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The Use of N-Hydroxy-5-norbornene-2,3-dicarboximide Active Esters in Peptide Synthesis[1)]

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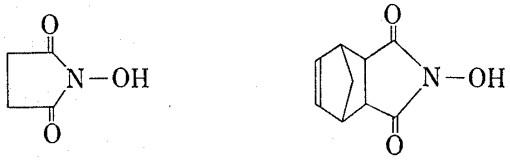
N-Hydroxy-5-norbornene-2,3-dicarboximide (HONB) was found to be an excellent reagent to be used in couple with N,N′-dicyclohexylcarbodiimide (DCC) for the peptide synthesis. Various racemization tests by the use of this reagent were conducted to see the degree of racemization during the peptide synthesis. The newly employed reagent (HONB) decreases racemization, prohibits formation of N-acylurea and affords peptides in excellent yields and a high state of purity. To evaluate this new reagent, luteinizing hormone releasing hormone (LH–RH) was prepared by the new active ester method.

Among various active esters currently used for peptide synthesis, N-hydroxysuccinimide (HOSu) active esters which were first reported in 1963 by Anderson, *et al.*[3)] have been of great value in the synthesis of peptides with any given sequences of amino acids avoiding racemization during the coupling processes of protected peptide fragments.[4)]

It has, however, been reported recently by Löw and Kisfaludy,[5)] and Gross and Bilk[6)] that the coupling method with N,N′-dicyclohexylcarbodiimide (DCC) and HOSu may often lead to an undesirable by–product, *i.e.*, succinimide–oxycarbonyl-β-alanine N-hydroxysuccinimide ester.

In order to circumvent the drawback inherent in the HOSu method we have directed our attention to an N-hydroxy–imide which has a rigid ring system. In contrast to HOSu, N-hydroxy-5-norbornene-2,3-dicarboximide (HONB, see Chart 1) has apparently a much rigid structure; furthermore, HONB is soluble in both water and organic solvents and can be easily prepared from the corresponding anhydride.[7)]



HOSu HONB

Chart 1

When HONB is stirred with DCC in dioxane or tetrahydrofuran, no dicyclohexylurea and β-alanine type by–product are formed at least within 10 hrs, whereas HOSu inevitably leads to formation of dicyclohexylurea and β-alanine derivative when treated with DCC.

1) Amino acids, peptides and their derivatives mentioned in this communication are L-configuration unless otherwise mentioned. Abbreviations used are those recommended by IUPAC–IUB Commission on Biochemistry Nomenclature in July 1965 and July 1966; *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967). Z=benzyloxycarbonyl, BOC=*t*-butoxycarbonyl, OBzl=benzyl ester, O^{t}Bu=*t*-butyl ester, NO_2=nitro.

2) Location: *Juso-Nishinocho, Higashiyodogawa-ku, Osaka.*

3) G.W. Anderson, J.E. Zimmerman and F.M. Callahan, *J. Am. Chem. Soc.*, **85**, 3039 (1963); *ibid.*, **86**, 1839 (1964).

4) F. Weygand, D. Hoffmann and E. Wünsch, *Z. Naturforsh.*, **21b**, 426 (1966); J.E. Zimmerman and G.W. Anderson, *J. Am. Chem. Soc.*, **89**, 7151 (1967).

5) M. Löw and L. Kisfaludy, *Acta Chim. Acad. Sci. Hung.*, **44**, 61 (1965).

6) H. Gross and L. Bilk, *Tetrahedron*, **24**, 6935 (1968).

7) L. Bauer and S.V. Miarka, *J. Org. Chem.*, **24**, 1293 (1959).

In the present paper we wish to report the application of HONB to the synthesis of various peptides including porcine or ovine luteinizing hormone releasing hormone (LH-RH).[8]

The HONB esters of acylamino acids or peptides which are routinely prepared by the DCC-method[9] are colorless crystalline or oily substances with good stability against hydrolysis, and the esters react readily with amines or esters of amino acids (or peptides), or even with salts of amino acids (or peptides) at room temperature or even at lower temperature to give the products in excellent yields. The yields and the physico-chemical properties of the HONB esters of acylamino acids prepared are listed in Table I, and the results of synthesis of some dipeptides are also summarized in Table II.

Table I. Yields and Properties of Acylamino Acid-HONB Esters

HONB esters of	Yield (%) (Cryst. solv.)	mp[a] (°C)	$[\alpha]_D^{22}$ (c=2, dioxane)	*Anal.* Calcd./Found C	H	N
Z-β-Ala	89 (acetonitrile)	125—126		62.49 62.52	5.24 5.18	7.29 7.29
Z-γ-Abu[b]	76 (AcOEt–pet. ether)	66—67		63.31 63.39	5.57 5.42	7.03 7.06
Z-Gly	89 (AcOEt–pet. ether)	114—116		61.61 61.69	4.90 4.70	7.56 7.43
Z-Glu(O^{t}Bu)	82 (AcOEt–pet. ether)	120—121	−32.0[c]	62.64 63.01	6.07 6.13	5.62 5.78
Z-pGlu	94 (AcOEt–pet. ether)	144—145	−41.9[d]	62.26 62.47	4.75 4.73	6.60 6.55
Z-Ile	86 (ether)	105—107	−19.8	64.77 64.75	6.15 6.08	6.57 6.58
Z-Leu	96 (ether–pet. ether)	85—86	−33.2	64.77 64.47	6.15 6.08	6.57 6.45
Z-D-Leu	84 (ether–pet. ether)	68—71	+31.7	64.77 64.43	6.15 6.06	6.57 6.48
Z-Phe	72 (AcOEt–pet. ether)	77—79	−17.7	67.81 67.77	5.25 5.53	6.08 5.81
Z-Pro	93 (ether)	119—121	−53.6	64.38 64.36	5.40 5.44	6.83 6.84
Z-Val	98 (ether–pet. ether)	99—101	−25.2	64.06 64.03	5.87 5.86	6.79 6.63
BOC-Phe	86 (AcOEt–pet. ether)	157—158	−20.1	64.77 64.80	6.15 6.15	6.57 6.50
BOC-Trp	92 (AcOEt–pet. ether)	159—161	−21.9	64.50 64.33	5.85 5.84	9.03 8.70

a) Melting points were determined by the capillary tube method and were uncorrected. *b*) Z-γ-aminobutyric acid *c*) in MeOH. *d*) in EtOH

The synthesis of peptide by this method can be accomplished practically by "one step reaction", without isolating the corresponding active esters, and in this case, peptides can be prepared with little or no racemization. In order to examine if the "one step method" provides a feasible process for the synthesis without racemization, Z-Gly-Phe-Gly-OEt (Anderson's tripeptide)[10] and Z-Phe-Ile-Gly-OBzl (detection of D-allo-Ile; a modification of the system of Bodanszky and Conklin[11]) were prepared by this method. Consequently,

8) H. Matsuo, Y. Baba, R. Nair, A. Arimura and A.V. Schally, *Biochem. Biophys. Res. Commun.*, **43**, 1334 (1971); R. Burgus, M. Butcher, M. Amoss, N. Ling, M. Monahan, J. Rivier, R. Fellows, R. Blackwell, W. Vale and R. Guillemin, *Proc. Nat. Acad. Sci.* (USA), **69**, 278 (1972).
9) D.F. Elliot and D.W. Russell, *Biochem. J.*, **66**, 49 (1957).
10) G.W. Anderson and R.W. Young, *J. Am. Chem. Soc.*, **74**, 5307 (1952); G.W. Anderson and R. Paul, *ibid.*, **82**, 4596 (1960).
11) M. Bodanszky and L.E. Conklin, *Chem. Commun.*, **1967**, 773.

Table II. Synthesis of Dipeptides

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (Conc., Solv.)	*Anal.* Calcd./Found C	H	N
Z-Ala-Gly-OBzl	92	109—110[a]		64.85 64.79	5.99 5.93	7.56 7.61
Z-Asn-Gly-OEt[b]	92	184—185[c]	−3.3° (1.0, AcOH)	54.69 54.63	6.02 6.11	11.96 12.04
Z-Gln-Ala-OBzl[b]	91	188—189	−18.5° (1.0, AcOH)	62.75 62.72	6.16 6.20	9.53 9.38
Z-Val-Val-OBzl	92	114—116[d]	−44.2° (2.1, MeOH)	68.16 67.76	7.32 7.08	6.36 6.47
Z-Gly-Phe-OH ($NaHCO_3$)[f]	100	125—126[e]	+35.2° (1.7, EtOH)	64.03 64.01	5.66 5.72	7.86 7.90
Z-Phe-Ile-OH (NaOH)[f]	94	152—154	−1.6° (1.1, EtOH)	66.97 66.95	6.84 6.89	6.79 6.79
Z-pGlu-Glu(OBzl)-OH (Et_3N)[f]	98	127—128	−17.9° (1.0, EtOH)	62.23 62.21	5.43 5.40	5.81 5.81

a) mp 111° (B. F. Erlanger and E. Brand, *J. Am. Chem. Soc.*, **73**, 3508 (1957)) *b*) The product was obtained by one step reaction. *c*) mp 184—185° (S.J. Leach and H. Lindley, *Australian J. Chem.*, **7**, 173 (1954)) *d*) mp 116°, $[\alpha]_D^{25}$ −44.3° (*c*=2.0 in MeOH) (T. Sugimura and W.K. Paik, unpublished data (*cf.* J.P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley & Sons, Inc,. 1961, p. 1139)) *e*) mp 124—125°, $[\alpha]_D^{24}$ +33.9° (*c*=4.7 in EtOH) (K. Nowak and J.Z. Siemion, *Rocz. Chem.*, **37**, 693 (1963)) *f*) Base shown in parenthesis was used for the blocking of the carboxylic residue of amine component.

it was found that in neither case was detected the isomerization in more than 1% as described in Experimental. Asn-peptides and Gln-peptides could also be obtained in good yields by the "one step method" as shown in Table II.

The usefulness of the HONB active ester in peptide synthesis was further confirmed by the synthesis of LH-RH (a decapeptide amide, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2).[8] The strategy for the synthesis is outlined in Chart 2.

Z-pGlu-ONB (I) was allowed to react with histidine to give Z-pGlu-His-OH (II) as fine prisms, and the dipeptide derivative was coupled with H-Trp-OBzl by the HONB method to give Z-pGlu-His-Trp-OBzl (III) in 86% yield. The blocking groups of III were removed in one step by hydrogenation using palladium black as catalyst to give the tripeptide IV in crystalline form.

The tripeptide ester VII, H-Ser-Tyr-Gly-O^{t}Bu, was prepared by acylation of H-Tyr-Gly-O^{t}Bu with Z-Ser-OH by the HONB method (89% yield) followed by catalytic hydrogenation to remove the amino-protecting group. The N-terminal tripeptide IV was then coupled with VII by the HONB method to yield pGlu-His-Trp-Ser-Tyr-Gly-O^{t}Bu (XI) in 75% yield. Acid solvolysis of this hexapeptide XI with trifluoroacetic acid afforded the corresponding acid XII in high yield.

For the synthesis of C-terminal tetrapeptide IX, Z-Arg(NO_2)-Pro-OH[12] was coupled with glycine amide by the HONB method to give the protected tripeptide amide VIII in 96% yield, which was treated with 25% hydrogen bromide in acetic acid to remove the Z-group. The resulting tripeptide hydrobromide was allowed to react with Z-Leu-ONB in the presence of triethylamine to give Z-Leu-Arg(NO_2)-Pro-Gly-NH_2 (IX) in 90% yield. The protected tetrapeptide amide IX was treated with 25% hydrogen bromide in acetic acid and the resulting tetrapeptide hydrobromide was passed through a column of Amberlite IR-4B (OH^-) to yield the corresponding free base X.

Coupling of the N-terminal hexapeptide XII and the C-terminal tetrapeptide amide X by the HONB method gave the protected decapeptide amide XIII, which was purified on a

12) R.A. Boissonnas, St. Guttmann and P.A. Jaquenoud, *Helv. Chim. Acta*, **43**, 1349 (1960).

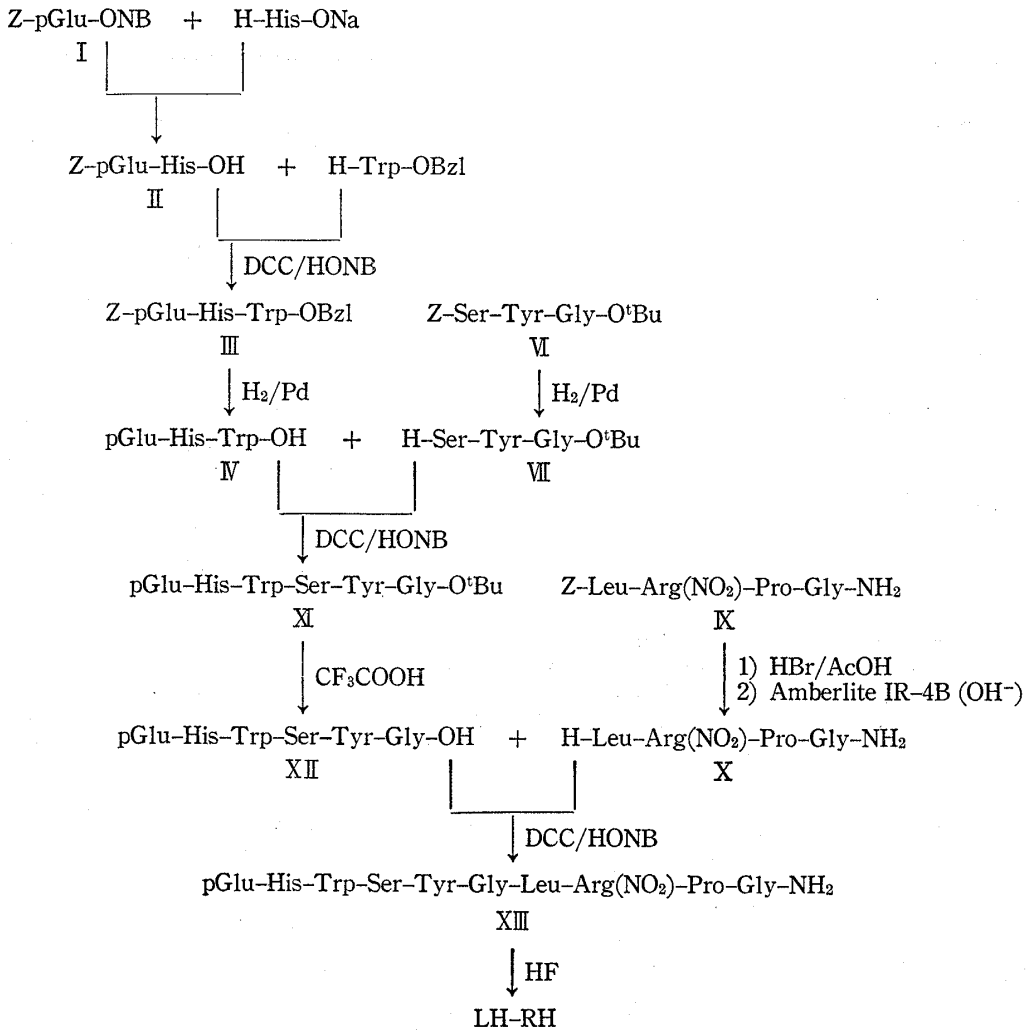


Chart 2 Synthesis of Luteinizing Hormone Releasing Hormone

column of Amberlite XAD-2 with 15% aqueous ethanol as eluent. The purified XIII was finally deblocked with the hydrogen fluoride method[13] to give the decapeptide amide, LH-RH. The decapeptide amide thus obtained was purified by column chromatography on CM cellulose with ammonium acetate buffer (pH 6.8) of linearly increasing ionic strength as eluent and on a column of Amberlite XAD-2 using 30% aqueous ethanol as eluting agent.

The final product synthesized was found to be homogeneous and identical with an authentic preparation[14] by paper chromatography, thin-layer chromatography and paper electrophoresis to Ehrlich, Sakaguchi and Pauli reagents, and the amino acid analysis and elemental analysis were also in good accordance with theoretical values.

The ovulation-inducing activity, measured in adult Sprague-Dawley rats,[15] of this material was found to possess the ED_{50} value comparable with that of the authentic preparation[14]: $ED_{50}=215\pm12$ ng/100 g of body weight.

All these results indicate that HONB active ester is promising for the synthesis of more complicated biological active peptides, and studies in this direction are now under progress in these laboratories.

13) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).
14) M. Fujino, T. Fukuda, S. Kobayashi, M. Obayashi and S. Shinagawa, *Chem. Pharm. Bull.* (Tokyo), **21**, 87 (1973).
15) I. Yamazaki and R. Nakayama, *Nippon Naibumpi Gakkai Zasshi*, **49**, 189 (1973).

Experimental

All melting points were taken by the capillary method and are uncorrected. Evaporations were carried out with a rotary evaporator. The purity of products was tested by thin-layer chromatography using Merck's silica gel G. Solvent systems used were: $CHCl_3$–MeOH–AcOH (9: 1: 0.5, *Rf*[1]), $CHCl_3$–MeOH (6: 1, *Rf*[2]), AcOEt–Pyridine–AcOH–H_2O (60: 20: 6: 10, *Rf*[3]), *n*-BuOH–AcOH–H_2O (4: 1: 1, *Rf*[4]), *n*-BuOH–AcOEt–AcOH–H_2O (1: 1: 1: 1, *Rf*[5]), *n*-BuOH–Pyridine–AcOH–H_2O (30: 20: 6: 24, *Rf*[6]).

Z–pGlu–ONB[I]——Z–pGlu–OH[16] (24.0 g, 90 mmoles) and HONB (17.9 g, 110 mmoles) were dissolved in a mixture of dioxane (200 ml) and tetrahydrofuran (THF) (200 ml), and to this was added DCC (21 g, 100 mmoles) at 2° with stirring. After stirring at 2° for 20 min and at room temperature for additional 40 min, the reaction mixture was filtered to remove the formed dicyclohexylurea (DCU), and the filtrate was concentrated *in vacuo* to give crystals which were collected by filtration and recrystallized from AcOEt–pet. ether; 36.0 g (94%), mp 144—145°, $[\alpha]_D^{22}$ −41.9° (*c*=0.2 in EtOH). *Anal.* Calcd. for $C_{22}H_{20}O_7N_2$: C, 62.26; H, 4.75; N, 6.60. Found: C, 62.47; H, 4.73; N, 6.55.

Other HONB esters of acylamino acids which were listed in Table I, were prepared in a similar manner.

Z–Ala–Gly–OBzl——To an ice-cold solution of Z–Ala–OH (4.5 g, 20 mmoles) and HONB (3.6 g, 20 mmoles) in a mixture of AcOEt (40 ml) and THF (30 ml) was added DCC (4.5 g) with stirring at 0°. After stirring at 0° for 30 min and additional 20 min at room temperature, the solution was filtered to remove the formed DCU. The filtrate was evaporated to dryness *in vacuo*. The resulting oily residue was dissolved in dioxane (40 ml), and to this solution were added H–Gly–OBzl *p*-toluenesulfonate (6.8 g, 20 mmoles) and triethylamine (3.0 ml) at room temperature, and the solution was stirred at room temperature for 4 hr. The reaction mixture was evaporated to dryness and the residue was dissolved in AcOEt (150 ml). The AcOEt solution was washed with 4% aqueous $NaHCO_3$, and with 1N HCl, dried over anhydr. Na_2SO_4, and evaporated to dryness *in vacuo*. The resulting needles were recrystallized from AcOEt–pet. ether; 6.12 g (92%), mp 109—110° (lit.[12] mp 111°), *Anal.* Calcd. for $C_{20}H_{22}O_5N_2$: C, 64.85; H, 5.99; N, 7.56. Found: C, 64.79; H, 5.93; N, 7.61.

Z–Val–Val–OBzl listed in Table II was prepared in a similar manner.

Z–pGlu–His–OH (II)——To a solution of H–His–OH monohydrochloride (71.5 g, 0.4 moles) and Na_2CO_3 (anhydr.; 42.4 g, 0.4 moles) in a mixture of water (800 ml) and dimethylformamide (DMF) (400 ml) was added I (140 g, 0.33 moles) together with dioxane (400 ml). After stirring vigorously for 4 hr at room temperature, dioxane (400 ml) was added to the reaction mixture, and the mixture was stirred for additional 10 hr. The reaction mixture was then evaporated to remove dioxane. The solution was cooled to 5° and neutralized with 1N HCl (400 ml). The solution was allowed to stand in a refrigerator to yield fine needles which were collected by filtration and recrystallized from aqueous MeOH; 89.5 g (67.7%), mp 146—147°, $[\alpha]_D^{21}$ −6.4° (*c*=1.12 in MeOH). *Anal.* Calcd. for $C_{19}H_{20}O_6N_4 \cdot 1.5H_2O$: C, 53.42; H, 5.42; N, 13.11. Found: C, 53.50; H, 5.23; N, 13.02.

Z–Gly–Phe–OH, Z–Phe–Ile–OH and Z–pGlu–Glu(OBzl)–OH were prepared in a similar manner (see Table II).

Racemization Test——a) Preparation of Z–Gly–Phe–Gly–OEt: To a solution of Z–Gly–Phe–OH (1.98 g, 5 mmoles) and H–Gly–OEt hydrochloride (700 mg, 5 mmoles) in DMF (10 ml) were added N-ethylmorpholine (0.64 ml), HONB (1.1 g, 6 mmoles) and DCC (1.1 g) with stirring at 0°. The reaction mixture was allowed to stand at 10° for 6 hr, and was diluted with AcOEt (10 ml). The formed DCU was filtered and the filtrate was washed with 4% aqueous $NaHCO_3$ and 1N HCl, and dried over anhydr. Na_2SO_4 and evaporated to dryness. The residue was then dried in a desiccator over P_2O_5 *in vacuo* to give needles; 2.20 g (100%), *Anal.* Calcd. for $C_{23}H_{27}O_6N_3$: C, 62.57; H, 6.16; N, 9.52. Found: C, 62.73; H, 6.34; N, 9.45. mp 113—115°, $[\alpha]_D^{19}$ −12.2° (*c*=2.0 in EtOH).

One gram of this product was dissolved in hot EtOH (50 ml), and the solution was cooled to 2°. To this solution was added a trace amount of crystalline Z–Gly–DL–Phe–OEt, and the solution was allowed to stand in a refrigerator (2°). No crystals appeared even after standing for one week. The solution was then concentrated to about 20 ml, and the solution was again allowed to stand in a refrigerator for 24 hr. The crystals separated were collected by filtration to give 650 mg of needles; mp 116—118°, $[\alpha]_D^{19}$ −12.5° (*c*=2.0 in EtOH) (lit. for the L-isomer; mp 118—119°, $[\alpha]_D^{24}$ −12.3° (*c*=2.0 in EtOH)). The above filtrate was diluted with ether to give some more needles (170 mg, mp 116—118°, $[\alpha]_D^{24}$ −12.3° (*c*=2.0 in EtOH); total recovery, 82%).

On the other hand, when the reaction was carried out without HONB, the total yield was 85% and the resulting tripeptide includes 7.05% of the racemate.

b) Preparation of Z–Phe–Ile–Gly–OBzl: To a solution of Z–Phe–Ile–OH (1.03 g, 2.5 mmoles), H–Gly–OBzl *p*-toluenesulfonate (840 mg, 2.5 mmoles) and N-ethylmorpholine (0.32 ml) in DMF (10 ml) were added HONB (540 mg) and DCC (600 mg) with stirring at 0°. The mixture was stirred at 0° for 2 hr and additional 4 hr at room temperature. The reaction mixture was filtered off to remove the formed DCU, and the filtrate

16) H. Gibian and E. Klieger, *Ann. Chem.*, **640**, 145 (1961).

was diluted with water (100 ml). The formed crystals were then extracted with AcOEt (80 ml×2). The AcOEt-extracts were combined and washed with 4% aqueous $NaHCO_3$ and 1N HCl, dried over anhydr. Na_2SO_4, and evaporated to dryness *in vacuo*. The resulting residue was dried well over P_2O_5 in a desiccator (yield; 1.39 g, 100%). The residue was hydrolyzed in 5.7N HCl at 110° for 20 hr for analyzing the amino acid compositions; Gly 1.00; Phe 0.96; Ile 0.98; allo-Ile 0.0048 (total recovery, 96%). This result indicates that the racemization during the reaction is less than 1%.

The control experiment of the reaction was carried out without HONB. The product (1.28 g, 84%) was hydrolyzed with HCl and analyzed for the amino acid compositions: Gly 1.00; Phe 0.93; Ile 0.62; allo-Ile 0.34 (total recovery, 89%).

Synthesis of LH–RH——Z–pGlu–His–Trp–OBzl (III): H–Trp–OBzl *p*-toluenesulfonate (37.3 g) was dissolved in AcOEt (300 ml), and the solution was washed with Na_2CO_3-saturated water (200 ml×3) and water (100 ml×3), dried over anhydr. Na_2SO_4 and evaporated to dryness *in vacuo*. The resulting oily residue was dissolved in DMF (400 ml) together with II (34.1 g, 0.08 mole) and the solution was cooled to 0°. To this solution were added HONB (17.2 g, 0.096 mole) and DCC (19.8 g, 0.096 mole) with stirring. After stirring at 0° for 2 hr and at room temperature for additional 6 hr, the reaction mixture was filtered to remove the formed DCU, and the filtrate was evaporated to dryness *in vacuo*. The residue was triturated with dry ether to give a fine powder which was collected by filtration and washed well with AcOEt. The powder was dissolved in $CHCl_3$ (200 ml) and purified by column chromatography on silica gel (500 g). The column was washed with $CHCl_3$ and $CHCl_3$–acetone (10: 3) and then eluted with $CHCl_3$–acetone–*t*-BuOH (10: 3: 4). The eluate containing the pure peptide was evaporated to dryness and the resulting residue was triturated with ether to give a fine powder; 46.75 g (86.5%), $[\alpha]_D^{25}$ −30.2° (c=1.0 in MeOH). Rf^4=0.64, Rf^5=0.79 (Pauly and Ehrlich reagents). *Anal.* Calcd. for $C_{37}H_{36}O_7N_6 \cdot 2H_2O$: C, 62.35; H, 5.66; N, 11.79. Found: C, 62.54; H, 5.82; N, 11.43.

pGlu–His–Trp–OH (IV): Compound III (60 g) was hydrogenated over a Pd catalyst in the usual manner in MeOH and the solution was filtered. The filtrate was evaporated to dryness to yield a crystalline product which was purified by recrystallization from water to give the free tripeptide as needles; 32.2 g (81.3%), mp 228° (sinter), $[\alpha]_D^{25}$ −21.8° (c=1.06 in H_2O), $[\alpha]_D^{23}$ −21.3° (c=1.025 in 10% AcOH), Rf^5=0.48. *Anal.* Calcd. for $C_{22}H_{24}O_5N_6 \cdot 1.5H_2O$: C, 57.25; H, 5.46; N, 18.21. Found: C, 57.57; H, 5.33; N, 18.12.

Di–Z–Tyr–Gly–O^{t}Bu (V): To a solution of H–Gly–O^{t}Bu (55 g, 0.375 mole) and di-Z–Tyr–OH (140 g, 0.382 mole) in a mixture of acetonitrile (300 ml) and $CHCl_3$ (200 ml) was added DCC (77.2 g, 0.35 mole) at 0°, and the mixture was stirred for 18 hr at room temperature. The reaction mixture was filtered to remove the formed DCU, and the filtrate was evaporated to dryness *in vacuo* to yield an oily residue which was then reprecipitated from AcOEt-ether; 112.8 g (52%), mp 87—89°, $[\alpha]_D^{26}$ −20.2° (c=0.76 in DMF). *Anal.* Calcd. for $C_{31}H_{34}O_8N_2$: C, 66.18; H, 6.09; N, 4.98. Found: C, 66.02; H, 6.05; N, 4.99.

Z–Ser–Tyr–Gly–O^{t}Bu (VI): Compound V (112 g, 0.2 mole) was hydrogenated over a Pd catalyst in dioxane (2 liters), and the mixture was filtered to remove the catalyst and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in a mixture of acetonitrile (250 ml) and AcOEt (150 ml) together with Z–Ser–OH (48 g, 0.2 mole) and HONB (38.4 g, 0.22 mole). To this solution was added DCC (45.4 g, 0.22 mole) at 0°, and the solution was stirred at room temperature for 20 hr. The reaction mixture was filtered to remove the formed DCU, and the filtrate was evaporated to dryness *in vacuo* to give an oily residue which was purified by column chromatography on silica gel (1 kg) (solvent: $CHCl_3$–MeOH, 9: 1).

The eluate containing the pure product was evaporated *in vacuo* to dryness, and the residue was crystallized from MeOH–AcOEt–ether to give fine needles; 94.2 g (89%), mp 141—142°, $[\alpha]_D^{26}$ −21.1° (c=0.62 in EtOH), Rf^1=0.42. *Anal.* Calcd. for $C_{26}H_{33}O_8N_3 \cdot 0.5H_2O$: C, 59.50; H, 6.53; N, 8.00. Found: C, 59.62; H, 6.22; N, 8.26.

H–Ser–Tyr–Gly–O^{t}Bu (VII): Compound VI (43.2 g) was dissolved in MeOH (500 ml) and hydrogenated over a Pd catalyst in the usual manner. The mixture was filtered to remove the catalyst and the filtrate was evaporated *in vacuo* to dryness to give the pure tripeptide as crystals; 28.7 g (100%), mp 135—136°, $[\alpha]_D^{24}$ +9.4° (c=0.85 in DMF), Rf^3=0.57. *Anal.* Calcd. for $C_{18}H_{27}O_6N_3 \cdot 0.5H_2O$: C, 55.34; H, 7.22; N, 10.76. Found: C, 55.66; H, 6.99; N, 10.82.

Z–Arg(NO_2)–Pro–Gly–NH_2 (VIII): To an ice-cold solution of Z–Arg(NO_2)–Pro–OH (24.8 g, 50 mmoles) and HONB (10.7 g) in DMF (100 ml) was added DCC (12.3 g) and the mixture was stirred for 6 hr. The reaction mixture was filtered to remove the formed DCU, and to this were added H–Gly–NH_2 acetate (7.3 g, 50 mmoles) and triethylamine (7.7 ml). After stirring at room temperature for 8 hr, the reaction mixture was evaporated to dryness *in vacuo*, and the resulting residue was dissolved in *n*-BuOH (200 ml). The *n*-BuOH solution was washed with 4% aqueous $NaHCO_3$ and 1N HCl, and evaporated to dryness. The residue was dissolved in EtOH, filtered, and the product was obtained by precipitation with dry ether; 26.5 g (96%), mp 100—116° (decomp.), $[\alpha]_D^{21}$ −25.9° (c=1.0 in DMF). *Anal.* Calcd. for $C_{21}H_{30}O_7N_3$: C, 49.79; H, 5.97; N, 22.12. Found: C, 49.81; H, 6.12; N, 21.75.

Z–Leu–Arg(NO_2)–Pro–Gly–NH_2 (IX): A solution of VIII (3.5 g, 7 mmoles) in 25% HBr–AcOH (25 ml) was allowed to stand at room temperature for 40 min. The product was precipitated by addition of dry ether to the reaction mixture followed by filtration and washing with dry ether. The precipitate thus ob-

tained was dried over P_2O_5 and NaOH pellets *in vacuo*. The dried powder then dissolved in a mixture of DMF (20 ml) and dioxane (20 ml), and to this solution was added triethylamine (1.4 ml).

On the other hand, Z-Leu-OH (2.12 g, 8 mmoles) and HONB (1.6 g) were dissolved in a mixture of THF (20 ml) and AcOEt (20 ml), and the solution was cooled to 0°. To this was added DCC (1.7 g) and the mixture was stirred for 30 min. After stirring for further 2 hr at room temperature, the reaction mixture was filtered to remove the formed DCU and evaporated to dryness to give an oily residue. The residue was dissolved in dioxane (10 ml) and the solution was added to the above-mentioned DMF solution which contained the amine component. After stirring for 8 hr, the solution was evaporated *in vacuo* and the residue was dissolved in water (300 ml). The aqueous solution was washed with ether–AcOEt (1: 1) and then extracted with *n*-BuOH (100 ml×3). The *n*-BuOH extracts were combined, washed with water and evaporated to dryness *in vacuo*. The residue was triturated with ether to yield a fine powder which was collected by filtration, and purified by reprecipitation from EtOH–ether; 3.8 g, (90%), mp 135—153° (decomp.), $[\alpha]_D^{25}$ −34.1° (c=1.09 in DMF), Rf^1=0.16, Rf^3=0.66. *Anal.* Calcd. for $C_{27}H_{41}O_8N_9$: C, 52.33; H, 6.66; N, 20.34. Found: C, 52.41; H, 6.83; N, 19.97.

H–Leu–Arg(NO_2)–Pro–Gly–NH_2 (X): Compound IX (3.62 g, 6 mmoles) was dissolved in 25% HBr–AcOH (20 ml) and the solution was stirred at 20° for 70 min. The product was precipitated by addition of dry ether to the reaction mixture followed by filtration and washing with dry ether. The precipitate thus obtained was dried over P_2O_5 and NaOH pellets *in vacuo*. The dried powder was dissolved in 50% aqueous MeOH and passed through a column of Amberlite IR-4B (OH^-). The passed solution and washings were combined and evaporated to a small volume and then lyophilized to give the pure free base as a powder; 2.70 g (95%), $[\alpha]_D^{22}$ −57.4° (c=1.0 in H_2O), Rf^2=0.06, Rf^5=0.57, Rf^6=0.58. *Anal.* Calcd. for $C_{19}H_{35}O_6N_9 \cdot H_2O$: C, 45.31; H, 7.41; N, 25.04. Found: C, 45.61; H, 7.22; N, 24.64.

pGlu–His–Trp–Ser–Tyr–Gly–O^{t}Bu (XI): Compound IV (23.1 g, 50 mmoles) and compound VII (19.1 g, 50 mmoles) were dissolved in DMF (500 ml) together with HONB (11 g), and the mixture was cooled to −5°. To this was added DCC (13 g) and the solution was stirred at 0° for 4 hr and at room temperature for additional 10 hr. The reaction mixture was filtered to remove the formed DCU, and the filtrate was evaporated to dryness *in vacuo*. The residue was triturated with a mixture of ether and AcOEt (1: 1) to give a fine powder which was collected by filtration. The powder was then triturated again with hot acetonitrile and the mixture was kept in a refrigerator to give a crystalline precipitate which was collected by filtration and washed well with ether; 31.0 g (75%), $[\alpha]_D^{24}$ −16.3° (c=1.0 in DMF), Rf^3=0.35, Rf^4=0.28, Rf^5=0.69. Amino acid *Anal.*: Glu 1.00; His 0.94; Ser 0.96; Tyr 1.01; Gly 1.01; Trp (UV) 1.03.

pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2 (LH–RH): A solution of XI (3.83 g, 4.5 mmole) in trifluoroacetic acid (40 ml) which contained mercaptoethanol (0.1 ml) and 4.4N HCl–dioxane (1.2 ml) was allowed to stand at room temperature for 50 min. The product was precipitated by addition of dry ether, collected by filtration and dried over NaOH pellets *in vacuo* to give the hexapeptide XII as a free acid: Rf^3=0.08, Rf^4=0.17, Rf^5=0.59. The dried free acid and compound X (2.67 g, 5.5 mmoles) were dissolved in DMF (50 ml) together with N-ethylmorphorine (0.58 ml) and HONB (1.3 g, 7.2 mmoles) and the solution was cooled to 0°. To this solution was added DCC (1.5 g, 7.2 mmoles) and the mixture was stirred for 2 hr at 0° and additional 20 hr at room temperature. The reaction mixture was then filtered to remove the formed DCU and evaporated to dryness. The resulting residue was triturated with ether (50 ml) to give a fine powder which was collected by filtration. The powder was dissolved in 10% aqueous EtOH in the presence of urea (2 g) (20 ml) and the solution was subjected to a column of Amberlite XAD-2 (200—300 mesh) (4×12 cm). The column was washed with 10% aqueous EtOH and then the protected decapeptide amide was eluted with 15% aqueous EtOH. The eluate was evaporated to remove EtOH and the concentrated solution was lyophilized to dryness; 4.5 g. Rf^5=0.41, Rf^6=0.58.

The dried powder (4 g) was dissolved in anhydr. hydrogen fluoride (*ca.* 40 ml) together with anisole (4 ml) and mercaptoethanol (1 ml) at −70°, and the mixture was stirred at 0° for 40 min. Volatile compounds were evaporated, and the residue was dissolved in water (70 ml). The solution was washed with ether (70 ml×2) and the aqueous layer was applied to a column of Amberlite CG-400 (AcO^-, 2×15 cm). The passed solution and washings were combined and lyophilized to give the crude LH–RH (4.0 g).

The crude product was dissolved in water (50 ml) and applied to a column (4×45 cm) of carboxymethyl (CM) cellulose which was eluted with pH 6.8 ammonium acetate buffer (gradient: 0.02M/0.2M=2 liters/2 liters). The fractions containing the pure product were combined and subjected to a column of Amberlite XAD-2 (4×12 cm). The column was washed well with water and the pure peptide was eluted with 30% aqueous EtOH. The eluate was evaporated to a small volume and then lyophilized to constant weight to give a white fluffy powder which was dried over P_2O_5 *in vacuo* at 50° for 5 hr; yield, 2.03 g, $[\alpha]_D^{23}$ −52.7° (c=0.5 in 5% AcOH), Rf^4=0.42, Rf^5=0.23. *Anal.* Calcd. for $C_{55}H_{75}O_{13}N_{17} \cdot 2AcOH \cdot 5H_2O$: C, 50.89; H, 6.73; N, 17.10. Found: C, 50.71; H, 6.12; N, 17.18. Amino acid *Anal.*: His 0.92; Arg 0.96; Ser 0.92; Glu 1.00; Pro 1.08; Gly 2.00; Leu 1.00; Tyr 0.96; Trp 1.04 (UV) (average recovery, 86%).

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