Solid Phase Peptide Synthesis by Oxidation-Reduction Condensation. Synthesis of Adrenocorticotropin(1—24) by Chain Elongation at the Carboxyl End on Solid Support

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Solid phase peptide synthesis by oxidation-reduction condensation *via* fragment condensation at the carboxyl end was established by a new monitoring method based on high pressure liquid chromatography. The merit of the method was demonstrated through the synthesis of ACTH (1—24). A resin-tetracosapeptide with quantitative amino acid ratios was easily prepared by coupling 6 fragments of 1—2, 3—7, 8—10, 11—14, 15—19, and 20—24 sequences. ACTH (1—24) with a full activity was obtained in 25% yield from the initial resin-dipeptide by hydrogen fluoride treatment and purification with carboxymethyl-cellulose chromatography.

The oxidation-reduction condensation reaction forming peptide linkage with use of triphenylphosphine (Ph_3P) and di-2-pyridyl disulfide ($(PyS)_2$) proceeds under mild conditions without basic or acidic catalysts. It has the advantage of minimizing the racemization of a carboxyl component and the side reactions of amino acids.[1] The condensation reaction has been successfully employed not only to syntheses of peptides,[2] nucleotides,[3] and coenzymes[4] but also to syntheses of various macrolides[5] *via* 2-pyridinethiol ester.[1,6] The active dehydrating agent in the reaction, phosphorane, is generated by mixing the carboxyl component with Ph_3P and $(PyS)_2$ whose melting points are 82 and 58 °C, respectively. Peptide bond formation would proceed through a cyclic transition state.[1] The high reactivity with high selectivity depends upon the stability of the intermediate of acyloxyphosphonium salt in which phosphorus atom exists largely in the pentacovalent form. This being the case, some modification is possible by changing the nature of the trivalent phosphorus compounds. As an example, the use of tris(*p*-bromophenyl)phosphite[7] in place of the triphenylphosphine increases the stability of the intermediate in polar solvents such as *N,N*-dimethylformamide. On the other hand, tributylphosphine decreases the stability of the intermediate which results in the decrease of selectivity in spite of the increase of reactivity.[8]

We have shown that the oxidation-reduction condensation can be successfully applied to the synthesis of LH-RH *via* fragment condensation on solid support by employing two types of chain elongation, from *C*-terminal amino acid to *N*-terminal amino acid and from *N*-terminal amino acid to *C*-terminal amino acid.[9]

The latter approach was further extended to a synthesis of ACTH(1—24).[10] This paper[11] contains a full report of the synthesis of ACTH(1—24) by a new technique to monitor the amount of amino component in solution based on high pressure liquid chromatography (HPLC). The general procedures for this approach are shown in Fig. 1.

The synthesis of ACTH(1—24) was carried out by coupling 6 fragments of 1—2, 3—7, 8—10, 11—14, 15—19, and 20—24 as shown in the following scheme.

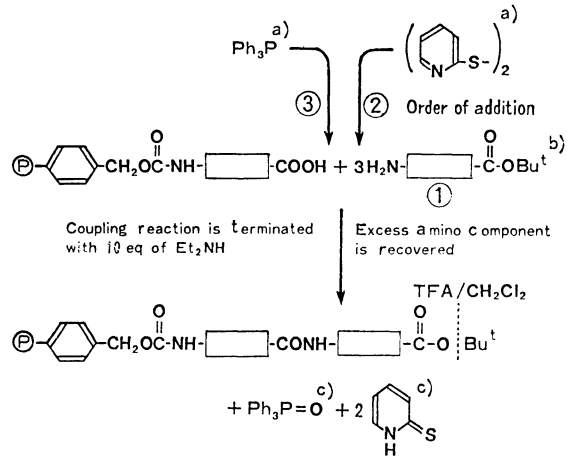
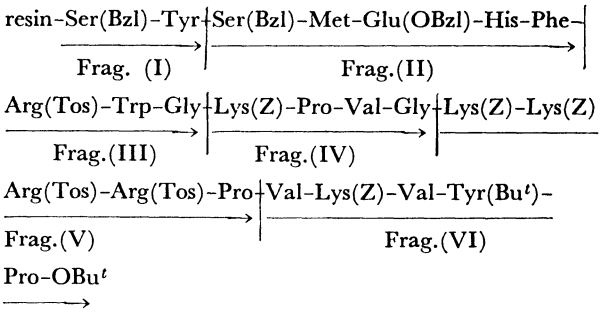


Fig. 1. General procedure for solid phase peptide synthesis by oxidation-reduction condensation.
Abbreviations, Ⓟ: copolymer of styrene-2% divinylbenzene, TFA : trifluoroacetic acid and -OBu^t : *t*-butyl ester.
a) The reagents are individually stable and safely stored but should not be mixed before addition.
b) Coupling reaction is monitored by measuring the amount of amino component in solution by means of high pressure liquid chromatography.
c) Very soluble in organic solvents and easily washed out with methylene chloride, ethanol, *etc.*



The following precautions were taken: 1) the number of amino acid residue in one fragment was chosen to be less than 5, considering the easiness of the syntheses of fragments, 2) each fragment contains a unique amino acid residue which makes it possible

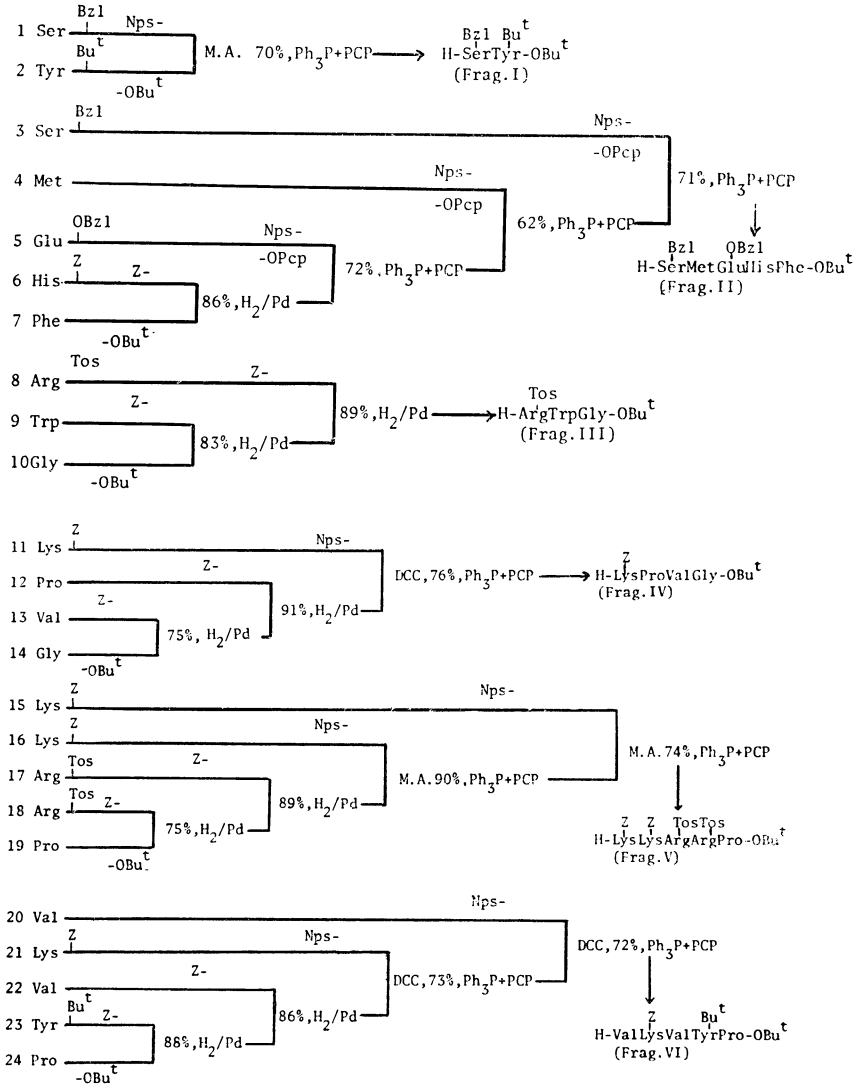


Fig. 2. The scheme for the preparation of peptide fragments.

TABLE 1. PHYSICAL PROPERTIES OF PEPTIDE FRAGMENTS

Fragment	Mp	$[\alpha]_D^{20}$ (c 1, methanol)	Amino acid ratios
Nps-SerTyr-OBu^t (I)	oil	—	Ser : Tyr 0.98 1
Nps-SerMetGluHisPhe-OBu^t (II)	158—160 °C	−26.3°	Ser : Met : Glu : His : Phe 1.01 0.94 0.97 1 0.99
Z-ArgTrpGly-OBu^t (III)	137—140 °C	−24.5°	Arg : Trp : Gly 0.99 0.87 1
Nps-LysProValGly-OBu^t (IV)	85—86 °C	−101.5°	Lys : Pro : Val : Gly 1.05 0.96 0.96 1
Nps-LysLysArgArgPro-OBu^t (V)	130—135 °C	−38.3°	Lys : Arg : Pro 2 1.96 0.96
Nps-ValLysValTyrPro-OBu^t (VI)	206—209 °C (dec)	−105.5°	Lys : Val : Tyr : Pro 0.96 1.96 1 1.03

to check the chain elongation by amino acid analysis, 3) each fragment has a UV absorbing moiety in order to monitor the coupling reaction by HPLC, 4) the biologically active center is included in one fragment V in order to ensure the incorporation of this fragment which could be proved by the activity. The *t*-butyl ester was selected for α-carboxyl protection since it is easily deprotected with trifluoroacetic acid. The protecting groups of side chains were benzyloxycarbonyl for ε-amino function of lysine, benzyl ester for γ-carboxyl function of glutamic acid, tosyl group for guanido function of arginine and benzyl ether for hydroxyl function of serine. In the syntheses of the fragments having these protecting groups, an α-amino protecting group must be removed without influencing the other groups. *o*-Nitrophenylsulfenyl(Nps-) protecting group was chosen for this purpose. Nps-protecting group was selectively removed under neutral conditions[10] by mixing the Nps-protected fragment with 5 fold excess each of Ph_3P and pentachlorophenol in methanol at room temperature. The pure fragment with the free amino function was quantitatively obtained by gel filtration on Sephadex LH-20 in methanol and drying *in vacuo* after evaporation of the solvent.

The syntheses of protected fragments were achieved stepwise in solution by oxidation-reduction condensation using the readily available benzyloxycarbonylamino acids except in cases of incorporation of Nps-amino acid residues which were coupled with dicyclohexylcarbodiimide (DCC) or by active ester and mixed anhydride method (M.A.) (Fig. 2). The physical properties of these intermediates are given in Table 1.

Starting with 1.5 g of 2% cross-linked resin containing 0.046 mmol of *O*-benzylseryltyrosine,[9] coupling steps of the incorporation of fragment II and III were carried out with a three-fold excess fragment and a 30 fold excess each of Ph_3P, $(PyS)_2$ and 2-pyridinethiol in methylene chloride, 6 h at −15 °C and overnight at room temperature. The steps of incorporation of fragments IV, V, and VI were carried out at room temperature in the absence of 2-pyridinethiol since carboxyl ends such as glycine and proline are free from racemization. Excess fragments were recovered in washing solvents since they did not change in the coupling reaction. They were easily purified by gel filtration on Sephadex LH-20 in methanol in 80—90% recovery yield. A successive coupling of 1 g of new resin–dipeptide with recovered fragments gave resin–tetracosapeptide with nearly quantitative amino acid ratios.

During the course of the synthesis, coupling rates were easily monitored by measuring the amount of fragment in solution by HPLC. Conditions suitable for effective separation have been found through the use of porous polymer Hitachi Gel 3010, a styrene-divinylbenzene polymer which is useful packing material for HPLC.[12]

With the use of aminated Hitachi Gel 3010,[13] eluting conditions were so chosen that the remaining fragment has a constant retention time irrespective of the fragment used for elongation, since it would not be practical to select eluting conditions in each coupling cycle with a different fragment. The problem was solved by elution with an acidic mobile phase by which each fragment was eluted always at or near the void volume separated from the other components in the coupling reaction. Taking these factors into consideration, analytical conditions were established as follows: column; aminated Hitachi Gel 3010 (5 mm i.d. × 50 cm length, Pyrex glass), mobile phase; 0.2 M hydrochloric acid, 0.1 M potassium chloride/methanol (1 : 9), flow rate; 2.0 ml/min, temperature; ambient, detector; UV (240 nm). A sample solution was prepared as follows: Add 20 μl of 0.2 M potassium hydroxide/80% methanol to 20 μl of reaction solution, and then neutralize with 20 μl of 0.2 M monopotassium citrate/80% MeOH. A definite volume (5—20 μl) of each solution was injected. For the

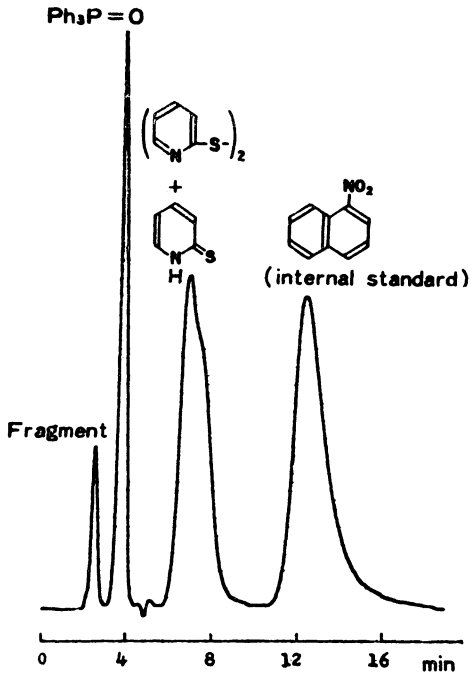


Fig. 3. A typical chromatogram by high pressure liquid chromatography.

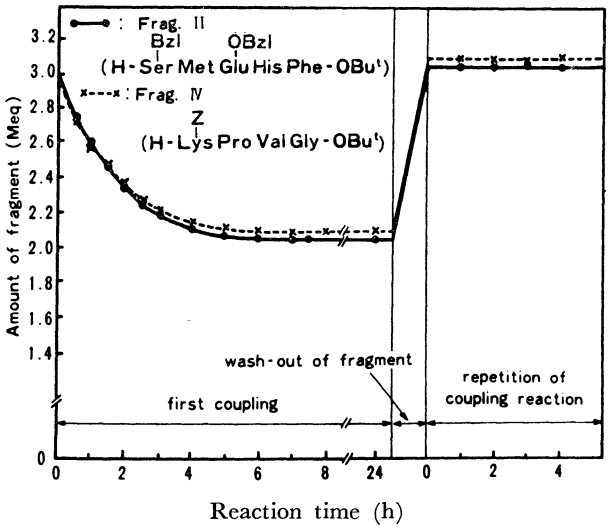


Fig. 4. Monitoring of coupling reaction by high pressure liquid chromatography.

sake of accurate determination and easy sampling, 1-nitronaphthalene was added to the reaction mixture as an internal standard. Each sample was analyzed within 15 min with an accuracy of ±1.0%.[14] A typical chromatogram is shown in Fig. 3. The remaining amino component was plotted against reaction time. Typical examples of monitoring results are shown in Fig. 4, one for the coupling of Frag. (II) and the other for that of Frag. (IV), wherein the coupling was carried out at −15 °C and room temperature, respectively. Reaction completion was principally determined by recording the time at which the curve indicates no further consumption of the fragment. Coupling was repeated until no fragment uptake was detected. It was also confirmed by determination of the amino acid ratios of the resulting peptide resin after the hydrolysis of a small portion of the resin with propionic acid–12 M hydrochloric acid (1 : 1 v/v).[15] The results at each step are given in Table 2. They indicate that fragment condensation proceeds efficiently with partial protection of side chains of amino acids.

The resin–tetracosapeptide was treated with hydrogen fluoride[16] for 30 min at 0 °C in the presence of anisole and 2-pyridinethiol. The yield of this cleavage step was 83%. The peptide was converted into acetate form and chromatographed on carboxymethyl-cellulose[17] after gel filtration on Sephadex G-25 in 1 M acetic acid. The pure ACTH(1—24)·7AcOH·$9H_2O$ which gave a single spot on thin layer chromatography and electrophoresis detected by Ehrlich, Pauly, Cl-tolidine, and ninhydrin reactions was obtained by precipitation from methanol–ethyl acetate–petroleum ether: dried *in vacuo* over phosphorus pentoxide; 41 mg, 25% yield from the initial resin–Ser(Bzl)–Tyr–OH, 43% yield from the liberated peptide from the resin. $[\alpha]_D^{20}$ −86.8° (*c* 0.5, 1% acetic acid) (lit,[18] $[\alpha]_D^{22}$ −88.6 ±2° (*c* 0.51, 1% acetic acid). Amino acid ratios after complete enzymatic digestion and acid hydrolysis[15] were in good agreement (Table 3). This shows that no detectable racemization occurred during the course of fragment condensation by oxidation-reduction process.

The ACTH activity of this purified peptide was assayed[19] by the ascorbic acid depletion test according to U.S.P. XVIII, and compared with the 2nd international standard: 96 (83—128) IU/mg identical with the value in literature.[18]

The new approach indicates that an oligopeptide as ACTH(1—24) is prepared in good yield by a simple purification procedure since the contamination of similar peptides with small difference in sequence is eliminated.

Particular merits of the method are as follows: 1) unreacted amino components can be recovered intact and recycled in the chain elongation procedure, 2) the number of protecting groups of the side chains can be reduced, 3) fragments are synthesized by the classical method from readily available Z-amino acid, 4) diacylation is avoided since excess amino components are used in solution, 5) the end peptide is homogeneous since similar contaminations are eliminated, 6) the number of acidolysis can be reduced, suitable for the acid sensitive tryptophan residue, 7) the progress and the completeness of each coupling reaction is easily analyzed by high pressure liquid chromatography.

The versatility of this approach, along with a combination of the classical synthesis in solution, promises a new possibility of facile preparation of pure peptides with long sequence, widening the extent of the usual solid phase peptide synthesis.

Experimental

All the melting points are uncorrected. Optical rotations were determined with a Perkin-Elmer Model 141 Polarimeter. Amino acid analyses were carried out on a Hitachi Amino Acid Analyzer, Model KLB-3B. High pressure liquid chromatography was performed on a Hitachi Chromatograph, Model 635 equipped with a variable UV detector. Solid phase peptide synthesis was carried out by shaking with a manual apparatus. Thin layer chromatography was

Table 2. Amino acid ratios[a] in each coupling step

	Ser	Tyr	Met	Glu	His	Phe	Arg	Gly	Lys	Pro	Val
Step I	0.97	1									
Step II	2.13	1.08	0.96	0.97	0.98	1					
Step III	2.16	1.11	0.95	0.98	0.96	1	0.97	1.01			
Step IV	2.08	1.08	0.91	1.02	0.96	1	0.99	2.01	0.99	0.96	1.02
Step V	2.11	1.06	0.92	1.01	0.98	1	2.91	2.01	2.79	1.91	0.95
Step VI	1.92	2.34	1.08	1.01	0.98	1	2.87	2.19	3.71	3.01	2.87
Theor.	(2)	(2)	(1)	(1)	(1)	(1)	(3)	(2)	(4)	(3)	(3)

a) Acid hydrolysis: propionic acid-hydrochloric acid(1 : 1 v/v), 130 °C, 2 h.

Table 3. Amino acid analyses of the synthetic ACTH (1—24)

	Ser	Tyr	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val	Content
(I)	1.91	2.07	0.96	1	0.87	1.07	3.32	—	2.18	4.39	3.28	3.14	81%
(II)	1.78	1.98	1.02	1.15	0.98	1.10	3.41	1	1.98	4.51	2.94	3.26	78%
Theor.	(2)	(2)	(1)	(1)	(1)	(1)	(3)	(1)	(2)	(4)	(3)	(3)	

(I): Propionic acid-hydrochloric acid (1 : 1 v/v) hydrolysis, 130 °C, 2 h. (II): Digestion with trypsin and chymotrypsin (37°, 24 h) and subsequently with aminopeptidase M and prolidase (37 °C, 24 h).

carried out on precoated plates of silica gel G (E. Merck) at 5 °C.

Nps-Ser(Bzl)-Tyr(Bu^t)-OBu^t (I. Fragment I). Z-Tyr(Bu^t)-OBu^t (4.70 g, 11 mmol) was hydrogenated in 300 ml of methanol over 10% Pd/C (0.5 g) as a catalyst; the solvent was removed after filtration of the catalyst and dried *in vacuo* over phosphorus pentoxide. The residue was used as H-Tyr(Bu^t)-OBu^t. Nps-Ser(Bzl)-OH·DCHA (5.29 g, 10 mmol) was suspended in 200 ml of ethyl acetate and washed with 10% citric acid in water. Ethyl acetate layer was separated and washed with water. After drying over magnesium sulfate, ethyl acetate was evaporated and the residue was used as Nps-Ser(Bzl)-OH. Ethyl chloroformate (1.08 g, 10 mmol) was added to a stirred mixture of Nps-Ser(Bzl)-OH, *N*-methylmorpholine (1.01 g, 10 mmol) in 200 ml of tetrahydrofuran at −15 °C. After stirring for 5 min, H-Tyr(Bu^t)-OBu^t in 50 ml of tetrahydrofuran was added at −15 °C and the reaction mixture was stirred at −15 °C for 30 min and at room temperature for 5 h. After filtration of the reaction mixture, the filtrate was evaporated and dissolved in 300 ml of ethyl acetate and washed successively with 10% citric acid, water, 5% sodium bicarbonate and water. After being dried over sodium sulfate, the solvent was evaporated *in vacuo* and Nps-Ser(Bzl)-Tyr(Bu^t)-OBu^t was obtained as an oil: yield 4.42 g (69.8%), R_f 0.89 on silica gel in *n*-BuOH : AcOH : H_2O (4 : 1 : 1) and R_f 0.85 in *n*-BuOH : AcOH : H_2O; pyridine (30 : 6 : 24 : 20). Amino acid ratios (propionic acid-12 M hydrochloric acid hydrolysis)[15]: Ser, 0.98; Tyr, 1.00 Nps-protecting group of this peptide was cleaved according to the following procedure: the above dipeptide (1.74 g, 2.8 mmol) was dissolved in 100 ml of methanol and 5 fold excess each of triphenylphosphine (3.66 g, 14 mmol), pentachlorophenol (3.72 g, 14 mmol) being added and stirred at room temperature for 30 min. The mixture was condensed after filtration of the precipitate and applied to Sephadex LH-20 column (2.5×60 cm) in methanol. H-Ser(Bzl)-Tyr(Bu^t)-OBu^t was eluted in the first fraction. The peptide was dried *in vacuo* over phosphorus pentoxide after evaporation of solvent and used for an attachment to the resin.

Z-His(Z)-Pro-OBu^t (II). To a stirred mixture of Z-His(Z)-OH with one molecule of methanol (9.11 g, 20 mmol) and di-2-pyridyl disulfide (5.28 g, 24 mmol) in 100 ml of dry methylene chloride was added triphenylphosphine (6.29 g, 24 mmol) and H-Phe-OBu^t (4.43 g, 20 mmol) in 30 ml of dry methylene chloride. The mixture was stirred at room temperature overnight and washed successively with 10% citric acid, water, 5% sodium bicarbonate and water and dried over magnesium sulfate. The solvent was evaporated after addition of silica gel (100 g) and coated residue was subjected to dry column chromatography[20] on silica gel and the peptide was eluted with diethyl ether and crystallized from ethyl acetate-diethyl ether-petroleum ether: yield 10.78 g (86.0%); mp 104—105 °C; $[\alpha]_D^{20}$ 1.7° (*c* 1, methanol).

Found: C, 66.55; H, 6.13; N, 9.09%. Calcd for $C_{35}H_{38}O_7N_4 \cdot 1/2\ H_2O$: C, 66,12; H, 6.11; N, 8.82%.

Nps-Glu(OBzl)-His-Phe-OBu^t (III). Nps-Glu(OBzl)-OH·DCHA (11.74 g, 20 mmol) was converted into a free form with citric acid as in the preparation of I and used as Nps-Glu(OBzl)-OH. To a stirred mixture of Nps-Glu(OBzl)-OH, pentachlorophenol (5.32 g, 20 mmol) in 200 ml of ethyl acetate was added dicyclohexylcarbodiimide (4.12 g, 20 mmol) with ice-cooling and the mixture was stirred at 0 °C for 1 h and kept stirring overnight. The precipitated dicyclohexylurea was filtered off and the filtrate washed with 10% citric acid, water, 5% sodium bicarbonate, water and dried over sodium sulfate. The solvent was evaporated *in vacuo* and the active ester was obtained as foam and used as Nps-Glu(OBzl)-OPcp. Compound II (6.26 g, 10 mmol) was hydrogenated over 10% Pd/C as in the preparation of I and used as H-His-Phe-OBu^t. To a stirred solution of H-His-Phe-OBu^t in 200 ml of *N,N*-dimethylformamide was added 6.54 g (10 mmol) of Nps-Glu(OBzl)-OPcp and stirred at room temperature for 48 h. The solvent was removed *in vacuo* and the residue was dissolved in ethyl acetate and washed successively with 10% citric acid, water, 5% sodium bicarbonate and water. The solvent was condensed after drying over sodium sulfate and the tripeptide was obtained by precipitation with the addition of diethyl ether: yield 5.31 g (72.4%); mp 78—80 °C, $[\alpha]_D^{20}$ −18.0° (*c* 1, methanol).

Found: C, 60.69; H, 6.10; N, 11.43; S, 4.38%. Calcd for $C_{37}H_{42}N_6O_8S$: C, 60.80; H, 5.79; N, 11.50; S, 4.39%.

Nps-Met-Glu(OBzl)-His-Phe-OBu^t (IV). Nps-Met-OPcp (2.99 g, 52.8%) was obtained as foam by the same procedure as for the preparation of Nps-Glu(OBzl)-OPcp from Nps-Met-OH·DCHA (4.38 g, 10 mmol), pentachlorophenol (2.66 g, 10 mmol) and dicyclohexylcarbodiimide (2.27 g, 11 mmol). Compound III (4.0 g, 5.48 mmol) was converted into amino free form as in the preparation of H-Ser(Bzl)-Tyr(Bu^t)-OBu^t of Fragment I. To a stirred solution of this amino free peptide in 150 ml of *N,N*-dimethylformamide was added the above Nps-Met-OPcp (3.10 g, 5.48 mmol) and kept stirring at room temperature for 48 h. After working up as in the preparation of Compound III, evaporation of the solvent gave a crude peptide. This was further purified by gel filtration on Sephadex LH-20 (2.5× 60 cm) in methanol, the tetrapeptide being obtained by crystallization from ethyl acetate-petroleum ether: yield 2.98 g (62.0%); mp 160—164 °C; $[\alpha]_D^{20}$ −42.3° (*c* 1, methanol).

Nps-Ser(Bzl)-Met-Glu(OBzl)-His-Phe-OBu^t (V, Fragment II). Nps-Ser(Bzl)-OPcp was prepared by the same procedure as for Nps-Glu(OBzl)-OPcp in the preparation of III starting from Nps-Ser(Bzl)-OH·DCHA (5.30 g, 10 mmol), pentachlorophenol (2.66 g, 10 mmol) and dicyclohexylcarbodiimide (2.27 g, 11 mmol): yield 3.85 g (64.6%); mp 117—118 °C; $[\alpha]_D^{20}$ −1.85° (*c* 1, methanol).

Found: C, 44.54; H, 2.74; N, 4.87; S, 5.45; Cl ,29.35%. Calcd for $C_{22}H_{15}N_2O_5SCl_5$: C, 44.28; H, 2.53; N, 4.70; S, 5.37; Cl, 29.71%.

Compound IV (2.06 g, 2.4 mmol) was converted into amino free form as in the preparation of H-Ser(Bzl)-Tyr(Bu^t)-OBu^t of Fragment I. To a stirred solution of this amino free peptide in 100 ml of *N,N*-dimethylformamide was added the above Nps-Ser(Bzl)-OPcp (1.43 g, 2.4 mmol) and the mixture was stirred at room temperature for 48 h. After working up as in the preparation of Compound IV, crystallization from ethyl acetate-petroleum ether gave the pentapeptide: yield 1.77 g (70.8%); mp 158—160 °C; $[\alpha]_D^{20}$ −25.6° (*c* 1, methanol).

Found: C, 59.96; H, 5.89; N, 10.49; S, 6.23%. Calcd for $C_{52}H_{62}N_8O_{11}S_2$: C, 60.10; H, 6.01; N, 10.73; S, 6.17%. R_f 0.84 on silica gel in *n*-BuOH : AcOH : H_2O (4 : 1 : 1) and R_f 0.88 in *n*-BuOH : AcOH : H_2O : pyridine (36 : 6 : 20 : 24). Amino acid ratios (propionic acid-12 M hydrochloric acid (1 : 1) hydrolysis): Ser, 0.97; Met, 0.92; Glu, 1.01; His, 1.00; Phe, 0.99. Nps-protecting group of this peptide was cleaved as in the preparation of I, the resulting amino free peptide being used for the chain elongation on the resin.

Z-Trp-Gly-OBu^t (VI). To a stirred mixture of Z-Trp-OH (33.8 g, 0.1 M), H-Gly-OBu^t (14.4 g, 0.11 M) and di-2-pyridyl disulfide (25.5 g, 0.12 M) in 300 ml of

ethyl acetate was added triphenylphosphine (31.4 g, 0.12 M) with ice-cooling and stirred at room temperature for 5 h. The mixture was worked up as in the preparation of II and the peptide was crystallized from ethyl acetate-petroleum ether: yield 37.5 g (83.2%); mp 60—70 °C (lit,[21] mp 60—70 °C); $[\alpha]_D^{20}$ −20.8° (*c* 1, methanol) (lit,[21] $[\alpha]_D^{20}$ −19.6° (*c* 1, methanol)).

Z-Arg(Tos)-Trp-Gly-OBu^t (VII, Fragment III). Compound VI (9.95 g, 22 mmol) was hydrogenated over 10% Pd/C as in the preparation of I and used as H-Trp-Gly-OBu^t. To a stirred mixture of Z-Arg(Tos)-OH (9.24 g, 20 mmol), the above H-Trp-Gly-OBu^t and di-2-pyridyl disulfide (5.10 g, 24 mmol) in 300 ml of ethyl acetate was added triphenylphosphine (6.28 g, 24 mmol) with ice-cooling and stirred at room temperature for 6 h. After working up as in the preparation of II, the coated residue was subjected to dry column chromatography. On being washed with diethyl ether, the coated layer was separated and the peptide was eluted with methanol-chloroform (1 : 2 v/v). After the solvent was removed, the residue was dissolved in ethyl acetate and washed with water. After being dried over sodium sulfate, the solution was condensed and the tripeptide was obtained by precipitation with the addition of petroleum ether: yield 13.57 g (89.3%); mp 137—140 °C; $[\alpha]_D^{20}$ −24.5° (*c* 1, methanol) (lit,[21] $[\alpha]_D^{20}$ −25.1° (*c* 1, methanol)). Amino acid ratios (3 M *p*-toluenesulfonic acid hydrolysis[22]): Arg. 0.99; Trp, 0.81; Gly, 1.00. The peptide was hydrogenated over 10% Pd/C as in the preparation of H-Tyr(Bu^t)-OBu^t of Fragment I and used for chain elongation on the resin.

Z-Val-Gly-OBu^t (VIII). To a stirred mixture of Z-Val-OH (6.30 g, 25 mmol), H-Gly-OBu^t (3.60 g, 27.5 mmol) and di-2-pyridyl disulfide (6.55 g, 30 mmol) in 250 ml of benzene was added triphenylphosphine (7.90 g, 30 mmol) in 100 ml of benzene with ice-cooling and stirred at room temperature overnight and worked up as in the preparation of II, the dipeptide being obtained by crystallization from methanol-diethyl ether: yield 6.85 g (75.2%); mp 147.3 °C; $[\alpha]_D^{20}$ −27.2° (*c* 1, methanol).

Found: C, 62.42; H, 7.63; N, 7.67%. Calcd for $C_{19}H_{28}N_2O_5$: C, 62.62; H, 7.74; N, 7.69%.

Z-Pro-Val-Gly-OBu^t (IX). Compound VIII (5.10 g 14 mmol) was hydrogenated over Pd/C as in the preparation of I and used as H-Val-Gly-OBu^t. To a stirred mixture of H-Val-Gly-OBu^t, Z-Pro-OH (4.72 g, 12.9 mmol) and di-2-pyridyl disulfide (4.17 g, 18.9 mmol) in 100 ml of methylene chloride was added triphenylphosphine (4.97 g, 18.9 mmol) in 20 ml of methylene chloride with ice-cooling and stirred at room temperature overnight. The solvent was removed and the residue was dissolved in 100 ml of ethyl acetate and worked up as in the preparation of II. The peptide was crystallized from methanol-petroleum ether after being subjected to dry column chromatography with chloroform-methanol (19 : 1 v/v): yield 5.30 g (91.2%); mp 120 °C; $[\alpha]^{20}$ −90.8° (*c* 1, methanol).

Found: C, 62.57; H, 7.67; N, 8.88%. Calcd for $C_{24}H_{35}N_3O_6$: C, 62.45; H, 7.64; N, 9.10%.

Nps-Lys(Z)-Pro-Val-Gly-OBu^t (X, Fragment IV). Compound IX (1.38 g, 3.0 m mol) was hydrogenated in methanol and Nps-Lys(Z)-OH·DCHA (1.80 g, 2.93 mmol) was converted into free form as in the preparation of I. To a stirred mixture of H-Pro-Val-Gly-OBu^t, Nps-Lys(Z)-OH in 100 ml of ethyl acetate was added dicyclohexylcarbodiimide (2.0 g, 10 mmol) with ice-cooling and stirred at room temperature overnight. The dicyclohexylurea was filtered off and the filtrate was worked up as in the preparation of II. The coated residue was subjected to dry column chromatography and eluted with ethyl acetate. The solution was washed with water, dried over sodium sulfate and condensed. Addition of petroleum ether precipitates the tetrapeptide: 1.66 g (76.2%); mp 85.6 °C; $[\alpha]_D^{20}$ −101.5° (*c* 1, methanol).

Found: C, 58.25; H, 6.75; N, 11.17; S, 4.60%. Calcd for $C_{36}H_{51}N_6O_9S$: C, 58.13; H, 6.91; N, 11.30; S, 4.31%. R_f 0.87 on silica gel in *n*-BuOH : AcOH : H_2O (4 : 1 : 1) and R_f 0.89 in *n*-BuOH : AcOH : H_2O : pyridine (30 : 6 : 24 : 20). Amino acid ratios (propionic acid-12 M hydrochloric acid hydrolysis): Lys, 1.05; Pro, 0.96; Val, 0.96; Gly, 1.00. The peptide was converted into amino free form as in the preparation of fragment I and used for chain elongation on the resin.

Z-Arg(Tos)-Pro-OBu^t (XI). To a stirred mixture of Z-Arg(Tos)-OH (13.92 g, 30 mmol), H-Pro-OBu^t (5.13 g, 30 mmol) and di-2-pyridyl disulfide (6.60 g, 30 mmol) in 100 ml of methylene chloride was added triphenylphosphine (7.86 g, 30 mmol) with ice-cooling and stirred at room temperature overnight. The mixture was worked up as in the preparation of VII and the peptide was crystallized from ethyl acetate-petroleum ether: yield 13.92 g (75%); mp 114—115 °C (lit,[23] 115—118 °C); $[\alpha]_D^{25}$ −42.5° (*c* 1, methanol) (lit,[23] −42.5°).

Z-Arg(Tos)-Arg(Tos)-Pro-OBu^t (XII). Compound XI (9.30 g, 15 mmol) was hydrogenated in methanol as in the preparation of I. To a stirred mixture of H-Arg(Tos)-Pro-OBu^t, Z-Arg(Tos)-OH (6.60 g, 14 mmol) and di-2-pyridyl disulfide (3.30 g, 15 mmol) in 50 ml of methylene chloride was added triphenylphosphine (3.94 g, 15 mmol) with ice-cooling and stirred at room temperature overnight. The mixture was worked up as in the preparation of VII, the peptide being eluted with methanol on dry column chromatography. The peptide was crystallized from methanol-diethyl ether after further filtration on Sephadex LH-20 in methanol: yield 12.41 g (89%); mp 100—102 °C (lit,[23] 100—120 °C); $[\alpha]_D^{25}$ −33.3° (*c* 1, methanol) (lit,[23] −33.3°).

Nps-Lys(Z)-Arg(Tos)-Arg(Tos)-Pro-OBu^t (XIII). Compound XII (10.20 g, 11 mmol) was hydrogenated in methanol and Nps-Lys(Z)-OH·DCHA (6.14 g, 10 mmol) was converted into free form as in the preparation of I. A mixed anhydride was prepared as in the preparation of I from Nps-Lys(Z)-OH, *N*-methylmorpholine (1.01 g, 10 mmol) and ethyl chloroformate (1.08 g, 10 mmol) in 50 ml of tetrahydrofuran. To this solution was added H-Arg(Tos)-Arg(Tos)-Pro-OBu^t in 30 ml of chloroform with stirring for 1 h with ice-cooling and kept standing overnight at room temperature. The mixture was evaporated and dissolved in 100 ml of ethyl acetate and washed successively with 2 M sodium hydroxide, water, 10% citric acid and water. After drying over magnesium sulfate, the solvent was removed and the residue was subjected to Sephadex LH-20 column (2.5×60 cm) in methanol. The first fraction was pooled and the peptide was precipitated by addition of petroleum ether after condensation of methanol: yield 10.90 g (90%); mp 88—92 °C; $[\alpha]_D^{20}$ −49.1° (*c* 1, methanol).

Found: C, 54.66; H, 6.43; N, 13.88; S, 7.84%. Calcd for $C_{55}H_{74}O_{13}N_{12}S_3$: C, 54.71; H, 6.19; N, 13.92; S, 7.97%.

Nps-Lys(Z)-Lys(Z)-Arg(Tos)-Arg(Tos)-Pro-OBu^t (XIV Fragment V). Compound XIII (8.10 g, 5.5 mmol) was converted into amino free form with triphenylphosphine (7.21 g, 27.5 mmol) and pentachlorophenol (7.30 g, 27.5 mmol) in 150 ml of methanol and 150 ml of methylene chloride as in the preparation of I. The H-Lys(Z)-Arg(Tos)-Arg(Tos)-Pro-OBu^t, Nps-Lys(Z)-OH·DCHA (3.07 g, 5 mmol), *N*-methylmorpholine (0.50 g, 5 mmol) and ethyl chloroformate (0.54 g, 5 mmol) were allowed to react in 50 ml of tetrahydrofuran and 50 ml of methylene chloride and

worked up as in the preparation of XII: yield 5.30 g (74%); mp 130—135 °C; $[\alpha]_D^{20}$ −38.3° (*c* 1, methanol).

Found: C, 56.46; H, 6.58; N, 13.44; S, 6.59%. Calcd for $C_{69}H_{92}O_{16}N_{14}S_3$: C, 56.38; H, 6.32; N, 13.35; S, 6.54%. R_f 0.96 on silica gel in *n*-BuOH : AcOH : H_2O (4 : 1 : 1) and R_f 0.89 in *n*-BuOH : AcOH : H_2O : pyridine (30 : 6 : 24 : 20). Amino acid ratios (propionic acid-12 M hydrochloric acid hydrolysis): Lys, 2.00; Arg, 1.96; Pro, 0.96. Nps-protecting group of the peptide was cleaved by the same procedure as in the preparation of I and the amino free peptide was used for chain elongation on the resin.

Z-Tyr(Bu^t)-Pro-OBu^t (XV). Z-Tyr(Bu^t)-OH·DCHA (5.52 g, 10 mmol) was converted into free form as in the preparation of I. To a stirred mixture of Z-Tyr(Bu^t)-OH, H-Pro-OBu^t (1.88 g, 11 mmol) and di-2-pyridyl disulfide (2.64 g, 12 mmol) in 100 ml of ethyl acetate was added triphenylphosphine (3.14 g, 12 mmol) with ice-cooling and stirred at room temperature for 8 h. The mixture was worked up as in the preparation of II, the peptide being obtained as an oil: yield 4.60 g (87.7%).

Z-Val-Tyr(Bu^t)-Pro-OBu^t (XVI). Compound XV (4.60 g, 8.77 mmol) was hydrogenated to H-Tyr(Bu^t)-Pro-OBu^t as in the preparation of I. To a stirred mixture of Z-Val-OH (2.0 g, 8 mmol), H-Tyr(Bu^t)-Pro-OBu^t and di-2-pyridyl disulfide (2.11 g, 9.6 mmol) in 200 ml of ethyl acetate was added triphenylphosphine (2.52 g, 9.6 mmol) with ice-cooling and stirred at room temperature overnight. The mixture was worked up as in the preparation of II, the peptide being obtained by the evaporation of diethyl ether: yield 4.30 g (86.3%); mp 45—49 °C.

Nps-Lys(Z)-Val-Tyr(Bu^t)-Pro-OBu^t (XIIV). Compound XVI (4.09 g, 6.57 mmol) was hydrogenated to H-Val-Tyr(Bu^t)-Pro-OBu^t and Nps-Lys(Z)-OH·DCHA (4.07 g, 6.57 mmol) was converted into a free form. To a stirred mixture of Nps-Lys(Z)-OH and H-Val-Tyr(Bu^t)-Pro-OBu^t in 200 ml of ethyl acetate was added dicyclohexylcarbodiimide (2.71 g, 13.1 mmol) and stirred for 2 h with ice-cooling and overnight at room temperature. Dicyclohexylurea was filtered off and the filtrate was worked up as in the preparation of X. Crystallization from ethyl acetate-petroleum ether gave the tetrapeptide: yield 3.92 g (72.8%); mp 155—156 °C; $[\alpha]_D^{20}$ −56.3° (*c* 1, methanol).

Found: C, 62.13; H, 7.16; N, 9.38; S, 3.42%. Calcd for $C_{47}H_{64}N_6O_{10}S$: C, 62.37; H, 7.13; N, 9.29; S, 3.54%.

Nps-Val-Lys(Z)-Val-Tyr(Bu^t)-Pro-OBu^t (XVIII, Fragment VI). Compound XVII (2.50 g, 2.76 mmol) and Nps-Val-OH·DCHA (1.49 g, 3.32 mmol) was converted into free form as in the preparation of I. H-Lys(Z)-Val-Tyr(Bu^t)-Pro-OBu^t, Nps-Val-OH and dicyclohexylcarbodiimide (1.14 g, 5.52 mmol) were allowed to react in 100 ml of ethyl acetate and worked up as in the preparation of X. The peptide was obtained by crystallization from ethyl acetate-petroleum ether: yield 2.08 g (72.3%); mp 206—209 °C (decomp.); $[\alpha]_D^{20}$ −105.5° (*c* 1, methanol).

Found: C, 61.87; H, 7.27; N, 9.41; S, 3.11%. Calcd for $C_{52}H_{73}N_7O_{11}S \cdot 1/2H_2O$: C, 61.64; H, 7.36; N, 9.68; S, 3.16%. R_f 0.95 on silica gel in *n*-BuOH : AcOH : H_2O (4 : 1 : 1) and R_f 0.88 in *n*-BuOH : AcOH : H_2O : pyridine (30 : 6 : 24 : 20). Amino acid ratios (propionic acid –12 M hydrochloric acid hydrolysis): Lys, 0.96; Val, 1.96; Tyr, 1.00; Pro, 1.03. Nps-protecting group of this peptide was cleaved by the same procedure as in the preparation of I and was used for chain elongation on the resin.

Attachment of H-Ser(Bzl)-Tyr(Bu^t)-OBu^t to the Methylchloroformylated Resin. The methylchloroformylated resin[9] (3 g, Cl: 1.87 mmol/g) was shaken 8 h at room temperature with 1.315 g (2.8 mmol) of H-Ser(Bzl)-Tyr(Bu^t)-OBu^t, triethylamine (0.28 g, 2.80 mmol) and 30 ml of chloroform in a vessel for solid phase peptide synthesis and washed with chloroform. The unreacted peptide was recovered in washing chloroform (2.24 mmol, 83% of the theoretical value). The remaining chloroformyl groups on the resin were covered by shaking with diethylamine (4.1 g, 56.1 mmol) and 30 ml of chloroform for 8 h and washed successively with chloroform (30 ml×3), ethanol (30 ml×3), and methylene chloride (30 ml×3). The resin was washed with trifluoroacetic acid-methylene chloride (1 : 1 v/v) and *t*-butyl group was deprotected by shaking for 30 min with 30 ml of trifluoroacetic acid-methylene chloride (1 : 1 v/v). The resulting resin was washed with methylene chloride (30 ml×3), chloroform (30 ml×3), and neutralized with 30 ml of triethylamine-chloroform (1 : 10 v/v) for 10 min. The resin was washed successively with chloroform (30 ml×3), ethanol (30 ml×3), and methylene chloride and dried *in vacuo*. An aliquot of the resin was hydrolyzed in propionic acid–12 M hydrochloric acid, the peptide content being determined by amino acid analyzer to be 0.031 mmol/g of the resin.

Chain Elongation. The resin-Ser(Bzl)-Tyr-OH prepared in the above experiment (1.5 g, 0.0465 mmol of dipeptide) was placed in a vessel for solid phase peptide synthesis. The following steps were used to couple each peptide fragment: 1) washing with methylene chloride (15 ml×3), 2) addition of 3 fold excess (0.1395 mmol) of amino free peptide fragment, 30 fold excess (1.395 mmol) of di-2-pyridyl disulfide (0.307 g) and 1-nitronaphthalene (internal standard for monitoring by HPLC) in 10 ml of methylene chloride followed by shaking for 5 min, 3) addition of 30 fold excess (1.395 mmol) of triphenylphosphine (0.366 g) in 5 ml of methylene chloride followed by a reaction period, 4) washing with methylene chloride (15 ml×3), 5) washing with ethanol (15 ml×3), 6) washing with methylene chloride (15 ml×3), 7) addition of 10 fold excess (0.465 mmol) of diethylamine (0.034 g) and 30 fold excess (1.395 mmol) each of di-2-pyridyl disulfide (0.307 g) and triphenylphosphine (0.366 g) in 15 ml of methylene chloride followed by shaking for 6 h, 8) washing with methylene chloride (15 ml×3), 9) washing with ethanol (15 ml×3), 10) washing with methylene chloride (15 ml×3), 11) wash with 15 ml of trifluoroacetic acid-methylene chloride (1 : 1 v/v) containing 5% of 2-mercaptoethanol, 12) removal of butyl ester with 15 ml of trifluoroacetic acid-methylene chloride (1 : 1 v/v) containing 5% of 2-mercaptoethanol for 30 min, 13) washing with methylene chloride (15 ml×3), 14) washing with chloroform (15 ml×3), 15) neutralization with 15 ml of triethylamine-chloroform (1 : 10 v/v) for 10 min, 16) washing with chloroform (15 ml×3), 17) washing with ethanol (15 ml×3), 18) washing with methylene chloride (15 ml×3). The first coupling of the initial resin-Ser(Bzl)-Tyr-OH with H-Ser(Bzl)-Met-Glu(OBzl)-His-Phe-OBu^t (fragment II) and second coupling with H-Arg(Tos)-Trp-Gly-OBu^t (fragment III) were carried out at −15 °C for 6 h and overnight at room temperature in the presence of 30 eq (1.395 mmol) of 2-pyridinethiol (0.155 g). Successive coupling of H-Lys(Z)-Pro-Val-Gly-OBu^t (fragment IV), H-Lys(Z)-Lys(Z)-Arg(Tos)-Arg(Tos)-Pro-OBu^t (fragment V) and H-Val-Lys(Z)-Val-Tyr(Bu^t)-Pro-OBu^t (fragment VI) were carried out at room temperature in the absence of 2-pyridinethiol. In each coupling step, an aliquot of the resulting resin-peptide was hydrolyzed in propionic acid–12 M hydrochloric acid (1 : 1 v/v).[15] The results of amino acid analyses are given in Table 2. The unreacted fragments were recovered in washing solvents of procedures 4), 5), and 6) and the quantities of fragments were determined by amino acid analyses of the

hydrolyzate of the portion of washing solvents: Fragment II, 0.075 mmol (recovery yield 81% of the theoretical value); Fragment III, 0.081 mmol (87%); Fragment IV, 0.084 mmol (90%); Fragment V, 0.076 mmol (82%); Fragment VI, 0.085 mmol (91%). These fragments were easily isolated by means of gel filtration on Sephadex LH-20 in methanol and complete evaporation of solvent *in vacuo*. With use of these recovered fragments, peptide chain was elongated by starting with 1 g of new resin-Ser(Bzl)-Tyr-OH (0.031 mmol) according to the procedure in the above chain elongation. Coupling reaction was continued until a monitoring curve by high pressure liquid chromatography showed no consumption of the fragment (*ca.* 6—8 h); repeated until no fragment uptake was detected. Amino acid analysis of the hydrolyzate of a portion of the resulting resin-tetracosapeptide by propionic acid–12 M hydrochloric acid showed nearly quantitative coupling reaction in each step: Ser, 2.11; Tyr, 2.02; Met, 0.91; Glu, 1.01; His, 1.00; Phe 0.97; Arg, 2.79; Gly, 1.94; Lys, 3.97; Pro, 2.75; Val, 2.93.

Monitoring of Coupling Reaction by High Pressure Liquid Chromatography. With the use of aminated Hitachi Gel 3010,[13] styrene-divinylbenzene polymer, the elution conditions were so established that each fragment has a constant retention time: column, 0.5×50 cm, Pyrex glass; mobile phase, 0.2 M hydrochloric acid, 0.1 M potassium chloride-methanol (1 : 9); flow rate, 2.0 ml/min; temperature, ambient; detector, UV (240 nm). Active dehydrating agent, phosphorane, in the reaction solution should be decomposed before injection to the column, since phosphorane is eluted near the fragment. A sample solution was prepared as follows: Add 20 μl of 0.2 M potassium hydroxide-80% methanol to 20 μl of the solution and then neutralize with 20 μl of 0.2 M monopotassium citrate-80% methanol. For the sake of accurate determination and easy sampling, 1-nitronaphthalene was added to the reaction mixture as an internal standard. A definite volume (5—20 μl) of each solution was injected, each sample being analyzed within 15 min with the accuracy of ±1.0%.[14] A typical chromatogram and examples of monitoring results are shown in Figs. 3 and 4, respectively. The amount of the consumed fragment (Fig. 4) was generally smaller than the actual amount consumed in chain elongation, since the initial peak height after shaking the resin with the fragment was arbitrarily scaled as 3 meq neglecting the small amount of adsorbed fragment on the resin. This small amount of the adsorbed fragment incorporated into the peptide chain makes up the difference in the quantity consumed as shown in the graph and the required value of 1 meq.

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH (ACTH(1—24)). The resin-tetracosapeptide started from 1.5 g of resin-dipeptide was treated with 40 ml of dry hydrogen fluoride[16] at 0° for 30 min in the presence of 2 ml of anisole and 2-pyridinethiol[9] (0.300 g). The yield of this cleavage step was 83%. After removal of hydrogen fluoride, the residue was dried over potassium hydroxide *in vacuo*. The resin was stirred in 30 ml of trifluoroacetic acid, filtered and washed with 30 ml of trifluoroacetic acid. The combined filtrate was evaporated and the residue was dissolved in 50 ml of acetic acid and evaporated *in vacuo*. The residue was dissolved in 30 ml of water, washed with diethyl ether and passed through a Dowex 1×2 (acetate form, 2.5×10 cm) with 300 ml of water to be converted into acetate form. The solution was condensed to 20 ml and stirred at 50 °C for 12 h after addition of 1 ml of mercaptoacetic acid. The solution was evaporated *in vacuo* and subjected to gel filtration on Sephadex G-25 (2×125 cm) in 1 M acetic acid in order to separate the peptide from 2-pyridinethiol and anisole. After evaporation of the Ehrlich and Pauly positive fractions of main peak, the peptide was purified on carboxymethylcellulose column (1×55 cm) by continuous gradient elution with ammonium acetate buffer at pH 6.7[17]: 0.01 M ammonium acetate (150 ml), 0.01—0.2 M ammonium acetate (270 ml), 0.2—0.4 M ammonium acetate (600 ml), 0.4 M ammonium acetate (330 ml). The main peak in 0.2—0.4 M ammonium acetate was proceeded by a small peak in 0.01—0.2 M ammonium acetate and followed by a very small peak in 0.4 M ammonium acetate. The fractions of the main peak were condensed and purified again on carboxymethylcellulose in the same manner after gel filtration on Sephadex G-25 in 1 M acetic acid. The fractions containing a single component were pooled and condensed *in vacuo*. Desalting was achieved by gel filtration on Sephadex G-25 in 1 M acetic acid and the peptide was precipitated from methanol containing 1% of acetic acid-ethyl acetate-petroleum ether after evaporation of solvent. The pure tetracosapeptide was obtained after drying *in vacuo* for 12 h at 40 °C over phosphorus pentoxide: yield 41 mg (25.2% yield based on Ser-Tyr attached on the resin and 42.8% yield from the liberated peptide from the resin); $[\alpha]^{20}$ −86.8° (*c* 0,5. 1% acetic acid) (lit,[18] $[\alpha]_D^{22}$ −88.6° ±2° (*c* 0.51, 1% acetic acid)); UV in 0.1 M sodium hydroxide, λ_{max} 283 mμ (ε=8640) and λ_{max} 289 mμ (ε=8880) (lit,[18] λ_{max} 283 mμ (ε=8550) and λ_{max} 289 mμ (ε=8810)). Thin layer chromatography gave a single spot detected by Ehrlich, Pauly, Cl-tolidine, and ninhydrin reactions: R_f 0.28 on silica gel in *n*-BuOH : AcOH : H_2O : pyridine (30 : 6 : 24 : 20) and R_f 0 in *n*-BuOH : AcOH : H_2O (4 : 1 : 1). Paper electrophoresis with lysine as reference also gave a single ninhydrin, Pauly, Ehrlich, and Cl-tolidine positive spot: R_m 0.72 in 0.5 M formic acid–2 M acetic acid (1 : 1 v/v), 60×10 cm, 900 V, 1.5 h and R_m 0.77 in pyridine-AcOH-H_2O (10 : 0.4 : 90 v/v), 60×10 cm, 1500 V, 1 h. The peptide was characterized as heptaacetate and nonahydrate by elemental analysis. Found: C, 51.19; H, 7.05; N, 15.93; S, 0.93%. Calcd for $C_{136}H_{210}O_{31}N_{40}S \cdot 7CH_3COOH \cdot 9H_2O$: C, 51.24; H, 7.33; N, 15.94; S, 0.91%. Two mg of the peptide dissolved in 0.5 ml of Tris buffer of pH 8.5 containing 0.01 M Mg^{2+} was incubated with 50γ of trypsin (Worthington Biochemical Co., TRL 100S) and 50 γ of chymotrypsin (Worthington Biochemical Co., CDI 2LX) at 37 °C for 24 h. The solution was boiled for 15 min, cooled and then incubated with 80 γ of aminopeptidase M (Rohm & Haas) and 80 γ of prolidase (Miles Laboratories Ltd., UK8) for 48 h at 37 °C. The results of amino acid analyses of the digestion mixture and propionic acid–12 M hydrochloric acid hydrolyzate of the peptide are shown in Table 3.

Bioassay. ACTH activity of the tetracosapeptide was assayed by the ascorbic acid depletion test according to USP XVIII, and compared with the 2nd international standard. The dose response regression lines for the synthetic peptide and the standard were parallel. The activity was calculated as 96 (83—128) IU/mg, identical with the value in literature.[18]

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11) Abbreviations recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (*Pure Appl. Chem.*, **40**, 315 (1974)) are used. DCC: dicyclohexylcarbodiimide, M. A.: mixed anhydride method, PCP: pentachlorophenol, —OPcp: pentachlorophenyl ester, and DCHA: dicyclohexyl amine. Amino acid symbols denote the L-configuration.
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