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Replacement of the Disulfide Bond in Oxytocin by an Amide Group. Synthesis and Some Biological Properties of [*cyclo*-(1-L-Aspartic acid,6-L-α,β-diaminopropionic acid)]oxytocin[1]

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As part of a continuing investigation of the steric and electronic functions of the disulfide group in neurohypophyseal hormones on their biological activity, the synthesis of "oxytocin lactam", [*cyclo*-(1-aspartic acid,6-α,β-diaminopropionic acid)]oxytocin, has been undertaken. The protected nonapeptide was prepared in a stepwise manner by solution techniques; after removal of side-chain protecting groups, formation of the bridging amide bonds was accomplished by oxidation–reduction condensation. The analogue possesses rat uterotonic, avian vasodepressor, and rat antidiuretic potencies of 16 ± 2, 6.6 ± 0.6, and 5.6 ± 3.8 units/mg, respectively.

The disulfide bridge present in the 20-membered ring portion of neurohypophyseal hormones carries a primary responsibility for the determination of the preferred conformation of the peptide backbone[2] and thereby for the topography of the peptides recognized by the various receptors responsive to these hormones. This point is dramatically illustrated by the nearly total lack of biological activity observed for the acyclic analogues, [$Ala^{1,6}$]oxytocin,[3] [$Ala^{1,6}$]arginine-vasopressin,[4] and [$Ser^{1,6}$]oxytocin.[3] The fact that the reactivity of the disulfide is not required for biological activity is amply demonstrated by the significant biological activities possessed by oxytocin analogues in which the 20-membered ring has been preserved, but one sulfur atom replaced by a methylene group[5-11] or both sulfur atoms by an ethylene group.[6,9,11-15] Changes of the dihedral angle of the bridging group exercise such a profound influence on the overall conformational integrity of the molecule that even a minor modification such as substitution of either one or both sulfur atoms by selenium evokes a definite change in the spectrum of biological activities.[16-18] A displacement of the disulfide bond within the four-atom bridge as in [1-mercaptoacetic acid,6-homocysteine]oxytocin[19] also dramatically alters the activities.

In an earlier investigation of the steric and electronic functions of the disulfide group on biological activity, an analogue of deamino-oxytocin, [*cyclo*-(1-β-alanine,6-aspartic acid)]oxytocin, in which the disulfide has been replaced by an amide group was synthesized.[20] As an extension of this work we wish to report the synthesis and some pharmacological properties of "oxytocin lactam", [*cyclo*-(1-aspartic acid,6-α,β-diaminopropionic acid)]-oxytocin, (Figure 1) in which the amide group is in reverse direction of acylation as that in the previously reported "deamino-oxytocin lactam".[20,21]

The synthetic route followed is summarized in Scheme I. In brief, the COOH-terminal tripeptide amide was extended in a stepwise manner using N^{α}-benzoxycarbonyl (Z) protected amino acids coupled with dicyclohexylcarbodiimide (DCC) mediated by 1-hydroxybenzotriazole[22] (HOBt) except for the NH_2-terminal aspartic acid residue which was coupled as the *N*-hydroxysuccinimide active ester. In all cases catalytic hydrogenation was used to remove the Z group. The side chains of the tyrosine and aspartic acid residues were protected by the *tert*-butyl group and the β-amino function of the α,β-diaminopropionic acid residue was protected by the *tert*-butyloxycarbonyl (Boc) group. These groups were removed from the linear nonapeptide by treatment with CF_3COOH and cyclization was accomplished by oxidation–reduction condensation.[23] The analogue was purified by gel filtration and partition chromatography[24] on Sephadex G-25.

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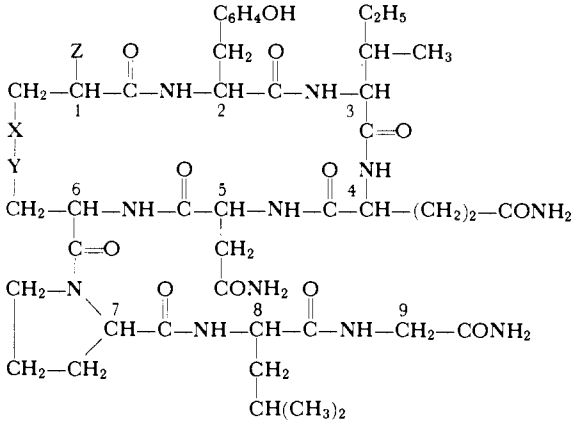


Figure 1. General structure of the oxytocin lactam analogues, [*cyclo*-(1-aspartic acid,6-α,β-diaminopropionic acid)]oxytocin (X = CO; Y = NH; Z = NH_2) and [*cyclo*-(1-β-alanine,6-aspartic acid)]oxytocin (X = NH; Y = CO; Z = H) (reported in ref 20). Numbers indicate position of amino acid residues.

Oxytocin lactam was tested for some of the biological activities characteristic of neurohypophyseal hormones.[25-30] The analogue possesses rat uterotonic, avian vasodepressor, and rat antidiuretic potencies of 16 ± 2, 6.6 ± 0.6, and 5.6 ± 3.8 units/mg, respectively, as compared to oxytocin with 540, 500, and 3 units/mg, respectively. It exhibits a very weak rat pressor response which is not parallel to the response of the standard, and no estimation of its specific activity could be made. The previously reported deamino-oxytocin lactam, [*cyclo*-(1-β-alanine,6-aspartic acid)]oxytocin,[20] exhibited milk-ejecting and uterotonic potencies of 2.93 ± 0.08 and 1.13 ± 0.04 units/mg, and was a weak inhibitor of the oxytocin-induced vasodepressor response. These data demonstrate that the substitution of the disulfide bond with an amide of either of the two isomeric forms results in analogues which retain sufficient conformational integrity to interact with receptors responsive to neurohypophyseal hormones.

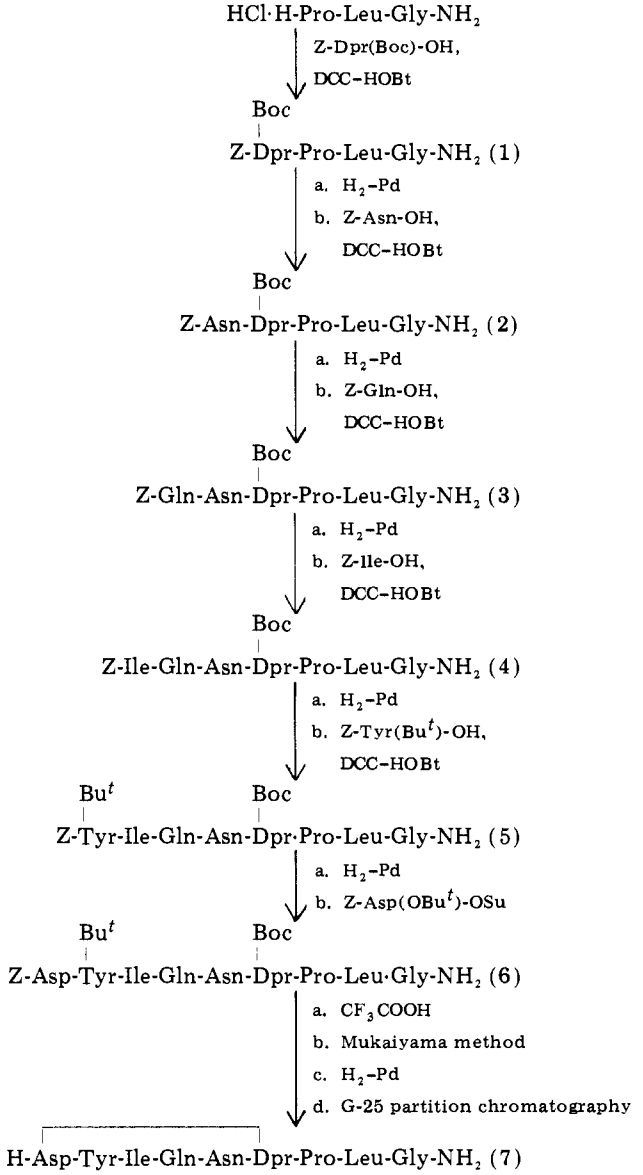
Experimental Section

Details on materials and experimental methods have been described previously.[31] Solvent systems for thin-layer chromatography (Merck silica gel 60) were A, *sec*-BuOH–HCOOH–H_2O (75:13.5:11.5); B, *sec*-BuOH–10% NH_4OH (85:15); C, $CHCl_3$–MeOH (5:1).

Rat uterine assays were performed on isolated horns from virgin rats in natural estrus according to the method of Holton, as modified by Munsick, with the use of Mg^{2+}-free van Dyke–Hastings solution as bathing fluid.[25] Avian vasodepressor assays were performed on conscious chickens by the method of Coon, as described in the U.S. Pharmacopeia, as modified by Munsick et al.[26] Pressor assays were carried out on anesthetized male rats as described in the U.S. Pharmacopeia.[27] Antidiuretic assays were performed on anesthetized male rats according to the method of Jeffers et al.,[28] as modified by Sawyer.[29] The four-point assay design of Schild[30] was used to obtain specific activities as compared to U.S.P. posterior pituitary reference standard.

Z-Dpr(Boc)-Pro-Leu-Gly-NH_2 (1). N^{α}-Benzyloxycarbonyl-N^{β}-*tert*-butyloxycarbonyl-L-α,β-diaminopropionic acid[32] (3.56 g, 10.5 mmol) was dissolved in DMF (20 mL), and a solution of HCl·H-Pro-Leu-Gly-NH_2[33,34] (3.36 g, 10.5 mmol) in DMF (20 mL) was added. The solution was cooled to 0 °C, and HOBt (2.56 g, 16.7 mmol) and DCC were added (2.4 g, 11.6 mmol), followed by NEt_3 (1.47 mL, 10.5 mmol). The reaction was stirred for 45 min at 0 °C and for 15 h at 25 °C. Dicyclohexylurea was removed by filtration and the product precipitated by pouring the filtrate into H_2O. This aqueous suspension was extracted with EtOAc (3 × 100 mL). The combined organic phases were washed with 1 M citric acid (three times), H_2O, 1 M $NaHCO_3$ (three times), and saline (twice), dried, and evaporated to a white residue which was crystallized from EtOAc–petroleum ether: yield 4.1 g (64.6%); mp 137–140 °C; $[\alpha]^{25}_D$ −48.9° (*c* 0.95, DMF); homogeneous on TLC (C). Anal. ($C_{29}H_{44}N_6O_8$) C, H, N.

Scheme I. Synthetic Pathway for the Preparation of [*cyclo*-(Asp^1,Dpr^6)]oxytocin[a]



[a] DCC, dicyclohexylcarbodiimide; Dpr, L-α,β-diaminopropionic acid; HOBt, hydroxybenzotriazole.

Z-Asn-Dpr(Boc)-Pro-Leu-Gly-NH_2 (2). Compound 1 (4.18 g, 6.9 mmol) was dissolved in CH_3OH (50 mL) and 5% Pd/C (~1 g) plus 2 drops of AcOH were added. The solution was hydrogenated for 7 h, the catalyst removed by filtration through a Celite pad, and the filtrate evaporated to dryness. The residue was dissolved in DMF (12 mL), Z-Asn-OH (1.84 g, 6.9 mmol) and HOBt (1.7 g, 11.1 mmol) were added, and the solution was cooled to 0 °C. DCC (1.57 g, 7.6 mmol) was added and the solution stirred for 1 h at 0 °C and 15 h at 25 °C. Dicyclohexylurea was removed by filtration and the filtrate evaporated. The residue was triturated with a small volume of H_2O, filtered, and crystallized from 2-propanol: yield 4.0 g (80.6%); mp 209–211 °C; $[\alpha]^{25}_D$ −36.4% (*c* 1.06, DMF); homogeneous on TLC (B, C). Anal. ($C_{33}H_{50}N_8O_{10}$) C, H, N.

Z-Gln-Asn-Dpr(Boc)-Pro-Leu-Gly-NH_2 (3). The Z group was cleaved from **2** (3 g, 4.1 mmol) as described above. The free base was dissolved in DMF (9 mL), Z-Gln-OH (1.16 g, 4.1 mmol) and HOBt (1.01 g, 6.6 mmol) were added, and the solution was cooled to 0 °C. DCC (0.84 g, 4.1 mmol) was added and the reaction mixture stirred at 0 °C for 1 h and at 25 °C for 15 h. Work-up as described above and crystallization from 95% EtOH yielded 2.5 g (72.0%) of **3**: mp 188 °C; $[\alpha]^{25}_D$ −45.9° (*c* 1, DMF); ho-

mogeneous on TLC (B). Anal. ($C_{38}H_{58}N_{10}O_{12}$) C, H, N.

Z-Ile-Gln-Asn-Dpr(Boc)-Pro-Leu-Gly-NH_2 (4). Compound 3 (2.5 g, 2.95 mmol) was dissolved in DMF (25 mL), Pd-black catalyst (0.5 g) added, and the reaction hydrogenated for 5 h. The catalyst was removed by filtration and the filtrate concentrated to 10 mL. This solution was cooled to 0 °C and Z-Ile-OH (0.86 g, 3.24 mmol), HOBt (0.52 g, 3.4 mmol), and DCC (0.67 g, 3.2 mmol) were added. The reaction was stirred for 1 h at 0 °C and for 15 h at 25 °C. After 24 h, TLC (A, B) indicated incomplete reaction and a further quantity of acylating agent [Z-Ile-OH (0.18 g, 0.68 mmol), HOBt (0.1 g, 0.68 mmol), and DCC (0.13 g, 0.63 mmol)] was added after preactivation for 45 min at 0 °C in DMF. The reaction mixture was stirred for a further 24 h, dicyclohexylurea removed by filtration, and the product precipitated by pouring the filtrate into H_2O. The precipitate was filtered and washed copiously with 1 M citric acid, H_2O, 1 M $NaHCO_3$, and H_2O. The residue was dried and crystallized from aqueous DMF: yield 1.6 g (56.5%); mp 174 °C; $[\alpha]^{25}_D$ −41.9° (c 1, DMF); homogeneous on TLC (A). Anal. ($C_{44}H_{69}N_{11}O_{13}$) C, H, N.

Z-Tyr(Bu^t)-Ile-Gln-Asn-Dpr(Boc)-Pro-Leu-Gly-NH_2 (5). Compound 4 (1.5 g, 1.56 mmol) was hydrogenated in DMF for 7 h as described for 4. The concentrated solution (5 mL) of the free base was cooled to 0 °C and Z-Tyr(Bu^t)-OH (0.64 g, 1.7 mmol), HOBt (0.38 g, 2.5 mmol), and DCC (0.35 g, 1.7 mmol) were added. The reaction was stirred at 0 °C for 1 h and at 25 °C for 24 h. Work-up and crystallization as described for 4 yielded 1.25 g (82%) of colorless crystals: mp 234–235 °C; $[\alpha]^{25}_D$ −32.8° (c 1, DMF); homogeneous on TLC (A, B). Anal. ($C_{57}H_{86}N_{12}O_{15}$) C, H, N.

Z-Asp(OBu^t)-Tyr(Bu^t)-Ile-Gln-Asn-Dpr(Boc)-Pro-Leu-Gly-NH_2 (6). Compound 5 (0.55 g, 0.466 mmol) was hydrogenated in DMF for 14 h, as described for 4. The concentrated solution (4 mL) was cooled to 0 °C, Z-Asp(OBu^t)-OSu added (0.2 g, 0.48 mmol), and the reaction stirred for 1 h at 0 °C and for 24 h at 25 °C. Work-up and crystallization as described for 4 yielded 0.45 g (71.5%) of colorless crystals: mp 218–219 °C; $[\alpha]^{25}_D$ −45.8° (c 0.1, DMF); homogeneous on TLC (A, B). Anal. ($C_{65}H_{99}N_{13}O_{18}$) C, H, N.

[*cyclo*-(1-Aspartic acid,6-α,β-diaminopropionic acid)]-oxytocin (7). Compound 6 (0.2 g, 0.148 mmol) was dissolved in CF_3COOH (5 mL) and the solution stirred for 1 h at 0 °C. After evaporation, the residual oil was triturated with ether, the trifluoroacetate salt was dissolved in AcOH (3 mL) and 1.6 M HCl–AcOH (0.4 mL), and the solution was poured into vigorously stirred anhydrous ether. The hydrochloride was filtered off and dissolved in DMF [10 mL, neutralized with NEt_3 (0.04 mL)], and a further quantity of DMF was added (90 mL). 2,2-Dipyridyl disulfide (0.12 g, 0.54 mmol) and triphenylphosphine (0.14 mg, 0.54 mmol) were added and the reaction was stirred for 72 h. The solvent was evaporated, the residual yellow oil dissolved in CH_3OH (2 mL), and H_2O (200 mL) added. The oily precipitate was extracted into EtOAc (3 × 75 mL) and the combined organic phases were dried ($MgSO_4$) and evaporated. The residue was reprecipitated from MeOH–EtOAc to yield 80 mg of crude N^{α}-Z-protected oxytocin lactam. A part (45 mg) of this material was dissolved in DMF (20 mL) and freshly prepared Pd-black (~0.1 g) added. The solution was hydrogenated for 4.5 h. The catalyst was removed by filtrations through a Celite pad, and the filtrate was evaporated to a light yellow residue (40 mg) which was chromatographed on a 2.5 × 100 cm column of Sephadex G-25 and eluted with 0.2 M AcOH. Fractions (5 mL) containing the major product with minor impurities were pooled and lyophilized to yield 34 mg of a colorless powder. This was further subjected to partition chromatography[24] on a 2.2 × 52 cm column of Sephadex G-25 (100–200 mesh block polymerizate) in the system 1-BuOH–H_2O containing 3.5% AcOH and 1.5% pyridine (1:1, v/v). The column, which had been equilibrated with both phases of the solvent system, was eluted with upper phase at 12–16 mL/h into 3.2-mL fractions. Peptide material was detected by the method of Lowry et al.[35] The product emerged as a symmetrical peak with a maximum at R_f 0.16; fractions corresponding to the peak area (86–100) were pooled and the product was isolated by lyophilization: wt 16.0 mg; $[\alpha]^{25}_D$ −106° (c 0.5, 1 N AcOH). TLC showed the product as a single spot, R_f 0.24 (BuOH–AcOH–H_2O, 4:1:1), R_f 0.53 (BuOH–pyridine–AcOH–H_2O, 15:10:3:6), in loads up to 50 μg developed for 120 mm. Anal. ($C_{44}H_{67}N_{13}O_{13}\cdot CH_3COOH\cdot H_2O$) (986.1 + 78.1) C, H, N. Amino acid analysis[36] (6 N HCl–phenol, 110 °C, 24 h): Dpr, 0.99; Asp, 1.95; Glu, 1.02; Pro, 1.00; Gly, 1.00; Ile, 0.97; Leu, 1.01; Tyr, 1.05.

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References and Notes

(1) Abbreviations follow generally the IUPAC–IUB Tentative Rules on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 577 (1972), except Dpr, L-α,β-diaminopropionic acid. Optically active amino acids are of the L configuration. Boc, *tert*-butyloxycarbonyl; Bu^t, *tert*-butyl; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; OSu, *N*-hydroxysuccinimide ester; Z, benzyloxycarbonyl.
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(33) Prepared from Boc-Pro-Leu-Gly-NH_2 (ref 34) by treatment with 2 M HCl in tetrahydrofuran for 1 h at room temperature: mp 181–184 °C; $[\alpha]^{25}_D$ −50.6° (*c* 0.1, 0.1 M HCl). Anal. ($C_{13}H_{25}N_4O_3Cl$) Cl.
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Inhibitory Analogues of the Luteinizing Hormone-Releasing Hormone Having D-Aromatic Residues in Positions 2 and 6 and Variation in Position 3[1]

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A single sc injection of 750 μg/rat of [D-Phe^2,Pro^3,D-Phe^6]-LH-RH on proestrus completely inhibited ovulation in 4-day cycling rats. Ovulation was inhibited partially at 375 μg/rat. At the 750 μg/rat dosage, analogues of LH-RH with D-Phe in positions 2 and 6, and with Sar, Arg, or Hyp in position 3, resulted in partial inhibitors and an inactive analogue with Thr substitution. In the same assay, analogues with D-Phe in position 2 and D-Trp in position 6 were partial inhibitors when D-Phe, Met, or Val were in position 3 and inactive with Ile substitution.

Since our report that a disubstituted LH-RH analogue, [Leu^2,Leu^3]-LH-RH, could inhibit the action of LH-RH in an in vitro assay using isolated whole rat pituitaries,[2] we have synthesized more analogues based on this sequence.[3,4] Of special interest was the design of an LH-RH inhibiting peptide that could effectively suppress ovulation.

The observations by Corbin and co-workers,[5] that [D-Phe^2,D-Ala^6]-LH-RH could inhibit ovulation in rats and rabbits and prevent pregnancy when appropriately administered, precoitally, at a dosage of 24 mg/kg sc, have stimulated the synthesis of analogues of LH-RH having a D-Phe residue in position 2. It was also realized that the incorporation of a D-amino acid residue in position 6, which followed from some of the work of Monahan et al.,[6] was seemingly essential for high antiovulatory activity in rats. However, when the modification of Fujino et al.[7] was applied to the [D-Phe^2,D-Ala^6]-LH-RH sequence, the resulting des-Gly^{10}-[D-Phe^2,D-Ala^6]-LH-RH ethylamide was found to be a superior inhibitor of LH-RH action, in vitro, using a monolayer technique but did not inhibit ovulation.[8]

Recently, Humphries et al.[9] reported that [D-Phe^2,-Pro^3,D-Trp^6]-LH-RH inhibited the release of LH and FSH by LH-RH, in vitro, at a ratio of analogue:LH-RH of 50:1. This analogue also completely inhibited ovulation in 4-day cycling rats at a single sc injection of 750 μg, and partial inhibition of ovulation was also observed at a 375-μg dosage. The analogues, [D-Phe^2,Leu^3,D-Trp^6]-LH-RH and [D-Phe^2,Leu^3,D-Phe^6]-LH-RH were less effective. Bowers and Folkers[10] have shown that the infusion into rats of [D-Phe^2,Pro^3,D-Trp^6]-LH-RH, from a sc implanted, osmotically driven minipump, at a rate of 375 μg/day for 4 days, completely inhibited ovulation in 4-day cycling rats.

This paper describes the synthesis and activities of some further analogues of LH-RH, having a D-Phe residue in position 2, amino acid substitution in position 3, and a D-aromatic amino acid in position 6, in a continuing effort to evaluate structure–activity relationships for inhibition of ovulation.

Experimental Section

The procedure of solid-phase peptide synthesis was essentially identical with that described.[4] Amino acid derivatives were supplied by Peninsula Laboratories, Beckman Bioproducts Division, or Sigma. Product yields (%) were estimated from the starting amino acid-resin. On chromatography, the product of the major peaks was examined by TLC. Usually only those fractions corresponding to the upper parts of the peak were taken, and consequently the percentage yields will be low. Completed, protected peptide-BHA resins were cleaved and deblocked by reaction for 1 h at 0 °C with CoF_3-dried liquid HF[11] containing ca. 20% anisole.

TLC on silica gel was used to evaluate product purity, with the systems R_f^1, EtOAc–H_2O–AcOH–1-BuOH (1:1:1:1 v/v); R_f^2, EtOAc–pyridine–AcOH–H_2O (5:5:1:3 v/v); R_f^3, propan-2-ol–1 N AcOH (2:1 v/v); and R_f^4, 0.1% AcOH–1-BuOH–pyridine (11:5:3 v/v, upper phase). Peptide spots were negative to ninhydrin and positive to chlorine–*o*-tolidine reagent. Amino acid analyses, on ca. 0.5-mg samples hydrolyzed in 6 N HCl in evacuated and sealed ampules for 18 h at 110 °C, were performed using a single column methodology on a Beckman Model 119 amino acid analyzer equipped with an Infotronics Model CRS-210 automatic digital integrator. Optical rotations were measured on a Perkin-Elmer 141 digital read-out polarimeter.

Synthesis of the 3-Substituted [D-Phe^2,D-Phe^6]-LH-RH Analogues. Benzhydrylamine (BHA)-resin hydrochloride (10 g, 0.44 mequiv/g, Beckman Bioproducts) was sized in CH_2Cl_2, neutralized, and coupled with Boc-Gly. The Boc-Gly-BHA resin was submitted to six cycles of deprotection, neutralization, and coupling to yield the heptapeptide BHA-resin, Boc-Ser(Bzl)-Tyr(BrZ)-D-Phe-Leu-Arg(Tos)-Pro-Gly-BHA resin (15.38 g).

Two-gram portions of the heptapeptide BHA-resin were submitted to further coupling cycles, with the appropriate position 3 amino acid derivative, then Boc-D-Phe, and finally Z-<Glu-OH. The protected decapeptide BHA-resins were simultaneously cleaved from the resin and deblocked with HF and purified over Sephadex G-25 (95 × 2.5 cm) with 10% AcOH, followed by ion-exchange chromatography on CM-Sephadex (26 × 1.5 cm) with an NH_4OAc gradient (1 mM, pH 4.5, to 125 mM). In the case of the Arg^3 analogue, 250 mM NH_4OAc was required to elute this highly basic peptide.

[D-Phe^2,Pro^3,D-Phe^6]-LH-RH: yield 423 mg (62%); amino acid analysis gave Glu 1.05, Phe 2 × 1.06, Pro 2 × 1.03, Ser 0.83, Tyr 0.94, Leu 0.91, Arg 0.98, Gly 0.93; R_f^1 0.80, R_f^2 0.91, R_f^3 0.87; $[\alpha]^{24}_D$ −71.60° (*c* 9.38, MeOH).

[D-Phe^2,Sar^3,D-Phe^6]-LH-RH: yield 348.5 mg (52.25%); amino acid analysis gave Glu 1.1, Phe 2 × 1.09, Ser 0.83, Tyr 0.86, Leu 0.97, Arg 1.01, Gly 0.92; R_f^1 0.71, R_f^2 0.80, R_f^3 0.76; $[\alpha]^{24}_D$ −63.59° (*c* 10.3, MeOH).

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