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Solid Phase Peptide Synthesis by Oxidation-Reduction Condensation. Synthesis of LH-RH by Fragment Condensation on Solid Support

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Solid phase peptide synthesis by oxidation-reduction condensation was investigated. The fragment condensation on solid support was examined by employing two types of chain elongation, that from *C*-terminal amino acid to *N*-terminal amino acid (A type elongation) and that from *N*-terminal amino acid to *C*-terminal amino acid (B type elongation). The feasibility of these two approaches A and B was demonstrated in two syntheses of LH–RH (luteinizing hormone-releasing hormone). The LH–RH prepared from the two different chain elongations showed identical mobilities on tlc, exhibiting full activities of natural LH–RH (AVS 77-33 ♯ 215-269).

Solid phase peptide synthesis has provided a rapid method for preparation of many biologically active peptides[1] in which peptide chains have been lengthened from *C*-terminal amino acid to *N*-terminal amino acid (A type elongation). However, only a few investigations[2] have been reported on the preparation of oligopeptides by solid phase synthesis in which the peptide chain elongation was carried out from *N*-terminal amino acid to *C*-terminal amino acid (B type elongation). As regards type A, many modifications[3] have been attempted by fragment condensation to minimize the contaminants which bear similar properties to those of the desired product. On the other hand, no development was attained as regards type B because of the danger of racemization both in the deprotection and in the coupling steps. Recently, a modification[4] of this method was attempted by the use of azide coupling, but it was found not to be a general procedure because of low yields and of restrictions on reaction solvents. In the present experiment, application of the oxidation-reduction process with triphenylphosphine (Ph_3P) and 2,2′-dipyridyl disulfide ($(PyS)_2$) to solid phase peptide synthesis was investigated, taking into consideration the fact that development of both types of chain elongations would be achieved by fragment condensation with oxidation-reduction process possessing the advantages of minimizing side reactions and the racemization of a carboxyl component.[5] This paper describes successful syntheses of porcine luteinizing hormone-releasing hormone (LH–RH)[6] with a sequence of <Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2 by em-

1) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964). G. R. Marshall and R. B. Merrifield, *ibid.*, **4**, 2394 (1965). A. Marglin and R. B. Merrifield, *J. Amer. Chem. Soc.*, **88**, 5051 (1966). H. Takashima, V. du Vigneaud, and R. B. Merrifield, *ibid.*, **90**, 1323 (1968). M. Manning, *ibid.*, **90**, 1348 (1968). J. Meienhofer and Y. Sano, *ibid.*, **90**, 2996 (1968). B. Gutte and R. B. Merrifield, *ibid.*, **91**, 501 (1969).

2) R. L. Letsinger and M. J. Kornet, *ibid.*, **85**, 3045 (1963).

3) F. Weygand and U. Rahnarsson, *Z. Naturforsch.*, **216**, 1141 (1966). G. S. Omenn and C. B. Anfinsen, *J. Amer. Chem. Soc.*, **90**, 6571 (1968). H. Yajima, H. Kawatani, and H. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **18**, 1333 (1970).

4) A. M. Felix and R. B. Merrifield, *J. Amer. Chem. Soc.*, **92**, 1385 (1970).

5) T. Mukaiyama, R. Matsueda, and N. Suzuki, *Tetrahedron Lett.*, **1970**, 1901. T. Mukaiyama, K. Goto, R. Matsueda, and M. Ueki, *ibid.*, **1970**, 5293.

6) H. Matsuo, Y. Baba, R. M. G. Nair, A. Arimura, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, **43**, 1334 (1971). Y. Baba, H. Matsuo, and A. V. Schally, *ibid.*, **44**, 459 (1971).

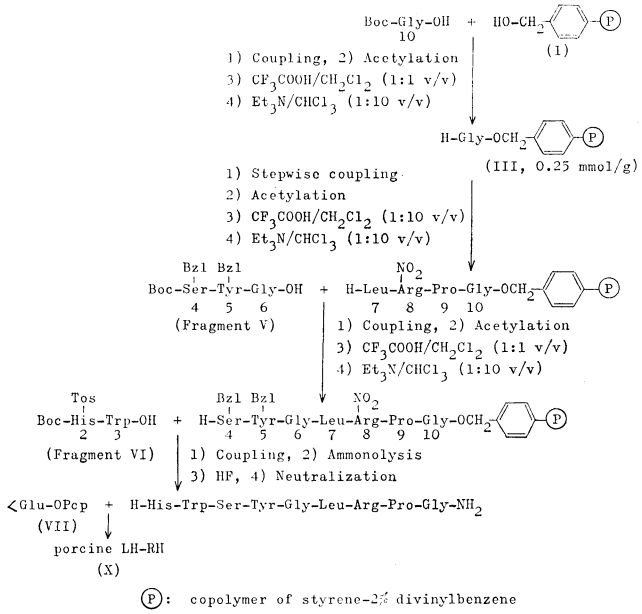

Fig. 1. Scheme for the synthesis of LH–RH by A type elongation.

ploying both A and B types of chain elongation.[7]

I. Chain Elongation from C-*Terminal Amino Acid to* N-*Terminal Amino Acid (Type A).* Attachment of the first amino acids or peptide fragments to the hydroxymethyl resin and the peptide chain elongation by fragment condensation have recently been achieved[8] by oxidation-reduction condensation. Synthesis of nonapeptide (2—10 sequence) of LH–RH was attempted by solid phase method as shown in Fig. 1. The chain elongation on solid support was designed to prepare nonapeptide since the tosyl group of the side chain of histidine was found to be partially cleaved by treatment with trifluoroacetic acid used for the removal of Boc-group. The syntheses of peptide fragments achieved by oxidation-reduction condensation in solution are outlined in Fig. 2 and physical properties of these intermediates are listed in Table 1. In the

Table 1. Physical properties of peptide fragments

Peptide fragment	Mp	$[\alpha]_D^t{}^\circ$ (c,solvent)
Fragment V	134°C	$[\alpha]_D^{20}$ −6.2° (c 1, MeOH)
Fragment VI	148—150°C	$[\alpha]_D^{20}$ 15.2° (c 1, DMF)
XV	172—175°C	$[\alpha]_D^{20}$ −9.6° (c 1, DMF)
XIX	168—171°C(decomp.)	$[\alpha]_D^{20}$ −43.2° (c 2, DMF)

first step, esterification of a 2% crosslinked hydroxymethyl resin which was synthesized from usual chloromethyl resin (Schwarz-Mann, New York, Cl content: 2.0 mmol/g) with the first Boc-glycine was undertaken by using 3 fold excess each of Boc-glycine, Ph_3P and $(PyS)_2$ in CH_2Cl_2. The remaining free hydroxyl groups were covered by acetylation[9] in DMF.[10] The Boc-

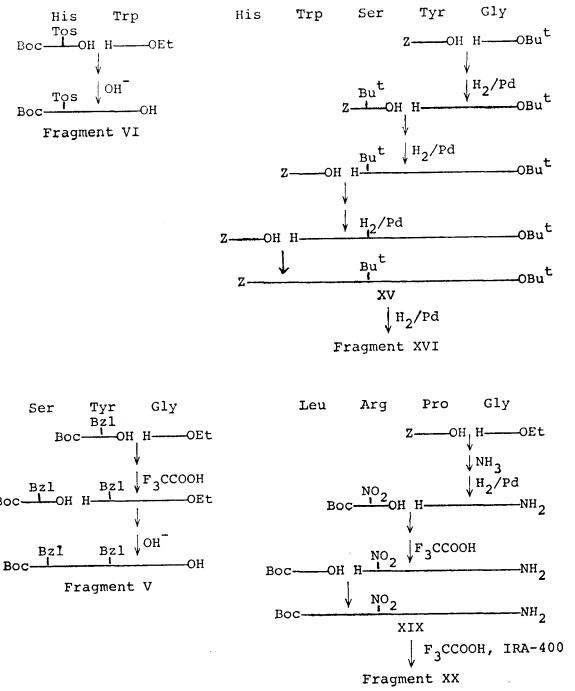

Fig. 2. Syntheses of peptide fragments.

glycine resin (III, 0.25 mmol/g) thus obtained was chain-lengthened to a tetrapeptide stage by stepwise elongation by oxidation-reduction condensation according to a similar procedure to that of the attachment of glycine to the resin. Removal of the Boc-group was performed by treatment with trifluoroacetic acid in CH_2Cl_2 (1 : 1 v/v) for 30 min, neutralization being carried out with triethylamine in $CHCl_3$ (1 : 10 v/v) for 10 min. The first coupling of tetrapeptide-resin with fragment V was achieved by treating the deprotected tetrapeptide-resin with a 1.5 fold excess of fragment V and 3 fold excess each of Ph_3P and $(PyS)_2$ in CH_2Cl_2 for 24 hr. The second coupling of heptapeptide-resin with fragment VI was also achieved by treating the deprotected heptapeptide-resin with 3 fold excess each of fragment VI, Ph_3P and $(PyS)_2$ in CH_2Cl_2 for 24 hr. A portion of this resulting resin was hydrolyzed in 3 M *p*-toluenesulfonic acid in the presence of 3-(2-aminoethyl)indole[11] after being treated with HF,[12] the amino acid analysis giving: His, 0.95; Trp, 0.84; Ser, 0.96; Tyr, 0.95; Gly, 2.12; Leu, 1; Arg, 0.93; Pro, 0.97.

Since triphenylphosphine oxide and 2-mercaptopyridine, coproducts produced in the above condensation, are very soluble in various organic solvents, they are washed away thoroughly in the washing steps. It is an important advantage over *N,N'*-dicyclohexylcarbodiimide-mediated coupling in which insoluble dicyclohexylurea are formed.

Removal of the completely protected peptide chain from the resin was undertaken by ammonolysis in absolute ethanol. The protected nonapeptide amide was purified by Avicel column in BuOH: AcOH: H_2O

7) The results were reported at the 10th Japanese Peptide Symposium held at Sapporo, 26, 27 September, 1972.

8) R. Matsueda, E. Kitazawa, H. Maruyama, H. Takahagi, and T. Mukaiyama, *Chem. Lett.*, **1972**, 379.

9) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," W. H. Freeman, San Francisco, Calif. (1969), p. 33.

10) *N,N*-dimethylformamide.

11) T. Y. Liu and Y. H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).

12) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, This Bulletin, **40**, 2164 (1967).

(4: 1: 1 v/v) and Sephadex LH-20 in DMF. The pure protected nonapeptide amide Boc–His(Tos)–Trp–Ser(Bzl)–Tyr(Bzl)–Gly–Leu–Arg(NO_2)–Pro–Gly–NH_2·H_2O (IX) was obtained as a white amorphous powder, weight 373 mg (48% yield based on the amount of glycine originally esterified to the resin), mp 197—201 °C (decomp.), $[\alpha]_D^{25}$ −27.6° (*c* 1, DMF). In order to check the racemization during fragment condensation, the nonapeptide amide was digested at 37 °C for 48 hr with aminopeptidase M after HF treatment and amino acid analysis of the digest gave: His, 1.06; Trp, 1.03; Ser, 1.02; Tyr, 1.02; Leu, 1; Gly, 1.04; Arg, and Pro were not observed. This shows that the fragment condensation by oxidation-reduction condensation was achieved with L-configuration being kept.

The nonapeptide amide was deprotected with HF in the presence of anisole and 2-mercaptopyridine[13] to avoid the decomposition of tryptophan residue. Conversion into LH–RH was attempted by coupling with <Glu–OPcp according to the procedure in the synthesis of thyrotropin-releasing hormone.[14] Since excess molar ratio of pyroglutamyl residue was observed to be incorporated to nonapeptide amide when 1.5 eq of <Glu–OPcp was used, the coupling was carried out in DMF by using 0.85 eq of <Glu–OPcp to nonapeptide amide after neutralization with Amberlite IRA-400 in MeOH. The product was applied to IRP-64 column in MeOH and eluted with 1% AcOH in MeOH. The peptide was purified on CM-Sephadex C-25[15c] column after gel filtration on Sephadex G-25 in 1 M AcOH. The decapeptide amide eluted with 0.15 M ammonium acetate was desalted by lyophilization three times and gel filtration on Sephadex G-25 in 1 M AcOH. Forty nine milligrams of pure LH–RH was obtained from 161 mg of the protected nonapeptide amide (IX) after precipitation from MeOH–Et_2O and drying at 40 °C *in vacuo* over P_2O_5. $[\alpha]_D^{20}$ −54.2° (*c* 1, 1% AcOH) lit, $[\alpha]_D^{28}$ −50.5±2° (*c* 1, 1% AcOH),[15a] $[\alpha]_D^{20}$ −52.9° (*c* 0.3, 1% AcOH),[15b] and $[\alpha]_D^{25}$ −48.2° (*c* 0.5, H_2O).[15c] This decapeptide amide was characterized as diacetate and trihydrate after further drying at 80 °C for 5 hr *in vacuo* over P_2O_5. The peptide was homogeneous on various tlc detected by Pauly, Ehrlich, Sakaguchi and Cl-tolidine reactions and gave the same R_f values as those reported.[15a] LH–RH activity of the purified decapeptide amide (amino acid content: 76%) was compared at 2 dose levels with that of natural LH–RH (AVS 77-33 # 215-269, amino acid content 67.5%) in ovariectomized, estrogen-progesterone treated rats. Serum LH levels were estimated by radioimmunoassay according to Niswender *et al.*[16] The synthetic LH–RH exhibited full activity[17] of the natural LH–RH.

II. Chain Elongation from N-*Terminal Amino Acid to* C-*Terminal Amino Acid (Type B).* Since Letsinger and Kornet[2] proposed the B type elongation of peptide chain, no successful developments have been reported in this type of chain elongation. Recently, a modification[4] of this method was attempted by the use of azide coupling. However, it was shown that this was not a general procedure because of low yields and restrictions on reaction solvents. Since the direction of B type chain elongation is similar to that in biosynthesis, it seemed attractive as an approach to biosynthesis by chemical means.

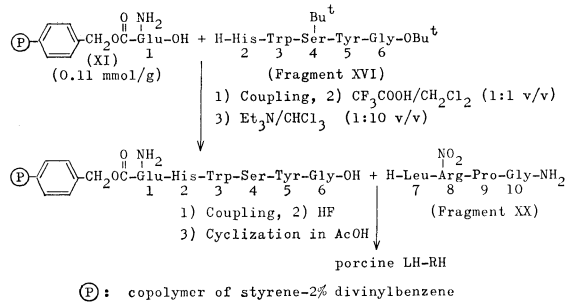

Fig. 3. Scheme for the synthesis of LH–RH by B type elongation.

The synthetic scheme for the B type elongation is shown in Fig. 3. In this approach, carboxyl protection was performed as *t*-butyl ester which is easily deprotected by trifluoroacetic acid. The building of decapeptide was designed to couple 3 fragments of 1,2—6 and 7—10 sequence in order to eliminate the trouble of preparing *t*-butyl ester of an acid-sensitive tryptophan and of racemization in fragment condensation with the use of glycine as a carboxyl component.

In the first step, an attachment of amino group of *t*-butyl glutaminate to a methylchloroformylated copolymer of styrene-2% divinylbenzene (Cl content: 1.4 mmol/g) was carried out in chloroform in the presence of triethylamine. The remaining chloroformyl group was covered by the reaction with diethylamine.[4] A resin–glutamine *t*-butyl ester containing 0.11 mmol/g was obtained. The first coupling of 2 g of resin-glutamine with fragment XVI was achieved by treating the deportected resin–glutamine with 3 fold excess each of fragment XVI, Ph_3P and $(PyS)_2$ in DMF and CH_2Cl_2 at room temperature for 10 hr and for an additional 24 hr after addition of 3 fold excess each of Ph_3P and $(PyS)_2$. The second coupling with fragment

13) Unpublished data.

14) G. Flouret, *J. Med. Chem.*, **13**, 843 (1970).

15) a) R. Geiger, W. König, H. Wissmann, K. Geisen, and F. Enzmann, *Biochem. Biophys. Res. Commun.*, **45**, 767 (1971). b) S. Sakakibara, *et al.*, "The 9th Japanese Symposium on Peptide Chemistry," Protein Research Foundation, Osaka, 1971, p. 90. c) N. Yanaihara, *et al.*, *ibid.*, p. 96.

16) G. D. Niswender, A. R. Midgley, Jr., S. Z. Monroe, and L. E. Reichert, Jr., *Proc. Soc. Exp. Biol. Med.*, **128**, 807 (1968).

17) Serum LH levels 15 min after jugular vein injection of 0.5 and 2.5 ng LH–RH in six rats in each dose level were estimated. The dose response regression lines for natural and synthetic LH–RH prepared by two procedures were parallel. Using four point factorial assay,[18] the LH–RH activity of synthetic LH–RH was calculated as follows: synthetic LH–RH of A type elongation (amino acid content 76%), 121% of the potency of natural LH–RH (AVS 77-33 # 215-269, amino acid content 67.5%); synthetic LH–RH of B type elongation (amino acid content 78%), 125% of the potency of natural LH–RH. These values show that synthetic LH–RH prepared by the two types of chain elongation have full activity of natural LH–RH taking into consideration the ratio of amino acid content of synthetic products to that of natural one.

18) C. I. Bliss, "The Statistics of Bioassay," Academic Press, Inc., (1952).

XX was carried out by the same procedure as that for the first coupling except that the deprotection of *t*-butyl group was carried out with CF_3COOH/CH_2Cl_2 (1: 1 v/v) containing 5% of 2-mercaptopyridine[13] to avoid decomposition of the acid-sensitive tryptophan residue. The amino acid ratios of the resulting resin–decapeptide amide after hydrolysis with 3 M *p*-toluenesulfonic acid[11] were as follows: Glu, 1.10; His, 1.01; Trp, 0.80; Ser, 0.95; Tyr, 1.03; Gly, 2.05; Leu, 1; Arg, 0.95;[19] Pro, 0.94; NH_3, 2.87. This shows that fragment condensation proceeds efficiently without protection of side chains of amino acids by this new method. The recovered amino components of fragments XVI and XX in the washing solvents, DMF and CH_2Cl_2, showed the same spots on tlc as with the starting fragments: the former was detected with ninhydrin, Pauly and Ehrlich reactions and the latter by ninhydrin and Cl-tolidine reactions. It was established that couplings of 1 g of new resin–glutamine with the recovered fragments XVI and XX gave a resin peptide with nearly the same amino acid ratios as those obtained in the above chain elongation.

The resin–decapeptide amide was treated with HF at room temperature for 30 min in the presence of anisole and 2-mercaptopyridine and the decapeptide amide was extracted with DMF. Cyclization of glutaminyl residue of the decapeptide amide to pyroglutamyl derivative (LH–RH) was achieved in acetic acid[20] at 80—100 °C for 30 min in the presence of aniosle and 2-mercaptopyridine. This decapeptide amide was purified on CM-Sephadex C-25[15c] as mentioned in A type elongation after separation of 2-mercaptopyridine by gel filtration on Sephadex G-25 in 1 M AcOH. The pure LH–RH (133 mg, 51% yield from the initial resin–glutamine) was obtained after desalting, lyophilization and precipitation from MeOH–Et_2O and drying at 40 °C *in vacuo* over P_2O_5. $[\alpha]_D^{20}$ −53.1° (*c* 1, 1% AcOH). The peptide was also characterized as diacetate and trihydrate after further drying at 80 °C for 5 hr *in vacuo* over P_2O_5. LH–RH activity of the purified decapeptide amide (amino acid content: 78%) which gave the same R_f values on various tlc as with the one prepared by A type elongation was compared at two dose levels with that of natural LH–RH (AVS 77-33 # 215-269), it exhibited full activity[17] of the natural LH–RH.

These new approaches demonstrate the advantages of preparing oligopeptides in good yields by a simple purification procedure since the contamination of similar peptides with small difference in sequence is eliminated. The introduction of tryptophan residue is more feasible than the usual solid phase synthesis since the number of acidolysis required for deblocking of protecting groups can be reduced.

Superiority of the oxidation-reduction process lies in the advantage it possesses of minimizing side reactions in carboxyl components. Thus, the side reaction, formation of acyl urea derivatives in dicyclohexylcarbodiimide method, and intensive restrictions on reaction solvent and temperature in azide method are eliminated.

Special practical merits of A and B types of elongation are as follows: type A, (1) it allows an attachment of the first amino acid in a rather homogeneous environment on the resin under milder conditions and in shorter reaction time than those carried out by ordinary methods and (2) the reaction conditions and operations are the same as for the subsequent chain-lengthening acylation and consequently incorporation of the esterification step in the automated process is possible. Type B, (1) chain elongation can be carried out in most cases with readily available Z-amino acids which can be used mostly without protection of side chains except carboxyl and amino groups, (2) excess amino components can be recovered unchanged and the formation of diacyl amide[21] can be avoided since the excess amino components are used in solution.

It is to be noted that solid phase peptide synthesis by oxidation-reduction condensation provides a versatile method which combines the advantages of the usual solid phase synthesis and the classical synthesis in solution.

Experimental

All melting points are uncorrected. Optical rotations were determined with a Perkin-Elmer Model 141 polarimeter. Amino acid analyses were performed on a Hitachi Amino Acid Analyzer, Model KLB-3B. Solid phase peptide synthesis was carried out by shaking with a manual apparatus. Thin layer chromatography was performed on precoated plates of silica gel G and cellulose F (E. Merck) at 5°.

Hydroxymethyl Resin (I) and Methylchloroformylated Resin (II). Chloromethylated copolystyrene-2% divinylbenzene (Schwarz-Mann, New York, Cl: 2.0 mmol/g, 50 g) was treated with potassium acetate, subsequently hydrolyzed and was converted into hydroxymethyl resin (I, Cl: 0%).[4] Similarly the hydroxymethyl resin (20 g) was allowed to react with phosgene and was further converted into methylchloroformylated resin (II). The resin of Cl content of 1.39 mmol/g was obtained.

I. A Type Elongation. Attachment of t*-Butyloxycarbonylglycine to the Hydroxymethyl Resin.* Hydroxymethyl resin (I) (4 g, OH: 8 mmol) was suspended in 40 ml of CH_2Cl_2 and shaken for 30 min in a vessel for solid phase synthesis and the resin was washed with CH_2Cl_2 (40 ml). After addition of 3 eq (24 mmol) each of *t*-butyloxycarbonyl glycine, 2,2′-dipyridyl disulfide in CH_2Cl_2 (20 ml) and shaking for a few min, 3 eq (24 mmol) of triphenylphosphine in CH_2Cl_2 (10 ml) was added at room temperature. The mixture was shaken for 24 hr at room temperature and washed successively with CH_2Cl_2 (30 ml × 3), EtOH (30 ml × 3) and DMF (30 ml × 3). The remaining free hydroxyl groups on the resin were covered by acetylation[10] with 10 eq (80 mmol) each of acetic anhydride and triethylamine in 30 ml of DMF for 2 hr and washed successively with DMF (30 ml × 3), EtOH (30 ml × 3) and CH_2Cl_2 (30 ml × 3). An aliquot of the resin (III) was hydrolyzed and glycine content was determined by amino acid analyzer to be 0.25 mmol/g of the resin.

Boc–Tyr(Bzl)–Gly–OEt (IV). To a stirred mixture of

19) The value was calculated from the amounts of Arg, Orn and $Arg(NO)_2$.

20) H. Matsuo, A. Arimura, R. M. G. Nair, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, **45**, 822 (1971).

21) M. Brenner, "Peptides, Proc. 8th Europ. Peptide Sympos.," North Holland Publ., Amsterdam, 1966, p. 1. E. Wünsch, *Angew. Chem.*, **83**, 773 (1971).

N-t-butyloxycarbonyl-*O*-benzyl-L-tyrosine (18.50 g, 50 mmol), ethyl glycinate (5.15 g, 50 mmol) and 2,2'-dipyridyl disulfide (11.00 g, 50 mmol) in 150 ml of dry CH_2Cl_2 was added triphenylphosphine (13.10 g, 50 mmol) in 50 ml of CH_2Cl_2 with ice-cooling. This was stirred for 6 hr at room temperature. After being left to stand overnight, the solvent was removed *in vacuo*, dissolved in 200 ml of ethyl acetate and washed successively with 10% citric-acid, water, 5% sodium bicarbonate and water. The solvent was evaporated after addition of silica gel (100 g) and the coated residue was applied to a dry column chromatography[22] on silica gel and the peptide was eluted with ether and crystallized from ethylacetate and petroleum ether; yield 20.0 g (87%); mp 123—126 °C; $[\alpha]_D^{20}$ 1.2° (*c* 2, MeOH).

Found: C, 65.91; H, 7.00; N, 6.25. Calcd for $C_{25}H_{32}O_6N_2$: C, 65.95; H, 7.02; N, 6.00.

Boc-Ser(Bzl)-Tyr(Bzl)-Gly-OEt (V). Compound IV (20 g, 40 mmol) was dissolved in 20 ml of CH_2Cl_2 and 50 ml of trifluoroacetic acid and stirred at room temperature for 1 hr. To the evaporated residue, 100 ml of ethyl acetate was added and washed first with saturated sodium bicarbonate, then water and dried over sodium sulfate. After evaporation of the solvent, the oily residue was used as ethyl *O*-benzyl-L-tyrosylglycinate. Dicyclohexylamine salt of *N-t*-butyloxycarbonyl-*O*-benzyl-L-serine (16.60 g, 43 mmol) was suspended in 10% citric acid (200 ml) and the mixture was extracted with ethyl acetate. The ethyl acetate layer was washed with water and dried over magnesium sulfate. The solvent was removed *in vacuo* and the residue was used as *N-t*-butyloxycarbonyl-*O*-benzyl-L-serine. To the stirred mixture of ethyl *O*-benzyl-L-tyrosylglycinate, *N-t*-butyloxycarbonyl-*O*-benzyl-L-serine and 2,2'-dipyridyl disulfide (11 g, 50 mmol) in 100 ml of CH_2Cl_2 was added triphenylphosphine (13.1 g, 50 mmol) in 50 ml of CH_2Cl_2 with ice-cooling and stirred at room temperature for 6 hr. After evaporation of solvent, the residue was dissolved in ethyl acetate and was washed successively with 10% citric acid, water, 5% sodium bicarbonate and water. Worked up as mentioned above, the tripeptide was obtained by dry column chromatography in ether and crystallization from ethyl acetate and petroleum ether; yield 19.00 g (71%); mp 63°C; $[\alpha]_D^{20}$ −9.0° (*c* 1, MeOH).

Found: C, 66.33; H, 6.87; N, 6.61. Calcd for $C_{35}H_{43}O_8N_3$: C, 66.33; H, 6.84; N, 6.63.

Boc-Ser(Bzl)-Tyr(Bzl)-Gly-OH (Fragment V). To a stirred mixture of 14.0 g (22 mmol) of IV in 100 ml of methanol was added 100 ml of water containing 1.0 g of sodium hydroxide. This was stirred at room temperature for 1 hr. The mixture was adjusted to pH 7 with acetic acid and evaporated *in vacuo*. The residue was dissolved in 100 ml of ethyl acetate and washed with 10% citric acid and water and dried over magnesium sulfate. The free acid peptide was obtained by crystallization from ethyl acetate–petroleum ether; yield 8.80 g (65%); mp 129—134°C; $[\alpha]_D^{20}$ −6.2° (*c* 1, MeOH).

Found: C, 65.51; H, 6.51; N, 7.10. Calcd for $C_{33}H_{39}O_8N_3$: C, 65.44; H, 6.49; N, 6.94. R_f 0.78 on silica gel G in *n*-BuOH: AcOH: H_2O (4: 1: 1) and R_f 0.72 on silica gel G in *n*-BuOH: AcOH: H_2O: Pyridine (30: 6: 24 :20). A portion of the peptide was deprotected with HF[12] for 2 hr at room temperature and hydrolysate in 6 M HCl gave the following amino acid ratios: Ser, 0.96; Tyr, 0.98; Gly, 1.

Boc-His(Tos)-Trp-OH (Fragment VI). Dicyclohexylamine salt of N^{α}-*t*-butyloxycarbonyl-N^{im}-tosyl-L-histidine (5.90 g, 10 mmol) and L-tryptophan ethyl ester hydrochloride (2.69 g, 10 mmol) were converted into free forms with citric acid and sodium bicarbonate respectively as in the preparation of IV. To a stirred mixture of N^{α}-*t*-butyloxycarbonyl-N^{im}-tosyl-L-histidine, L-tryptophan ethyl ester and 2,2'-dipyridyl disulfide (2.20 g, 10 mmol) in 30 ml of CH_2Cl_2 was added triphenylphosphine (2.62 g, 10 mmol) in 20 ml of CH_2Cl_2 with ice-cooling and the mixture was stirred at room temperature for 8 hr. The residue, after evaporation of solvent, was dissolved in ethyl acetate and washed successively with 10% citric acid, water, 5% sodium bicarbonate and water and dried over sodium sulfate. The solvent was evaporated and the residue was hydrolyzed with sodium hydroxide in methanol and water as in the preparation of V. After evaporation of methanol, the water layer was adjusted to pH 3 with acetic acid and extracted with *n*-buthanol saturated with water. The *n*-buthanol layer was washed with water saturated with *n*-buthanol and evaporated to dryness *in vacuo*. The dipeptide acid (4.05 g; 68%) was obtained from the residue by adding ether: mp 148—150 °C; $[\alpha]_D^{20}$ 15.2° (*c* 1, DMF).

Found: C, 58.54; H, 5.81; N, 11.80; S, 5.42%. Calcd for $C_{29}H_{33}O_7N_5S$: C, 58.47; H, 5.58; N, 11.75; S, 5.38%. R_f 0.80 on silica gel G in *n*-BuOH: AcOH: H_2O (4: 1: 1) and R_f 0.73 on silica gel G in *n*-BuOH: AcOH: H_2O: Pyridine (30: 6: 24: 20). Amino acid ratios (3M *p*-toluenesulfonic acid in the presence of 3-(2-aminoethyl)indole[11]): His, 1; Trp, 0.85.

⟨Glu-OPcp (VII). To a stirred mixture of L-pyroglutamic acid (1.29 g, 10 mmol), pentachlorophenol 2.66 g (10 mmol) and 2,2'-dipyridyl disulfide (2.20 g, 10 mmol) in 40 ml of DMF was added triphenylphosphine (2.62 g, 10 mmol) in 20 ml of DMF with ice-cooling. The resulting mixture was stirred at room temperature for 5 hr and kept standing overnignt. After evaporation of solvent, the residue was crystallized from ethanol–ether and 2.32 g (62%) of the active ester was obtained: mp 196—198 °C, $[\alpha]_D^{28}$ +20.8° (*c* 2, DMF) lit,[14] mp 196—199 °C, $[\alpha]_D^{26}$ +21° (*c* 2, DMF).

Found: C, 35.26; H, 1.67; N, 3.96; Cl, 47.06%. Calcd for $C_{11}H_6Cl_5O_3N$: C, 35.00; H, 1.60; N, 3.71; Cl, 46.97%.

Boc-His(Tos)-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO$_2$)-Pro-Gly-resin (VIII). Two grams of *t*-butyloxycarbonylglycine-resin (III, 0.25 mmol/g) were added to the vessel for solid phase peptide synthesis and the following steps were used to couple each new amino acid or peptide fragment: (1) washing with CH_2Cl_2 (15 ml×3); (2) prewash with trifluoroacetic acid/CH_2Cl_2 (1: 1 v/v); (3) removal of *t*-butyloxycarbonyl group with 15 ml of trifluoroacetic acid/CH_2Cl_2 (1: 1 v/v) for 30 min; (4) washing with CH_2Cl_2 (15 ml×3); (5) washing with $CHCl_3$ (15 ml×3); (6) neutralization with 15 ml of $Et_3N/CHCl_3$ (1: 10 v/v) for 10 min; (7) washing with $CHCl_3$ (15 ml×3); (8) addition of 3 fold excess (1.5 mmol) each of protected new amino acid or peptide fragment and 2,2'-dipyridyl disulfide in 10 ml of reaction solvent (CH_2Cl_2 or DMF); (9) addition of 3 eq (1.5 mmol) of triphenylphosphine in 5 ml of reaction solvent followed by a reaction period; (10) washing with reaction solvent (CH_2Cl_2 or DMF, 15 ml×3); (11) washing with ethanol (15 ml×3). The following *t*-butyloxycarbonyl amino acids were successively coupled at room temperature for 6 hr; Boc-Pro in CH_2Cl_2 Boc-Arg(NO$_2$)-OH in DMF[10] and Boc-Leu-OH·H_2O in CH_2Cl_2. In the case of Boc-Leu-OH·H_2O, 6 fold excess each of triphenylphosphine and 2,2'-dipyridyl disulfide was used. After the coupling of leucine, the peptide resin was treated with 10 fold excess (5 mmol) each of acetic anhydride and triethylamine in 15 ml of DMF for 2 hr[9] and was washed successively with DMF (15 ml×3) and ethanol (15 ml×3). The coupling of tetrapeptide–resin with Boc-Ser(Bzl)-Tyr-(Bzl)-OH (fragment V) was carried out with a 1.5 fold excess of fragment V and 3 fold excess each of triphenylphosphine and 2,2'-dipyridyl disulfide in CH_2Cl_2 for 24 hr, followed by

22) B. Loev and K. M. Snader, *Chem. Ind.*, **1965**, 15. B. Loev and M. M. Goodman, *ibid.*, **1967**, 2026.

acetylation[9] with 10 fold excess each of acetic anhydride and triethylamine in DMF for 2 hr. The coupling of the resulting heptapeptide–resin with Boc–His(Tos)–Trp–OH (tragment VI) was achieved with 3 fold excess each of fragment VI, 2,2′-dipyridyl disulfide and triphenylphosphine in CH_2Cl_2 for 24 hr. A portion of this resin was treated with HF[12] at room temperature for 2 hr in the presence of 2-mercaptopyridine[13] and anisole and hydrolyzed in 3M *p*-toluenesulfonic acid[11] containing 0.2% of 3-(2-aminoethyl)indole for 24 hr and the hydrolysate gave the following amino acid ratios: His, 0.95; Trp, 0.84; Ser, 0.96; Tyr, 0.95; Gly, 2.12; Leu, 1; Arg, 0.93; Pro, 0.97.

Boc–His(Tos)–Trp–Ser(Bzl)–Tyr(Bzl)–Gly–Leu–Arg(NO_2)–Pro–Gly–NH_2 (IX). The nonapeptide–resin (VIII) was suspended in abs. ethanol and the stirred suspension was bubbled with a stream of ammonia from a refluxing solution of ammonia, which contained sodium as a drying agent, at −15—−30 °C for 8 hr with exclusion of moisture. The mixture was stirred overnight at −15—0 °C and subsequently at room temperature for 8 hr. After the evaporation of solvent, the residue was dried *in vacuo* over P_2O_5 and KOH. The cleaved material was extracted with DMF and methanol and the resin was removed by filtration. The combined filtrate was concentrated and applied to Sephadex LH-20 (2×130 cm) in DMF. The main peak was followed by two very small peaks detected by the absorption at 280 nm. The main peak was further purified with Avicel (Funakoshi Pharmaceutical Co., Tokyo) column (2.5×115 cm) in *n*-BuOH: AcOH: water (4: 1: 1) and followed by the filtration on Sephadex LH-20 in DMF. The fractions were condensed and the protected nonapeptide amide monohydrate was precipitated by adding ethyl acetate and ether. The precipitate was filtered, thoroughly washed with ethyl acetate and ether and dried *in vacuo* (P_2O_5 and KOH) to give an amorphous powder (373 mg, 48% based on Boc–glycine attached to the resin), mp 197—201 °C (decomp.), $[\alpha]_D^{25}$ −27.6° (*c* 1, DMF).

Found: C, 57.91; H, 6.35; N, 15.29; S, 1.81%. Calcd for $C_{76}H_{95}O_{17}N_{17}S \cdot H_2O$: C, 58.18; H, 6.23; N, 15.18; S, 2.04%. R_f 0.66 on silica gel G in *n*-BuOH: AcOH: H_2O (4: 1: 1) and R_f 0.75 on silica gel G in *n*-BuOH: AcOH: H_2O: Pyridine (30: 6: 24: 20). Amino acid analysis (3 M *p*-toluenesulfonic acid after HF treatment): His, 1.01; Trp, 0.82; Ser, 0.94; Tyr, 0.96; Gly, 2.04; Leu, 1; Arg, 0.88; Pro, 0.94; NH_3, 1.38. Amino acid analysis of an aminopeptidase M (Rohm and Hass Darmstadt) digest of the nonapeptide amide which was completely deprotected with HF in the presence of anisole and 2-mercaptopyridine gave: His, 1.06; Trp, 1.03; Ser, 1.02; Tyr, 1.02; Gly, 1.04; Leu, 1. No detection of Arg and Pro by the above amino acid analysis is due to the well-known resistance to enzymatic digestion of peptide bond involving proline.

〈Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2 (LH–RH, X). In the coupling of completely deprotected nonapeptide with pentachlorophenyl L-pyroglutamate, 1.39 molar ratio of pyroglutamyl residue was found to be incorporated into nonapeptide when 1.5 eq of pentachlorophenyl pyroglutamate was used. The reaction conditions were so determined as not to use excess pentachlorophenyl glutamate. The protected nonapeptide (IX) 161 mg (0.123 mmol) was deprotected with 30 ml of dry HF for 1.5 hr at room temperature in the presence of 20 mg of 2-mercaptopyridine an i 1 ml of anisole. After the removal of HF and anisole *in vacuo*, the residue was washed with ether and dissolved in 100 ml of methanol and neutralized to pH 7 with Amberlite IRA-400 in methanol. The solvent was removed *in vacuo* and the residue was dissolved in 20 ml of DMF. To this mixture was added 0.85 eq of pentachlorophenyl L-pyroglutamate (VII) (35.5 mg, 0.942 mmol) and the mixture was stirred at room temperature for 2 hr and allowed to stand overnight. The solvent was evaporated and the peptide was precipitated by adding ethyl acetate and ether. The precipitate was dissolved in methanol and charged on the column of Amberlite IRP-64 (H^+) (1.5×8 cm) which was eluted first with methanol (200 ml) and then with 1% AcOH in methanol (700 ml). The elute of 1% AcOH in methanol was evaporated *in vacuo* and applied to Sephadex G-25 column (2.0×133 cm) in 1 M AcOH. After the evaporation of Ehlrich and Pauly positive fractions of the main peak, the decapeptide was precipitated from methanol–ethyl acetate–ether. The precipitate was dissolved in 150 ml of water and was further purified on CM-Sephadex C-25 (2.5×11 cm) as reported by N. Yanaihara *et al.*:[15] eluted successively with H_2O (150 ml), 0.1 M $AcONH_4$ (500 ml), 0.15 M $AcONH_4$ (500 ml) and 0.2 M $AcONH_4$ (500 ml). The main peak in 0.15 M $AcONH_4$ which was negative to ninhydrin was followed by a small peak in 0.2 M $AcONH_4$ positive to ninhydrin. The fractions containing a single component evaluated by Ehlrich, Pauly and Cl-tolidine reactions on tlc were pooled and evaporated *in vacuo* to 100 ml after acidification with AcOH. Desalting was achieved by lyophilization 3 times and further gel filtration on Sephadex G-25 in 1 M AcOH. The pure decapeptide amide was obtained by precipitation from methanol–ethyl acetate–ether after evaporation of solvent and dried *in vacuo* over P_2O_5 at 40°C for 12 hr: yield 46 mg (38% yield based on nonapeptide (IX)) $[\alpha]_D^{20}$ −54.2° (*c* 1, 1% AcOH) lit, $[\alpha]_D^{20}$ −50.5 ±2° (*c* 1, 1% AcOH)[15a], $[\alpha]_D^{20}$ −52.9° (*c* 1, 1% AcOH)[15b], $[\alpha]_D^{25}$ −48.2° (*c*0.5, H_2O)[15b]. Amino acid ratios (3 M *p*-toluenesulfonic acid[12]): Glu, 1.02; His, 0.98; Trp, 0.84; Ser, 1.01; Tyr, 0.99; Gly, 2.06; Leu, 1; Arg, 0.92; Pro, 0.96; NH_3, 1.35 and amino acid content was calculated to be 76% from the recovery value of leucine. The peptide was characterized as diacetate and trihydrate after further drying *in vacuo* at 80° over P_2O_5 for 5 hr.

Found: C, 52.32; H, 6.37; N, 17.56%. Calcd for $C_{55}H_{75}N_{17}O_{13} \cdot 2CH_3COOH \cdot 3H_2O$: C, 52.24; H, 6.61; N, 17.56%.

The product showed a single spot on various systems of tlc to Pauly, Ehlrich, Sakaguchi and Cl-tolidine reagents, the R_f values being identical with those reported in the literature[15a]: R_f 0.19 (lit, 0.16) on silica gel G and R_f 0.43 (lit, 0.44) on cellulose F in *n*-BuOH: AcOH: H_2O (4: 1: 5); R_f 0.51 (lit, 0.54) on silica gel G and R_f 0.61 (lit, 0.64) on cellulose F in $CHCl_3$: MeOH: 32%AcOH (12: 9: 4); R_f 0.44 (lit, 0.41) on silica gel G in *n*-BuOH: AcOH: Pyridine: H_2O (4: 1: 1: 2); R_f 0.79 (lit, 0.80) on cellulose F in $CHCl_3$: MeOH: 28%-NH_4OH (12: 9: 4).

II. B Type Elongation. Attachment of t-*Butyl* L-*Glutaminate to the Methylchloroformylated Resin.* The methylchloroformylated resin (II) 4 g (Cl: 1.39 mmol/g) was treated with 0.5 eq (2.78 mmol) each of *t*-butyl L-glutaminate and triethyl amine in 40 ml of $CHCl_3$ for 4 hr and successively with 10 eq (55.6 mmol) of diethylamine for 4 hr.[4] The content of glutamine of the resin (XI) was determined by amino acid analyzer to be 0.11 mmol/g.

Z–Tyr–Gly–OBu^t (XII). To a stirred mixture of 0.1 mol each of benzyloxycarbonyl-L-tyrosine (31.50 g), *t*-butyl glycinate (13.1 g) and 2,2′-dipyridyl disulfide (22 g) in 300 ml of DMF was added triphenylphosphine (26.2 g, 0.1 M) in 100 ml of CH_2Cl_2 and the mixture was stirred at room temperature for 9 hr. After working up as in the preparation of IV, crystallization from ethyl acetate–petroleum ether gave the dipeptide: 35.6 g (83%); mp 113—114°; $[\alpha]_D^{28}$ −22.4° (*c* 2, DMF).

Found: C, 64.25; H, 6.63; N, 6.70%. Calcd for $C_{23}H_{28}O_6N_2$: C, 64.47; H, 6.59; N, 6.54%.

Z-Ser(Bu^t)-Tyr-Gly-OBu^t (XIII). Compound XII 34.8 g (72 mmol) in 300 ml of methanol was hydrogenated over 10% Pd/C as a catalyst. The solvent was removed after filtration of the catalyst and dried *in vacuo* over P_2O_5. The residue, benzyloxycarbonyl-*O*-*t*-butyl-L-serine (17.7 g, 60 mmol) and 2,2'-dipyridyl disulfide 13.2 g (60 mmol), were dissolved in 200 ml of CH_2Cl_2. To this stirred mixture was added triphenylphosphine (15.70 g, 60 mmol) in 100 ml of CH_2Cl_2 with ice-cooling and the mixture was stirred at room temperature for 8 hr. After working up as in the preparation of IV, evaporation of solvent gave an amorphous powder: 27.5 g (80%); mp 105—115 °C; $[\alpha]_D^{20}$ −20.5° (*c* 1, MeOH).

Found: C, 60.92; H, 7.15; N, 7.30%. Calcd for $C_{30}H_{41}O_8N_3 \cdot H_2O$: C, 61.10; H, 7.35; N, 7.13%.

Z-Trp-Ser(Bu^t)-Tyr-Gly-OBu^t (XIV). Compound XIII (25.1 g, 44 mmol) was hydrogenated in methanol over 10% Pd/C as in the preparation of XIII. To a stirred mixture of the above *t*-butyl *O*-*t*-butyl-L-seryl-L-tyrosylglycinate, benzyloxycarbonyl-L-tryptophan (13.5 g, 40 mmol) and 2,2'-dipyridyl disulfide (8.80 g, 40 mmol) in 200 ml of DMF was added triphenylphosphine (10.5 g, 40 mmol) in 100 ml of CH_2Cl_2 with ice-cooling and the mixture was stirred at room temperature for 6 hr and kept standing overnight. The solvent was removed *in vacuo* and the residue was dissolved in 200 ml of ethyl acetate. The mixture was washed with 10% citric acid, water, 5% sodium bicarbonate and water and dried over sodium sulfate. The solvent was removed *in vacuo* after the addition of 100 g of silica gel. The coated residue was subjected to dry column chromatography and developed with ether. The coated layer was separated and the peptide was eluted with MeOH/$CHCl_3$ (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 tetrapeptide was obtained by precipitation with the addition of petroleum ether: 23.5 g (78%); mp 138—140°; $[\alpha]_D^{20}$ −14.8° (*c* 1, DMF).

Found: C, 65.13; H, 6.89; N, 9.15%. Calcd for $C_{41}H_{51}O_9N_5$: C, 64.97; H, 6.78; N, 9.24%.

Z-His-Trp-Ser(Bu^t)-Tyr-Gly-OBu^t (XV). Compound XIV (16.65 g, 22 mmol) was hydrogenated over 10% Pd/C as in the preparation of XIII. To a stirred mixture of the above tetrapeptide, benzyloxycarbonyl-L-histidine (5.78 g, 20 mmol) and 2,2'-dipyridyl disulfide (4.40 g, 20 mmol) in 150 ml of DMF was added triphenylphosphine (5.24 g, 20 mmol) in 100 ml of CH_2Cl_2 with ice-cooling and the mixture stirred at room temperature for 8 hr. After being left to stand overnight, the solvent was evaporated *in vacuo* and the residue was dissolved in *n*-buthanol. The mixture was washed successively with 10% citric acid, water and 5% sodium bicarbonate and evaporated *in vacuo*. From the residue, pentapeptide was precipitated from methanol–ethyl acetate–ether and was further filtered with Sephadex LH-20 in DMF. It was crystallized from methanol–ethyl acetate–ether and dried *in vacuo* over P_2O_5: yield 13.90 g (78%); mp 172—175 °C; $[\alpha]_D^{20}$ −9.6° (*c* 1, DMF).

Found: C, 61.57; H, 6.58; N, 12.10%. Calcd for $C_{47}H_{58}O_{10}N_8 \cdot H_2O$: C, 61.83; H, 6.62; N, 12.27%. R_f 0.72 on silica gel G in *n*-BuOH: AcOH: H_2O (4:1:1) and R_f 0.74 on silica gel G in *n*-BuOH: AcOH: H_2O: Pyridine (30:6:24:20). Amino acid ratios (3M *p*-toluenesulfonic acid hydrolysis): His, 1.01; Trp, 0.84; Ser, 0.98; Tyr, 0.97; Gly, 1.

H-His-Trp-Ser(Bu^t)-Tyr-Gly-OBu^t (Fragment XVI). Compound XV (679 ml, 0.66 mmol) was hydrogenated over Pd/C in methanol and the solvent was removed after filtration of the catalyst. The residue was dried *in vacuo* over P_2O_5 and was used as fragment XVI: R_f 0.47 on silica gel G in *n*-BuOH: AcOH: H_2O (4:1:1) and R_f 0.66 on silica gel G in *n*-BuOH: AcOH: H_2O: Pyridine (30:6:24:20).

Z-Pro-Gly-NH$_2$ (XVII). To a stirred mixture of 50 mmol each of benzyloxycarbonyl-L-proline (12.50 g), ethyl glycinate 5.15 g and 2,2'-dipyridyl disulfide (11.00 g) in 150 ml of CH_2Cl_2 was added triphenylphosphine (13.10 g, 50 mmol) in 50 ml of CH_2Cl_2 with ice-cooling and the mixture was stirred at room temperature for 8 hr. After being left to stand overnight and worked up as in the preparation of XII, ethyl benzyloxycarbonyl-L-prolylglycinate was obtained as an oily substance. This product was dissolved in 1000 ml of abs. ethanol to which was added 500 ml of dry ammonia at −30 °C. After stirring was continued at room temperature for 58 hr, the solvent was removed and the dipeptide amide was obtained by crystallization from ethyl acetate: yield 13.80 g (89%); mp 120 °C; $[\alpha]_D^{20}$ −37.1° (*c* 1, MeOH).

Found: C, 58.88; H, 6.23; N, 13.92%. Calcd for $C_{15}H_{19}N_3O_4$: C, 59.02; H, 6.29; N, 13.77%.

Boc-Arg(NO$_2$)-Pro-Gly-NH$_2$ (XVIII). Compound XVII (10.10 g, 33 mmol) was hydrogenated as in the preparation of XIII. To a stirred mixture of L-prolylglycine amide, *t*-butyloxycarbonyl-N^G-nitro-L-arginine (9.60 g, 30 mmol) in 150 ml of DMF was added triphenylphosphine (7.86 g, 30 mmol) in 50 ml of DMF with ice-cooling and the mixture stirred at room temperature for 8 hr. After being left to stand overnight, the solvent was removed and dry column chromatography was developed with ethyl acetate. The coated layer was separated and the tripeptide was eluted with MeOH/$CHCl_3$ (1:2). The solvent was removed and crystallization of the residue from ethyl acetate gave tripeptide: 11.00 g (78%); mp 157 °C; $[\alpha]_D^{20}$ −33.8° (*c* 1, MeOH).

Found: C, 45.62; H, 6.73; N, 23.45%. Calcd for $C_{18}H_{32}O_7N_8$: C, 45.66; H, 6.78; N, 23.68%.

Boc-Leu-Arg(NO$_2$)-Pro-Gly-NH$_2$ (XIX). Compound XVIII (10.40 g, 22 mmol) was dissolved in 30 ml each of trifluoroacetic acid and CH_2Cl_2 and stirred at room temperature for 1 hr. To the evaporated residue was added 500 ml of ether and the precipitate was filtered. it was dried over potassium hydroxide *in vacuo*. To a stirred mixture of the above trifluoroacetic acid salt of tripeptide, triethylamine (2.50 g, 25 mmol), 2,2'-dipyridyl disulfide (8.80 g, 40 mmol) and *t*-butyloxycarbonyl-L-leucine monohydrate (5.00 g, 20 mmol) in 100 ml of DMF was added triphenylphosphine (10.48 g, 40 mmol) in 50 ml of DMF. The mixture was stirred at room temperature for 6 hr and left to stand overnight. After evaporation of the solvent, the residue was dissolved in *n*-buthanol and washed successively with 10% citric acid, water, 5% sodium bicarbonate and water. The solvent was removed and the residue was subjected to dry column chromatography developed with ethyl acetate. From the coated layer the peptide was eluted with MeOH/$CHCl_3$ (1:3) and crystallization from methanol–ethyl acetate–petroleum ether, after evaporation of the solvent, gave the tetrapeptide amide: 11.00 g (94%); mp 168—171 °C (decomp.); $[\alpha]_D^{20}$ −43.2° (*c* 2, DMF).

Found: C, 48.82; H, 7.42; N, 21.18%. Calcd for $C_{24}H_{43}O_8N_9$: C, 49.15; H, 7.34; N, 21.50%. R_f 0.56 on silica gel G in *n*-BuOH: AcOH: H_2O (4:1:1) and R_f 0.70 on silica gel G in *n*-BuOH: AcOH: H_2O: Pyridine (30:6:24:20).
Amino acid ratios: Leu, 1; Arg, 0.96[19]; Pro, 0.99; Gly, 1.03; NH_3, 1.39.

H-Leu-Arg(NO$_2$)-Pro-Gly-NH$_2$ (Fragment XX). Compound XIX (387 mg, 0.66 mmol) was dissolved in 20 ml of trifluoroacetic acid with ice-cooling and stirred at room temperature for 1 hr. Ether was added and the precipitate was collected by filtration. The precipitate was dissolved in methanol and neutralized with IRA-400 in methanol. The evaporated residue was dried over KOH *in vacuo* and used as

fragment XX: R_f 0.23 on silica gel G in *n*-BuOH: AcOH:H_2O (4: 1: 1) and R_f 0.61 on silica gel G in *n*-BuOH: AcOH: H_2O: Pyridine (30: 6: 24: 20).

Resin–Glu(NH_2)–His–Trp–Ser–Tyr–Gly–Leu–Arg(NO_2)–Pro–Gly–NH_2 (XXI). Two grams of resin–L-glutamine *t*-butyl ester (XI, 0.11 mmol/g) was added to the vessel for solid phase peptide synthesis and chain elongation was carried out according to the procedure for the preparation of VIII. The first coupling of the initial resin–glutamine with H–His–Trp–Ser(Bu^t)–Tyr–Gly–OBu^t (fragment XVI) was carried out by adding 3 eq (0.66 mmol) of triphenylphosphine in 5 ml of CH_2Cl_2 to a mixture of the resin and 3 eq (0.66 mmol) each of fragment XVI and 2,2′-dipyridyl disulfide in 10 ml of DMF. The mixture was shaken at room temperature for 10 hr followed by shaking for 24 hr after the further addition of 3 eq (0.66 mmol) each of triphenylphosphine and 2,2′-dipyridyl disulfide. A portion of the resulting resin was hydrolyzed in 3 M *p*-toluenesulfonic acid and the amino acid analysis gave the following ratios: Glu, 1.09; His, 0.98; Trp, 0.84; Ser, 0.96; Tyr, 0.95; Gly, 1; NH_3, 1.42. The recovered fragment XVI in the washing solvent of DMF and CH_2Cl_2 showed the same spot on tlc as the starting fragment XVI detected with ninhydrin, Pauly and Ehrlich reaction. Fragment XVI was isolated by gel filtration on Sephadex LH-20 in DMF: 160 mg (0.35 mmol). The second coupling of the resulting resin–hexapeptide with 3 eq (0.66 mmol) of H–Leu–Arg(NO_2)–Pro–Gly–NH_2 (fragment XX) was carried out by the same procedure as described in the first coupling except that the deprotection of *t*-butyl group (steps (2) and (3) in the preparation of VIII) was carried out with trifluoroacetic acid/CH_2Cl_2 (1: 1 v/v) containing 5% of 2-mercaptopyridine. The amino acid analysis (3 M *p*-toluenesulfonic acid) of an aliquot of the resin–decapeptide gave the following results: Glu, 1.10; His, 1.01; Trp, 0.80; Ser, 0.95; Tyr, 1.03; Gly, 2.05; Leu, 1; Arg, 0.95;[19] Pro, 0.94; NH_3, 2.87. The recovered fragment XX in the washing solvent was also identical on tlc with the starting material. It was established by the following experiment that the recovered fragments were used successfully in the next coupling. The coupling of 1g of a new resin–glutamine *t*-butyl ester (XI, 0.11 mmol/g) with the recovered fragment XVI (0.35 mmol) isolated as above and the recovered fragment XX in washing solvent after the evaporation of solvent gave similar results. The amino acid analysis gave: Glu, 1.07; His, 0.97; Trp, 0.80; Ser, 0.99; Tyr, 0.96; Gly, 2.06; Leu, 1; Arg, 0.94;[19] Pro, 0.98; NH_3, 2.93.

〈Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2 (LH–RH, X). The resin–decapeptide (XXI) 2 g was treated with 30 ml of dry HF[13] for 30 ml at room temperature in the presence of 1 ml of anisole and 300 mg of 2-mercaptopyridine. After the removal of HF and anisole *in vacuo*, 150 ml of DMF was added and the resin was filtered off. The solution was condensed and the peptide was precipitated by adding ethyl acetate and ether. The glutaminyl residue of this decapeptide was cyclized[20] in 40 ml of acetic acid containing 200 mg of 2-mercaptopyridine and 20 ml of anisole at 80—100 °C for 30 min. After evaporation of solvent, the peptide was precipitated from methanol–ether. The peptide was dissolved in 200 ml of water and passed through a Dowex 1×2 (OAc) column to be converted into acetate form. The peptide was also purified on CM-Sephadex as in the preparation of X by A type elongation after the separation of 2-mercaptopyridine by gel filtration on Sephadex G-25; the main peak in 0.15 M $AcONH_4$ was proceeded by a small peak in 0.1 M $AcONH_4$. The fraction containing a single component was pooled and the pure decapeptide amide was obtained after desalting, precipitation and drying *in vacuo* over P_2O_5 at 40°: yield 133 mg (51% based on the glutamine attached to the resin); $[\alpha]_D^{20}$ −53.1° (*c* 1, 1% AcOH). Amino acid analysis (3 M *p*-toluenesulfonic acid hydrolysate: Glu, 1.02; His, 0.99; Trp, 0.81; Ser, 0.96; Tyr, 0.97; Gly, 2.02; Leu, 1; Arg, 0.96; Pro, 0.97; NH_3, 1.32 and amino acid content was calculated to be 78% from the recovery value of leucine. The peptide was homogeneous on various tlc described in the preparation of X by detection with Pauly, Ehrlich, Sakaguchi and Cl-tolidine reagents, the R_f values being identical with those of natural LH–RH and the synthetic LH–RH by A type elongation. The peptide was also characterized as diacetate and trihydrate after further drying *in vacuo* at 80°C over P_2O_5.

Found: C, 52.26; H, 6.64; N, 17.14%. Calcd for $C_{55}H_{75}N_{17}O_{13}\cdot 2CH_3COOH\cdot 3H_2O$: C, 52.24; H, 6.61; N, 17.56%.

Bioassay. LH–RH activities of the decapeptides were compared at 2 dose levels with that of natural LH–RH (AVS 77-33 #215-269, amino acid content 67.5%) in ovariectomized, estrogen-progesterone treated rats. Serum LH levels 15 min after the jugular vein injection of 0.5 and 2.5 ng LH–RH in six rats in each dose level were estimated by radioimmunoassay according to Niswender *et al.*[16] and expressed in terms of NIH-LH-S17. The dose response regression lines for natural and synthetic two decapeptides were parallel. Using four point factorial assay,[18] the LH–RH activities of synthetic LH–RH were calculated as follows: synthetic LH–RH of A type elongation (amino acid content 76%), 121% of the potency of natural LH–RH with 95% fiducial limits of 87—182%; synthetic LH–RH of B type elongation (amino acid content 78%), 125% of the potency of natural LH–RH with 95% fiducial limits of 91—173%. The values were in good agreement with the ratios of the amino acid contents of synthetic LH–RH to that of the natural one.

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