A Synthesis of Human Proinsulin C-Peptide[1)]

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A peptide corresponding to the thirty-one amino acid sequence of human proinsulin C-peptide (positions 33—63 of proinsulin) was synthesized by the solid-phase method. The product was purified consecutively by gel filtration, DEAE-cellulose chromatography, and high-performance liquid chromatography (HPLC). The purified material behaved as a single component in reversed-phase HPLC, gave correct amino acid ratios, and was not distinguished from natural human C-peptide in terms of immunoreactivity and chromatographic behaviors. The $\alpha \rightarrow \beta$ transpeptidation at the Asp–Leu sequence, possible to occur associated with the HF cleavage, was studied using model peptides to demonstrate that the formation of β-peptide was 3—4% regardless of whether the β-carboxylic acid is free or protected as a benzyl ester.

Proinsulin synthesized in the β cells of the pancreas is transferred to the Golgi apparatus, where this precursor is degraded into insulin and C-peptide by certain proteolytic processes and both are released into the circulation.[2,3)]

The measurement of the concentration of C-peptide in peripheral venous plasma is of increasing importance for estimating the endogenous function of the pancreas, particularly in patients receiving insulin therapy. For this purpose Melani *et al.* first established a radioimmunoassay system using natural human C-peptide.[3)] However, the use of natural peptide is extremely limited and in addition human C-peptide can not be substituted by the animal peptides because C-peptides are highly species-specific in their primary structure. The chemical synthesis is thus the only means to meet the requirement for this peptide.

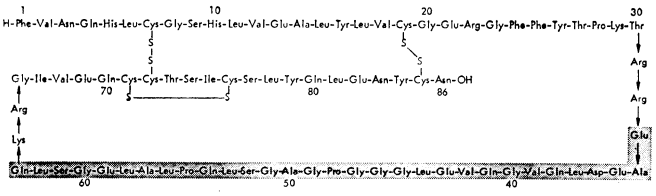

Fig. 1. Primary structure of human proinsulin.[4a)]
The shadowed portion indicates the region of C-peptide.

Human C-peptide (Fig. 1)[4)] is a thirty-one amino acid peptide corresponding to positions 33—63 of human proinsulin and contains none of aromatic amino acids, basic amino acids, and sulfur-containing amino acids. The synthesis by the conventional solution method has already been done by Naithani *et al.*,[5,6)] Geiger *et al.*,[7)] and Yanaihara *et al.*[8)] Yanaihara *et al.*[9)] also synthesized a derivative of human connecting peptide (Arg–Arg–C-peptide–Lys–Arg, positions 31—65 of proinsulin). These synthetic products were used in establishment of the radioimmunoassay system for C-peptide.[7,10,11)] The present paper describes our synthesis of human C-peptide by the solid-phase method.[12)] Synthesis of as large a peptide as C-peptide, particularly by the solid-phase technique, often presents considerable difficulty because it usually affords a highly heterogeneous product. Recently, however, HPLC has become a powerful tool for separation of closely related polypeptides[13,14)] including synthetic corticotropins and semisynthetic insulins.[14)] In the present synthesis of C-peptide also, reversed-phase HPLC played a major role in purification and characterization of the product.

Results and Discussion

The $\alpha \rightarrow \beta$ Rearrangement at the Aspartic Acid Residue. Human C-peptide contains a single Asp residue in position 36 (as numbered for proinsulin sequence) within the sequence Glu–Asp–Leu–Gln. It has been of special concern, particularly in the solid-phase synthesis, that Asp(OBzl)–Ser(Bzl)[15)] and Asp(OBzl)–Gly[16,17)] sequences are highly susceptible to cyclic imide formation when treated with HBr in TFA or with HF. The aspartimide formed is subject to nucleophilic attack at either carbonyl to give a mixture of α-Asp and β-Asp peptides. It is known, however, that Asp–Ser(Bzl) or Asp–Gly sequences containing a free β-COOH have little tendency to undergo this $\alpha \rightarrow \beta$ rearrangement.[15,18)] Thus, Yang and Merrifield[19)] proposed the use of the Pac group for temporary protection of the β-COOH. The Pac can be removed selectively after the completion of peptide synthesis but before the HF cleavage. This rearrangement is also known with other Asp peptides.[20)] Therefore, we tried to estimate the occurrence of the rearrangement in the present synthesis of C-peptide using Boc–Glu(OBzl)–Asp(OR)–Leu–Gly–OBzl (**1**: R=Bzl, **2**: R=H, **3**: R=Pac) as model compounds. The authentic samples of α-peptide, H–Glu–Asp(OH)–Leu–Gly–OH (**18**), and β-peptide, H–Glu–Asp(Leu–Gly–OH)–OH (**23**), were synthesized as illustrated in Fig. 2. They were well distinguished from each other and could, therefore, be determined easily on an amino acid analyzer.

The model compounds **1**—**3** were treated with HF–anisole in the usual manner and the product was

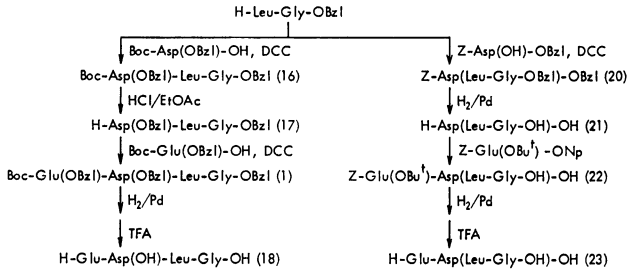

Fig. 2. Synthesis of model peptides.

Table 1. The $\alpha \rightarrow \beta$ rearrangement of aspartyl peptides, as examined with model compounds

Boc–Glu(OBzl)–Asp(OR)–Leu–Gly–OBzl[a)]

R	Percent formation of	
	α-Peptide	β-Peptide
Bzl	96.9	3.1
H	95.9	4.1
Pac	49.4	50.6

a) For details see Experimental.

subjected to an amino acid analyzer before and after treatment with triethylamine. Before the base treatment β-peptide was not found in the product, but the chromatogram showed the presence of an unknown material. When treated with base, β-peptide became detectable, while the unknown peak had disappeared completely, indicating the latter to be an aspartimide, H–Glu–Asp> Leu–Gly–OH, the intermediate to β-peptide. The results are summarized in Table 1. The data show that compound **1** is not more susceptible to the α→β rearrangement than compound **2**, indicating that in compound **1** the rate of cyclization is much slower than the rate of cleavage of the benzyl ester, that is, the protecting group is removed first, then cyclization occurs very slowly as in compound **2**. Since the Asp(OBzl)–Leu sequence was thus found not to be more susceptible than the Asp–Leu sequence, which gave 4% of β-peptide under the conditions used, we decided to use the benzyl ester as a protecting group of Asp-36 in the present synthesis of C-peptide.

Compound **3** containing a β-Pac ester has a great tendency to undergo rearrangement as shown in Table 1. Therefore, the β-Pac ester has to be removed completely prior to the HF cleavage,[19)] or otherwise its incomplete removal will lead to ready formation of β-peptide.

Solid-phase Synthesis of Human C-Peptide. The synthesis was started with the introduction of Boc–Gln to the solid support using perhydrodibenzo-18-crown-6 as catalyst according to Roeske and Gesellchen.[22)] The resulting Boc–Gln-resin had a substitution of 0.35 mmol Gln/g. A 2.5-g portion (0.87 mmol) of the resin was placed in a peptide synthesizer and subjected to cycles of synthesis according to the procedure outlined in Table 2. The amino acid residues were introduced stepwise except for positions 50—51, 44—49, and 38—40, which were introduced by using Boc–Ala–Gly–OH (**4**), Boc–Leu–Gly–Gly–Gly–Pro–Gly–OH (**5**), and Boc–Gln–Val–Gly–OH (**6**), respectively.

The α-amino function was temporarily blocked by the Boc group and the side chain functional groups of Asp, Glu, and Ser were protected by the benzyl group. The Boc group was removed with TFA–CH_2Cl_2 (1 : 1 by vol).[23)] The coupling reactions were mediated by DCC throughout except for glutamines which were incorporated by the active ester method using Boc–Gln–ONp with DMF as solvent (steps 11—13 in Table 2).

The DCC couplings were performed with 3 equiv of reactants (reactons) (6 equiv in the case of Boc–Pro or Boc–Val) at room temperature for 2 h, while the active ester couplings were carried out for 16 h with 6 equiv of Boc–Gln–ONp. All the coupling reactions were performed twice by repeating steps 11—20 and the second coupling reaction was followed by acetylation with acetic anhydride to block any remaining amino function

Table 2. Schedule for solid-phase synthesis of peptide

Step	Operation and reagent[a)]	Number of operation	Mixing time (min)
1	50% TFA in CH_2Cl_2, 15 ml	1	3
2	50% TFA in CH_2Cl_2, 15 ml	1	20
3	CH_2Cl_2, 15ml	2	3
4	2-Propanol, 15 ml	2	3
5	CH_2Cl_2, 15 ml	3	3
6	5% DIEA in CH_2Cl_2, 15 ml	2	3
7	Repeat step 3		
8	Repeat step 4		
9	Repeat step 5		
10	Monitoring[b)]		
11	Boc–amino acid or Boc–peptide (3 equiv.)[c)] in CH_2Cl_2, 6 ml[d)]		
12	CH_2Cl_2 rinse, 6 ml		
13	DCC (3 equiv.) in CH_2Cl_2, 6 ml		120
11*	DMF, 15 ml	2	3
12*	Boc–Gln–ONp (6 equiv.) in DMF, 15 ml	1	960
13*	DMF, 15 ml	2	3
14—20	Repeat steps 3—9		
21	Monitoring[b)]		
22—31	Repeat steps 11—20		
32	Monitoring[b)]		
33	15% Ac_2O in CH_2Cl_2, 6 ml		
34	CH_2Cl_2 rinse, 6 ml		
35	15% DIEA in CH_2Cl_2, 6 ml		60
36—42	Repeat steps 3—9		
43	Monitoring[b)]		

a) For introduction of Boc–Gln follow steps 11*—13* in place of steps 11—13. b) See Table 3 for detailed prodcedure. Delete step 10 when it follows deprotection of a Boc–Gln derivative. c) Use 6 equiv. in case of Boc–Pro or Boc–Val. d) DMF–CH_2Cl_2 mixtures are used in cases of Boc–peptides which are of low solubility in CH_2Cl_2.

Table 3. Procedure for monitoring solid-phase synthesis by the picrate method

Step	Operation and reagent	Number of operation	Mixing time (min)
1	0.1 M Picric acid in CH_2Cl_2, 15 ml	1	3
2	CH_2Cl_2,15 ml	7	3
3	2-Propanol	2	3
4	CH_2Cl_2, 15 ml	3	3
5	5% DIEA in CH_2Cl_2, 15 ml	2	3
6	CH_2Cl_2, 15 ml	2	3
7	2-Propanol, 15 ml	2	3
8	CH_2Cl_2, 15 ml	3	3

a) The washings collected in steps 5—8 are combined and subjected, after appropriate dilution with 95% ethanol, to manual measurement of absorption at 358 nm. The procedure basically follows Gisin's.[24)]

completely (steps 33—42).

The progress of solid-phase synthesis was monitored by the picrate method,[24] which determines resin-bound free amino group by picric acid salt formation. The determination was carried out, according to the procedure shown in Table 3, after every reaction of the deprotection, couplings, and acetylation. Only when a Gln came to the N-terminal, the determination after deprotection was omitted to prevent the chain termination by possible formation of the pyrrolidone ring. The picric acid bound to the resin was displaced by DIEA and measured on a spectrophotometer based on the molar absorptivity of 16100 at 358 nm.[24] The results are shown in Fig. 3. The total free amino group, as determined after deprotection, decreased progressively from 0.87 mmol for Gln-63 to 0.32 mmol for Ala-34 at an average rate of 4.5% per cycle.

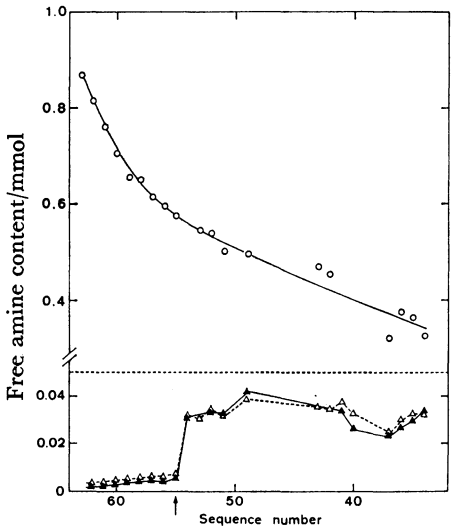

Fig. 3. Progress of solid-phase synthesis of human C-peptide as monitored by the picrate method.[24]
○—○: After deprotection (step 10 in Table 2), △---△: after 1st coupling (step 21 in Table 2), ▲—▲: after acetylation (step 43 in Table 2). Note that the scale is different between the upper part and the lower part of the ordinate. Sequence number (abscissa) represents position in the human proinsulin sequence. Positions 51—50, 49—44, and 40—38 were introduced as fragments. Glutamine residues occupy positions 54, 41, and 38, where the determination after deprotection (step 10 in Table 2) was eliminated. The arrow indicates the position of Pro-55. For details see text.

The free amino group was also determined after both first and second couplings. The values for the two determinations were nearly identical and were little affected by the subsequent acetylation, indicating that the reaction had been achieved satisfactorily by a single coupling operation. Therefore, the second coupling and acetylation might not be essential in the present synthesis. It is worthy of note that the picrate value showed a sudden rise by 0.02—0.03 mmol when Boc-Gln was introduced next to Pro-55. Schou *et al.*[25] suggested that an N-alkylation takes place between Pro and surplus chloromethyl group on the resin to form a tertiary amine. Such amine may be a cause for the apparent increase in free amino group which is seen in Fig. 3.

The protected peptide-resin obtained was found to contain 0.49 mmol of peptide, as calculated from the content of Leu. This value is significantly higher than the picrate value (0.32 mmol) obtained for Ala-34. The big difference might be a reflection of the accumulation of incomplete couplings not easily detectable by the picrate method.

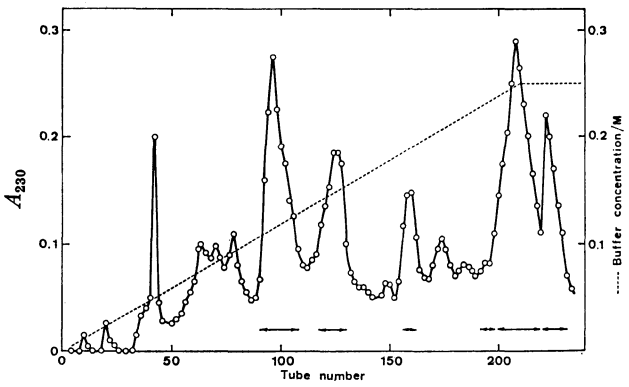

Fig. 4. Fractionation of crude human C-peptide preparation on DEAE-cellulose column.
Material: solid-phase synthetic product (gel-filtered through a Sephadex G-50 column), 237 mg; column: Whatman DE-52, 2.2×33 cm; eluent: 0—0.25 M NH_4HCO_3 (3000 ml) with a linear concentration gradient; fractionation: 14 ml/tube. Fractions, as indicated by the horizontal arrows, were collected.

The peptide-resin was deprotected with HF–anisole in the usual manner[21] and the resulting crude product was gel-filtered first and then subjected to DEAE-cellulose chromatography (Fig. 4). The fractions indicated by the horizontal arrows were collected and lyophilized. Tubes 199—220 and 221—234 had virtually the same and the simplest HPLC profile consisting of one major (**b**) and three minor components (**a**, **c**, and **d**) (Fig. 6A). These fractions were also shown to resemble human C-peptide more closely than any other fractions in the amino acid ratios. The combined amount of tubes 199—234 represented 25% of the crude product. Rechromatography of this preparation on a DEAE-cellulose column afforded a major peak (tubes 174—200, Fig. 5), from which a preparation containing components **a** and **b** but not **c** and **d** was obtained (Fig. 6B). For final purification, this was subjected to HPLC under conditions similar to those used for analytical HPLC. The eluate corresponding to component **b** was collected and desalted by gel filtration. The resulting preparation was shown to be fairly homogeneous by the criteria of HPLC (Fig. 6D) and TLC. The amino acid composition was consistent with that expected for human C-peptide and the optical rotation was identical with those reported in the literature.[5,6] Components **a**, **c**, and **d** have remained unidentified.

The synthetic peptide thus obtained was then compared with natural C-peptide preparations in order to establish their identity. For this purpose C-peptide was isolated from human urine and purified to a single

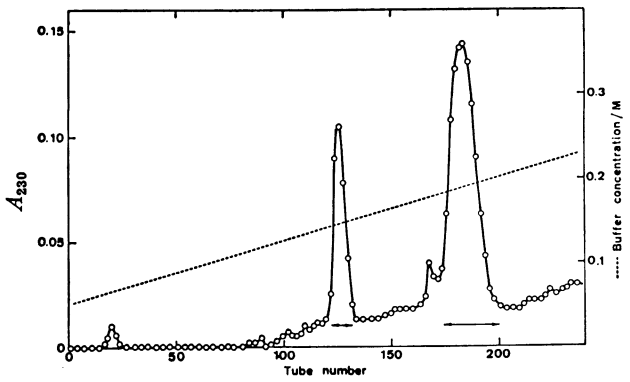

Fig. 5. Rechromatography of synthetic human C-peptide. Material: preparation from tubes 199—234 in Fig. 4, 21.3 mg; column: Whatman DE-52, 1.74× 33 cm; eluent: 0.05—0.25 M NH_4HCO_3 (2000 ml) with a linear concentration gradient; fractionation: 6 ml/tube. Fractions, as indicated by the horizontal arrows, were collected.

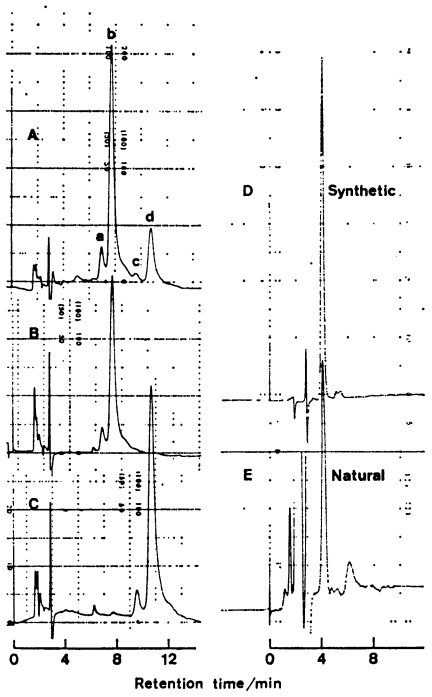

Fig. 6. HPLC of human C-peptide preparations.
A, tubes 199—220 in Fig. 4; B, tubes 174—200 in Fig. 5; C, tubes 122—132 in Fig. 5; D, synthetic C-peptide (component **b**, isolated by HPLC from tubes 174—200 in Fig. 5); E, natural C-peptide isolated from human urine. Peaks seen in E, but not in D, are those derived from a phosphate buffer used as solvent of natural C-peptide. HPLC conditions: column, Nucleosil $5C_{18}$, 0.4×25 cm; eluant, 0.1 M sodium phosphate (pH 7.0)–CH_3CN (79 : 21 by vol), 1 ml/min; detection, at 220 nm (0.04 AUFS).

component. This preparation contained all the constituent amino acids of human C-peptide in correct ratios. The isolation and purification of this urinary C-peptide will be described elsewhere. The identical HPLC profiles of the synthetic and natural preparations are shown in Fig. 6. Comparison between these two

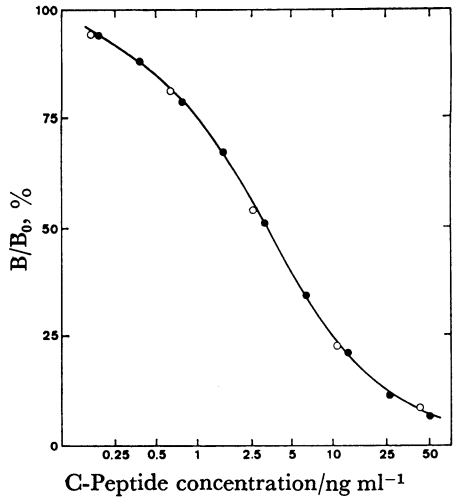

Fig. 7. Radioimmunoassay of human C-peptide preparations.
●—●: Synthetic C-peptide (component **b**), ○—○: natural C-peptide isolated from human urine. The assay was performed using a C-peptide assay system described elsewhere.[26]

preparations was also made in terms of the ability to displace [^{125}I]iodotyrosylated C-peptide specifically bound to the antibodies using a radioimmunoassay system developed by Kono *et al.*[26] As illustrated in Fig. 7, the displacement curves of the synthetic and natural preparations are perfectly superimposable. The identical results were also obtained in the radioimmunoassay performed with human pancreatic C-peptide as reference (data not shown).

These observations permit us to conclude that the synthetic peptide prepared in the present work is identical to human C-peptide.

Experimental

Protected amino acid derivatives, DCC, TFA, and chloromethylated copoly(styrene–2%-divinylbenzene) resin (200—400 mesh, 0.73 mmol Cl/g) were obtained from Protein Research Foundation, Osaka. DIEA (Aldrich) was distilled before use (bp 129—129.5 °C). Other reagents and solvents were of reagent grade and used without further purification. All melting points are uncorrected. TLC was performed on precoated silica gel plates (Kieselgel $60F_{254}$, Merck), unless otherwise specified, with the following solvent systems (ratios by vol): A, $CHCl_3$–MeOH (9 : 1); B, $CHCl_3$–MeOH (8 : 2); C, $CHCl_3$–MeOH–AcOH (90 : 10 : 3); D, $CHCl_3$–MeOH–AcOH (80 : 20 : 3); E, EtOAc–AcOH–H_2O (4 : 1 : 1); F, EtOAc–AcOH–H_2O (3 : 1 : 1). For detection the plate developed was sprayed with concd HCl, heated at 150 °C, and then sprayed with 0.2% ninhydrin in H_2O-saturated *n*-BuOH. Silica gel used for column chromatography was Kieselgel 60 (Merck). Amino acid analyses were performed on a Hitachi amino acid analyzer KLA-5 equipped with a Shimadzu data processor Chromatopac-ElA. Acid hydrolysis of peptide-resin samples was carried out in sealed evacuated tubes with concd HCl–propionic acid (1 : 1 by vol)[27] at 110 °C for 20 h. The hydrolysis of free peptides was carried out with 6 M HCl under similar conditions. Digestion with leucine aminopeptidase (Worthington) was performed as described by Hofmann *et al.*[28] in a substrate-enzyme ratio

of 10 (by wt). For HPLC a Waters Associates Model 6000A solvent delivery system, equipped with a Waters U6K injector, and a Japan Spectroscopic UVIDEC-100-II variable-wavelength UV detector were used throughout.

Peptide Synthesizer. A microcomputer-controlled semiautomated peptide synthesizer was designed and constructed in these Laboratories. The whole design basically followed that of Brunfeldt[29] except for the reaction vessel and its shaking device, which are rather similar to those of Merrifield's instrument.[30] The apparatus performed all the operations required for introduction of at least one amino acid residue or equivalent according to the schedule shown in Table 2. Although the apparatus is not equipped with any monitoring system, it performed transfer of reagents and solvents, for monitoring by the picrate method,[24] along the reservoir-reaction vessel-collecting bottle line. The spectrophotometric determination of the displaced picric acid was carried out manually.

Solid-phase Synthesis of Human C-Peptide. Neutralization of Boc-Gln-OH (2.1 g, 8.5 mmol) with KOH in 60% EtOH yielded Boc-Gln-OK, which was dried over P_2O_5 and then dissolved in DMF (100 ml). To this were added perhydrodibenzo-18-crown-6 (3.21 g, 8.6 mmol)[22] and chloromethylated copoly(styrene-2%-divinylbenzene) resin (10 g, 0.73 mmol Cl/g) and the mixture was gently stirred at 50 °C for 18 h. The Boc-Gln-resin thus obtained was filtered off, washed with DMF (30 ml×3), 75% DMF (30 ml×3), water (30 ml×2) and EtOH (30 ml×4) and dried over P_2O_5 *in vacuo* (11.2 g); Gln content: 0.35 mmol/g resin.

The Boc-Gln-resin (2.5 g, 0.87 mmol Gln) was placed in the peptide synthesizer and the synthesis was performed at room temperature according to the procedure shown in Table 2. On completion of the synthetic cycles the protected peptide-resin obtained was dried over P_2O_5 *in vacuo* (3.61 g).

A 1.08-g sample of the protected peptide-resin was treated with HF (7 ml) at −3—0 °C for 60 min in the presence of anisole (3 ml). After evaporation of the HF at the same temperature, the residue was triturated with EtOAc and the supernatant was discarded by decantation. The residue was washed with EtOAc two more times and then extracted with 0.05 M NH_4HCO_3. Lyophilization of the extract gave 320 mg of crude product.

The crude material (305 mg) was subjected to gel filtration on a column (3.3×91 cm) of Sephadex G-50 (superfine) with 0.05 M NH_4HCO_3 as eluent. Fractions (7.5 ml/tube) collected were monitored by absorbance at 230 nm and by TLC on a cellulose plate (Cellulose F_{254}, Merck) with *n*-BuOH–AcOH–pyridine–H_2O (30 : 6 : 20 : 24) as solvent. Tubes 40—83, which showed similar TLC patterns, were pooled and lyophilized (237 mg). This material was then fractionated on a DEAE-cellulose column (Whatman DE-52), as shown in Fig. 4, to give tubes 90—108 (40 mg), 118—130 (22 mg), 156—161 (11 mg), 191—198 (7 mg), 199—220 (60 mg), and 221—234 (17 mg). Amino acid ratios in acid hydrolysate of tubes 199—220: Asp 1.0 (1), Ser 1.8 (2), Glu 7.1 (8), Pro 2.5 (2), Gly 6.9 (7), Ala 3.0 (3), Val 1.9 (2), Leu 6.0 (6) (theoretical values for human C-peptide are given in parentheses).

Tubes 199—220 and 221—234 were combined and its small portion (21.3 mg) was rechromatographed on a DE-52 column (Fig. 5). The major peak at tubes 174—200 afforded 13.8 mg of peptide upon lyophilization. This was then subjected in ten separate portions (1.4 mg each) to HPLC on a column of Nucleosil $5C_{18}$ (1.0×30 cm) with a mixture of 0.01 M sodium phosphate buffer (pH 7.1) and CH_3CN (84.5 : 15.5 by vol) as eluent. The flow rate was 6 ml/min. The eluete corresponding to a major peak, as monitored by absorbance at 220 nm, was collected. Evaporation of the solvent gave a residue, which was desalted by gel filtration on a column (2.4×120 cm) of Sephadex G-25 (medium) with 5 mM NH_4HCO_3 as eluent. The peptide emerged was lyophilized and dried over P_2O_5; 7.6 mg (9% based on crude deprotection product), $[\alpha]_D^{26}$ −95.4±6.2° (*c* 0.2, 0.05 M NH_4HCO_3). Lit: $[\alpha]_D^{22}$ −95.4° (*c* 0.3, 0.05 M NH_4HCO_3),[5] $[\alpha]_D^{23}$ −96.2° (*c* 0.4, 0.05 M NH_4HCO_3),[6] $[\alpha]_D^{22}$ −92.3° (*c* 0.3, 0.1 M NH_3aq),[7] $[\alpha]_D^{20}$ −103.2° (*c* 1.00, 50% AcOH).[9] Amino acid ratios in acid hydrolysate: Asp 1.04 (1), Ser 1.88 (2), Glu 7.90 (8), Pro 2.24 (2), Gly 7.01 (7), Ala 3.09 (3), Val 1.95 (2), Leu 6.00 (6). TLC (cellulose): homogeneous in *n*-BuOH–AcOH–pyridine–H_2O (30 : 6 : 20 : 24). For HPLC see Fig. 6.

Synthesis of C-Peptide Fragments. *Boc-Ala-Gly-OBzl (**7**)*: Coupling of Boc-Ala-OH and H-Gly-OBzl (free base) with DCC in CH_2Cl_2 yielded compound **7**; 92%, mp 85—85.5 °C, $[\alpha]_D^{24.5}$ −28.1±0.7° (*c* 1.0, MeOH). Lit:[31] mp 83—84 °C, $[\alpha]_D^{20}$ −23.9° (*c* 1.0, $CHCl_3$). TLC: homogeneous in system C.

*Boc-Ala-Gly-OH (**4**)*: Catalytic hydrogenolysis of **7** gave **4** in 83% yield; mp 82—86 °C, $[\alpha]_D^{23.5}$ −25.1±0.6° (*c* 1.0, MeOH). Lit:[32] mp 78—80 °C, $[\alpha]_D$ −23° (MeOH). TLC: homogeneous in system C.

*Boc-Pro-Gly-OBzl (**8**)*: Coupling of Boc-Pro-OSu with H-Gly-OBzl (free base) in DMF yielded **8**; 88%, mp 71 °C, $[\alpha]_D^{25}$ −64.3±1° (*c* 1.0, MeOH). Lit:[33] mp 68—70 °C. TLC: homogeneous in system B.

*Boc-Gly-Pro-Gly-OBzl (**9**)*: Treatment of **8** with 1 M HCl in AcOH yielded H-Pro-Gly-OBzl·HCl, which was coupled with Boc-Gly-OSu in DMF in the presence of Et_3N to give **9**; 78%, mp 150—151 °C, $[\alpha]_D^{24}$ −81.3±1.6° (*c* 1.0, MeOH). Lit:[33] mp 145—147 °C. TLC: homogeneous in system B.

*Boc-Leu-Gly-Gly-OH (**10**)*: Coupling of Boc-Leu-OSu with H-Gly-Gly-OH (solubilized by Triton B) in DMF gave **10** as hygroscopic amorphous powder; 82%, $[\alpha]_D^{27}$ −8.1±0.5° (*c* 1.0, MeOH). TLC: homogeneous in system D. Found: C, 50.92; H, 7.74; N, 11.02%. Calcd for $C_{15}H_{27}N_3O_6\cdot 0.5H_2O$: C, 50.84; H, 7.96; N, 11.86%.

*Boc-Leu-Gly-Gly-NHNH$_2$ (**11**)*: Coupling of Boc-Leu-OSu with H-Gly-Gly-OBzl gave Boc-Leu-Gly-Gly-OBzl, from which compound **11** was derived by hydrazinolysis; 81%, $[\alpha]_D^{24}$ −8.3±0.5° (*c* 1.0, MeOH). TLC: homogeneous in system B. Found: C, 49.51; H, 7.83; N, 19.08%. Calcd for $C_{15}H_{29}N_5O_5\cdot 0.5H_2O$: C, 48.90; H, 8.21; N, 19.01%.

*Boc-Leu-Gly-Gly-Gly-Pro-Gly-OBzl (**12**)*: Treatment of **9** with 1 M HCl in EtOAc gave H-Gly-Pro-Gly-OBzl·HCl, which was coupled to **10** with DCC-HOSu[34] in DMF in the presence of Et_3N to yield **12** as amorphous solid; 87%, $[\alpha]_D^{24}$ −56.6±1.0° (*c* 1.0, MeOH). TLC: homogeneous in system B. Found: C, 56.61; H, 7.52; N, 12.56%. Calcd for $C_{31}H_{46}N_6O_9$: C, 56.78; H, 7.22; N, 12.81%.

Coupling of H-Gly-Pro-Gly-OBzl with the azide derived from **11** by the treatment with isopentyl nitrite[35] also produced **12** in 96% yield; $[\alpha]_D^{24.5}$ −57.0±0.9° (*c* 1.0, MeOH).

*Boc-Leu-Gly-Gly-Gly-Pro-Gly-OH (**5**)*: Catalytic hydrogenolysis of **12** in AcOH gave **5** as amorphous solid; 96%, $[\alpha]_D^{24.5}$ −55.9±1.0° (*c* 1.0, MeOH). TLC: homogeneous in system E. Found: C, 51.72; H. 7.18; N, 14.93%. Calcd for $C_{24}H_{40}N_6O_9$: C, 51.79; H, 7.24; N, 15.10%.

*Boc-Val-Gly-OBzl (**13**)*: Coupling of Boc-Val-OH and H-Gly-OBzl with DCC yielded **13**; 76%, mp 73—75 °C, $[\alpha]_D^{24}$ −27.1±0.7° (*c* 1.0, MeOH). Lit: mp 63—69 °C;[36] mp 74.5—76.5 °C, $[\alpha]_D^{20}$ −8.3° (*c* 2.0, DMF).[37] TLC: homogeneous in system A.

Boc-Gln-Val-Gly-OBzl (14): Treatment of **13** with TFA yielded H-Val-Gly-OBzl·TFA, which was coupled with Boc-Gln-ONp in DMF in the presence of DIEA to give **14**; 61%, mp 208—209 °C, $[\alpha]_D^{23}$ −47.9±1.3° (*c* 1.0, MeOH). TLC: homogeneous in system D. Found: C, 58.56; H, 7.10; N, 11.36%. Calcd for $C_{24}H_{36}N_4O_7$: C, 58.52; H, 7.34; N, 11.37%.

Boc-Gln-Val-Gly-OH (6): Catalytic hydrogenolysis of **14** in AcOH yielded **6** as amorphous solid; 76%, $[\alpha]_D^{26}$ −45.6 ±1.5° (*c* 0.5, MeOH). TLC: homogeneous in system F. Found: C, 50.47; H, 7.68; N, 13.76%. Calcd for $C_{17}H_{30}N_4O_7$: C, 50.47; H, 7.51; N, 13.92%.

Synthesis of Model Peptides. H-Leu-Gly-OBzl·HCOOH (15): Coupling of Boc-Leu-OH and H-Gly-OBzl with DCC followed by treatment with HCOOH for 5 h yielded **15**; 74%, mp 93—94 °C, $[\alpha]_D^{22.5}$ +7.4±0.5° (*c* 1.0, MeOH). Lit:[39] mp 92.5—93.5 °C, $[\alpha]_D^{20}$ +5.0° (*c* 1.0, MeOH). TLC: homogeneous in system E.

Boc-Asp(OBzl)-Leu-Gly-OBzl (16): Coupling of Boc-Asp-(OBzl)-OH and H-Leu-Gly-OBzl (free base derived from **15**) with DCC yielded **16**; 80%, mp 111—115 °C, $[\alpha]_D^{23}$ −38.6±0.8° (*c* 1.0, MeOH). TLC: homogeneous in system A. Found: C, 63.88; H, 7.19; N, 7.10%. Calcd for $C_{31}H_{41}N_3O_8$: C, 63.79; H, 7.08; N, 7.19%.

H-Asp(OBzl)-Leu-Gly-OBzl·HCl (17): Treatment of **16** with 1 M HCl in EtOAc produced **17**; 91%, mp 172—174 °C, $[\alpha]_D^{23}$ −19.7 ±0.6°, (*c* 1.0, MeOH). TLC: homogeneous in system B. Found: C, 59.83; H, 6.61; N, 8.10; Cl, 6.96%. Calcd for $C_{26}H_{33}N_3O_6$·HCl: C, 60.05; H, 6.59; N, 8.08; Cl, 6.82%.

Boc-Glu(OBzl)-Asp(OBzl)-Leu-Gly-OBzl (1): Boc-Glu-(OBzl)-OH and **17** were coupled with DCC in CH_2Cl_2 in the presence of DIEA to give compound **1** as amorphous solid after purification on a silica gel column with EtOAc-hexane (1 : 1) as solvent; 62%, $[\alpha]_D^{23}$ −30.1±0.7° (*c* 1.0, MeOH). TLC: homogeneous in system B. Found: C, 64.04; H, 6.80; N, 6.92%. Calcd for $C_{43}H_{54}N_4O_{11}$: C, 64.32; H, 6.78; N, 6.98%.

H-Glu-Asp-Leu-Gly-OH (18): Catalytic hydrogenolysis followed by treatment with TFA of compound **1** produced the TFA salt of **18**. This was treated with Amberlite CG-400 (acetate form) with 1 M AcOH as solvent. The resulting solution was lyophilized and the residue was subjected to chromatography on a column (1.2×29 cm) of DEAE-cellulose (Whatman DE-52) using an NH_4HCO_3 buffer (pH 7.8, 2000 ml) with a linear concentration gradient of 0—0.2 M. The fractions (10 ml/tube) were monitored by absorbance at 230 nm and those corresponding to a major peak were pooled and lyophilized. The resulting material was desalted by gel filtration on a Sephadex G-10 column with 0.1 M AcOH as eluent. A pure preparation of **18** was obtained as colorless powder upon lyophilization; 88%, $[\alpha]_D^{24}$ −36.2±1.4° (*c* 0.5, H_2O). TLC: homogeneous in system F. Found: C, 44.42; H, 6.82; N, 12.18%. Calcd for $C_{17}H_{28}N_4O_9$·1.5H_2O: C, 44.44; H, 6.80; N, 12.19%. Amino acid ratios in: acid hydrolysate: Asp 1.03 (1), Glu 1.03 (1), Gly 0.96 (1), Leu 1.00 (1); leucine aminopeptidase digest: Asp 0.97 (1), Glu 0.98 (1), Gly 0.97 (1), Leu 1.00 (1).

Z-Asp(OH)-OBzl (19): The Cs salt of Z-Asp(OBu^t)-OH was allowed to react with benzyl bromide at 25 °C for 3 d. The resulting Z-Asp(OBu^t)-OBzl was treated with 1 M HCl in EtOAc to give **19**; 39%, mp 83—84 °C, $[\alpha]_D^{25}$ −18.1±0.6° (*c* 1.0, MeOH). Lit:[40] mp 82—85 °C, $[\alpha]_D^{25}$ −15.3° (*c* 1.60, EtOH). TLC: homogeneous in system B.

Z-Asp(Leu-Gly-OBzl)-OBzl (20): Compound **19** and H-Leu-Gly-OBzl (free base derived from **15**) were coupled with DCC to yield **20**; 74%, mp 150 °C, $[\alpha]_D^{25}$ −35.2±0.8° (*c* 1.0, MeOH). TLC: homogeneous in system B. Found: C, 66.22; H, 6.53; N, 7.01%. Calcd for $C_{34}H_{39}N_3O_8$: C, 66.11; H, 6.63; N, 6.80%.

H-Asp(Leu-Gly-OH)-OH (21): Crystalline **21** was obtained from **20** by catalytic hydrogenolysis in AcOH; 93%, mp 236—237 °C dec, $[\alpha]_D^{26}$ −30.0±0.7° (*c* 1.0, H_2O). Found: C, 47.42; H, 7.09; N, 13.52%. Calcd for $C_{12}H_{21}N_3O_6$: C, 47.52; H, 6.98; N, 13.85%.

Z-Glu(OBu^t)-Asp(Leu-Gly-OH)-OH (22): Compound **21** was coupled with Z-Glu(OBu^t)-ONp in DMF in the presence of DIEA to give **22** as amorphous solid; 89%, $[\alpha]_D^{24}$ −24.9 ±0.6° (*c* 1.0, MeOH). TLC: homogeneous in system E. Found: C, 54.98; H, 6.88; N, 8.64%. Calcd for $C_{29}H_{42}N_4O_{11}$·0.5H_2O: C, 55.14; H, 6.86; N, 8.87%.

H-Glu-Asp(Leu-Gly-OH)-OH (23): Catalytic hydrogenolysis and subsequent treatment with TFA of **22** yielded **23**, which was purified in the same manner as described for **18**; 86%, $[\alpha]_D^{24.5}$ −8.0±0.5° (*c* 1.0, H_2O). TLC: homogeneous in system E. Found: C, 43.99; H, 6.71; N, 11.73%. Calcd for $C_{17}H_{28}N_4O_9$·2H_2O: C, 43.59; H, 6.89; N, 11.96%.

Boc-Asp(OPac)-Leu-Gly-OBzl (24): Boc-Asp(OPac)-OH derived from the dicyclohexylamine salt[19] and H-Leu-Gly-OBzl derived from **15** were coupled with DCC to give **24**; 84%, mp 233—233.5 °C, $[\alpha]_D^{22}$ −36.5±0.8° (*c* 1.0, MeOH). TLC: homogeneous in system B. Found: C, 62.89; H, 6.91; N, 6.78%. Calcd for $C_{32}H_{41}N_3O_9$: C, 62.83; H, 6.76; N, 6.78%.

H-Asp(OPac)-Leu-Gly-OBzl·HCl (25): Treatment of **24** with M HCl in EtOAc yielded **25**; 90%, mp 79—80 °C, $[\alpha]_D^{23}$ −7.4±0.5° (*c* 1.0, MeOH). TLC: homogeneous in system D. Found: C, 58.93; H, 6.17; N, 7.65; Cl, 6.55%. Calcd for $C_{27}H_{33}N_3O_7$·HCl: C, 59.17; H, 6.25; N, 7.67; Cl ,6.47%.

Boc-Glu(OBzl)-Asp(OPac)-Leu-Gly-OBzl (3): Coupling of Boc-Glu(OBzl)-OH and H-Asp(OPac)-Leu-Gly-OBzl (free base derived from **25**) with DCC yielded **3**, which was purified on a silica gel column with 20—30% EtOAc in CH_2Cl_2 as solvent; 68%, mp 142—143.5 °C, $[\alpha]_D^{23}$ −31.6 ±0.7° (*c* 1.0, MeOH). TLC: homogeneous in system B. Found: C, 63.33; H, 6.55; N, 6.91%. Calcd for $C_{44}H_{54}N_4O_{12}$: C, 63.60; H, 6.55; N, 6.74%.

Boc-Glu(OBzl)-Asp(OH)-Leu-Gly-OBzl (2): Compound **3** was treated with Zn in AcOH at 25 °C overnight to give the Zn salt of **2** as amorphous solid. TLC: almost homogeneous in system C. Found: C, 51.88; H, 6.25; N, 7.01%. Calcd for $C_{36}H_{47}N_4O_{11}Zn$: C, 50.95; H, 6.10; N, 7.21%.

The α→β Rearrangement of Aspartyl Peptides. The model compound (**1**, **2**, or **3**; 108 mg) was treated with HF (2 ml) in the presence of anisole (0.3 ml) at 0 °C for 60 min. The HF was evaporated at 0 °C *in vacuo* and the residue was dissolved in water. The solution was washed twice with Et_2O and then lyophilized (80 mg). A 30-mg portion of this product was dissolved in 1% Et_3N (5 ml) and the solution was kept at 37 °C for 16 h, after which the solvent was removed by lyophilization. The resulting material was subjected to determination on an amino acid analyzer. The contents of α-peptide [H-Glu-Asp(OH)-Leu-Gly-OH] and β-peptide [H-Glu-Asp(Leu-Gly-OH)-OH] were calculated with the authentic samples (**18** and **23**) as reference. The α-peptide and β-peptide had retention times of 103 and 91 min, respectively, and the ninhydrin color intensity of β-peptide was found to be 0.96 relative to that of α-peptide under the following conditions: column, Hitachi Custom No. 2613, 0.9× 55 cm; buffer, 0.2 M sodium citrate (pH 3.25), 30 ml/h; temp, 55 °C. The results are shown in Table 1.

The peptide synthesizer used in the present work was

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References

1) All the amino acid residues mentioned in this communication are of the L-configuration. Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [*Biochemistry*, **5**, 2585 (1966); **6**, 362 (1967); **11**, 1726 (1972)], and include: Boc, *t*-butoxycarbonyl; Z, benzyloxycarbonyl; Bu^t, *t*-butyl; Bzl, benzyl; Pac, phenacyl; ONp, *p*-nitrophenoxy; OSu, succinimidooxy; DCC, dicyclohexylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide.

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