Study of the Synthesis of Histidine Peptides. II. Use of N^{im}-Tosylhistidine Derivatives in Conventional Solution Synthesis of Peptides[1,2]

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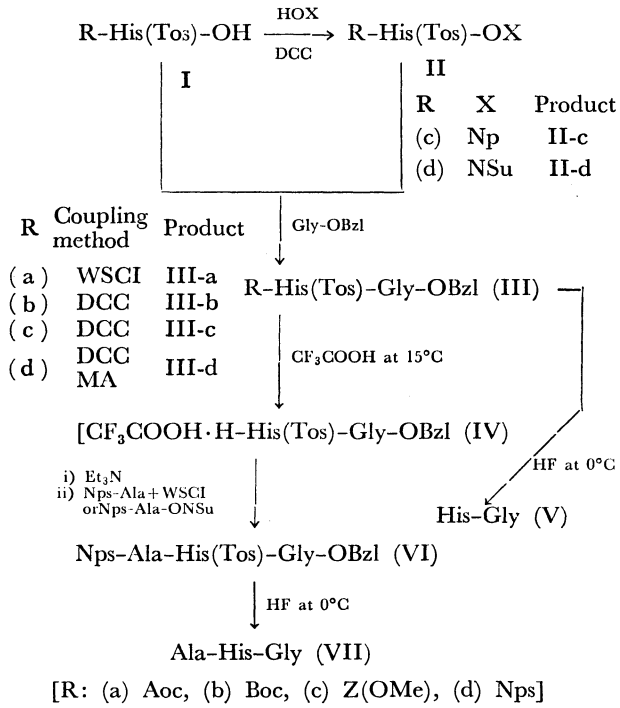
His–Gly and Ala–His–Gly were synthesized by the conventional solution methods using various N^{α}-protected-N^{im}-tosyl-L-histidines as the key intermediates, and handling conditions of these His(Tos)-derivatives were examined. During treatment of an Nps-peptide with HF, the formation of a strongly photosensitive by-product was observed when anisole alone was used as the scavenger, however the side reaction was completely suppressed when skatole was added to the reaction mixture. In order to evaluate the usefulness of these His(Tos)-derivatives in general peptide synthesis, TRH and LH–RH were synthesized, and both products were obtained in pure form without any difficulty. Thus, it can be emphasized that the use of these His(Tos)-derivatives is efficient and convenient for introducing His-residues into peptides even in solution procedures.

Previously, the present authors published the basic ideas on the use of the tosyl group for the protection of histidyl residues in peptide synthesis,[1] and the usefulness of Aoc–His(Tos) was demonstrated in the solid-phase synthesis of IIe^5-Angiotensin II.[3] Since then, this procedure has been tested by many other researchers, and it is widely recognized that the procedure is the most promising one for the incorporation of histidyl residues into peptides by the solid-phase method.[4] In a preceding paper,[5] detailed conditions for the synthesis of various N^{α}-protected His(Tos)-derivatives were presented, and the behavior of the tosyl group was examined under ordinary conditions which are generally used for peptide synthesis. The present study is the evaluation of the N^{im}-protection method in conventional peptide synthesis.



[R: (a) Aoc, (b) Boc, (c) Z(OMe), (d) Nps]

Scheme 1. Peptide Synthesis.

First, the coupling conditions of various N^{α}-protected His(Tos)-derivatives with glycine benzyl ester were examined applying different coupling methods (Scheme 1). From these experiments, it was confirmed that the mixed anhydride method, the dicyclohexylcarbodiimide (DCC) method, and the active ester method gave reasonable results, but the application of the azide method was impractical because of the instability of the tosyl group during the conversion of the hydrazide to the azide followed by the coupling reactions with amine components.

In the case of applying DCC as the coupling reagent, methylene chloride gave the most promising results as the reaction solvent. When tetrahydrofuran (THF), *N,N*-dimethylformamide (DMF), or acetonitrile was used as the reaction solvent under ordinary conditions, the formation of acyl urea was not negligible; particularly the use of an excess amount of base is known to enhance acyl urea formation.

There was no problem in synthesizing the active esters of N^{α}-protected N^{im}-tosylhistidines such as *p*-nitrophenyl ester and *N*-hydroxysuccinimide ester, as far as the DCC method was applied. However, an attempt to synthesize Aoc–His(Tos)–ONSu by the trifluoroacetate method[6] was unsuccessful; in the reaction, the *N*-hydroxysuccinimide ester of *p*-toluenesulfonic acid was formed as the major product.

Aoc- or Z(OMe)–His(Tos)–Gly–OBzl which was obtained by the above-mentioned procedures was treated with trifluoroacetic acid to remove the N^{α}-protecting group, and the product was further coupled with Nps–Ala using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (WSCI)[7] as the reagent or with Nps–Ala–ONSu. Both procedures gave the same product, Nps–Ala–His(Tos)–Gly–OBzl, in reasonable yields.

The removal of the protective groups from His(Tos)-containing peptides was tested under several different conditions, and the most promising results were obtained using the HF-method.[8] As had been expected, a single treatment of Z(OMe)–His(Tos)–Gly–OBzl with HF in the presence of anisole gave His–Gly without any problem. However, a peculiar phenomenon was observed when Nps–His(Tos)–Gly–OBzl or Nps–Ala–His(Tos)–Gly–OBzl was treated with HF using anisole as the scavenger: during processing of the reaction mixture, the formation of a yellow-colored material was observed, which was water-soluble and extremely

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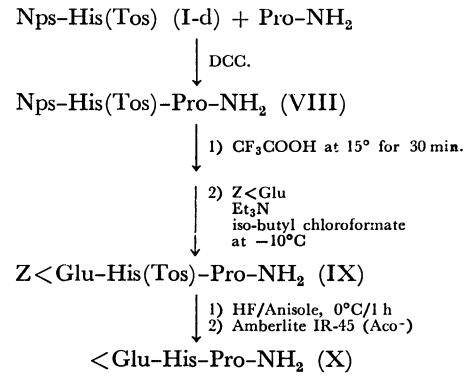
photosensitive. This side product had a solubility similar to free peptide, quickly turned to brownish violet, and finally became black within two or three hours during the purification process of the peptide. Removal of the colored material from the principal product was not impossible, but the purification procedures reduced the yield of the final product considerably. The primarily-formed photo-sensitive material should be an Nps–anisole adduct, and it may have further changed to a complex compound through intermediates such as nitrosophenyl sulfoxide in an ordinary lighted room. The addition of excess skatole together with anisole in the HF-reaction mixture completely suppressed the formation of the black; a yellow compound was also formed in the reaction, but this colored material was soluble in chloroform and not so sensitive to visible light. This material was isolated from the reaction mixture as crystals, and the structure was confirmed to be 2-Nps-skatole. Recovery of His–Gly from Nps–His(Tos)–Gly–OBzl using the HF-reaction using skatole as the scavenger was about 80% efficient. The efficiency of the HF-Skatole procedure was also confirmed by obtaining Ala–His–Gly from Nps–Ala–His(Tos)–Gly–OBzl in a 64% yield after final purification.

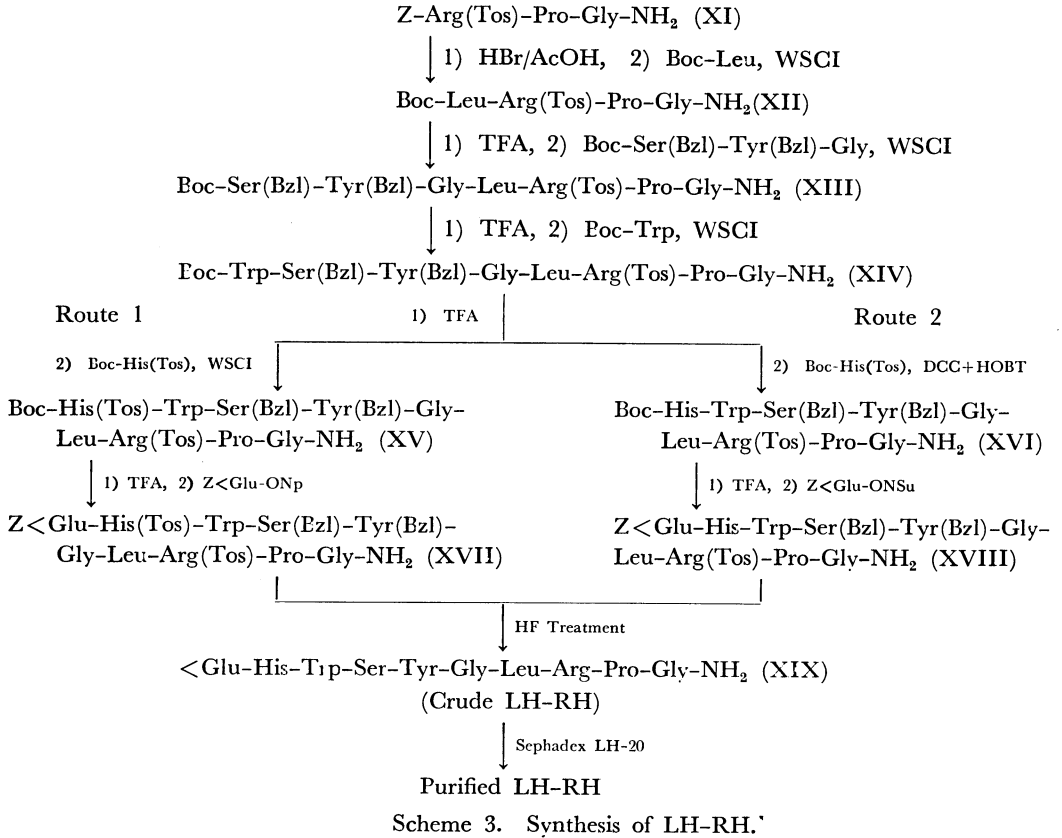
As shown above, the introduction of a tosyl group into the histidyl residue is advantageous not only in reducing the formation of side products during the coupling reactions, but also in enhancing the solubility of the histidine derivatives in organic solvents. On the other hand, the tosyl group is known to be susceptible to HOBT, HBr, HCl, NaOH, NH_3 and even to the amine-components to be coupled.[5] Therefore, partial



Scheme 2. Synthesis of Thyrotropin-releasing Hormone

cleavage of the tosyl groups is frequently observed during coupling reactions. Thus, in such cases, it may be favorable to remove all of the tosyl groups after the incorporation of the His-residues into peptides, because the major purpose of the protection of the histidyl residue will have been fulfilled after it has been incorporated into a peptide without forming considerable side products. These techniques were applied to the synthesis of biologically-active peptides, thyrotropin-releasing hormones (TRH)[9] and luteinizing-hormone releasing hormones (LH-RH),[10] in order to determine its efficiency.

The synthesis of TRH was carried out as shown in Scheme 2. Nps–His(Tos)[5] was coupled with proline amide[11] in a mixture of acetonitrile and DMF using DCC as the reagent. The Nps-dipeptide amide thus obtained was then treated with trifluoroacetic acid to remove the Nps-group, and the N^{α}-deprotected



Scheme 3. Synthesis of LH-RH.

dipeptide amide was coupled with Z<Glu using the mixed anhydride method. Since the N^{im}-tosyl group had been kept stable throughout the synthesis, the final protected tripeptide could be purified simply by means of silica-gel column chromatography. The purified, fully-protected tripeptide was treated with HF in the presence of anisole, the excess HF was removed under reduced pressure, and the residue was treated with a short column of Amberlite IR-45 (AcO^- form) to remove the remaining HF. Then, the final product was obtained by lyophilization as an amorphous powder which proved to be homogeneous without the application of any additional purification procedures.

The synthesis of LH-RH was carried out *via* two different routes as shown in Scheme 3. In route 1, Boc–His(Tos)[5] was coupled with Trp–Ser(Bzl)–Tyr(Bzl)–Gly–Leu–Arg(Tos)–Pro–Gly–NH_2 using WSCI[7] alone as the coupling reagent. The reaction proceeded smoothly, and Boc–His(Tos)–Trp–Ser(Bzl)–Tyr(Bzl)–Gly–Leu–Arg(Tos)–Pro–Gly–NH_2 was obtained as the major product, but partially formed de-tosylated material had to be removed to obtain a single product. Then, the Boc-group was removed by treatment with trifluoroacetic acid, and Z<Glu–ONp was coupled to obtain fully-protected LH-RH. In route 2, the same reaction was carried out in the presence of HOBT analogously to the Geiger procedure.[12] In this case, the tosyl group was completely removed during the coupling reaction,[5] and detosylated nonapeptide was obtained in a high yield. Since no problem was observed in the final coupling reaction with Z<Glu–ONSu,[13] route 2 was considered to be more practical for large-scale synthesis of LH-RH. The final protected decapeptide amide was treated with HF, in the presence of anisole together with tryptophan, followed by purification on Sephadex LH-20, and highly-purified LH-RH was obtained in good yield. The chromatogram of the HF-reaction product indicated the formation of a side product (Fraction II in Fig. 1.). Amino acid analysis of an acid hydrolyzate of the side product indicated a remarkable decrement in the recovery of Tyr. Thus, this material was considered to be modified LH-RH at the Tyr-residue, which should form during the HF-treatment.

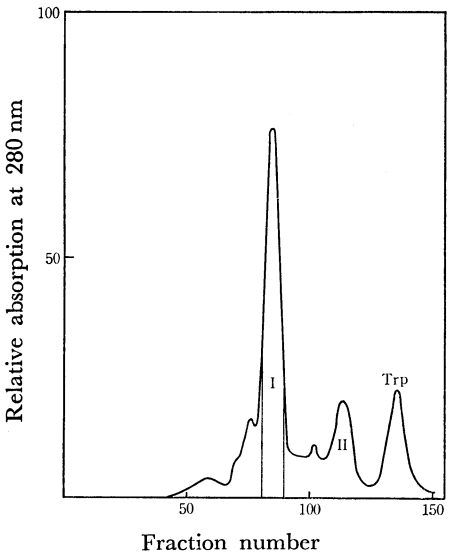


Fig. 1. Purification of crude LH-RH on Sephadex LH-20 column. Column size: 2.8×130 cm (Resin volume, 800 ml). Sample, 0.6 g; Eluent, 1% AcOH; Fraction volume, 6.3 ml; Flow rate, 75 ml/h.

As demonstrated above, the present method for the protection of histidyl residues by the tosyl group has proved to be useful not only in solid-phase synthesis, but also in conventional solution procedures.

Experimental

Melting points were measured by the capillary method and are uncorrected. Paper chromatography was carried out on Toyo filter paper No. 51, and thin layer chromatography on cellulose powder using the following solvent systems: R_f^1; 1-butanol : acetic acid : water (4 : 1 : 1), R_f^2; chloroform : methanol : 30% acetic acid (3 : 2 : 1), R_f^3; 1-butanol : acetic acid : water (4 : 1 : 5, upper phase). All materials obtained were dried over P_2O_5 *in vacuo* for 24 h at room temperature before being applied to the analysis. The IR spectra were recorded on a Shimadzu IR-27G spectrometer, and the UV spectra on a Hitachi EPS-2U spectrometer. The NMR spectra were recorded at 60 MHz with tetramethylsilane as the internal standard using a Hitachi R-20A spectrometer. The HF reactions were carried out using a PRF HF-Reaction Apparatus Type I.

Z(OMe)-His(Tos)-ONp (II-c). DCC (4.4 g, 21 mmol) was stirred into a solution of I-c[5] (9.5 g, 21 mmol) and *p*-nitrophenol (2.8 g, 21 mmol) in THF (100 ml) at −5 °C. The stirring was continued for 1 h at −5—0 °C and for additional 2 h at room temperature. After adding a few drops of glacial acetic acid at 0 °C, the mixture was stirred for 30 min, and then *N,N'*-dicyclohexylurea was removed by filtration; the parent solution was concentrated to a residue under reduced pressure. The residual oil was dissolved in ethyl acetate, the solution was washed successively with 5% aqueous sodium hydrogencarbonate and a saturated sodium chloride solution, and dried over magnesium sulfate. The dried solution was concentrated under reduced pressure, and the residue was recrystallized from methanol to obtain needles; wt. 10.0 g (84.7%), mp 93—5 °C, $[\alpha]_D^{23}$+41° (*c* 1, THF). Found: C, 56.43; H, 4.49; N, 9.48; S, 5.40%. Calcd for $C_{28}H_{26}O_9N_4S$: C, 56.56; H, 4.40; N, 9.42; S, 5.38%.

Nps-His(Tos)-ONSu (II-d). DCC (700 mg, 3.4 mmol) was stirred into a solution of I-d[5] (1.4 g, 3 mmol) and *N*-hydroxysuccinimide (350 mg, 3 mmol) in a mixture of ethyl acetate (50 ml) and THF (50 ml) at −5 °C. The stirring was continued for 1 h at −5—0 °C and for additional 2 h at room temperature, and then a few drops of glacial acetic acid were added to the reaction mixture at 0 °C. After 30 min, *N,N'*-dicyclohexylurea was removed by filtration, and the filtrate was concentrated under reduced pressure. The residue was dissolved in a small amount of ethyl acetate, the solution was charged at the top of a column (0.9×75 cm) of silica gel which had been equilibrated with a mixture of benzene and ethyl acetate (5 : 3). The column was developed using the same solvent mixture and fractions containing the second yellow band were collected and evaporated. The residue was treated with hexane producing amorphous solid; wt 1.5 g (89%).

Reaction of I-a with N-*(Trifluoroacetoxy)succinimide (Formation of* N-*Tosyloxy-succinimide).* *N*-(Trifluoroacetoxy)succinimide[6] (1.2 g, 5 mmol) was stirred into a solution of compound I-a[5] (2.1 g, 5 mmol) in dry pyridine (5 ml), and

the mixture was allowed to react overnight at room temperature. Then, the reaction mixture was poured into ice-water which precipitated colorless plates which were collected by filtration, washed with ice-water and dried. Then, the dried crystals were recrystallized from ethyl acetate; wt. 0.85 g (60.5%), mp 142.5—145 °C. IR (in Nujol) 1745 (C=O), 1595 (p-CH_3-C_6H_4), 1390, 1180 (SO_2-O-) cm^{-1}. NMR ($CDCl_3$, δ) 2.0—2.2 (d, 2H), 2.55—2.75 (d, 2H), 7.2 (s, 4H), 7.5 (s, 3H). Found: C, 49.10; H, 4.00; N, 5.29%. Calcd for $C_{11}H_{11}O_5NS$: C, 49.07; H, 4.12; N, 5.20%. An authentic sample was synthesized as follows: A solution of tosic anhydride[14] (3.2 g, 10 mmol) and *N*-hydroxysuccinimide (2.3 g, 20 mmol) in dry THF (30 ml) was refluxed for 5 h. Then, the solvent was evaporated out under reduced pressure, the residue was dissolved in ethyl acetate, the solution was washed well with water, and dried over anhydrous sodium sulfate. Concentration of the dried solution gave colorless plates; wt. 1.3 g (24%) mp 142—144 °C. This material showed the same IR and NMR spectra as the above obtained material.

Aoc-His(Tos)-Gly-OBzl (III-a). WSCI (190 mg, 1.2 mmol) was stirred into a solution of I-a (423 mg, 1 mmol) and Gly-OBzl·Tos-OH[15] (400 mg, 1.2 mmol) in THF (13 ml) at −5 °C; the mixture was allowed to react for 1 h at −5—0 °C and then for additional 15 h at room temperature. The solvent was exchanged with ethyl acetate, the solution was washed with 0.5 M sulfuric acid, 5% aqueous sodium hydrogencarbonate and water, and dried over anhydrous magnesium sulfate. Evaporation of the solvent under reduced pressure gave an oil; wt. 540 mg.

Boc-His(Tos)-Gly-OBzl (III-b). DCC (2.1 g, 10 mmol) was stirred into a mixture of compound I-b***,[5] (4.1 g, 10 mmol), Gly-OBzl·Tos-OH[15] (4.0 g, 12 mmol) and triethylamine (1.7 ml), in methylene chloride (40 ml) at −5—0 °C; the stirring was continued for 1 h at the same temperature and then for additional 15 h at room temperature. The *N,N'*-dicyclohexylurea formed was removed by filtration, and washed well with chloroform (100 ml); the solution and the washing were combined, washed successively with 0.5 M sulfuric acid, 5% aqueous sodium hydrogencarbonate and water, and dried over anhydrous magnesium sulfate. The dried solution was concentrated to dryness under reduced pressure, and the residue was washed with a mixture of ether and hexane, and dried. Recrystallization from ethyl acetate-hexane gave colorless needles; wt. 4.4 g (77.7%), mp 51—54 °C, $[\alpha]_D^{22}$ −1.1° (*c* 2, DMF); Found: C, 58.39; H, 6.27; N, 9.60%. Calcd for $C_{27}H_{32}O_7N_4S$: C, 58.26; H, 5.80; N, 10.07%.

Z(OMe)-His(Tos)-Gly-OBzl (III-c). *The DCC Method*: Compound I-c[5] (1.4 g, 3 mmol) and Gly-OBzl·Tos-OH[15] (1.2 g, 3.6 mmol) were coupled in chloroform (30 ml) at −5—0 °C in the presence of triethylamine (0.5 ml) using DCC (680 mg, 3.6 mmol) as described above. The reaction mixture was treated in the same manner, and the final product was recrystallized from methanol-ethyl acetate producing needles; wt. 1.5 g (80%), mp 166—167 °C, $[\alpha]_D^{22}$ +4.6° (*c* 2, DMF); Found: C, 60.13; H, 5.08; N, 9.12%. Calcd for $C_{31}H_{32}O_8N_2S$: C, 59.99; H, 5.20; N, 9.12%.

The Active Ester Method: A mixture of II-c (1.2 g, 2.1 mmol), Gly-OBzl·Tos-OH[15] (1.0 g, 3 mmol) and triethylamine (0.42 ml) in DMF (10 ml) was allowed to react for 15 h at room temperature. Then, it was diluted with chloroform (50 ml), and the solution was washed and treated as in the case of the synthesis of III-b; wt. 950 mg (76.6%), mp 166—167 °C, $[\alpha]_D^{22}$ +4.3° (*c* 1, DMF). The melting point of this compound showed no depression upon mixing with the product synthesized by the DCC method.

Nps-His(Tos)-Gly-OBzl (III-d). *The Mixed Anhydride Method*: A solution of isobutyl chloroformate (137 mg, 1 mmol) in toluene (1 ml) was stirred for 10 min into a mixture of compound I-d[5] (462 mg, 1 mmol) and triethylamine (0.14 ml) in chloroform (2 ml) and toluene (1 ml) at −10 °C. After stirring for additional 10 min, the solution was mixed slowly with a mixture of Gly-OBzl·Tos-OH[15] (350 mg, 1.05 mmol) and triethylamine (0.15 ml) in chloroform (2 ml) at −10——5 °C. The stirring was continued for 2 h at room temperature; the reaction mixture was treated similarly to the case of the synthesis of III-b. Recrystallization of the final product from ethyl acetate gave yellow needles; wt. 470 mg (77.2%), mp 162—163 °C, $[\alpha]_D^{18}$ +56.7° (*c* 1, DMF). Found: C, 55.16; H, 4.19; N, 11.24; S, 10.44%. Calcd for $C_{28}H_{27}O_7N_7S_2$: C, 55.16; H, 4.46; N, 11.48; S, 10.51%.

The DCC Method: Compound I-d[5] (925 mg, 2 mmol) was subjected to a DCC reaction with Gly-OBzl·Tos-OH[15] (700 mg, 2.1 mmol) in methylene chloride as described above for the synthesis of III-b, and the same compound was obtained; wt. 850 mg (69.9%), mp 164—165 °C, $[\alpha]_D^{18}$ +57.5° (*c* 1, DMF). The melting point of this compound showed no depression upon mixing with the compound obtained by the MA method.

The Active Ester Method: Compound II-d (1.2 g, 2.1 mmol) was coupled with Gly-OBzl·Tos-OH (700 mg, 2.1 mmol) in DMF as in the case of the synthesis of II-c; wt. 910 mg (74.5%), mp 164—165 °C, $[\alpha]_D^{18}$ +57.7° (*c* 1, DMF). The melting point of this compound showed no depression upon mixing with the compound synthesized by the MA method.

Nps-Ala-His(Tos)-Gly-OBzl (VI). *The WSCI Method*: Compound III-a (450 mg, 0.8 mmol) was dissolved in trifluoroacetic acid (1 ml) at 15 °C. After 30 min, excess trifluoroacetic acid was evaporated out under reduced pressure, the residue was treated with dry ether (50 ml) to obtain an amorphous solid. After drying over sodium hydroxide *in vacuo*, this material was dissolved in methylene chloride (10 ml) together with Nps-Ala (160 mg, 1.1 mmol), and WSCI (100 mg, 0.7 mmol) was stirred into the solution at −5—0 °C for 30 min. The stirring was continued overnight at room temperature, then the solvent was removed by evaporation under reduced pressure, and the residue was dissolved in ethyl acetate. The solution was washed successively with 0.5 M sulfuric acid, aqueous 5% sodium hydrogencarbonate and water, and dried over anhydrous magnesium sulfate. The dried solution was concentrated under reduced pressure, the residue was triturated with hexane, and then recrystallized from ethyl acetate and hexane to obtain yellow needles; wt. 338 mg (62%), mp 88—89 °C, $[\alpha]_D^{17}$ −30.0° (*c* 1, AcOEt). Found: C, 54.34; H, 4.53; N, 12.35; S, 9.43%. Calcd for $C_{31}H_{32}O_8N_6S_2$: C, 54.69; H, 4.73; N, 12.34; S, 9.42%.

The Active Ester Method: Compound III-c (609 mg, 1 mmol) was treated with trifluoroacetic acid (1.2 ml) in the presence of anisole (0.4 ml) at 15 °C for 30 min. Excess trifluoroacetic acid was evaporated out under reduced pressure, the residue was treated with dry ether (50 ml) resulting in its solidification, and the solid (IV) was further washed with dry ether. The solid was dissolved in DMF (2 ml) together with Nps-Ala-ONSu (680 mg, 2 mmol)[16] and *N,N*-diethylglycine ethyl ester[17] (1 g), and the mixture was allowed to react overnight at room temperature. The reaction mixture was treated as described above, and the final product was recrystallized from ethyl acetate and hexane;

*** The melting point presented in the Table 1 in Ref. 5 should read 123—125 °C.

wt. 538 mg (79%), mp 87—88 °C, $[\alpha]_D^{18}$ −30.5° (*c* 1, AcOEt). The melting point of this compound showed no depression upon mixing with the compound synthesized by the WSCI method.

His–Gly (V). *A) The HF Treatment of III-c with Anisole*: Compound III-c (1.24 g, 2 mmol) was mixed well in an HF reaction vessel together with anisole (2.8 ml), and anhydrous HF (5 ml) was added to the vessel at −70 °C using an HF-reaction apparatus. The mixture was allowed to react at 0 °C for 1 h, and then excess HF was removed under reduced pressure. After remaining under vacuum for 3 more hours at 0 °C, the residue was extracted with water (10 ml), and the extract was washed well with ether (10 ml). The aqueous layer was treated with a small amount of active charcoal, and then the solution was passed through a column of Amberlite IR-45 (OH^- form) (10 ml), which was washed with water and with aqueous 5% acetic acid. The eluate and washing were combined and lyophilized, and the residue was crystallized from aqueous ethanol to obtain colorless needles; wt. 355 mg (84%), mp 190 °C (decomp.), $[\alpha]_D^{17}$ +24.7° (*c* 1, water). Reported:[18] mp 190 °C (decomp.), $[\alpha]_D$ +25±1° (*c* 2, water). The material showed a single spot of both ninhydrin and Pauly positive on paper chromatography (R_f^1, 0.115), and on paper electrophoresis using a pyridine-acetate buffer (pH 4.8) as the solvent. Found: C, 43.61; H, 5.76; N, 25.4%. Calcd for $C_8H_{12}O_3N_4 \cdot {}^1/_2H_2O$: C, 43.43; H, 5.92; N, 25.32%.

B) The HF Treatment of III-d with Anisole: Compound III-d (1.22 g, 2 mmol) was treated in the same manner as described above except that the charcoal treatment was repeated until colorless material was obtained. Yield; 250 mg (56.6%), mp 190 °C (decomp.), $[\alpha]_D^{17}$ +24.5° (*c* 1, water), R_f^1 0.115.

C) The HF Treatment of III-d with Skatole: Compound III-d (1.22 g, 2 mmol) was treated with anhydrous HF (5 ml) in the presence of anisole (2 ml) together with skatole (600 mg, 4 mmol), as in the case of the synthesis of V, at 0 °C for 1 h. After removal of the excess HF under reduced pressure, the residue was dissolved in a mixture of water and chloroform. The aqueous phase was washed with ether, and then passed through a column of Amberlite IR-45 (OH^- form) (10 ml). The column was eluted with water and then washed with aqueous 5% acetic acid; the eluate and washing were combined and lyophilized. The residue was washed with methanol, and recrystallized from aqueous ethanol to obtain colorless needles; wt. 340 mg (80.5%), mp 189 °C (decomp.), $[\alpha]_D^{17}$ +24.3° (*c* 1, water), R_f^1 0.115.

Separation of the Side Product, 2-Nps-skatole: The chloroform layer, which was separated from the water-chloroform extract of the HF-reaction mixture, was washed with water and dried over anhydrous magnesium sulfate. The dried solution was concentrated to dryness, the residue was dissolved in a small amount of ethyl acetate, and the solution was charged at the top of a silica gel column (0.7×70 cm) which had been equilibrated with a solvent system of ethyl acetate and benzene (1 : 1). The column was developed with the same solvent mixture, the fractions containing the main peak were collected and evaporated. The residue was recrystallized from aqueous methanol; wt. 380 mg (67%), mp 139—141 °C, UV λ_{max}^{EtOH} 224 nm (ε 55200), 283 (ε 21400), 261 nm (ε 5600): NMR ($CDCl_3$, δ) 2.20—2.60 (s, 3H), 6.57—6.82 (m, 1H), 7.00—7.50 (m, 5H), 7.53—7.75 (m, 1H), 7.95—8.32 (m, 2H). Found: C, 63.60; H, 4.16; N, 9.60; S, 10.94%. Calcd for $C_{15}H_{12}O_2N_2S$: C, 63.38; H, 4.26; N, 9.89; S, 11.25%.

Ala–His–Gly (VII). Compound VI (1.36 g, 2 mmol) was mixed well with anisole (2 ml) and skatole (600 mg, 4 mmol) in an HF reaction vessel, and the mixture was treated with HF (7 ml) as above at 0 °C for 1 h. After removal of the excess HF, the residue was treated in the same manner as in the case of the synthesis of V (Procedure C). Compound VII thus obtained was recrystallized from aqueous ethanol producing colorless needles; wt. 360 mg (63.4%), mp 230 °C (decomp.), $[\alpha]_D^{18}$ +36.3° (*c* 1, AcOH). The homogeneity of this material was confirmed by ninhydrin and Pauly reagent on paper electrophoresis and paper chromatography; R_f^1 0.23. Amino acid ratios in an acid hydrolyzate: Ala 1.00, His 1.00, Gly 1.00. Ratio in an APM digest: Ala 1.00, His 1.00, Gly 0.98. Found: C, 46.87; H, 6.00; N, 24.70%. Calcd for $C_{11}H_{17}O_5N_4$: C, 46.63; H, 6.05; N, 24.72%.

Nps-His(Tos)–Pro–NH_2 (VIII). DCC (1.1 g, 5.3 mmol) was stirred into a solution of I-d[5] (2.3 g, 5 mmol) and Pro–NH_2[10] (0.57 g, 5 mmol) in a mixture of acetonitrile (50 ml) and DMF (20 ml) at −5 °C. Stirring was continued for 1 h at −5—0 °C and for additional 15 h at room temperature. The *N,N'*-dicyclohexylurea formed was removed by filtration, the filtrate was evaporated under reduced pressure, and the residue was redissolved in ethyl acetate. The ethyl acetate solution was washed successively with 0.5 M sulfuric acid, aqueous 5% sodium hydrogencarbonate, and water, and dried over anhydrous magnesium sulfate. The dried solution was concentrated to dryness, and the residue was washed with a mixture of methanol and ether, and dried. Recrystallization of the dried residue from ethyl acetate gave yellow needles; wt. 2.0 g (72%), mp 188 °C, $[\alpha]_D^{20}$ +15.1° (*c* 0.5, DMF). Found: C, 51.78; H, 4.62; N, 14.85; S, 11.07%. Calcd for $C_{24}H_{26}O_6N_6S_2$: C, 51.59; H, 4.69; N, 15.04; S, 11.47%.

Z<Glu–His(Tos)–Prc–NH_2 (IX). Compound VIII (0.79 g, 1.5 mmol) was dissolved in a mixture of trifluoroacetic acid (1.5 ml) and methylene chloride (1.5 ml) together with anisole (2 ml) at 15 °C. After 30 min, the methylene chloride and excess trifluoroacetic acid were removed by evaporation under reduced pressure, and the residue was treated with dry ether (50 ml) to give an amorphous powder. To a solution of Z<Glu (0.4 g, 1.5 mmol) and triethylamine (0.21 ml) in THF (4 ml), a solution of isobutyl chloroformate (0.21 g, 1.5 mmol) in toluene (2 ml) was added drop by drop at −10 °C for 10 min. After additional 10 min, the reaction mixture was stirred at once into an aqueous 5% sodium hydrogencarbonate solution of the powder obtained above at −5—0 °C; the pH of the solution was confirmed to be 7. After stirring for 1 h at room temperature, the solution was evaporated under reduced pressure, and the residue was extracted with chloroform (20 ml). The chloroform extract was washed successively with 0.5 M sulfuric acid, aqueous 5% sodium hydrogencarbonate, and dried over anhydrous magnesium sulfate. The solvent was evaporated to dryness, and the residue (0.63 g) was charged at the top of a column of silica gel (0.8×50 cm) which had been equilibrated with a solvent system of chloroform, ethyl acetate and ethanol (5 : 4 : 2). The column was developed with the same solvent mixture, and fractions containing the main band were combined and evaporated to dryness. The residue was washed well with dry ether to obtain an amorphous powder; wt. 450 mg (52.3%), mp 113—116 °C (decomp.), $[\alpha]_D^{15}$ −29.1° (*c* 1, DMF). Found: C, 57.09; H, 5.17; N, 12.86%. Calcd for $C_{31}H_{34}O_8N_6S$: C, 57.22; H, 5.27; N, 12.92%.

<Glu–His–Pro–NH_2 (X) (TRH). Compound IX (65 mg, 0.1 mmol) was treated with anhydrous HF (1 ml) in the presence of anisole (0.15 ml) at 0 °C for 1 h, as in the case of the synthesis of V, and then the excess HF was removed

under reduced pressure. After being kept under vacuum for 4 h at 0 °C, the residue was dissolved in water, the solution was washed with ether, and the aqueous layer was passed through a column (0.5×10 cm) of Amberlite IR-45 (AcO^- form). The column was eluted with water and washed with aqueous 5% acetic acid. The eluate and washing were combined and lyophilized to obtain a residual powder; wt. 37.6 mg (96.5%), $[\alpha]_D^{25}$ −44.8° (*c* 1, 95% AcOH). Reported:[19] $[\alpha]_D^{15}$ −45.1° (*c* 1, AcOH). The homogeneity of this material was confirmed by paper electrophoresis using a 0.1 M pyridine-acetic acid buffer (pH 4.7) and by paper chromatography, which were located using the Pauly reagent; R_f^2 0.55. The amino acid ratio in an acid hydrolyzate: Glu 1.00, His 1.06, Pro 0.94. Found: C, 49.40; H, 6.34; N, 21.54%. Calcd for $C_{16}H_{22}O_4N_6\cdot{}^3/_2H_2O$: C, 49.34; H, 6.47; N, 21.58%.

Z-Arg(Tos)-Pro-Gly-NH$_2$ (XI). A solution of Z-Arg(Tos)-Pro-Gly-OEt[20] (6.5 g, 0.01 mol) in methanol (150 ml) was saturated with ammonia gas at 0 °C. After being kept for 2 days at room temperature, the solution was concentrated to dryness and was triturated with ethyl acetate to obtain an amorphous powder: wt. 5.3 g (82%) mp 145.5—147 °C; $[\alpha]_D^{23}$ −20.2° (*c* 1, DMF). Found: C, 54.52; H, 6.13; N, 15.69; S, 5.13%. Calcd for $C_{28}H_{37}O_7N_7S$: C, 54.60; H, 6.05; N, 15.92; S, 5.20%.

Boc-Leu-Arg(Tos)-Pro-Gly-NH$_2$ (XII). Compound XI (3.7 g, 6 mmol) was dissolved in a 25% HBr solution in acetic acid (17 ml), and the solution was allowed to react for 50 min at room temperature. Then, the product was precipitated by adding a large volume of dry ether. The supernatant was removed by decantation, the precipitates were washed several times with dry ether, and dried over sodium hydroxide *in vacuo*. The dried material was dissolved in DMF (10 ml), the pH of the solution was adjusted to 7 with *N*-ethylmorpholine, and Boc-Leu-ONSu[21] (2.4 g, 7.2 mmol) was added to the solution. After being kept for 2 days at room temperature, the solution was diluted with excess chloroform. The chloroform solution was washed with 1 M hydrochloric acid, 10% sodium carbonate, and a saturated solution of sodium chloride, successively, and dried over anhydrous magnesium sulfate. The dried solution was concentrated under reduced pressure to a residue, which was crystallized from ethanol-hexane: wt. 3.1 g (75%), mp 152—153 °C, $[\alpha]_D^{27}$ −33° (*c* 1, DMF). Found: C, 52.81; H, 7.33; N, 15.78%. Calcd for $C_{31}H_{50}O_8N_8S\cdot{}^1/_2H_2O$: C, 52.89; H, 7.31; N, 15.92%.

Boc-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-NH$_2$ (XIII). Compound XII (3.1 g, 4.4 mmol) was dissolved in trifluoroacetic acid (10 ml) at 0 °C, and the solution was allowed to react for 1 h at room temperatdre. The solution was concentrated to a residue under reduced pressure; and the residue was dried over sodium hydroxide *in vacuo*. The dried material was dissolved in 30 ml of dried THF, and Boc-Ser(Bzl)-Tyr(Bzl)-Gly[22] (2.4 g, 4 mmol) was added to the solution together with HOBT (0.6 g, 4.4 mmol). Then, WSCI (0.8 ml, 4.4 mmol) was added to the mixture at 0 °C. The whole mixture was allowed to react overnight, and then, the solvent was evaporated out under reduced pressure. The residue was dissolved in chloroform (100 ml), the solution was washed well with water and dried for a short time over anhydrous magnesium sulfate. The dried solution was concentrated to a residue, which was purified by reprecipitation from chloroform-ethyl acetate; 4.6 g (97%), mp 118.5—123 °C, $[\alpha]_D^{20}$ −29.5° (*c* 1, DMF). Found: C, 59.35; H, 6.97; N, 12.22%. Calcd for $C_{60}H_{82}O_{13}N_{11}S\cdot 2H_2O$; C, 59.29; H, 6.97; N, 12.67%.

Boc-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-NH$_2$ (XIV). Compound XIII (3.8 g, 3.1 mmol) was dissolved in trifluoroacetic acid (12 ml) at 0 °C, and the mixture was allowed to react for 30 min at room temperature. Then, it was concentrated to a residue, which was washed with ether and dried over sodium hydroxide *in vacuo*. The dried material was dissolved in DMF (5 ml) together with HOBT (0.2 g), the pH of the solution was adjusted to 7 by adding triethylamine, and then, Boc-Trp-ONSu[21] (1.9 g, 4.7 mmol) was stirred into the solution. The whole mixture was allowed to react for 3 days, and then the product was precipitated out as a gelatinous powder by adding ethyl acetate. The product was collected by filtration, reprecipitated twice from DMF-ethyl acetate, washed with ether, and dried; wt. 3.9 g (90%), mp 142—149 °C (decomp.), $[\alpha]_D^{29}$ −27.4° (*c* 1, DMF). Found: C, 59.67; H, 6.60; N, 12.71%. Calcd for $C_{70}H_{89}O_{14}N_{13}S\cdot 2H_2O$: C, 59.85; H, 6.67; N, 12.96%.

Boc-His(Tos)-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-NH$_2$ (XV). Compound XIV (2.8 g, 2 mmol) was mixed well with 0.5 ml each of anisole and mercaptoethanol, and 10 ml of trifluoroacetic acid was added to the mixture at 0 °C; then, the solution was allowed to react for 30 min at room temperature. The solution was concentrated to a residue, which was triturated with dry ether and dried over sodium hydroxide *in vacuo*. The dried material was dissolved in a mixture of DMF and THF (10 ml each) together with I-b[5] (1.6 g, 4 mmol), and 0.62 ml of WSCI (4 mmol) was stirred into the mixture at 0 °C. Then, the mixture was allowed to react overnight at room temperature. THF was removed by distillation under reduced pressure, and the product was precipitated by the addition of water, which was collected by filtration and purified by reprecipitation from DMF-ethyl acetate; wt. 2.7 g (80%), mp 156—158.5 °C (decomp.), $[\alpha]_D^{20}$ −36.8° (*c* 1, DMF). Found: C, 58.40; H, 6.11; N, 13.00%. Calcd for $C_{83}H_{103}O_{17}N_{16}S_2\cdot 2H_2O$: C, 58.74; H, 6.35; N, 13.2%.

Z<Glu-His(Tos)-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-NH$_2$ (XVII). Trifluoroacetic acid (3 ml) was stirred into a mixture of compound XV (252 mg, 0.15 mmol) and mercaptoethanol (0.05 ml) at 0 °C, and then the whole mixture was allowed to react for 40 min at room temperature. Excess trifluoroacetic acid was removed by distillation under reduced pressure, and the residue was treated with ether to precipitate the product as a powder, which was collected by filtration, washed with ether and dried over sodium hydroxide *in vacuo*. The dried material was dissolved in 1 ml of DMF, the pH of the solution was adjusted to 7 with *N*-methylmorpholine, and Z<Glu-ONp[23] (86 mg, 0.22 mmol) was stirred into the solution; the mixture was allowed to react for 2 days at room temperature. The reaction mixture was diluted with chloroform, the solution was washed successively with 1 M hydrochloric acid, water, 1 M sodium carbonate, and water, and dried over anhydrous magnesium sulfate. The dried solution was concentrated to a residue, which was purified by passage through a column of silica gel using a mixture of chloroform, methanol and acetic acid (95 : 5 : 3) as the solvent. Fractions containing the main band were collected and concentrated to a residue, which was purified by reprecipitation from chloroform-ether; wt. 150 mg (54.3%), mp 133.5—138.5 °C (decomp.), $[\alpha]_D^{20}$ −40.5° (*c* 1, DMF). Found: C, 59.39; H, 5.89; 13.41%. Calcd for $C_{91}H_{106}O_{19}N_{17}S_2\cdot 3H_2O$: C, 59.33; H, 6.02; N, 12.93%.

Boc-His-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-NH$_2$ (XVI). Compound (XIV) (2.8 g, 2 mmol) was treated with trifluoroacetic acid as in the case of the synthesis of XV. The des-Boc-product was dissolved in

DMF (10 ml); HOBT (0.544 g, 4 mmol), Boc-His(Tos) (1.6 g, 4 mmol) and WSCI (0.62 ml, 4 mmol) were stirred successively into the DMF solution at 0 °C. After being allowed to react overnight at room temperature, the mixture was diluted with water to precipitate the product, which was collected by filtration and dried. Finally, the dried product was purified by reprecipitation twice from DMF–ethyl acetate; wt. 2.7 g (90%), mp 143.5—147.5 °C (decomp.), $[\alpha]_D^{25}$ −26.9° (*c* 1, DMF). Found: C, 58.95; H, 6.76; N, 14.23%. Calcd for $C_{76}H_{96}O_{15}N_{16}S \cdot 2H_2O$: C, 59.21; H, 6.54; N, 14.54%.

Z < Glu–His–Trp–Ser(Bzl) – Tyr(Bzl) – Gly–Leu–Arg(Tos)–Pro–Gly–NH_2 (XVIII). Compound XVI (2.3 g, 1.5 mmol) was treated with trifluoroacetic acid to remove the Boc-group as shown in the case of the synthesis of XVII. The des-Boc-compound thus obtained was dissolved in DMF (4 ml), together with HOBT (0.3 g, 2.2 mmol), the pH of the solution being adjusted to 7 by the addition of *N*-ethylmorpholine at 0 °C, and then Z<Glu–ONSu[12] (0.81 g, 2.25 mmol) was added. The whole mixture was allowed to react for 3 days at room temperature, and then excess water was added to precipitate the product, which was collected by filtration and dried. The dried material was reprecipitated twice from DMF–ethyl acetate for purification: wt. 2.2 g (88%), mp 132—138.5 °C (decomp.), $[\alpha]_D^{25}$ −25.4° (*c* 1, DMF). Found: C, 60.19; H, 6.18; N, 14.0%. Calcd for $C_{84}H_{99}O_{17}N_{17}S \cdot H_2O$: C, 60.45; H, 6.10; N, 14.27%.

<Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2 (LH–RH). (a): Compound XVIII (1 g, 0.58 mmol) was mixed well with tryptophan (0.1 g), phenol (0.5 g) and anisole (1 ml) in an HF reaction vessel; then, the mixture was treated with HF (10 ml) for one h at 0 °C as in the case of the synthesis of V. After removel of the excess HF under reduced pressure, the residue was extracted with 0.1 M acetic acid, the extract was washed with ether, and then passed through a Dowex 1×2 column (AcO^- form, 3×10 cm). The column was washed well with 0.1 M acetic acid, and the effluent and washing were combined and lyophilized to obtain 0.6 g of the crude product. The crude material was charged at the top of a column of Sephadex LH-20 (2.8×130 cm) which had previously been equilibrated with 1% acetic acid, and the column was eluted with the same solvent. Fractions containing the major product (Peak I in Fig. 1) were collected and lyophilized; the residue was further fractionated on the same type of column under the same conditions to obtain a homogenous product; wt. 220 mg, $[\alpha]_D^{25}$ −49.4° (*c* 0.4, H_2O), −51.0° (*c* 0.3, 1% AcOH). Reported: $[\alpha]_D^{28}$ −50.5° (*c* 1, 1%AcOH).[24] The homogeneity of this material was confirmed by paper electrophoresis using a 0.1 M pyridine-acetic acid buffer (pH 4.7) and by thin layer chromatography on cellulose powder, which were located using the Pauly reagent; R_f[3] 0.41. Found: C, 51.37; H, 6.58; N, 17.59%. Calcd for $C_{55}H_{75}O_{13}N_{17} \cdot 2C_2H_4O_2 \cdot 4H_2O$: C, 51.55; H, 6.67; N, 17.33%. The amino acid ratio in an acid hydrolyzate: His 1.00, Arg 0.96, Ser 1.00, Glu 1.04, Pro 1.01, Gly 1.98, Leu 0.96, Tyr 1.00, NH_3 1.06, Trp 0.97. The average recovery rate of the amino acids was 95%; Trp was determined by spectrophotometry. The amino acid ratio in an acid hydrolyzate of Fraction II: His 0.93, Arg 0.96, Ser 0.94, Glu 1.03, Pro 1.01, Gly 1.88, Leu 1.00, Tyr 0.28. *(b)*: Compound XVII (1 g, 0.53 mmol) was treated with HF in the same manner as described above. No major difference was observed at each step of the production of the final product; wt. 230 mg, $[\alpha]_D^{22}$ −48.7° (*c* 0.4, H_2O). Paper electrophoresis and thin layer chromatography showed this compound to be homogeneous and identical with the compound obtained above. Found: C, 51.74; H, 6.65; N, 17.67%. The amino acid ratio in an acid hydrolyzate: His 0.91, Arg 0.98, Ser 0.99, Glu 1.03, Pro 1.01, Gly 2.00, Leu 0.95, Tyr 1.00, NH_3 1.15, Trp 0.94. The average recovery rate of the amino acids was 94%.

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2) The abbreviations used in this report are those recommended by the IUPAC-IUB: *J. Biol. Chem.*, **247**, 977 (1972); Aoc, *t*-amyloxycarbonyl; DCHA, dicyclohexylamine; HOBT, 1-hydroxybenzotriazole; APM, Amino Peptidase M.

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