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Analogues of Luteinizing Hormone-Releasing Hormone with Modification in Position 8[1]

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Eight analogues of LH–RH, in which arginine residue in position 8 was modified, were synthesized and evaluated for their LH–RH activity. The results suggest that both the size and basicity of the guanidino group, as well as the length of the methylene chain in Arg^8 residue are important factors for generating full hormonal activity.

After the primary structure of porcine hypothalamic luteinizing hormone-releasing hormone (LH–RH) was determined to be the decapeptide amide, <Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2,[3,4] many analogues of this hormone have been reported by various investigators. Several groups have already inferred that the basicity of arginine residue in position 8 was one of important factors for eliciting the full activity.[5–7]

In order to evaluate the role of the arginine moiety in detail, we synthesized and tested for biological activity the following LH–RH analogues substituted in the 8-position: Nar^8–, Dab^8–, Har^8–, Lys^8–, Orn^8–, Cit^8–, N^δ–Ac–Orn^8– and N^δ–Bz–Orn^8–LH–RH.

All of the analogues in this study were synthesized by the conventional classical method as depicted in Chart 1 and 2. The α-amino groups were generally protected by the Z group, while the δ-amino group of Orn, ε-amino group of Lys and γ-amino group of Dab were protected by the PHT group. The hydroxyl groups of Ser and Tyr were also masked by the Bu^t group.

<Glu–His–Trp–Ser(Bu^t)–Tyr(Bu^t)–Gly–OH and H–Leu–X–Pro–Gly–NH_2 (X=PHT–Orn, PHT–Lys, PHT–Dab, Cit, Ac–Orn and Bz–Orn), synthesized stepwise, were coupled by dicyclohexylcarbodiimide (DCCD) in the presence of 1-hydroxybenzotriazole (HOBt)[8] to give the protected decapeptide amides, <Glu–His–Trp–Ser(Bu^t)–Tyr(Bu^t)–Gly–Leu–X–Pro–Gly–NH_2. The protected decapeptide amides, thus prepared, were treated with anhydrous hydrogen fluoride[9] to remove the Bu^t group. The PHT group, which is not split by hydrogen fluoride, was removed by hydrazine hydrate before the treatment with hydrogen fluoride.

1) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission of Biochemical Nomenclature in May 1971: *J. Biol. Chem.*, **247**, 977 (1972). Abbreviations of the less-common amino acids are as follows: Har, Homoarginine; Nar, Norarginine; Dab, α, γ-Diaminobutyric acid. The other abbreviations are as follows: Z, carbobenzoxy; PHT, phthalyl; Ac, acetyl; Bz, benzoyl; Bu^t, *tert*-butyl; ONSu, N-hydroxysuccinimide ester; OTCP, 2,4,5-trichlorophenyl ester; DCHA, dicyclohexylamine.

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4) Y. Baba, H. Matsuo, and A.V. Schally, *Biochem. Biophys. Res. Commun.*, **44**, 459 (1971).

5) J-K. Chang, H. Sievertsson, B.L. Currie, C. Bogentoft, and K. Folkers, *J. Med. Chem.*, **15**, 623 (1972).

6) J-K. Chang, R.H. William, A.J. Humphries, N-G. Johansson, and K. Folkers, *Biochem. Biophys. Res. Commun.*, **47**, 727 (1972).

7) M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, I. Yamazaki, R. Nakayama, W.F. White, and R.H. Rippel, *Biochem. Biophys. Res. Commun.*, **49**, 698 (1972).

8) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).

9) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).

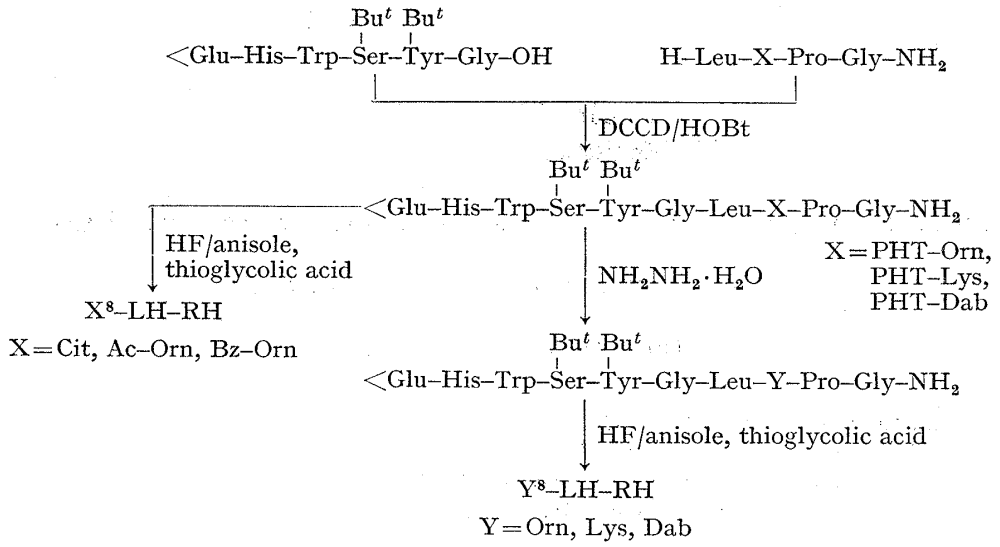


Chart 1

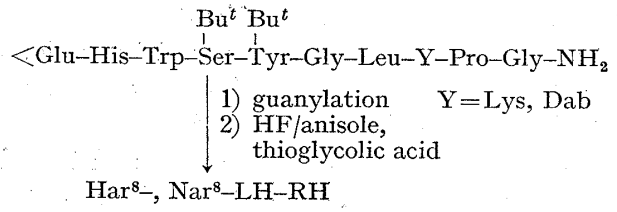


Chart 2

Nar^8– and Har^8–LH–RH were prepared by the reaction of the partially protected decapeptide amides, <Glu–His–Trp–Ser(Bu^t)–Tyr(Bu^t)–Gly–Leu–Lys(and Dab)–Pro–Gly–NH$_2$, with 1-guanyl-3,5-dimethylpyrazole,[10] followed by the treatment with hydrogen fluoride.

The crude peptides obtained were purified by the column partition chromatography on Sephadex G-25,[11] using the solvent system constituting of *n*-butanol–0.1 M ammonium acetate (1:1) or *n*-butanol–0.1 M ammonium acetate buffer pH 4.5 (1:1). These analogues exhibited a single spot on thin-layer chromatogram by three different solvent systems, and their acid hydrolysates showed the correct amino acid ratios by the automatic amino acid analyzer as depicted in Table I.

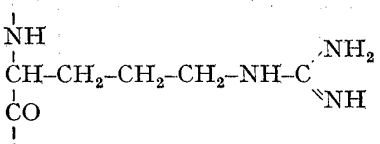
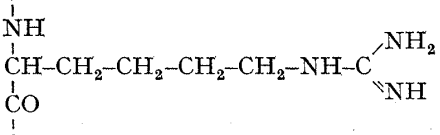
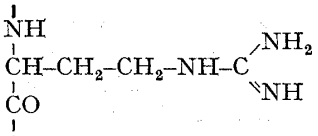
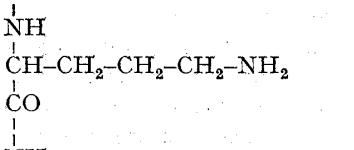
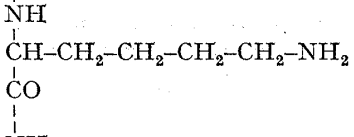
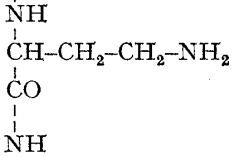
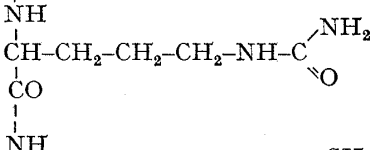
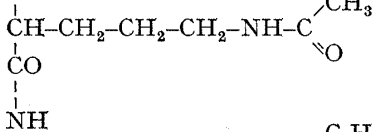
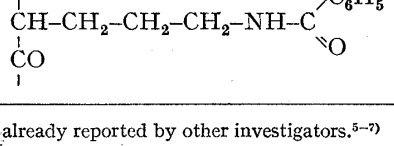
Table I. Chemical and Physical Properties of LH–RH Analogues with Modification in Position 8

Compounds	$[\alpha]_D^{21}$ (c=0.3, 0.1 N AcOH)	*Rf*[a]			Amino acid analysis[b]								
		I	II	III	Glu	His	Trp	Ser	Tyr	Gly	Leu	Pro	
Synthetic LH–RH	−52.9	0.33	0.44	0.52									
Har^8–LH–RH	−47.1	0.32	0.41	0.52	0.90	0.89	0.68	0.56	1.01	1.95	1.00	0.90	1.00(Har)
Nar^8–	−49.5	0.34	0.44	0.51	0.95	0.96	0.79	0.75	0.99	1.97	1.00	0.99	0.86(Nar)
Orn^8–	−49.2	0.29	0.41	0.48	0.91	0.88	0.60	0.67	0.88	1.83	1.00	0.92	0.93(Orn)
Lys^8–	−60.2	0.29	0.41	0.49	0.95	0.83	0.84	0.74	0.99	2.03	1.00	0.96	1.04(Lys)
Dab^8–	−58.8	0.33	0.41	0.49	0.97	0.99	0.70	0.84	1.05	2.04	1.00	0.98	1.45(Dab)
Cit^8–	−46.1	0.37	0.47	0.55	0.96	1.01	0.86	0.63	0.99	1.93	1.00	0.94	0.41(Orn)
N$^\delta$–Ac–Orn^8–	−58.2	0.36	0.50	0.55	0.84	0.90	0.74	0.63	0.87	1.85	1.00	1.01	0.94(Orn)
N$^\delta$–Bz–Orn^8–	−40.4	0.46	0.58	0.60	0.98	0.97	0.70	0.72	0.85	2.04	1.00	0.98	0.99(Orn)

a) *Rf*-I, II and III values refer to the solvent systems *n*-BuOH–AcOH–H_2O (60:15:25), *n*-BuOH–AcOH–H_2O–pyridine (30:6:24:20) and $CHCl_3$–MeOH–32% AcOH (60:45:20), respectively.
b) acid hydrolysate (6 N HCl, 110°, 24 hr, in the presence of thioglycolic acid)

10) R.A.B. Bannard, A.A. Casselman, W.F. Cuckburn, and G.M. Brown, *Can. J. Chem.*, **36**, 1541 (1958).
11) D. Yamashiro, *Nature*, **201**, 76 (1964).

Table II. LH–RH Activity of LH–RH Analogues with Modification in Position 8 as Compared with Synthetic LH–RH

Compounds	Structure in position 8	% LH–RH activity *in vivo* with 95% confidence limits
Synthetic LH–RH		assumed 100%
Har^8–LH–RH		12.6% (12.2—13.0)
Nar^8–		15.1 (10.1—20.9)
Orn^8–[a]		2.0 (1.3— 3.3)
Lys^8–[a]		6.0 (5.2— 6.9)
Dab^8–		2.0 (1.1— 3.9)
Cit^8–		7.5 (5.0—11.1)
N^δ–Ac–On^8–		5.1 (1.0—24.7)
N^δ–Bz–On^8–		1.1 (0.8— 1.7)

a) These analogues were already reported by other investigators.[5–7]

All these analogues were tested for LH–RH activity and compared with synthetic LH–RH at two dose levels. The evaluation of LH–RH activity was performed *in vivo* by the stimulation of release of LH in ovariectomized rats pretreated with estrogen and progesterone,[12] followed by radioimmunoassay for rat LH according to Niswender, *et al.*[13]

As shown in Table II, marked decrease in LH–RH activity was observed in both Nar^8– and Har^8–LH–RH. However relative potencies of these two analogues were found much higher comparing with Lys^8–, Orn^8– or Dab^8–LH–RH, in which the guanidino group of arginine

12) V.D. Ramirez and S.M. McCann, *Endocrynology*, **73**, 193 (1963).
13) G.D. Niswender, A.R. Midgley, Jr., S.E. Monroe, and E. Reichert, Jr., *Proc. Soc. Exp. Biol. Med.*, **128**, 807 (1968).

residue of LH–RH was converted to the amino group, another basic substituent, and the methylene chain length was varied. These results indicate the importance of both the guanidino group and the methylene chain length of Arg[8] moiety to the appearance of full LH–RH activity. On the other hand, significant activity was shown in Cit[8]– and N^{δ}–Ac–Orn[8]–LH–RH, where the size of Arg[8] residue was not greatly altered but its basicity was completely abolished. In particular, Cit[8]–LH–RH was more active than Lys[8]– or Orn[8]–LH–RH. The size of the guanidino group in Arg[8] residue, as well as its basicity, appears to contribute to elicit full hormonal activity.

Experimental

Melting points are uncorrected. Optical rotations were measured with a Perkin–Elmer polarimeter 141. Thin-layer chromatographies (TLC) were performed on Merck Silica gel $60F_{254}$. *Rf* values to the following solvent systems: Rf^{I} $CHCl_3$–EtOH (10: 1), Rf^{II} $CHCl_3$–EtOH (8: 1), Rf^{III} $CHCl_3$–EtOH–AcOH (95: 5: 3) and Rf^{IV} $CHCl_3$–EtOH–AcOH (20: 5: 1). Amino acid analyses of peptides were carried out on samples that had been hydrolyzed with constant boiling HCl for 24 hr in evacuated, sealed tubes at 110°, and were recorded on a Hitachi amino acid analyzer KLA-2.

Z-Orn(PHT)-Pro-OBu^t (8—9a)——A mixed anhydride was prepared in the usual manner from Z–Orn(PHT)–OH[14] (59.5 g) and isobutyl chloroformate (20.5 g) in anhydrous THF (300 ml) with N-methylmorpholine (15.2 g). The resulted mixed anhydride solution was added slowly with stirring to a chilled solution of H–Pro–OBu^t[15] (28.3 g) in THF (120 ml). The mixture was stirred for 1 hr in an ice bath and for additional 2 hr at room temperature. After evaporation of the solvent, the residue was acidified with 1% citric acid and extracted by AcOEt. The extract was washed with 2% Na_2CO_3 and H_2O, dried over Na_2SO_4, and concentrated to a syrupy residue: Yield 75.9 g (92.1%). Rf^{I} 0.61. This material was used in the next step without further purification.

Z-Orn(PHT)-Pro-OH (8—9b)——Z–Orn(PHT)–Pro–OBu^t (8—9a, 45.0 g) was dissolved in trifluoroacetic acid (TFA, 100 ml) and the mixture was stirred for 30 min at room temperature. TFA was removed under reduced pressure and the residue was dissolved in a small volume of benzene. The solvent was evaporated *in vacuo*. This operation was repeated 3 times. The oily residue was chromatographed on silica gel (Wakogel C-200, 900 g). The first fraction, eluted with $CHCl_3$ (100 ml), gave a small amount of unidentified product. Elution with $CHCl_3$–EtOH (8: 1, 1000 ml) and evaporation of the solvent gave a colourless oil as the second fraction: Yield 36.5 g (96.6%). Rf^{II} 0.41.

Z-Orn(PHT)-Pro-Gly-NH_2 (8—10a)——Isobutyl chloroformate (10.3 g) was added with stirring at −35 to −40° to a solution of Z–Orn(PHT)–Pro–OH (8—9b, 36.3 g) in a mixture of anhydrous N,N-dimethylformamide (DMF, 30 ml) and THF (300 ml) containing N-methylmorpholine (7.6 g). The mixture was kept at −15 to −20° for 20 min, and H–Gly–NH_2·HCl (9.1 g) and Et_3N (8.4 g) in DMF (100 ml) were added. The stirring was continued without cooling and the reaction mixture was stirred for 2 hr after the temperature had reached room temperature. The solvent was evaporated and the residue was dissolved in AcOEt, which was washed with 1% citric acid, 2% $NaHCO_3$ and H_2O. The solvent was removed after drying over Na_2SO_4. The solid residue obtained on addition of ether was collected and recrystallized from AcOEt–ether: Yield 39.0 g (98.0%). mp 88—90°. $[\alpha]_D^{21}$ −17.5° (c=0.3, MeOH). Rf^{II} 0.19, Rf^{III} 0.58. *Anal.* Calcd. for $C_{28}H_{31}O_7N_5 \cdot H_2O$: C, 59.25; H, 5.86; N, 12.34. Found: C, 59.48; H, 5.61; N, 12.53.

Z-Leu-Orn(PHT)-Pro-Gly-NH_2 (7—10a)——Z–Orn(PHT)–Pro–Gly–NH_2 (8—10a, 31.8 g) was dissolved in AcOH (130 ml) with warming. A 25% solution of HBr in AcOH (210 ml) was added and the solution was kept for 1 hr at room temperature. The addition of dry ether (1500 ml) resulted precipitate which was collected by filtration and washed with ether. This powder was dissolved in DMF (190 ml) containing Et_3N (5.7 g) and N-methylmorpholine (5.0 g). To this solution, Z–Leu–ONSu (24.6 g) was added at 0° and the mixture was stirred at room temperature for 22 hr. The excess Z–Leu–ONSu was coupled with N,N-dimethylaminopropylamine[16] (1.0 g) for 1 hr. After evaporation of the solvent, the oily residue was dissolved in AcOEt. The solution was washed with 10% citric acid and H_2O, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was solidified on addition of ether containing a small volume of AcOEt. Recrystallized from AcOEt–ether: Yield 33.1 g (83.6%). mp 102—104°. $[\alpha]_D^{21}$ −34.9° (c=0.3, AcOEt). *Anal.* Calcd. for $C_{34}H_{42}O_8N_6 \cdot \frac{1}{2}H_2O$: C, 60.79; H, 6.45; N, 12.51. Found: C, 60.90; H, 6.28; N, 12.43.

14) M. Bodanszky, M.A. Ondetti, C.A. Birkhimer, and P.L. Thomas, *J. Am. Chem. Soc.*, **86**, 4452 (1964).
15) G.W. Anderson and F.M. Callahan, *J. Am. Chem. Soc.*, **82**, 3359 (1960).
16) M. Löw and C. Kisfaludy, *Acta Chim. Acad. Sci. Hung.*, **44**, 61 (1965).

Z-Lys(PHT)-Pro-Gly-NH_2 (8—10b)——Isobutyl chloroformate (2.5 g) was added with stirring at −35 to −40° to a solution of Z-Lys(PHT)-Pro-OH[17] (9.0 g) in AcOEt (60 ml) containing N-methylmorpholine (1.8 g). The mixture was kept at −15 to −20° for 20 min, and H-Gly-NH_2·HCl (9.1 g) and Et_3N (2.2 g) in DMF (20 ml) were added. The stirring was continued without cooling and the reaction mixture was stirred for 2 hr after the temperature had reached room temperature. The solvent was evaporated and the residue was dissolved in AcOEt, which was washed with 1% citric acid, 2% $NaHCO_3$ and H_2O. The solvent was removed after drying over Na_2SO_4. The solid residue obtained on addition of ether was collected and recrystallized from AcOEt–ether: Yield 3.3 g (33.7%). mp 102—105°. $[\alpha]_D^{21}$ −13.9° (c=0.3, DMF). *Anal.* Calcd. for $C_{29}H_{33}O_7N_5 \cdot \frac{1}{2}H_2O$: C, 60.83; H, 5.98; N, 12.23. Found: C, 61.42; H, 6.14; N, 11.81.

Z-Leu-Lys(PHT)-Pro-Gly-NH_2 (7—10f)——Z-Lys(PHT)-Pro-Gly-NH_2 (8—10b, 2.3 g) was dissolved in AcOH (10 ml) with warming. A 25% solution of HBr in AcOH (20 ml) was added and the solution was kept for 1 hr at room temperature. The addition of dry ether (150 ml) resulted precipitate which was collected by filtration and washed with ether. This powder was dissolved in DMF (20 ml) containing Et_3N (0.4 g) and N-methylmorpholine (0.4 g). To this solution, Z-Leu-ONSu (2.0 g) was added at 0° and the mixture was stirred at room temperature for 20 hr. The excess Z-Leu-ONSu was coupled with N,N-dimethylpropylamine (0.15 g) for 1 hr. The solvent was evaporated under reduced pressure and the oily residue was dissolved in AcOEt. The solution was washed with 10% citric acid and H_2O, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was solidified on addition of ether. Recrystallized from AcOEt–ether: Yield 1.8 g (48.6 %). mp 81—86°. $[\alpha]_D^{21}$ −27.2° (c=0.3, AcOH). *Anal.* Calcd. for $C_{35}H_{44}O_8N_6 \cdot \frac{1}{2}H_2O$: C, 61.30; H, 6.61; N, 12.25. Found: C, 61.62; H, 6.61; N, 11.88.

Z-Dab(PHT)-Pro-OBu^t (8—9c)——DCCD (4.9 g) dissolved in AcOEt (5 ml) was added to a mixture of Z-Dab(PHT)-OH (from Z-Dab(PHT)-OH DCHA salt[18] (11.3 g)), H-Pro-OBu^t (3.4 g) and N-hydroxysuccinimide (HOSu, 2.8 g) in AcOEt (50 ml) at −40 to −50°. The reaction mixture was stirred at room temperature for 20 hr and cooled to 0°. The precipitated DC urea was filtered off and the filtrate was washed with 10% citric acid, 5% Na_2CO_3 and the saturated brine and dried over Na_2SO_4. The solvent was evaporated *in vacuo* to dryness. The resulted solid residue was recrystallized from AcOEt–*n*-hexane: Yield 2.5 g (21.9%). mp 157—159°. $[\alpha]_D^{21}$ −44.8° (c=0.3, DMF). *Anal.* Calcd. for $C_{29}H_{33}O_7N_3$: C, 65.03; H, 6.21; N, 7.85. Found: C, 64.95; H, 6.17; N, 7.99.

Z-Dab(PHT)-Pro-Gly-NH_2 (8—10c)——Z-Dab(PHT)-Pro-OBu^t (8—9c, 2.3 g) was treated with TFA (25 ml) at room temperature for 10 min. After evaporation of TFA, the oily residue was dissolved in a small volume of benzene and concentration was carried out again. This operation was repeated 3 times. The oily residue was combined with a solution of H-Gly-NH_2·HCl (0.53 g), Et_3N (0.5 g) and HOSu (0.6 g) in DMF (20 ml). To this solution, a solution of DCCD (0.8 g) in DMF (1 ml) was added with stirring at 0°. The reaction mixture was stirred at room temperature for 24 hr and cooled to 0°. The precipitated DC urea was filtered off and the filtrate was concentrated under reduced pressure. The residue dissolved in AcOEt was washed with 10% citric acid, 5% Na_2CO_3 and the saturated brine, dried over Na_2SO_4 and concentrated. The solid product was collected by filtration and washed with ether: Yield 1.5 g (73.9%). mp 127—130°. $[\alpha]_D^{21}$ −15.6° (c=0.3, DMF). *Anal.* Calcd. for $C_{27}H_{29}O_7N_5 \cdot \frac{1}{2}H_2O$: C, 59.55; H, 5.55; N, 12.86. Found: C, 59.34; H, 5.55; N, 12.82.

Z-Leu-Dab(PHT)-Pro-Gly-NH_2 (7—10g)——Z-Dab(PHT)-Pro-Gly-NH_2 (8—10c, 0.5 g) was dissolved in AcOH (2 ml) with warming. A 25% solution of HBr in AcOH (4 ml) was added and the solution was kept for 1 hr at room temperature. The addition of dry ether (50 ml) resulted precipitate which was collected by filtration and washed with ether. This powder was dissolved in DMF (5 ml) containing Et_3N (0.09 g) and N-methylmorpholine (0.09 g). To this solution, Z-Leu-ONSu (0.4 g) was added at 0° and the mixture was stirred at room temperature for 20 hr. The solvent was removed under reduced pressure and the oily residue was dissolved in AcOEt. The solution was washed with 10% citric acid and H_2O, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was solidified on addition of ether. Recrystallized from AcOEt–ether: Yield 0.5 g (86.2%). mp 127—129°. $[\alpha]_D^{21}$ −39.3° (c=0.3, AcOH). *Anal.* Calcd. for $C_{33}H_{40}O_8N_6 \cdot 2H_2O$: C, 57.88; H, 6.48; N, 12.27. Found: C, 57.59; H, 6.79; N, 12.29.

Z-Leu-Orn-Pro-Gly-NH_2 (7—10b)——Z-Leu-Orn(PHT)-Pro-Gly-NH_2 (7—10a, 4.5 g) was dissolved in MeOH (20 ml). A 2 M methanolic solution of hydrazine hydrate (12 ml) was added and the solution was stirred at room temperature for 3 hr. A 4 M solution of AcOH in MeOH (30 ml) was added and the mixture was kept cold overnight. The precipitated tetrahydrophthalazine-1,4-dione was filtered off and the filtrate was concentrated to dryness *in vacuo*. The residue was extracted with three 10 ml portions of 20% aq. AcOH. The combined extracts were lyophilized to yield colourless solid: Yield 3.5 g (97.2%). *Rf* 0.48 (*n*-butanol: AcOH: H_2O=60: 15: 25). This material was used in the next step without further purification.

17) S. Hase, S. Sakakibara, M. Wahrenberg, M. Kirchberger, I.L. Schwartz, and R. Walter, *J. Am. Chem. Soc.*, **94**, 3590 (1972).

18) Z-Dab(PHT)-OH DCHA salt was prepared by the method of M. Bodanszky, M.A. Ondetti, C.A. Birkhimer, and P.L. Thomas, *J. Am. Chem. Soc.*, **86**, 4452 (1964).

Z-Leu-Cit-Pro-Gly-NH_2 (7—10c)——Z–Leu–Orn–Pro–Gly–NH_2 (7—10b, 1.22 g) and potassium cyanate (0.53 g) were dissolved in a mixture of 1 N KOH (2.8 ml), H_2O (1.8 ml) and MeOH (15 ml). Two additional portions of potassium cyanate (0.7 and 0.3 g, each) were added after 24 and 48 hr. The mixture was stirred at room temperature and examined from time to time by TLC. When a ninhydrin positive spot practically disappeared (3 days), the reaction mixture was acidified with 1 N HCl and the insoluble material was filtered off. The filtrate was extracted by *n*-butanol and *n*-butanol extract was washed with H_2O. The solvent was removed under reduced pressure to give the solid product. Recrystallized from MeOH–AcOEt: Yield 0.7 g (58.3%). mp 182—190°. $[\alpha]_D^{21}$ −35.0° (c=0.3, AcOH). *Anal.* Calcd. for $C_{27}H_{41}O_7N_7 \cdot \frac{1}{2}H_2O$: C, 55.47; H, 7.24; N, 16.77. Found: C, 55.18; H, 7.18; N, 16.96.

Z-Leu-Orn(Ac)-Pro-Gly-NH_2 (7—10d)——Z–Leu–Orn–Pro–Gly–NH_2 (7—10b, 1.5 g) was dissolved in DMF (16 ml) containing Et_3N (0.5 ml). To this solution, acetic anhydride (0.3 g) was added slowly at room temperature and the mixture was examined by TLC. When a ninhydrin positive spot was practically disappeared (1 hr), the solvent was removed under reduced pressure. The oily residue chromatographed on silica gel (Wakogel C-200, 30 g). Elution with $CHCl_3$–MeOH (5: 1) gave a solid on addition of AcOEt. Recrystallized from MeOH–AcOEt: Yield 0.9 g (56.3%). mp 92—94°. $[\alpha]_D^{21}$ −38.0° (c=0.3, AcOH). *Anal.* Calcd. for $C_{28}H_{42}O_7N_6 \cdot 2H_2O$: C, 55.06; H, 7.59; N, 13.76. Found: C, 54.89; H, 8.13; N, 13.23.

Z-Leu-Orn(Bz)-Pro-Gly-NH_2 (7—10e)——Benzoyl chloride (0.56 g) was added to a solution of Z–Leu–Orn–Pro–Gly–NH_2 (7—10b, 1.15 g) in $CHCl_3$ (15 ml) containing Et_3N (1.45 g) at 0°. The mixture was stirred at room temperature for 2 hr (a ninhydrin positive spot was practically disappeared), and the solvent was evaporated *in vacuo*. The oily residue was dissolved in *n*-butanol and the solution was washed with H_2O. The organic layer was separated and the solvent was removed under reduced pressure. Ether was added to the oily residue and the resulted precipitate was collected by filtration and washed with AcOEt: Yield 0.57 g (53.6%). mp 97—104°. $[\alpha]_D^{21}$ −32.9° (c=0.3, AcOH). *Anal.* Calcd. for $C_{33}H_{44}O_7N_6 \cdot \frac{5}{2}H_2O$: C, 58.14; H, 7.24; N, 12.33. Found: C, 58.35; H, 7.98; N, 12.00.

Z-Tyr(Bu^t)-Gly-OEt (5—6a)——Isobutyl chloroformate (2.73 g) was added with stirring at −40 to −50° to a solution of Z–Tyr(Bu^t)–OH[19] (7.3 g) in anhydrous THF (40 ml) containing N-methylmorpholine (2.02 g). The mixture was kept at −30 to −35° for 10 min, and H–Gly–OEt (prepared from 3.37 g of the hydrochloride with 2.43 g of Et_3N) in CH_2Cl_2 (40 ml) was added. The reaction mixture was stirred without cooling and stirring was continued for 1.5 hr after the temperature had reached room temperature. The solvent was evaporated and the residue was dissolved in AcOEt, which was washed with 1% citric acid, 2% $NaHCO_3$ and H_2O. After evaporation of the solvent, the solid residue was collected and recrystallized from AcOEt–*n*-hexane: Yield 7.8 g (86.7%). mp 75—76.5°. $[\alpha]_D^{20}$ −19.3° (c=0.3, MeOH). *Anal.* Calcd. for $C_{25}H_{32}O_6N_2$: C, 65.77; H, 7.07; N, 6.14. Found: C, 65.95; H, 7.11; N, 6.27.

Z-Ser(Bu^t)-Tyr(Bu^t)-Gly-OEt (4—6a)——Z–Tyr(Bu^t)–Gly–OEt (5—6a, 7.0 g) was hydrogenated for 2.5 hr over 10% palladium–on–charcoal (2.0 g) in MeOH (100 ml) containing AcOH (1.2 g). The catalyst was filtered off and the solvent was evaporated *in vacuo*. The oily residue was dissolved in DMF (50 ml). To this solution, Z–Ser(Bu^t)–ONSu[20] (6.3 g) and N-methylmorpholine (1.5 g) were added at 0°. Stirring was continued at 0° for 1 hr and then at room temperature overnight. The solvent was removed under reduced pressure. The residue was dissolved in AcOEt and washed with 10% citric acid, 5% Na_2CO_3 and the saturated brine. The solution dried over Na_2SO_4 was concentrated to about 15 ml. Addition of *n*-hexane caused precipitation of the product: Yield 9.3% (94.9%). mp 68—71°. $[\alpha]_D^{21}$ −3.9° (c=0.3, DMF). Rf^{II} 0.69. *Anal.* Calcd. for $C_{32}H_{45}O_8N_3$: C, 64.09; H, 7.56; N, 7.01. Found: C, 64.39; H, 7.50; N, 7.17.

Z-Trp-Ser(Bu^t)-Tyr(Bu^t)-Gly-OEt (3—6a)——Z–Ser(Bu^t)–Tyr(Bu^t)–Gly–OEt (4—6a, 2.8 g) was hydrogenated for 3 hr over 10% palladium–on–charcoal (0.5 g) in MeOH (30 ml) containing AcOH (0.5 g). The catalyst was filtered off and the filtrate was concentrated to dryness *in vacuo*. The oily residue was dissolved in DMF (20 ml) and cooled to 0°. To this solution, Z–Trp–ONSu (2.2 g) and N-methylmorpholine (0.5 g) were added. The reaction mixture was kept at 0° for 2.5 hr and then at room temperature overnight. The solvent was removed under reduced pressure and the oily residue was dissolved in AcOEt. The solution was washed with 10% citric acid, 5% Na_2CO_3 and the saturated brine, dried over Na_2SO_4 and concentrated *in vacuo*. Addition of *n*-hexane caused precipitation of the product: Yield 3.1 g (83.4%). mp 156—158°. $[\alpha]_D^{21}$ −8.0° (c=0.3, DMF). Rf^{II} 0.55. *Anal.* Calcd. for $C_{43}H_{55}O_9N_5$: C, 65.71; H, 7.05; N, 8.91. Found: C, 66.08; H, 7.15; N, 9.05.

Z-His-Trp-Ser(Bu^t)-Tyr(Bu^t)-Gly-OEt (2—6a)——Z–Trp–Ser(Bu^t)–Tyr(Bu^t)–Gly–OEt (3—6a, 1.3 g) was hydrogenated for 3 hr over 10% palladium–on–charcoal (0.3 g) in the mixture of MeOH (25 ml) and DMF (5 ml) containing AcOH (0.12 g). The catalyst was filtered off and the filtrate was concentrated under reduced pressure. The residue was dissolved in DMF (10 ml) containing Et_3N (0.2 g) and cooled to 0°. A solution of Z–His–N_3[21] (prepared from Z–His–N_2H_3 (606 mg)) in AcOEt was added with stirring at 0°. After

19) E. Wünsch and J. Jentsch, *Chem. Ber.*, **97**, 2490 (1964); E. Wünsch and A. Zwick, *ibid.*, **99**, 105 (1966).
20) E. Wünsch, A. Zwick, and A. Fontana, *Chem. Ber.*, **101**, 326 (1968).
21) R.W. Holley and E. Sondheimer, *J. Am. Chem. Soc.*, **76**, 1326 (1954).

20 hr, the solvent was removed under reduced pressure and AcOEt was added to the oily residue. The resulted precipitate was collected by filtration and washed with AcOEt: Yield 1.3 g (83.3%). mp 120—123°. $[\alpha]_D^{21}$ −4.8° (c=0.3, DMF). Rf^{II} 0.33. *Anal.* Calcd. for $C_{49}H_{62}O_{10}N_8 \cdot 3H_2O$: C, 60.23; H, 7.01; N, 11.47. Found: C, 60.04; H, 6.81; N, 11.22.

Z-<Glu-His-Trp-Ser(Bu^t)-Tyr(Bu^t)-Gly-OEt (1—6a)——Z-His-Trp-Ser(Bu^t)-Tyr(Bu^t)-Gly-OEt (2—6a, 1.2 g) was hydrogenated for 18 hr over 10% palladium-on-charcoal (0.2 g) in 80% aq. MeOH (100 ml) containing AcOH (0.3 g). The catalyst was filtered off and the filtrate was concentrated to dryness. The residue was dissolved in DMF (10 ml) containing Et_3N (0.24 g) and cooled to 0°. Z-<Glu-OTCP[22] (0.6 g) was added to this solution at 0°. The reaction mixture was kept at 0° for 2 hr and then at room temperature for 20 hr. The solvent was removed under reduced pressure and the residue was dissolved in *n*-butanol. The solution was washed with 1% citric acid, 5% Na_2CO_3, the saturated brine and H_2O, and concentrated. The solid residue was collected by filtration and washed with AcOEt and a small volume of MeOH: Yield 0.6 g (46.1%). mp 165—170°. $[\alpha]_D^{21}$ −4.8° (c=0.3, DMF). Rf^{III} 0.20. *Anal.* Calcd. for $C_{54}H_{67}O_{12}N_9 \cdot 3H_2O$: C, 59.60; H, 6.76; N, 11.58. Found: C, 59.41; H, 6.47; N, 11.43.

<Glu-His-Trp-Ser(Bu^t)-Tyr(Bu^t)-Gly-OH (1—6b)——Z-<Glu-His-Trp-Ser(Bu^t)-Tyr(Bu^t)-Gly-OEt (1—6a, 0.5 g) was hydrogenated for 3 hr over 10% palladium-on-charcoal (0.1 g) in a mixture of DMF (22 ml) and MeOH (6 ml). The catalyst was removed by filtration and the filtrate was concentrated to dryness. The residue was dissolved in DMF (10 ml) and 2 N NaOH (0.5 ml) was added. After 70 min, the reaction mixture was neutralized with 1 N HCl (1.2 ml) and the solvent was removed under reduced pressure. *n*-Butanol extract of the residue was washed with H_2O and evaporated *in vacuo*. The solid residue obtained on addition of AcOEt was collected by filtration and washed with AcOEt: Yield 0.4 g (98.4%). mp 242° (decomp.). Rf^{IV} 0.54. $[\alpha]_D^{21}$ +1° (c=0.3, AcOH). *Anal.* Calcd. for $C_{44}H_{56}O_{10}N_9 \cdot 7/2H_2O$: C, 56.52; H, 6.90; N, 13.48. Found: C, 56.20; H, 6.31; N, 13.10.

Syntheses of the Protected Decapeptide Amides

General Procedure——The protected tetrapeptide amides, Z-Leu-X-Pro-Gly-NH_2 (X=PHT-Orn, PHT-Lys, Cit, Ac-Orn and Bz-Orn; 0.3 mmole), were decarbobenzoxylated by hydrogenolysis over 10% palladium-on-charcoal or the treatment with 25% HBr solution in AcOH. DCCD (0.3 mmole) dissolved in DMF (0.5 ml) was added to a solution of <Glu-His-Trp-Ser(Bu^t)-Tyr(Bu^t)-Gly-OH (1—6b, 0.25 mmole), H-Leu-X-Pro-Gly-NH_2 (prepared from Z-Leu-X-Pro-Gly-NH_2 (0.3 mmole))and HOBt[8] (0.5 mmole) in DMF (6 ml) with stirring at −20 to −25°. After 1 hr, the reaction temperature was elevated to room temperature and the reaction was continued for 3 to 5 days. A mixture of CH_2Cl_2 (20 ml) and ether (150 ml) was added to the reaction mixture and the resulted precipitate was collected by filtration. No further purification was carried out at this stage.

Hydrazinolysis——<Glu-His-Trp-Ser(Bu^t)-Tyr(Bu^t)-Gly-Leu-X-Pro-Gly-NH_2 (X=PHT-Orn, PHT-Lys and PHT-Dab; 0.3 mmole) was dissolved in DMF (3 ml) and hydrazine hydrate (100%; 0.9 mmole) was added with stirring at room temperature. After 2 hr, the reaction mixture was acidified with AcOH and allowed to stand overnight. The precipitated tetrahydrophthalazine-1,4-dione was filtered off and the filtrate was concentrated under reduced pressure. The solid material, which was obtained on addition of AcOEt to the residue, was collected by filtration and washed with AcOEt.

<Glu-His-Trp-Ser(Bu^t)-Tyr(Bu^t)-Gly-Leu-Har(and Nar)-Pro-Gly-NH_2 (1—10a and 1—10b)——<Glu-His-Trp-Ser(Bu^t)-Tyr(Bu^t)-Gly-Leu-Lys(or Dab)-Pro-Gly-NH_2 (100 mg) was dissolved in DMF (0.5 ml). To this solution was added a solution of 1-guanyl-3,5-dimethylpyrazole nitrate[10] (40 mg) in DMF (0.5 ml) containing Et_3N (70 mg) and MeOH (1 ml) with stirring at room temperature. When a ninhydrin positive spot was practically disappeared on TLC (4 days), the solvent was removed under reduced pressure. The solidified residue, which was obtained on addition of CH_2Cl_2, was collected by filtration and washed with CH_2Cl_2 and ether: Yield 97.9 mg (1—10a); 99.1 mg (1—10b). No further purification was carried out at this stage.

<Glu-His-Trp-Ser(Bu^t)-Tyr(Bu^t)-Gly-Leu-Y-Pro-Gly-NH_2 (Y=Orn, Lys, Dab, Ac-Orn, Bz-Orn, Cit, Har or Nar; 150 mg) was treated with dry HF[9] (10 ml) in the presence of anisole (0.5 ml) and thioglycolic acid (0.1 ml) in an ice bath for 30 min. After evaporation of HF, the residue was dissolved in cold H_2O, passed through a column of Dowex 1-X4 (acetate form; 1.5×10 cm) and lyophilized.

Purification of the Decapeptide Amides——Partition column chromatography on Sephadex G-25 (fine, Pharmacia Fine Chemicals AB, Uppsala, Sweden; 3×80 cm) was performed. The solvent system was *n*-butanol–0.1 M ammonium acetate (1:1) (solvent system A) or *n*-butanol–0.1 M ammonium acetate buffer pH 4.5 (1:1) (solvent system B). 0.1 M ammonium acetate buffer pH 4.5 was prepared as follows. Ammonium acetate (7.7 g, 0.1 mole) was dissolved in H_2O (950 ml) and the solution was adjusted to pH 4.5 with glacial AcOH. This solution was filled up to 1000 ml with H_2O. A complete cycle of column operation was consisted of five stages: I, equilibration of the column with the lower phase of the solvent system (500 ml); II, equilibration with the upper phase of the solvent system (150 ml); III, chromatography. Material to be purified (40—60 mg) was dissolved in a small amount of the upper phase, applied and eluted with the upper

22) P.H. Bentley, H. Gregory, A.H. Laird, and J.S. Morley, *J. Chem. Soc.*, **1964**, 6130.

phase (750—1000 ml); IV, washing of the column with 5% AcOH (2000 ml); V, the column was ready to return to stage I of the next cycle. The completion of a cycle usually required 5 to 6 days. Eluent was collected in 7—8 ml portions and absorbancy at 280 nm was measured after the addition of MeOH (1 ml) to eliminate turbidity. The necessary fractions were combined and concentrated to dryness under reduced pressure. The residue was dissolved in 0.1 M AcOH (5 ml), the solution was treated with a small amount of active charcoal and lyophilized to give fluffy powder. The obtained powder was dried to constant weight in desiccator. Har^8-, Nar^8-, Cit^8-, N^δ-Ac-Orn^8- and N^δ-Bz-Orn^8-LH-RH were purified by the solvent system A. The other decapeptide amides, Orn^8-, Lys^8- and Dab^8-LH-RH, were purified by the solvent system B. Acid hydrolysates of these purified peptides showed the correct amino acid ratios as depicted in Table I.

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