaction times) for coupling of Z-Gly-L-Phe-OH and H-Gly-OEt by MA method[9] were applied for the present experiment. To Z-Gly-L-Ala-OH (L-III) (1 mmol) in THF (5 m*l*) was added isobutyl chloroformate. After 12 min at −15°C, a mixture of H-L-Leu-OBzl·TsOH and TEA in THF (5 m*l*) was added, and the reaction mixture was left at 25°C for 15 hr. The mixture was treated as described in the preparation of DL-III, and the ethyl acetate solution was evaporated; yield of a crude solid (VIII), 474 mg. A part (47.4 mg) of VIII was hydrogenated in 90% acetic acid, and the filtrate was evaporated. The residue was dissolved in 0.2 M citrate buffer at pH 4.25 (10 m*l*), and a part (0.7 m*l*) of it was submitted to the analyzer; the yield of H-Gly-Ala-Leu-OH (LL plus DL) from L-III was calculated as 87%, and the extent of racemization was calculated as 9.5. Similar experiment with NMM instead of TEA was carried out, and the yield of the tripeptide and the extent of racemization were calculated (see, Table 1).

(c) Racemization in other Methods: Z-Gly-L-Ala-OH was condensed with H-L-Leu-OBzl by a method using the reagent of DCC, NEPIS or EEDQ. An isolation of crude Z-Gly-Ala-Leu-OBzl (VIII), the hydrogenation of VIII, and an analysis of the hydrogenated material were identical with the procedures described above. The results are summarized in Table 1.

We wish to express our thanks to Dr. K. Noda of this laboratory for his help with an amino acid analyzer and to Professor B. Belleau for the generous gift of EEDQ.