Rapid Peptide Synthesis in Liquid Phase. Preparation of Angiotensin II and Delta-sleep-inducing Peptide by the "Hold-in-Solution" Method[1,2]

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A new technique for rapid peptide synthesis has been demonstrated by the preparation of a protected angiotensin II, Z-Asp(OBzl)-Arg(NO_2)-Val-Tyr(Bzl)-Ile-His(Bzl)-Pro-Phe-OBzl (**1**), and a protected delta-sleep-inducing peptide, Z-Trp-Ala-Gly-Gly-Asp(OBzl)-Ala-Ser(Bzl)-Gly-Glu(OBzl)-OBzl (**2**). Elongation of the peptide chain was carried out in 1,2-dichloroethane by repeating the following series of operations: acylation of a benzyl ester of amino acid or peptide with Boc-amino acid by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 1-hydroxybenzotriazole; washing of the organic layer; acidolysis of the α-amino protector; neutralization; and washing. Throughout the synthesis, the growing peptides were held in the organic layer without being isolated. The protected peptides, **1** and **2**, were obtained in overall yields of 78% and 53% based on their *C*-terminal amino acid esters, and were then hydrogenated to give angiotensin II and delta-sleep-inducing peptide, respectively, thus demonstrating the usefulness of this method for rapid peptide synthesis.

The liquid phase method for peptide synthesis is superior to the solid phase method especially with respect to variety of synthetic routes available and facility for purification of the intermediates. The former method, however, usually involves a considerable amount of time-consuming work; hence various attempts have been made to reduce the time required.[3] The present paper describes a new technique, tentatively named the "hold-in-solution" method, for rapid peptide synthesis in the liquid phase, in which a water-containing environment can be tolerated throughout the entire procedure for peptide elongation. Essentially, this method consists of a series of operations, the series being repeated in as many cycles as necessary to obtain the desired elongation (Fig. 1). The details of the operations, designated herein as (A), (B), (C), (D), and (E), are as follows: (A) Acylation of an amino acid benzyl ester with a Boc-amino acid by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSCD·HCl)[4] and 1-hydroxybenzotriazole (HOBt)[5] as the coupling reagents in a water-immiscible organic solvent, 1,2-dichloroethane (DCE). The coupling reaction is carried out at room temperature. Completion of the acylation is easily detected by disappearance of the amine component on TLC. Excess amounts (usually 2 mol equivalents per mole of the amine component) of a Boc-amino acid, HOBt, and WSCD·HCl are used for the acylation. (B) Successive washing of the reaction mixture with 0.1 M (1 M=1 mol dm^{-3}) HCl, water, 0.5 M Na_2CO_3, and water. Complete removal of the excess Boc-amino acid is sometimes difficult by the washing but is not necessary in this step. (C) Acidolysis of the α-amino protector by HCl in dioxane (concentration of HCl in the mixture; 1.7—2.2 M. 0 °C, 0.5 h). In this step the excess Boc-amino acid is converted to the free amino acid, which is easily removed from the organic layer in the following steps. (D) Neutralization of the mixture by 2 M Na_2CO_3 below 0 °C. (E) Removal of the aqueous layer and washing of the organic layer with water, 0.5 M Na_2CO_3, and water in that order. All the treatments are carried out in a single vessel. When a mixture emulsifies, the two layers are separated by centrifugation.

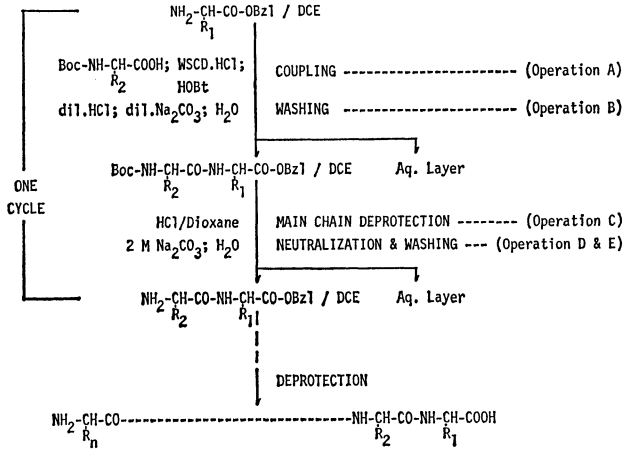

Fig. 1. Scheme of the synthesis of an oilgopeptide by the "hold-in-solution" method.

By repeating the series of operations through two or more cycles, a peptide chain can be elongated stepwise. During the preparation, the intermediate peptide is held in the organic layer without being isolated. It is desirable for best results that the *N*-terminal free peptide have low solubility in the aqueous layer.

Table 1 shows the effect of some additives on the preparation of a model peptide, Boc–Val–Ile–OBzl,

Table 1. Effect of additives on a one-hour coupling reaction of Boc-Val-OH and H-Ile-OBzl[a]

Additive	Yield of Boc-Val-Ile-OBzl (%) Solvent (ml)			
	DCE (12)	DCE/H_2O (10/2)	DMF (12)	DMF/H_2O (10/2)
None	58	30	16	12
HOBt	82	71	80	90
HOSu	21	33	9	20
HONb	27	35	11	23

a) See Experimental.

Table 2. Amino acid ratios in each cycle of acylation in the preparation of angiotensin II

Cycle No.	Pro	Phe	His[a]	Ile	Tyr[b]	Val	Arg	Asp
1	0.95	1.05						
2	0.98	1.02	—					
3	1.02	1.06	—	0.92				
4	1.02	1.06	—	0.92	0.82			
5	1.05	1.15	—	1.00	0.84	0.97		
6	1.03	1.01	—	0.99	0.37	0.99	0.88[c]	
Isolated **1** (crude)	1.03	1.02	—	0.78	0.35	1.03	1.33[c]	0.92
After hydrogenolysis (crude)	1.04	1.08	0.93	0.92	0.99	1.04	0.94	1.07

a) im-Benzyl group was not cleaved by the hydrolysis. b) Removal of the benzyl ether substituted on the phenyl group was incomplete. c) Sum of Arg and Orn.

Table 3. Amino acid ratios in each cycle of acylation in the preparation of DSIP

Cycle No.	Glu	Gly	Ser	Ala	Asp	Gly	Gly	Ala	Trp
1	1.00	1.00							
2	1.07	1.04	0.89						
3	1.08	1.05	0.92	0.95					
4	1.05	1.02	0.91	0.93	1.09				
5	1.06	0.96[a]	0.93	0.98	1.11	0.96[a]			
6	1.10	0.94[a]	0.95	1.04	1.10	0.94[a]	0.94[a]		
7	1.12	0.98[a]	0.93	0.94[b]	1.13	0.98[a]	0.98[a]	0.94[b]	
Isolated **2**	1.07	0.99[a]	0.93	0.97[b]	1.10	0.99[a]	0.99[a]	0.97[b]	—[c]

a) Average value for Gly. b) Average value for Ala. c) Destroyed by the hydrolysis.

by a one-hour coupling reaction of Boc–Val–OH and H–Ile–OBzl, both of which are rather less reactive because of the steric hindrance of their bulky side chains, in several solvent systems. Without the additives, the coupling yield was low even in the anhydrous solvents. The presence of water further lowered the yield. Addition of HOBt markedly improved the yield in the water-containing solvents as well as in the anhydrous media. It is interesting that the use of other additives, *N*-hydroxysuccinimide (HOSu)[6] and *N*-hydroxy-5-norbornene-2,3-dicarboximide (HONb),[7] rather lowered the yield in the anhydrous solvents. In the water-containing systems, they improved the yield but the effect was not as significant as that of HOBt. Thus, peptide elongation with sufficient yield is possible in water-containing media by addition of HOBt.

As a model peptide synthesis by this new method, preparation of a protected angiotensin II, Z–Asp(OBzl)–Arg(NO_2)–Val–Tyr(Bzl)–Ile–His(Bzl)–Pro–Phe–OBzl (**1**), and a protected delta-sleep-inducing peptide,[8] Z–Trp–Ala–Gly–Gly–Asp(OBzl)–Ala–Ser(Bzl)–Gly–Glu(OBzl)–OBzl (**2**), was carried out in DCE. Two equimolar amounts of the acylating reagents were used per mole of the amine components. Tables 2 and 3 show the amino acid ratios in the acid hydrolysates of the intermediate peptides held in the organic layer at the end of operation E of each cycle. Complete acylation was achieved in every coupling reaction This fact indicates that the acylation in this method proceeds more smoothly than that in the solid phase method employing the usual polystyrene resin as the support.[9] Centrifugation was required in operation E after introduction of valine at position 3 in the preparation of **1**, and of alanine at position 6 in the case of **2**. The protected peptides, **1** and **2**, were obtained in overall yields of 78% and 53%, respectively, based on their *C*-terminal esters. HPLC profiles of the products are shown in Fig. 2. After the catalytic hydrogenolysis of **1** and **2**, the free peptides were easily purified by partition chromatography on Sephadex[10] to give angiotensin II and DSIP, respectively. Homogeneities of the synthetic peptides were confirmed by HPLC analyses (Fig. 3).

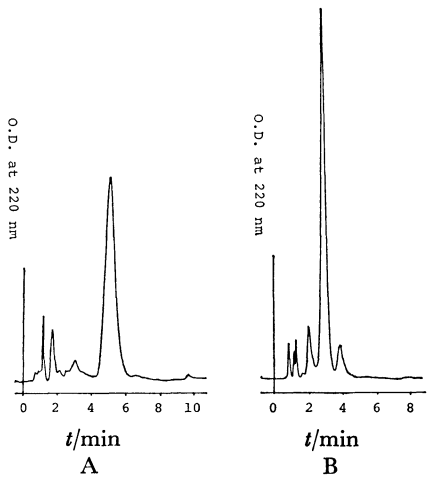

Fig. 2. HPLC profiles of (A) crude **1** ($MeOH/H_2O$, 8/1) and (B) crude **2** ($MeOH/H_2O$, 5/1).

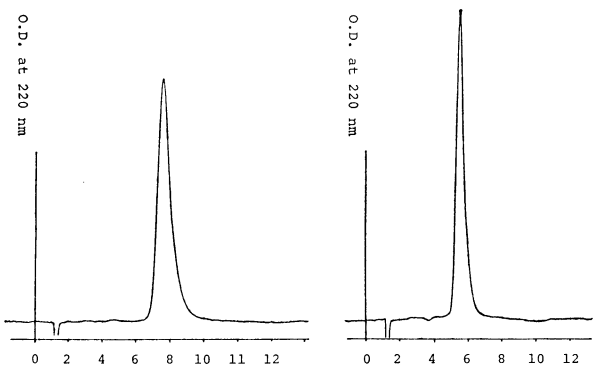

Fig. 3. HPLC profiles of (A) synthesized angiotensin II (MeOH/1% ammonium acetate, pH 6.90, 7/10) and (B) synthesized DSIP (MeOH/1% ammonium acetate, pH 6.90, 1/8).

Preparation of the octa- and nonapeptide demonstrated the utility of the new method for rapid synthesis of oligopeptides. The method has been applied successfully to the preparation of the fragment peptides which served as the building blocks in the construction of granuliberin R[11] and the active site of troponin I.[12]

Advantages of the "hold-in-solution" peptide synthesis are (1) that it is fast, (2) that it permits the intermediates to be characterized easily, and (3) that it does not require large excesses of the acylating reagents nor any special apparatus.

Experimental

The peptides were hydrolyzed in constantly boiling HCl (110 °C, 16 h) or in 3 M 2-mercaptoethanesulfonic acid (110 °C, 20 h). Amino acid analyses were carried out on a JLC-6AS automatic amino acid analyzer (JEOL). HPLC analyses were performed on a steel column (4 mm×250 mm) packed with LiChrosorb RP-18 (10 μm, Merck & Co.) and optical density at 220 nm was monitored at a flow rate of 1.83 ml/min. An authentic sample of angiotensin II was purchased from the Protein Research Foundation, Osaka, Japan.

Effect of Additives. Boc–Val–OH (0.5 mM, derived from the DCHA salt), H–Ile–OBzl tosylate (0.5 mM), triethylamine (0.5 mM), and an additive (0.5 mM) were dissolved in a solvent. To this solution, solid WSCD·HCl (0.5 mM) was added and the mixture was stirred for 1 h at room temperature. The solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate (50 ml) and washed successively with 0.1 M HCl (10 ml×2), water (10 ml), 0.5 M Na_2CO_3 (20 ml), and water (10 ml×4). After removal of the solvent, the residue was dissolved in methanol/water (3/1) of a known volume (50—110 ml) and an aliquot of the solution (5—30 μl) was applied to HPLC (solvent: methanol/water, 3/1). The yield of the dipeptide was determined by use of the calibration curve of a standard solution of Boc–Val–Ile–OBzl (9.56 mg in 30 ml of methanol/water, 3/1).

Preparation of Angiotensin II. H–Phe–OBzl tosylate (222 mg, 0.5 mM), triethylamine (0.07 ml), Boc–Pro–OH (1.0 mM, 215 mg), and HOBt (135 mg, 1.0 mM) were dissolved in DCE (25 ml). WSCD·HCl (192 mg, 1.0 mM) in water (1 ml) was added to the solution and the mixture was stirred for 2 h at room temperature. The reaction mixture was washed successively with 0.1 M HCl (5 ml×3), water (5 ml), 0.5 M Na_2CO_3 (5 ml), and water (5 ml×4) below 10 °C. Each of the aqueous phases was removed by pipetting. To the organic layer, HCl in dioxane (7.2 M, 8 ml) was added at −10—0 °C and the mixture was stirred at 0 °C for 0.5 h, then chilled to −10 °C, and 2 M Na_2CO_3 (18 ml) was added to it. The aqueous layer was removed and the organic layer was washed successively with water (4 ml×2), 0.5 M Na_2CO_3 (4 ml), and water (4 ml×2). An aliquot (*ca.* 0.05 ml) of the organic layer was evaporated and the residue was hydrolyzed to determine the amino acid ratio (Table 2). In the second cycle, Boc–His(Bzl)–OH (345 mg, 1.0 mM) and HOBt (135 mg, 1.0 mM) were added to the organic layer followed by WSCD·HCl (192 mg, 1.0 mM) dissolved in water (1 ml). The mixture was stirred for 4.5 h, then otherwise subjected to the procedures described above for the first cycle. Further acylation was carried out successively by Boc–Ile–OH, Boc–Tyr(Bzl)–OH, Boc–Val–OH, Boc–Arg(NO_2)–OH, and Z–Asp(OBzl)–OH (1.0 mM each) using HOBt and WSCD·HCl (1.0 mM each) as the condensation reagents. The coupling reactions were carried out for 3 h to overnight. Oily Boc-amino acids, Boc–Ile–OH and Boc–Val–OH, were added as DCE solution (2 ml). Boc–Arg(NO_2)–OH was added as a DMF solution (2 ml). Centrifugation was required in operation E after addition of Boc–Val–OH. In the final acylation, the reaction mixture was diluted with DMF (10 ml) after addition of WSCD·HCl. After the acylation was completed, the mixture was further diluted with DCE (100 ml) and water (40 ml). The organic layer was separated and washed successively with 0.1 M HCl (10 ml×2), 0.5 M Na_2CO_3 (20 ml), and water (40 ml×2, 20 ml×3). The organic layer was concentrated to give an oil, which was then solidified by addition of ether. **1**: mp 169—178 °C decomp, 609 mg (78% based on H–Phe–OBzl tosylate). Calcd for $C_{86}H_{100}O_{16}N_{14}$: C, 65.14; H, 6.36; N, 12.37%. Found: C, 65.10; H, 6.43; N, 12.16%. R_f: 0.29 (Merck precoated silicagel plate, $CHCl_3$/MeOH, 95/5). A part of the product (200 mg) was hydrogenated in a mixture of DMF (30 ml), water (20 ml), and acetic acid (1 ml) for 42 h using Pd black. After removal of the catalyst and the solvent, a crude product was obtained. R_f: 0.54 (Avicel precoated cellulose plate, 1-butanol/pyridine/acetic acid/water, 16/10/3/12). Amino acid ratio in HCl–hydrolysate, Asp 1.07, Pro 1.04, Val 1.04, Ile, 0.92, Tyr 0.99, Phe 1.08, His 0.93, Arg 0.94. The product was purified by partition chromatography on a Sephadex G-25 column (1.6 cm×36 cm, 1-butanol/pyridine/acetic acid/water, 16/10/3/12). Fractions of 3 g each were collected and fractions 15—18 were combined and concentrated. The residue was applied to a Sephadex G-10 column (1.2 cm×24 cm) and eluted with 5% acetic acid. Fractions of 2 g each were collected and the desired peptide was recovered from fractions 5—9 by lyophilization: 94 mg; 63% from **1**. Amino acid ratio in HCl–hydrolysate (for 24 h): Asp 1.09, Pro 0.98, Val 1.02, Ile 0.91, Tyr 1.01, Phe 1.09, His 0.94, Arg 0.96. Calcd for $C_{50}H_{71}O_{12}N_{13}\cdot CH_3COOH\cdot 4H_2O$: C, 53.01; H, 7.10; N, 15.45%. Found: C, 53.24; H, 6.57; N, 15.14%. The biological activity (oxytocic activity on an isolated rat uterus) of the prepared peptide was the same as that of the authentic sample.

Preparation of DSIP. H–Glu(OBzl)–OBzl tosylate (250 mg, 0.5 mM) in DCE (25 ml) was acylated successively by Boc–Gly–OH, Boc–Ser(Bzl)–OH, Boc–Ala–OH, Boc–Asp(OBzl)–OH, Boc–Gly–OH, Boc–Gly–OH, Boc–Ala–OH, and Z–Trp–OH (1.0 mM each) using HOBt and WSCD·HCl

(1.0 mM each) as described above. The following reagents were used:

Operation B: 0.1 M HCl (5 ml×3), water (5 ml), 0.5 M Na_2CO_3 (5 ml), water (5 ml×4).

Operation C: 8.8 M HCl/dioxane (8 ml).

Operation D: 2 M Na_2CO_3 (18 ml).

Operation E: water (4 ml×2), 0.5 M Na_2CO_3 (4 ml), water (4 ml×4 to 6).

Centrifugation was required in operation E after introduction of Boc–Ala–OH at position 6 and in both operation B and E after introduction of Boc–Ala–OH at position 2. After the final acylation, the reaction mixture was washed with water (10 ml×3, the aqueous phases being separated by centrifugation). The organic layer was concentrated to give an oil, which was solidified in ether. The solid collected was dissolved in DMF, decolorized with charcoal, and reprecipitated by addition of ether. **2**: 284 mg. 53% based on H–Glu(OBzl)–OBzl tosylate. Mp 210—213 °C decomp. Calcd for $C_{71}H_{78}O_{17}N_{10}\cdot H_2O$: C, 62.26; H, 5.92; N, 10.29%. Found: C, 62.59; H, 5.88; N, 10.26%. Amino acid ratio in HCl–hydrolysate: Asp 1.10, Ser 0.93, Glu 1.07, Gly 2.96, Ala 1.94. In 2-mercaptoethanesulfonic acid–hydrolysate: Asp 1.12, Ser 0.96, Glu 1.10, Gly 2.90, Ala 1.92, Trp 0.73. A part of the product (166 mg) was hydrogenated for 7 h in DMF (60 ml) and water (20 ml) using Pd black. After the usual work-up, the crude product was applied to partition chromatography on a Sephadex G-25 column (1.6 cm×36 cm, 1-butanol/pyridine/acetic acid/water, 16/10/3/12). Fractions of 3 g each were collected and fractions 47—53 were combined, concentrated, and lyophilized from water (67 mg). The peptide was further applied to a Sephadex G-10 column (1.2 cm×26 cm), and eluted with water. Fractions of 1 g each were collected and fractions 12—19 were combined and lyophilized to give the desired peptide: 52 mg, 39% from **2**. R_f: 0.11 (silica gel, 1-butanol/acetic acid/water, 4/1/1). Calcd for $C_{35}H_{48}O_{15}N_{10}\cdot 4H_2O$: C, 45.65; H, 5.12; N, 15.20%. Found: C, 45.12; H, 5.58; N, 15.41%. Amino acid ratio in HCl-hydrolysate: Asp 1.01, Ser 0.92, Glu 1.09, Gly 2.96, Ala 2.01, Trp 0.85. In 2-mercaptoethanesulfonic acid–hydrolysate: Asp 0.91, Ser 0.97, Glu 1.08, Gly 2.99, Ala 2.06, Trp 0.98.

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2) Amino acids used are of L-configuration. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IBU Commission of Biochemical Nomenclature. Other abbreviations are as follows: Boc= butoxycarbonyl, Z = benzyloxycarbonyl, Bzl = benzyl, NO_2=nitro.

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