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Tactics for Simplification of Purification Process in the Solid Phase Peptide Synthesis[1]

KENJI SUZUKI, YUSUKE SASAKI, and NOBUYOSHI ENDO

Tohoku College of Pharmacy[2]

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These tactics depend primarily on coupling of a highly polar compound, as a highly polar handle in the purification process, with the terminal amino group of the desired sequence assembled on a solid support. Lysine was used as the highly polar compound in this study. All protecting groups labile towards hydrogen fluoride were removed as usual and the resulting crude peptide was purified through a carboxymethyl cellulose column. The amino terminal lysine residue was removed by Edman degradation to obtain the desired peptide. The technique thus developed has permitted the syntheses of peptide fragments of the B chain of human insulin, H-Gly-Phe-Phe-Tyr-Thr-Pro-Lys(Tfa)-Thr-OH and H-Gly-Ser-His-Leu-Val-OH, with a simple purification process in good yield and in high purity.

Solid phase peptide synthesis[3] is now well established for the syntheses of those oligopeptides up to 10—15 residues which can be purified with ease.[4] However, when the purification of the product is not easy, this method does not always give a satisfactory result. Consequently, the purification of the product is still an important problem in solid phase synthesis.[5]

This study dealing with simplification of the purification process in the solid phase peptide synthesis stemmed from the observation made during solid phase synthesis of an encephalitogenic peptide, H–Arg–Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg–OH, by stepwise chain elongation and fragment condensation procedure.[6] In this synthesis, it was expected that there were, as usual, possibilities of the formation of damaged peptides in the terminology of Bayer,[7] truncated and deletion sequences in the terminology of Marshall,[8] and difficulty

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1) Symbols for amino acid derivatives and peptides used in this text are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature; *Biochem. J.*, **126**, 773 (1972). Other abbreviations: DCC= dicyclohexylcarbodiimide, DMF=dimethylformamide, CMC=carboxymethyl cellulose, AP–M=aminopeptidase M, IR=infrared.
2) Location: *Komatsushima, Sendai, 983, Japan.*
3) R.B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
4) J.H. Jones and B. Ridge, "Amino acids, Peptides, and Proteins," Vol. 4, ed. by G.T. Young, The Chemical Society, London, 1972, p. 375.
5) R.C. Sheppard, "Peptides 1971," ed. by H. Nesvadba, North-Holland, Amsterdam, 1973, p. 111.
6) K. Suzuki and Y. Sasaki, *Chem. Pharm. Bull.* (Tokyo), **21**, 2634 (1973).
7) E. Bayer, "Peptides: Chemistry and Biochemistry," ed. by B. Weinstein, Marcel Dekker, New York, 1970, p. 99.
8) W.S. Hancock, D.J. Prescott, P.R. Vagelos, and G.R. Marshall, *J. Org. Chem.*, **38**, 774 (1973).

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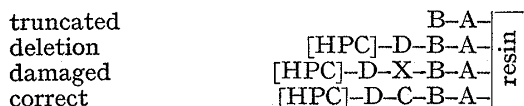
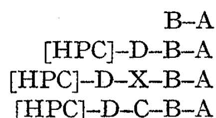
of purification. However, liquid hydrogen fluoride treatment[9] of the protected peptide resin followed by purification using a CMC column revealed that a main peak contained the desired decapeptide and a minor truncated peptide, which were easily separated by preparative thin-layer chromatography. Another main peak eluted earlier than that of the desired fraction gave two to three ninhydrin-positive spots on paper chromatography in either of the synthetic approaches described above. Thus, the decapeptide was highly purified with almost similar simplicity as that of the decapeptide prepared by the conventional solution method.[10] These findings suggest that the arginine residue of N-terminus in the decapeptide may be effective as a "highly polar handle" to simplify the isolation of the desired peptide from presumably some lower basic truncated or damaged peptides in this case.

On the basis of these considerations, we are examining new tactics to overcome the problem of purification in the solid phase peptide synthesis. The present communication deals with the basic idea behind the new tactics and with demonstrations of its feasibility through the synthesis of the carboxyl terminal octapeptide derivative of B chain of human insulin, H-Gly-Phe-Phe-Tyr-Thr-Pro-Lys(Tfa)-Thr-OH, which is presumably a key intermediate for the semisynthesis of human insulin,[11] and the synthesis of peptide fragment of B chain of human insulin, H-Gly-Ser-His-Leu-Val-OH, which is presumably useful as building unit for the total synthesis of B chain of human insulin. The general concept underlying these tactics is outlined in Chart 1. The term "highly polar compound" used in this paper signifies basic or acidic compound and basicity of the basic compound is required to be stronger than that of one α-amino group of the peptide chain. Naturally, the modification of amino group of the N-terminal amino acid residue of the desired peptide resin is not necessary when the N-terminal amino acid residue of the desired peptide is constituted with acidic or basic amino acid which functions itself as a highly polar handle. Some possible sequences in the synthesis of tetrapeptide, D-C-B-A, according to these tactics are shown in Table I, for the aid of a full understanding of the validity of these tactics. The sequences in Table I deal with a more detailed explanation of the step 2 in Chart 1. We will assume that A, B, C, and D in Table I are neutral amino acids to facilitate the understanding. It is obvious that sequences modified with a highly polar compound, [HPC], including the correct sequence would be easily separated

1) Coupling of highly polar compound with amino group of N-terminal amino acid residue of peptide resin



2) Deblocking of the above substance with commonly used reagent will give the following mixture of peptide derivatives



3) Purification based on differences of the charge in peptide molecules
4) Removal of highly polar compound to release the desired peptide

A, B, C, and D stand for amino acid residues or in which functional group(s) are protected; X stands for damaged amino acid residue; [HPC] stands for highly polar compound or in which its functional group(s) are protected; - stands for covalent bond.

Chart 1. Tactics for the Simplification of Purification Process in the Solid Phase Peptide Synthesis

9) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan.*, **40**, 2164 (1967).
10) K. Suzuki, T. Abiko, N. Endo, Y. Sasaki, and J. Arisue, *Chem. Pharm. Bull.* (Tokyo), **21**, 2627 (1973).
11) M.A. Ruttenberg, *Science*, **177**, 623 (1972).

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Table I. Some Possible Sequences in the Synthesis of Tetrapeptide, D–C–B–A, according to These Tactics

Correct	[HPC]-D-C-B-A		D ······ B-A
Truncated	D-C-B-A		D············A
	C-B-A		C······A
	B-A	Damaged	[HPC]-D-X-B-A
	A	Damaged and	
Deletion	[HPC]-D-C······A	truncated	D-X-B-A
	[HPC]-D······B-A		X-B-A
	[HPC]······C-B-A	Damaged and	
	[HPC]-D ········· A	deleted	[HPC]-D-X······A
	[HPC]······C······A		[HPC]······X-B-A
	[HPC] ········· B-A		[HPC]······X······A
	[HPC]···············A		D-X······A
	D-C······A		X-B-A
		etc.	X······A

from other [HPC]-lacking failure sequences by purification based on differences of the charge in peptide molecules, such as ion-exchange chromatography. In other words, it would be possible to remove about half of failure sequences in number in this example by these tactics. Consequently, further purification which follows the purification of step 3 in Chart 1, if necessary, would be easier in general compared with that of the solid phase synthesis which has been widely used. Isolation of the desired [HPC]-peptide from other failure [HPC]-peptides due to mainly deletion sequence is difficult by purification method based on difference of the charge in peptide molecule. Accordingly, for prevention of the formation of deletion sequences, acetylation of eventual unchanged amino group and deblocking of the protecting group for α-amino group during the peptide chain elongation cycle shown in Table II should be as exhaustive as possible.

Table II. Schedule for Solid Phase Peptide Synthesis on Boc-Thr(Bzl)-resin (I)

Step	Reagent and purpose	Time (min)
1	dioxane, washing (3×)	2
2	4N HCl–dioxane, prewashing (1×)	5
3	4N HCl–dioxane, deblocking (1×)	30
4	dioxane, washing (3×)	2
5	DMF, washing (3×)	2
6	10% Et_3N–DMF, prewashing (1×)	5
7	10% Et_3N–DMF, neutralization (1×)	10
8	DMF, washing (3×)	2
9	CH_2Cl_2, washing (3×)	2
10	Boc–amino acid–CH_2Cl_2, (1×)	5
11	DCC–CH_2Cl_2, coupling (1×)	300
12	DMF, washing (3×)	2
13	Ac_2O–Et_3N–DMF (0.5: 0.3: 10), acetylation (1×)	60
14	DMF, washing (3×)	2
15	EtOH, washing (1×)	2
16	AcOH, washing (3×)	2
17	EtOH, washing (3×)	2

For the construction of Boc–Gly–Phe–Phe–Tyr(Bzl)–Thr(Bzl)–Pro–Lys(Tfa)–Thr(Bzl)–resin (II), Boc–Thr(Bzl)–OH[12] was first esterified onto chloromethylated copolymer of styrene with 2% divinylbenzene in dimethylformamide and triethylamine to obtain Boc–Thr(Bzl)–resin (I). Generally, the cycle shown in Table II was used for the stepwise condensation of

12) T. Mizoguchi, G. Levin, D.W. Woolley, and J.M. Stewart, *J. Org. Chem.*, 33, 903 (1968).

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N^{α}-Boc–amino acid derivatives onto I, one at a time. Boc–Lys(Tfa)–OH[13] was prepared in high yield by a different approach from that in the literature. In each coupling reaction, 3 equivalents of Boc–amino acid and DCC were used. The weight gain represented 91% incorporation on the resin. II was treated with hydrogen fluoride in the presence of anisole to obtain the crude product. Elution pattern of the product on Sephadex G-25 column eluted with 1N acetic acid seemed to be a single peak, when the eluates were monitored at 280 nm. However, when the eluates were analyzed by paper chromatography, the fractions eluted earlier in the peak contained the desired peptide with minor contaminations, while the fractions eluted later contained only the desired peptide, H–Gly–Phe–Phe–Tyr–Thr–Pro–Lys(Tfa)–Thr–OH (III). Thus, complete separation of the desired peptide from the contaminants was unsuccessful by a single purification process, although the desired peptide was obtained in high purity in part and the yield was 24% based on I. The IR spectrum showed the presence of a C–F bond. For removal of trifluoroacetyl group in III, a sample of III was treated with 1M piperidine for 1 hr at room temperature[13] followed by neutralization with 1M hydrogen chloride and the mixture was lyophilized. The optical purity of the de-trifluoroacetylated peptide was assessed by AP–M digest.[14]

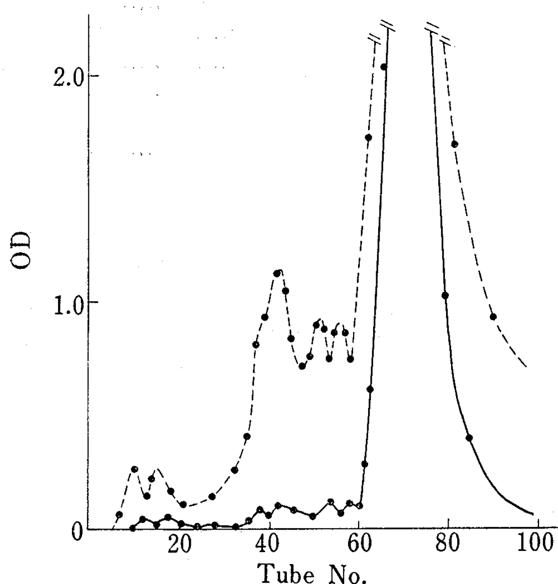

Fig. 1. Purification of V on a Column of CMC

The column (2×15 cm), applied with the crude material (162 mg), was eluted with a linear gradient from H_2O (300 ml) in a mixing chamber to 0.07M NH_4OAc buffer (pH 6.5, 300 ml) in the reservoir, at the flow rate of 5 ml/4 min. Fractions of 5 ml each were collected.

—•—•—: OD at 280 nm ---•---•---: OD at 230 nm

Boc–Lys(Boc)–OH[15] was condensed with II according to the cycle shown in Table II to obtain Boc–Lys(Boc)–Gly–Phe–Phe–Tyr(Bzl)–Thr(Bzl)–Pro–Lys(Tfa)–Thr(Bzl)–resin (IV). IV was treated with hydrogen fluoride in the usual manner and the crude product thus obtained was purified on CMC column by linear gradient elution between water and 0.07M ammonium acetate buffer of pH 6.5 to obtain H–Lys–Gly–

13) C.B. Anfinsen, D. Ontjes, M. Ohno, L. Corley, and A. Eastlake, *Proc. Natl, Acad. Sci. U.S.*, **58**, 1806 (1967).
14) AP–M (Lörm & Haas Co., Darmstadt) was purchased through Protein Research Foundation, Minoh, Osaka. Two enzyme preparations of reference Nos. 710807 and 481112, codified by the distributor in Osaka, exhibited different enzymic activity; namely, AP–M (ref. No. 710807) exhibited a slight prolidase activity, as reported in the literature [E.C. Jorgensen, G.C. Windrige, and W. Patton, *J. Med. Chem.*, **12**, 733 (1969)], and AP–M (ref. No. 481112, lot No. 4330139 of the manufacturer) digested synthetic bradykinin completely in 45 hr. Digestion was carried out according to the procedure reported in the literature [K. Hofmann, F.M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966)]. Amino acid ratios of 2 day digest of the de-trifluoroacetylated peptide with AP-M (ref. No. 710807) were Lys 0.93, Thr 1.11, Pro trace, Gly 1.00, Tyr 0.75, and Phe 1.03. When the fresh enzyme was added after 2 day digestion and digestion was continued for additional 2 days, amino acid ratios were Lys 1.09, Thr 1.56, Pro 0.62, Gly 1.00, Tyr 0.95, and Phe 2.00. Thus the peptide was almost completely digested in 4 days except for Thr-Pro bond which was digested about 62% in 4 days. The hydrophobic cluster of –Phe–Phe–Tyr– in the molecule resisted digestion by the enzyme preparation. However, the peptide bond including Thr–Pro was completely hydrolyzed in 23 hr with the enzyme preparation (lot No. 4330139) of a different lot purchased from the same manufacturer as described in the experimental part. It is noteworthy that the proteolytic activity as well as prolidase activity of AP–M differs from lot to lot. Consequently, care must be taken when this enzyme is used for assessment of the optical purity of synthetic peptides.
15) E. Sandrin and R.A. Boissonnas, *Helv. Chim. Acta*, **46**, 1637 (1963); T. Nagasawa, K. Kuroiwa. K. Narita, and Y. Isowa, *Bull. Chem. Soc. Japan.*, **46**, 1269 (1973).

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Phe–Phe–Tyr–Thr–Pro–Lys(Tfa)–Thr–OH (V). The elution pattern obtained is illustrated in Fig. 1. Fractions 62 to 80 containing the desired peptide were pooled and lyophilized to obtain V, which was homogeneous on paper chromatography and its purity was assessed by amino acid analysis, yield 56% based on I.

Removal of the N-terminal lysine residue in V was accomplished by Edman procedure.[16] Coupling of V with phenyl isothiocyanate was carried out at pH 8.5 to 9.0. It is important to avoid keeping the reaction mixture in higher pH, since trifluoroacetyl group is sensitive to strong alkalinity. Phenylthiocarbamoyl derivative of V was treated in trifluoroacetic acid for 1 hr at 40° and the solvent was evaporated in vacuum. The residue was dissolved in 30% acetic acid and the solution was washed with methylene chloride. The acetic acid solution was passed through Sephadex G-25 column and the peptide-containing fractions were pooled and lyophilized to obtain H–Gly–Phe–Phe–Tyr–Thr–Pro–Lys(Tfa)–Thr–OH (IIIa), yield 43% based on I. IIIa thus obtained was identical to III in physical and chemical data.

A 43% yield of IIIa by these tactics was superior to a 24% yield of III by general solid phase synthesis. The superiority of the yield can be attributed to the efficient separation of V and the reasonable yield of Edman degradation reaction.

Analogously, H–Gly–Ser–His–Leu–Val–OH (VIII) was prepared from H–Lys–Gly–Ser–His–Leu–Val–OH (VII), which was derived from the corresponding protected peptide resin, Z–Lys(Z)–Gly–Ser(Bzl)–His(Tos)–Leu–Val–resin (VI). In this case, however, coupling procedure was modified in a manner reported in a previous communication.[17] Namely, steps 5 to 7 in Table I were eliminated and in step 10 three equivalents of N-methylmorpholine salt of Boc-amino acid derivative was added, followed by step 11. VII was purified on CMC column, and its elution pattern is illustrated in Fig. 2. VII thus obtained was homogeneous

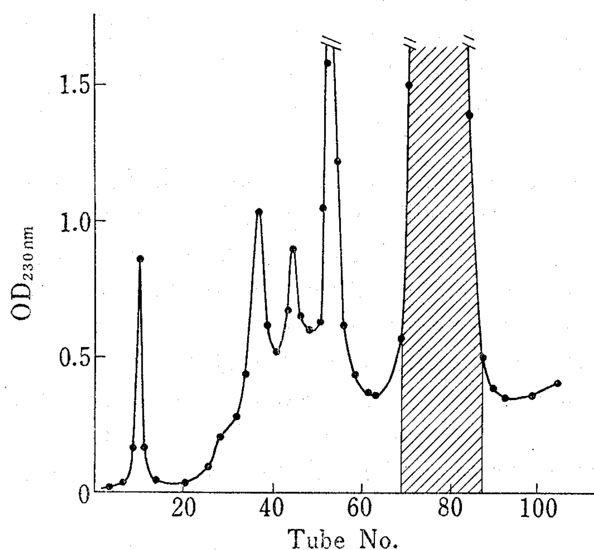

Fig. 2. Purification of VII on a Column of CMC

The column (2×18.5 cm), applied with the crude material (200 mg), was eluted with a linear gradient of H_2O (300 ml) in the mixing chamber to 0.2M NH_4OAc buffer (pH 6.5, 300 ml) in the reservoir, at the flow rate of 5 ml/4 min. Fractions of 5 ml each were collected. Shaded part indicates the desired fractions.

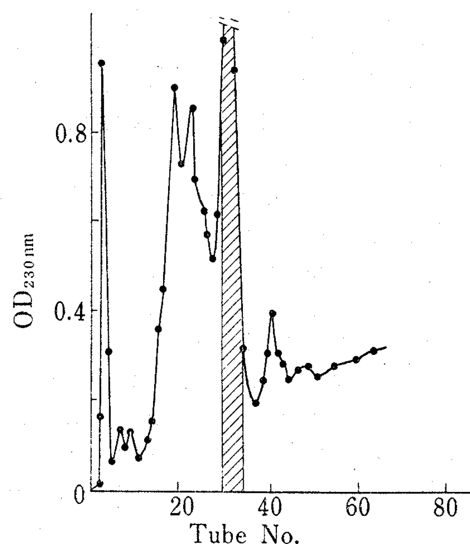

Fig. 3. Chromatographic Pattern of Crude VIII prepared from IX on a Column of CMC

The column (1.8×15 cm), applied with the crude material (85 mg), was eluted with a linear gradient of H_2O (300 ml) in the mixing chamber to 0.2M NH_4OAc buffer (pH 6.5, 300 ml) in the reservoir, at the flow rate of 5 ml/4 min. Fractions of 5 ml each were collected. Shaded part indicates the desired fraction.

16) P. Edman, *Acta Chem. Scand.*, 4, 277 (1950).
17) K. Suzuki, K. Nitta, and N. Endo, *Chem. Pharm. Bull.* (Tokyo), 23, 222 (1975).

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on paper chromatography and its optical purity was assessed by amino acid analysis of the AP-M digest. Removal of the N-terminal lysine residue in VII was accomplished by the Edman procedure to obtain VIII, yield 37% based on Boc–Val–resin. Preparation of VIII from Boc–Gly–Ser(Bzl)–His(Tos)–Leu–Val–resin (XI), which was the intermediate of VI, was attempted to compare with the result of highly polar handle procedure. IX was treated with hydrogen fluoride and the resulting crude peptide was passed through a CMC column, and its elution pattern is illustrated in Fig. 3. Fractions 33 to 35 contained the desired peptide with two minor contaminants on paper chromatography using two different solvent systems. Further purification was not attempted. Differences in the retention volume of the desired and undesired fractions were greater in Fig. 2 than that in Fig. 3.

Discussion

These tactics for the simplification of the purification process in the solid phase peptide synthesis may offer many advantages over current techniques in certain cases. Neutral peptides are frequently purified on Dowex 50 column using a volatile pyridinium acetate buffer solution,[18] but a peptide isolated in this way is contaminated with unknown material presumably consisting of the soluble from the ion-exchanger.[19] In this respect, these tactics may be favorable for the purification of neutral peptides. The other advantage of these tactics is that solubility of the N-terminal modified peptide in water tends to increase depending on the hydrophilic group(s) of the highly polar compound and, consequently, it may be favorable for the purification of peptides which are only slightly soluble in water. The synthesis of IIIa is such a case. These tactics would be applicable for the synthesis of pyroglutamyl-peptide, since the modified glutaminyl-peptide could be converted to the desired peptide by removal of the modifying group followed by cyclization[20] of the resulting glutaminyl-peptide. These tactics would also be applicable to a continuous peptide synthesis by the conventional solution method as well in which the intermediates are not purified during the synthesis.[21,22] Although these tactics, of course, do not seem to be as universal as would be expected from Chart 1 and Table I, it seems that these tactics have merits to be given the first choice in the solid phase peptide synthesis.

In our own opinion, an ingenious combination of all of the conventional solution method, the solid phase method, and the semisynthetic approach will be of great advantage in overcoming, at least in part, disadvantages of the procedures at the present state of peptide synthesis. Studies reported so far in the direction of a combination of conventional solution method and solid phase method can be classified into two categories at the present time. The first one comprises synthesis of peptide fragments by the conventional method, followed by condensation of the fragments on a solid phase.[6,23,24] The second comprises stepwise synthesis of peptide fragments which, for instance, are composed of 3 to 12 amino acids on a solid phase, according to the suggestion of Wieland,[25] and condensation of extensively purified

18) J.M. Stewart and J.D. Young, "Solid Phase Peptide Synthesis," W.H. Freeman, San Francisco, 1969, p. 51.
19) K. Suzuki and T. Abiko, unpublished data. See also K. Titani and Y. Yaoi, *Tanpakushitsu, Kakusan, Koso*, **10**, 250 (1965).
20) J. Rudinger and Z. Pravda, *Collect. Czech. Chem. Commun.*, 23, 1947 (1958); H. Matsuo, A. Arimura, R. M.G. Nair, and A.V. Schally, *Biochem. Biophys. Res. Commun.*, **45**, 822 (1971).
21) M.A. Tilak, *Tetrahedron Letters*, **1970**, 849.
22) C. Birr, "Peptides 1972," ed. by H. Hanson and H.-D. Jakubke, North-Holland, Amsterdam, 1973, p. 72.
23) S. Sakakibara, Y. Kishida, Y. Kikuchi, R. Sakai, and K. Kakachi, *Bull. Chem. Soc. Japan.*, **41**, 1273 (1968) and references cited in ref. 28).
24) H. Yajima, Y. Kiso, Y. Okada, and H. Watanabe, *Chem. Commun.*, **1974**, 106; H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **22**, 1087 (1974).
25) Th. Wieland, "Peptides 1972," ed. by H. Hanson and H.-D. Jakubke, North-Holland, Amsterdam, 1973, p. 38.

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fragments by the conventional methods.[22,26,27] However, it is obvious that other combinations of the two methods are theoretically possible and will be designed as needed. Under these circumstances, extensively purified peptide fragments synthesized by the solid phase method would become important as building units for the synthesis of a long peptide chain, and this consideration is in accord with the principle that the converging process is better than the linear process with respect to the overall yield in the synthesis of a long peptide.[28] Purification process would be simplified, if in the planning of a synthetic procedure for a long peptide chain the intermediate-sized peptides would be selected from the completed sequence in such a way that their N-terminal amino acid is arginine, histidine, aspartic acid, or glutamic acid.

These tactics described in the present paper also have been successfully applied for the syntheses of ACTH-(15—24)-decapeptide derivative,[29] human insulin B chain-(1—6)-hexapeptide and human encephalitogenic basic protein-(159—164)-hexapeptide.[30]

Experimental

All melting points are uncorrected. Paper chromatography was performed on filter paper, Toyo Roshi No. 51, at room temperature and solvent systems used were (A) BuOH–AcOH–H_2O (4: 1: 5, upper phase),[31] (B) BuOH–AcOH–pyridine–H_2O (15: 3: 10: 12).[32] IR spectrum of III was recorded on a Hitachi Model EPI-G2 grating infrared spectrophotometer. Amino acid analysis was carried out on a Hitachi Model KLA-3B amino acid analyzer according to the directions given by Moore, *et al.*[33] For amino acid analysis, a sample was hydrolyzed by constant boiling HCl in an evacuated sealed tube at 110° for 22 hr, unless otherwise mentioned, and AP–M digestion was carried out by the procedure given by Hofmann, *et al.*[34]

Boc-Lys(Tfa)-OH——To a solution of N^{α}-Boc-Lys-OH[35] (3.95 g) in 1N NaOH (14.3 ml) and 0.1 M $NaHCO_3$–Na_2CO_3 buffer (pH 9.2, 55 ml) was added ethyl thioltrifluoroacetate[36] (3.62 g). The solution was stirred vigorously at room temperature for 48 hr under a well-ventilated hood. The solution was chilled, acidified to Congo Red with solid citric acid, and extracted with EtOAc (30 ml × 3). The extract was washed with saturated NaCl solution, dried over $MgSO_4$, and evaporated in vacuum. The resulting oily residue was crystallized by addition of petr. ether and storing in the cold. The product was recrystallized from CH_2Cl_2 to colorless needles, mp 102—103° (lit.[13] mp 103°); yield, 4.10 g (74%).

Boc-Thr(Bzl)-resin (I)——I was prepared from Boc-Thr(Bzl)-OH (2.48 g, 8 mmoles), Et_3N (0.81 ml, 7.2 mmoles), and chloromethylated copolymer (5 g, 1.6 mmoles of Cl/g, 100—200 mesh) in DMF (60 ml) according to the procedure described by Sakakibara[37] and Marglin.[37] Yield, 5.34 g. The amino acid content in the resin so obtained was 0.245 mmole/g of the resin from the result of the amino acid analysis in the usual acid hydrolysate of the dry resin.

Boc-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Tfa)-Thr(Bzl)-resin (II)——I (2.67 g, threonine content = 0.655 mmole) was placed in the reaction vessel and the construction of II followed the schedule shown in Table II. Volumes of solvent and solution of reagent (s) used were 30 ml in each operation. N^{α}-Boc-amino acids and their derivatives with side chain-protecting groups of Lys(Tfa), Thr(Bzl), and Tyr(Bzl),[15] were used for the incorporation of each amino acid. In steps 10 and 11, 1.97 mmoles (3 eq.) of Boc-amino acid and DCC were used. Boc-Lys(Tfa)-OH was dissolved in DMF–CH_2Cl_2 (1: 4). After the coupling of Boc-Gly-OH, an additional

26) M.A. Barton, R.U. Lemieux, and J.Y. Savoie, *J. Am. Chem. Soc.*, **95**, 4501 (1973).
27) C. Birr, *Ann. Chem.*, **1973**, 1652.
28) I. Fleming, "Selected Organic Syntheses," John Wiley & Sons, London, 1973, p. 101.
29) K. Suzuki, K. Nitta, and N. Endo, "Proceedings of the 12th Symposium on Peptide Chemistry," ed. by H. Yajima, Protein Research Foundation, Osaka, 1974, p. 57.
30) K. Suzuki, Y. Sasaki, and N. Endo, "Proceedings of the 12th Symposium on Peptide Chemistry," ed. by H. Yajima, Protein Research Foundation, Osaka, 1974, p. 61.
31) S.M. Partridge, *Biochem. J.*, **42**, 238 (1948).
32) S.G. Waley and G. Watson, *Biochem. J.*, **55**, 328 (1953).
33) S. Moore, D.H. Spackman, and W.H. Stein, *Anal. Chem.*, 30, 1185 (1958).
34) Reference cited in footnote 14.
35) R. Schwyzer, A. Costopanagiotis, and P. Sieber, *Helv. Chim. Acta*, **46**, 870 (1963).
36) M. Hauptschein, C.S. Stokes, and E.A. Nodiff, *J. Am. Chem. Soc.*, **74**, 4005 (1952).
37) A. Kishi, Y. Kishida, and S. Sakakibara, "Proceedings of the 7th Symposium on Peptide Chemistry," ed. by S. Akabori, Protein Research Foundation, Osaka, 1969, p. 36; A. Marglin, *Tetrahedron Letters*, **1971**, 3145.

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acetylation was performed by washing with DMF (3×2 min) followed by second acetylation (60 min). After all the reaction cycles were over, the resin was dried over P_2O_5 in vacuum; yield, 3.45 g.

H-Gly-Phe-Phe-Tyr-Thr-Pro-Lys(Tfa)-Thr-OH (III)——II (500 mg) was treated with anhyd. HF (10 ml) in the presence of anisole (1.5 ml) at 0° for 60 min and then the excess HF was removed in vacuum. The residue was extracted with 1% AcOH (30 ml×2) and the extract was washed well with EtOAc. Combined extract was concentrated to a small volume in vacuum and passed through a column (1.8×10 cm) of Dowex 1×2 (acetate form) which was eluted with 2% AcOH. Eluates positive to the Cl-*o*-tolidine reagent were pooled and evaporated to dryness in vacuum; yield, 46 mg. The crude octapeptide derivative thus obtained was dissolved in 1N AcOH (3 ml) and a small amount of insoluble material was removed by centrifugation. The clear supernatant was submitted to the column (2×50 cm) of Sephadex G-25 which was equilibrated with 1N AcOH and eluted with 1N AcOH at a flow rate of 20 ml/30 min. Fractions of 5 ml each were collected and absorbancy at 280 nm was determined on each fraction. The eluates in tubes No. 16 to 19 were pooled and lyophilized; yield, 31 mg (24% based on I); slightly soluble in water; mp 173—176°; $[\alpha]_D^{15}$ −22.5° (c=1.3, 30% AcOH); the IR spectrum in Nujol mull showed a peak at 1190 cm^{-1} (C–F); *Rf* 0.79 (A), *Rf* 0.91 (B) single spot positive to ninhydrin, Pauly, and Cl-*o*-tolidine reagents; amino acid ratios in the acid hydrolysate: Lys 1.06, Thr 2.01, Pro 1.04, Gly 1.00, Tyr 0.92, Phe 1.92 (average recovery 97%); Tfa group in III was removed with 1M piperidine according to the procedure reported in the literature[13] and the product was digested with AP–M (lot No. 4330139) for 23 hr, amino acid ratios in the AP-M digest: Lys 0.84,[38] Thr 2.00, Pro 1.14, Gly 1.07, Tyr 0.95, Phe 1.95 (average recovery 83%).

H-Lys-Gly-Phe-Phe-Tyr-Thr-Pro-Lys(Tfa)-Thr-OH(V)——II (800 mg) was placed in the reaction vessel and Boc-Lys(Boc)-OH[15] (0.6 mmole) was incorporated according to the cycle shown in Table II to yield Boc-Lys-(Boc)-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Tfa)-Thr(Bzl)-resin (IV). Volumes of solvent and solution of reagent (s) used were 10 ml in each operation; yield, 820 mg.

All protecting groups of IV, except for Tfa-group of Lys(Tfa)-residue, were removed by HF treatment in the same manner as described above. The crude peptide (162 mg) thus obtained was dissolved in H_2O (2 ml) and submitted to a column of CMC and chromatographed as shown in Fig. 1. The eluates in tubes No. 62 to 80 containing the desired peptide were pooled and lyophilized to constant weight; yield, 135 mg (56% based on I); mp 160—165°; $[\alpha]_D^{20}$ −36.0° (c=1.0, H_2O); *Rf* 0.51 (A), *Rf* 0.72 (B) single spot positive to ninhydrin, Pauly, and Cl-*o*-tolidine reagents; amino acid ratios in the acid hydrolysate: Lys 2.05, Thr 1.71, Pro 1.13, Gly 1.09, Tyr 0.83, Phe 2.00 (average recovery 90%).

Edman Degradation (Preparation of IIIa from V)——V (53 mg) was dissolved in 50% pyridine (15 ml) and the solution was adjusted to pH 8.6 with 25% Et_3N in pyridine. Phenyl isothiocyanate (1 ml) was added to this solution and stirred at 40° for 2 hr. The pH of the reaction mixture was maintained between 8.5 and 9.0 by the addition of 25% Et_3N in pyridine during the reaction. Completeness of the reaction was determined by Kaiser's ninhydrin test.[39] Excess phenyl isocyanate and pyridine were extracted with benzene (5 ml×4). Vigorous shaking caused emulsification of the solvents, so that a few drops of MeOH was added and the emulsion was centrifuged. The aqueous phase so obtained was evaporated to dryness in vacuum and dried completely over P_2O_5; slightly yellow sticky material. This material was dissolved in dry CF_3COOH (2 ml) and stirred at 40° for 60 min under N_2 gas. After removal of excess CF_3COOH in vacuum, the oily residue was dissolved in 30% AcOH (15 ml) and the solution was washed with CH_2Cl_2 (5 ml×5). The aqueous phase was concentrated to approximately 2 ml, submitted to a column (3×60 cm) of Sephadex G-25, and chromatographed in the same manner as described for the preparation of III. The eluates in tubes No. 41 to 49 were pooled and lyophilized; yield, 45 mg (43% based on I); its chemical and physical properties agreed well with those of III; *Anal.* Calcd. for $C_{50}H_{64}O_{13}N_9F_3 \cdot 4AcOH \cdot 9H_2O$: C, 47.76; H, 6.77; N, 8.64. Found: C, 47.89; H, 6.41; N, 8.87.

Z-Lys(Z)-Gly-Ser(Bzl)-His(Tos)-Leu-Val-resin (VI)——Boc-Val-resin was prepared from Boc-Val-OH (4.03 g, 17.42 mmoles), Et_3N (2.19 ml), 15.68 mmoles), and chloromethylated copolymer (13 g, 1.34 mmoles of Cl/g, 100—200 mesh) in the same manner as described for the preparation of I; yield, 14.7 g. The amino acid content in the resin so obtained was 0.339 mmole/g. Each amino acid was incorporated to Boc-Val-resin(5.00 g) according to the schedule shown in Table II, except for the coupling steps which were modified as follows: Steps 5 to 7 were eliminated and in step 10, three equivalents of N-methylmorpholine salt of Boc-amino acid derivative in CH_2Cl_2 was added and followed by step 11. Volumes of solvent and solution of reagent (s) used were 30 ml in each operation. Amino acid derivatives used for incorporation were Boc-Leu-OH, Boc-His(Tos)-OH, Boc-Ser(Bzl)-OH, Boc-Gly-OH, and Z-Lys(Z)-OH. After all the reaction cycle were over, the resin was dried over P_2O_5 in vacuum; yield, 6.32 g.

H-Lys-Gly-Ser-His-Leu-Val-OH (VII)——All the protecting groups in VI (2.0 g) were removed by HF treatment in the same manner as described above. The crude peptide (200 mg) thus obtained was dissolved in H_2O (5 ml) and submitted to column chromatography over CMC as shown in Fig. 2. The eluates in tubes

38) Because the Tris buffer itself gave a ninhydrin-positive minor peak at the elution position of lysine, the analytical value of lysine was corrected by the value due to the buffer solution.
39) E. Kaiser, R.L. Colescott, C.D. Bossinger, and P.I. Cook, *Anal. Biochem.*, 34, 595 (1970).

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No. 68 to 87 containing the desired peptide were pooled and evaporated to dryness in vacuum. The residue was passed through a column (3 × 57 cm) of Sephadex G-25 which was eluted with 1N AcOH to remove NH_4-OAc; yield, 87 mg (44% based on Boc-Val-resin); mp 125—158° (decomp.); $[\alpha]_D^{18}$ −34.3° (c=0.7, H_2O); *Rf* 0.13 (A), *Rf* 0.35 (B), single spot positive to ninhydrin and Pauly reagents; amino acid ratios in the acid hydrolysate: Lys 0.92, His 0.94, Ser 0.97, Gly 1.12, Val 1.05, Leu 1.08 (average recovery 81%); amino acid ratios in the AP–M (lot No. 4330139) digest (20 hr): Lys 0.97, His 0.94, Ser 1.00, Gly 1.02, Val 1.03, Leu 1.04 (average recovery 73%).

H-Gly-Ser-His-Leu-Val-OH (VIII)——VII (30 mg) was treated and purified in the same manner as described for the preparation of IIIa; yield, 20 mg (37% based on Boc-Val-resin); mp 164—190° (decomp.); $[\alpha]_D^{18}$ −57.8° (c=0.9, H_2O); *Rf* 0.40 (A), *Rf* 0.53 (B), single spot positive to ninhydrin and Pauly reagents; amino acid ratios in the acid hydrolysate: His 1.08, Ser 0.90, Gly 1.01, Val 0.99, Leu 1.02 (average recovery 82%); amino acid ratios in the AP–M (lot No. 4330139) digest (20 hr): His 0.90, Ser 0.99, Gly 1.04, Val 1.13, Leu 1.14 (average recovery 90%).

Attempt for the Direct Synthesis of VIII——Boc-Gly-Ser(Bzl)-His(Tos)-Leu-Val-resin (IX) was prepared in the same manner as described for the preparation of VI. All the protecting groups in IX (480 mg) were removed by HF treatment in the same manner as described above. The crude peptide (85 mg) thus obtained was dissolved in H_2O (2 ml) and submitted to column chromatography over CMC as shown in Fig. 3. The eluates in tubes No. 33 to 35 were pooled and lyophilized. This product contained two minor compounds on paper chromatography, wt. 32 mg.

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