Synthesis of Gonadotropin-Releasing Hormone III Analogs. Structure−Antitumor Activity Relationships

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Following the observation that the activity of gonadotropin-releasing hormone III (GnRH-III) in the suppression of growth of MDA-MB-231 and MCF-7 breast cancer cells surpasses that of GnRH and other analogs thereof, analogs of GnRH-III were synthesized to investigate the structural basis for the improved antitumor activity. Compounds synthesized include analogs with changes in the central sequence in which GnRH-III differs from GnRH and in the C- and N-terminal regions. The results indicate that a salt bridge between Asp^6 and Lys^8 stabilizes the active conformation of GnRH-III and show the importance of the Trp^7. Replacement of the C-terminal Gly-NH_2 with D-Ala-NH_2 was not well tolerated, but replacement with ethylamide was. Replacement of $pGlu^1$ with Ac-D-Trp appears to have a significantly deleterious effect on a unique conformation of GnRH-III which is responsible for its binding to the receptors on cancer cell lines and the resultant antitumor activity.

Introduction

Hormonal therapy with steroids can play an important role in the treatment of hormone-dependent malignant tumors. Agonists and antagonists of the hypothalamic peptide gonadotropin releasing hormone (GnRH; Figure 1) are used to treat breast and prostate tumors.[1−4] The GnRH analogs can inhibit the growth of tumors indirectly by causing estrogen deprivation. The primary site of action of GnRH in the pituitary gland is the cells that express GnRH-receptors and secrete gonadotropic hormones.[5] Paradoxically, continuous exposure of the pituitary gland to long-acting GnRH agonists causes down-regulation of GnRH-receptors, leading to a profound decrease in gonadotropin secretion and even chemical castration.[6−8] GnRH analogs can also exert their antitumor activity by affecting the tumor cells directly. The presence of GnRH binding sites in human mammary cancer,[9,10] Ishikawa endometrium,[11] LNCaP prostate carcinoma,[12] and Dunning R3327H prostate tumor[13] cells has been reported.

The direct antitumor activity of the GnRH variant GnRH-III (Figure 1) of the sea lamprey[14] *Petromyzon marinus* surpasses[15] that of the pituitary agonist Buserelin ([D-Ser(^{t}Bu)]^6-hGnRH-(1−9)-EA) and of the antagonists SB-30[16] and MI-1544.[17,18] GnRH-III has 1000-fold weaker activity than has GnRH in releasing LH from superfused rat pituitary cells, indicating selective antitumor activity.[19] The superior antitumor activity of GnRH-III compared with other GnRH analogs may be dependent on the structural features, sequence 5−8, which are different in GnRH-III. Thus, analogs synthesized (Table 1) to establish the structural basis for the improved antitumor activity of GnRH-III include compounds with changes in sequence 5−8.

GnRH: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

GnRH-III: pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2

Figure 1. Primary structure of gonadotropin releasing hormones.

Compounds **1**−**8** (Table 1) were synthesized to study electrostatic interference with a putative salt bridge between residues Asp^6 and Lys^8. Compounds **11** and **12** were intended to have stability to proteolysis by carboxypeptidases and compound **13** by both carboxypeptidase and aminopeptidases.[20−22] Compounds **10** and **15** were synthesized as precursors of tritiated analogs, compounds **16** and **17**, respectively, for receptor binding studies to be published elsewhere, but they were also biologically tested. The formyl groups were introduced to protect the indole side chains of $Trp^{3,7}$ during the catalytic reduction of the double bond in $\Delta^{3,4}$-Pro^9 of **10** with tritium gas during formation of **16**. The antitumor activity of analogs was assessed by studying the effects of peptides on cell proliferation and colony formation by human MCF-7 and MDA-MB-231 breast, Ishikawa endometrium, and PC3 prostate cancer cell lines (Tables 2 and 3).

Results and Discussion

All the analogs of GnRH-III were prepared by solid-phase peptide synthesis and purified as described previously for GnRH and related derivatives[23] (see the Experimental Section). The structure of the cyclic peptides was verified using FAB-MS combined with proteolytic digestion and collision-induced dissociation (CID). Tryptic digestion of **3** resulted in hydrolytic

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Table 1. Chromatographic Characterization of GnRH-III Analogs

compound	TLC[a] R_f	k' in HPLC[b] a	k' in HPLC[b] b
1, [Lys^5]GnRH-III	0.4 (A), 0.15 (B)	6.50 (30)	1.19 (30)
2, [ϵ-*N*-Fmoc-Lys^5]GnRH-III	0.5 (B)		
3, [Lys^5,cyclo(Asp^6,Lys^8)]GnRH-III	0.35 (B), 0.3 (D)	3.57 (38)	
4, [cyclo(Asp^6,Lys^8)]GnRH-III	0.76 (A), 0.3 (D)	4.83 (38)	1.57 (30)
5, [Lys^4,ϵ-*N*-Fmoc-Lys^8]GnRH-III	0.45 (B), 0.17 (D)	9.28 (55)	28.2 (30)
6, [Lys^4]GnRH-III	0.5 (A), 0.07 (B)	3.57 (30)	1.69 (30)
7, [ϵ-*N*-Ac-Lys^4]GnRH-III	0.07 (B), 0.09 (D)	11.57 (30)	1.54 (30)
8, [Glu^6]GnRH-III		7.57 (30)	1.50 (30)
9, [Phe^7]GnRH-III	0.65 (A)		2.9 (17)
10, [N^{in}-For-Trp^{3,7},$\Delta^{3,4}$-Pro^9]GnRH-III	0.68 (A), 0.04 (D)		2.58 (70)
11, GnRH-III-(1−9)-EA	0.78 (A), 0.26 (D)	3.33 (45)	2.27 (30)
12, [D-Ala^{10}]GnRH-III	0.7 (A)	9.29 (30)	1.50 (30)
13, Ac[D-Trp^1,D-Ala^{10}]GnRH-III	0.8 (A), 0.36 (D)	10.9 (40)	10.9 (30)
14, [Asu^6]GnRH-III[27]			
15, [$\Delta^{3,4}$-Pro^9]GnRH-III			3.13 (16)
16, [N^{in}-For-Trp^{3,7},(^{3}H)Pro^9]GnRH-III	see text		−
17, [(^{3}H)Pro^9]GnRH-III	−	see text	

[a] Solvents are described in the Experimental Section. [b] Solvents: a, % (v/v) methanol in 0.1 M NaH_2PO_4 (pH = 2.22); b, % (v/v) acetonitrile in 0.1 (v/v) TFA in water. $k' = (t_R - t_0)/t_0$.

Table 2. Effect of GnRH-III Analog (50 mM) on Colony Formation by Human Tumor Cell Lines

compound	% inhibition MCF-7	MDA-MB-231	Ishikawa	PC3
1	5 ± 0.5	4 ± 0.3	6 ± 0.5	0
3	44 ± 5.6	27 ± 3.1	13 ± 1.4	11 ± 1.3
4	44 ± 4.7	27 ± 2.6	16 ± 2.0	18 ± 2.6
6	40 ± 4.0	35 ± 3.9	21 ± 1.6	15 ± 2.1
7	52 ± 5.3	42 ± 5.1	14 ± 1.5	12 ± 1.6
8	44 ± 3.1	25 ± 2.8	5 ± 0.3	20 ± 3.0
9	26 ± 3.0	21 ± 3.4	nt[a]	nt
10	31 ± 3.9	32 ± 4.1	24 ± 2.8	20 ± 2.8
11	28 ± 2.1	27 ± 3.0	13 ± 1.6	22 ± 1.9
12	4 ± 0.4	15 ± 1.1	6 ± 0.5	3 ± 0.2
13	14 ± 1.6	20 ± 1.8	5 ± 0.8	11 ± 0.9
14	18 ± 2.0	nt	nt	nt
15	42 ± 4.9	42 ± 5.2	21 ± 2.3	19 ± 17
GnRH-III	45 ± 4.9	44 ± 4.3	18 ± 2.0	21 ± 2.3
[D-Trp^6]GnRH (decapeptyl)	27 ± 2.2	29 ± 2.7	17 ± 1.8	15 ± 1.3

[a] nt = not tested.

Table 3. Effect of GnRH-III Analogs (50 mM) on Cell Proliferation by Human Tumor Cell Lines Using the Sulforhodamine B Assay

compound	% inhibition MCF-7	MDA-MB-231	PC3	LNCaP
1	4 ± 0.5	2 ± 0.3	nt[a]	nt
3	19 ± 4.7	21 ± 5.0	16 ± 4.1	nt
4	20 ± 4.8	16 ± 3.6	11 ± 2.8	nt
8	17 ± 4.0	27 ± 4.8	12 ± 2.6	nt
11	20 ± 4.0	32 ± 3.9	15 ± 3.1	nt
12	8 ± 2.2	8 ± 2.0	2 ± 04	nt
13	14 ± 4.1	16 ± 4.6	7 ± 1.8	nt
14	18 ± 2.0	18 ± 3.9	14 ± 3.6	32 ± 2.9
15	19 ± 4.7	18 ± 3.9	14 ± 3.6	nt
GnRH-III	20 ± 3.8	19 ± 4.6	12 ± 3.0	40 ± 5.0

[a] nt = not tested.

products containing the amino acid residues of sequences 1−5 and 6−10. The lack of cleavage after Lys^8 verified the expected involvement of the ϵ-NH_2 group in the cyclic structure. [*Cyclo*(Asp^6,Lys^8)]GnRH-III, **4**, did not yield any tryptic product, presumably due to the ring closure between the Asp^6 and Lys^8 side chains, while chymotryptic digestion resulted in a hydrolytic product containing the residues of sequence 4−10 (peak at m/z 807) presumably due to cleavage after Trp^3. The cyclic structure was also verified by CID measurement; when Ar collided with the peptide, an intense molecular ion peak (m/z 1070) due to residues 1−8 was observed.

Tritium labeling of **10** and **15** resulted in **16** and **17** with specific activity of 82 and 57 Ci/mmol, respectively. Chromatographic profiles were not altered due to tritiation so further purifications were not necessary.

Replacement of His^5 with Lys in **1** caused loss of the antitumor activity displayed by GnRH-III in suppressing both colony formation and cell proliferation (Tables 2 and 3). While the opportunity for hydrogen-bonding is altered when the weakly basic and aromatic histidyl residue is replaced by a more basic residue with an ionic side chain, the loss of antitumor activity might also be due to interference by the Lys^5 side chain with an ionic interaction between the side chains of Asp^6 and Lys^8. To examine the conformational and biological importance of such an interaction, a steric conformer (**3**), in which the conformational constraint, made possible by electrostatic interaction between the side chains of Asp^6 and Lys^8 in **1**, is ensured by a covalent bond between the same side chains, was prepared. **3** had significant though lower antitumor activity than GnRH-III even though residue 5 still had been changed from His to Lys. The comparable activity of **4**, however, shows that the Lys insertion in place of His at position 5 is of no significance *per se* whereas Lys insertion can cause abolition of activity when, as in **1**, it should have the opportunity to disrupt the conformation maintained by the putative salt bridge. Lys inserted at position 4, in place of Ser, as in **6** and **7**, rather than in postion 5, as in **1**, apparently is too far away to disrupt the salt bridge, since comparable significant antitumor activity is detected with and without acetylation of the lysyl side chain in **7** and **6**, respectively (Table 2).

Displacement of the side chain carboxyl group from a β to a γ position, in residue 6 of **8**, decreased the antitumor activity measured by the clonogenic assay with MDA-MB-231 breast and Ishikawa endometrial cancer cell lines (Table 2) whereas in cell proliferation the antitumor activity increased with MDA-MB-231 breast cancer cells (Table 3). It appears that the displacement did not alter the bioactive conformation of GnRH-III. Replacement of Trp^7 with Phe in **9** caused a dramatic decrease in antitumor activity, suggesting

the involvement of the indole ring in the interaction of the peptide with the receptor.

The C-terminal replacement of Gly-NH_2 with ethyl amide in **11** was tolerated with some loss of antitumor activity, whereas the D-Ala[10] replacement in **12** resulted in loss of activity. Similar loss of activity was observed when the N-terminal pGlu residue was replaced with Ac-D-Trp.

The double bond in the $\Delta^{3,4}$-Pro[9] of **15** did not interfere with the biological activity (Tables 2 and 3), probably because it does not affect the conformation of the peptide. The slight reduction of activity in **10**, however, is attributed to the formyl substitution of the indole rings and is further evidence of the involvement of the indole rings in the interaction of GnRH-III with the receptor.

The GnRH-III analogs were more effective in the clonogenic assay than in the antiproliferation experiments. In the latter case, the lower inhibitory values are possibly due to the much higher initial cell numbers which, in turn, produced a higher amount of autocrine growth factors than did the cells in the clonogenic assay.[24]

Conclusions

The results indicate that an ionic interaction between the Asp[6] and Lys[8] residues stabilizes the biologically active conformation of GnRH-III. The lower antitumor activity of the cyclic analogs emphasizes the significance of ionic side chains. The antitumor activity of some analogs (**11**, **7**, and **4**) was close to that of the GnRH-III, but the latter was still the most potent compound.

The importance of the indole rings of the Trp[3,7] residues was also demonstrated. Replacement of the C-terminal Gly-NH_2 with D-Ala-NH_2 was not well-tolerated, but replacement with ethylamide was. Replacement of pGlu[1] with Ac-D-Trp also reduced antitumor activity. An increase in activity, however, might have been expected because of possible protection from any pyroglutamase present in the medium and because this replacement facilitates retention of the endocrine functions of other GnRH analogs.[20−22] Thus, it appears that the Ac-D-Trp replacement has a significantly deleterious effect on a unique conformation of GnRH-III, which is responsible for its binding to the receptors on cancer cell lines and the resultant antitumor activity.

Experimental Section[28]

Benzhydrylamine resin (0.6 mmol g^{-1}) and Merrifield resin (ca. 1.0 mmol g^{-1}) were obtained from Bachem (Bubendorf, Switzerland). 4-(2′,4′-Dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin (0.4 mmol g^{-1}) was provided by Bachem California (Torrence, CA). Boc-D-Ala, Boc-Lys(2-ClZ), Boc-Lys(Fmoc), Boc-Ser(Bzl), Boc-His(Tos), Boc-Asp(OcHex), Boc-Asp(OFm), Boc-Glu(OcHex), Boc-D-Trp, Boc-Trp(For), and Boc-$\Delta^{3,4}$-Pro were purchased from Bachem (Bubendorf, Switzerland). Boc-Gly, Boc-Pro, and Boc-Trp were supplied by Reanal, Inc. (Budapest, Hungary). Fmoc-Asp(OtBu), Fmoc-Gly, Fmoc-His(Trt), Fmoc-Lys(tBoc), Fmoc-Phe, Fmoc-Pro, Fmoc-Ser(tBu), and Fmoc-Trp were purchased from ABI (Foster City, CA). Fmoc-$\Delta^{3,4}$-Pro was purchased from Advanced ChemTech (Louisville, KY). Pyroglutamic acid was obtained from Fluka Chemie AG (Buchs, Switzerland). BOP and TBTU reagents were purchased from Richelieu Biotechnologies, Inc. (Montreal, Canada). Pd/$BaSO_4$ (10% Pd) was supplied by Merck-Schuchardt (Hohenbrunn, Germany). All solvents and other reagents were of reagent grade or better and used without further purification. A Synostat P synthesizer was bought from Biotronic GmbH (Maintal, Germany), and ABI 430 A and 432 A synthesizers were bought from ABI (Foster City, CA). An HF apparatus for cleavage and deprotection was bought from Peptide Institute, Inc. (Osaka, Japan). DC-Alufolien Kieselgel 60 (Merck) was used for TLC analysis of synthetic products with the following solvents: A, EtOAc/pyridine/AcOH/H_2O 15:20:6:11(v/v/v/v); B, EtOAc/pyridine/AcOH/H_2O 30:20:6:11 (v/v/v/v); C, EtOAc/pyridine/AcOH/H_2O 36:30:9:16.5 (v/v/v/v); D, *n*-butanol/AcOH/H_2O 4:1:2 (v/v/v).

1−**8** and **10**−**14** were synthesized on the Synostat P synthesizer using *N*-α-Boc-protected amino acids as previously described.[23] **9** and **15** were synthesized on ABI 430A and ABI 432A synthesizers, respectively, using *N*-α-Fmoc-protected amino acids and the standard synthesis protocols provided by the manufacturer. Side chain cyclization in **3** and **4** was performed using BOP with DIEA as catalyst. Peptides were purified as required by gel filtration, unless otherwise stated below, on columns of Sephadex G-25 (2.5 cm x 100 cm) equilibrated with a solution of acetic acid (33% v/v) in water followed either by medium pressure liquid chromatography (MPLC) on columns of Prepex RPC18 (20−44 mm particle size) using the solvents described below for the individual peptides or by HPLC.

The decapeptide precursors **10** and **15**, containing $\Delta^{3,4}$-Pro[9], were tritiated by catalytic saturation of the double-bond using $^3H_2$ gas and Pd/$BaSO_4$ catalyst (10% Pd, Merck Art, 807105) in a specially designed vacuum manifold.[25] Radioactive purity of the products was determined using HPLC.

The FAB-MS and FAB-MS/MS experiments were performed with a VG VAB-2SEQ hybrid tandem mass spectrometer coupled to an OPUS 2000 data system. The instrument was equipped with a liquid secondary ion source (Cs^+ ion gun used at 30 keV) and a second field-free region collision cell. The CID experiments were carried out at 8 keV ion energy, using argon as collision gas. For analyses, peptides were dissolved in DMSO and mixed with glycerol. For proteolytic digestion, performed directly on the FAB probe tip, the peptides were dissolved in 50 mM NH_4HCO_3 buffer, pH 8, and mixed on the stainless steel target with the appropriate enzyme in the same buffer. After 5 min, the reaction was stopped by adding 1 mL of either 0.5 M HCl or 0.5 M TFA. This mixture was diluted with 1 mL of glycerol and subjected to FAB-MS analysis.

The effects of peptides on growth of human MCF-7 and MDA-MB-231 breast, Ishikawa endometrium, and PC3 prostate cancer cell lines was examined using clonogenic, cell proliferation, and sulforhodamine assays as described previously.[24] In the clonogenic assay, colony formation reflects survival of cells. In the cell proliferation assay, cells are directly counted after release from the Petri dishes by trypsinization and in the sulforhodamine assay, dye bound to cells is quantified spectrophotometrically, with results expressed as percentage of control values.

[Lys[5]]GnRH-III (1). The peptide was synthesized in 1 mmol scale. After gel filtration, the peptide was purified by MPLC using the linear solvent gradient formed by adding a solution (400 mL) of methanol (50% v/v) in 0.05 M ammonium acetate buffer, pH 4.0, to a solution (400 mL) of methanol (30% v/v) in 0.05 M ammonium acetate buffer, pH 4.0. Residual ammonium acetate was removed from the peptide preparation by rechromatography on the same MPLC column using a linear gradient formed by adding 400 mL of a mixture (4:1 v/v) of a solution of acetic acid (10% v/v) in water and 2-propanol to 400 mL of a solution of acetic acid (10% v/v) in water. The yield was 209 mg. FAB-MS $(M + H)^+$: calcd 1250.5, found 1250.5. Amino acid analysis: Asp 0.93 (1), Glu 0.95 (1), Gly 0.98 (1), His 0.93 (1), Lys 1.89 (2), Pro 0.90 (1), Ser 0.88 (1).

[ε-*N*-Fmoc-Lys[5]]GnRH-III (2). The peptide was synthesized in 0.5 mmol scale. Solvent gradient for MPLC was formed by adding 400 mL of a mixture (1:4 v/v) of 0.05 M ammonium acetate buffer, pH 4.0, and methanol to 400 mL of a mixture (1:1 v/v) of 0.05 M ammonium acetate buffer, pH 4.0, and methanol. Residual ammonium acetate was removed by repeated lyophilization, exchanging traces of acetate with chloride by lyophilization from 0.02 M HCl followed by water. A 102 mg sample of peptide was obtained.

[Lys^5,*cyclo*(Asp^6,Lys^8)]GnRH-III (3). A solution (400 μL) of 1% $NaHCO_3$ was added, with stirring, at 0 °C, to a solution of **2** (58 mg) in DMF (50 mL). Then, a solution of BOP (20 mg) and HOBt (20 mg) in DMF (10 mL) was similarly added. A solution of DIEA (38.5% v/v) in DMF (400 μL) was added dropwise. The mixture was stirred overnight and removed by evaporation. The residue was triturated with ethyl acetate and collected by filtration. The solid (54 mg) was dissolved in DMF (10 mL) and piperidine (400 μL) was added to remove the Fmoc group. Peptide was purified by gel filtration on a column (1.5 cm × 50 cm) of Sephadex G-25 equilibrated with a solution of acetic acid (33% v/v) in water, followed by MPLC with a solvent gradient formed by adding 400 mL of a mixture (30% v/v) of 0.05 M ammonium acetate buffer, pH 4.0, in methanol to 400 mL of a mixture (70% v/v) of 0.05 M ammonium acetate buffer, pH 4.0, in methanol. Peptide product was collected by lyophilization of peak fractions, redissolved in water and collected once more by lyophilization. The yield was 28 mg. FAB-MS $(M + H)^+$: calcd 1232.6, found 1232.9. Amino acid analysis: Asp 0.92 (1), Glu 0.95 (1), Gly 0.97 (1), His 0.91 (1), Lys 1.85 (2), Pro 0.91 (1), Ser 0.87 (1).

[*cyclo*(Asp^6,Lys^8)]GnRH-III (4). GnRH-III·HOAc (135 mg) was dissolved in 0.02 M HCl. Lyophilization yielded 128 mg of GnRH-III·HCl. Cyclization was as for **3**, but the peptide was first dissolved in 10 mL of DMF and 800 μL of 1% $NaHCO_3$, and 800 μL of the solution of DIEA (38.5% v/v) in DMF was used. Pure peptide (80 mg) was recovered after gel filtration as for **3**. FAB-MS $(M + H)^+$: calcd 1241.5, found 1241.6. Amino acid analysis: Asp 0.92 (1), Glu 0.94 (1), Gly 0.98 (1), His 1.85 (2), Lys 0.92 (1), Pro 0.92 (1), Ser 0.82 (1).

[Lys^4,ϵ-*N*-Fmoc-Lys^8]GnRH-III (5). Peptide synthesis was carried out in 1 mmol scale. After gel filtration, 1.04 g crude peptide **5** preparation was obtained, of which 530 mg was applied to the MPLC column. This was irrigated with 200 mL of a solution of acetic acid (20% v/v) in water followed by a solvent gradient formed by adding 400 mL of a mixture (1:3 v/v) of 2-propanol and a solution of acetic acid (20% v/v) in water to 400 mL of a solution of acetic acid (20% v/v) in water. FAB-MS $(M + H)^+$: calcd 1341.6, found 1342.5.

[Lys^4]GnRH-III (6). A solution (15 mL) of piperidine (10% v/v) in DMF was added dropwise to a stirred solution (10 mL) of crude peptide **5** preparation (200 mg) at 0 °C. The mixture was stirred for 1 h at 0 °C. Solvent was evaporated and the residue was dissolved in a solution of acetic acid (33% v/v) in water prior to gel filtration. The solvent gradient during MPLC was formed by adding 400 mL of a mixture (2:1 v/v) of a solution of acetic acid (10% v/v) in water and methanol to 400 mL of a solution of acetic acid (10% v/v) in water. Lyophilization of the peak fraction yielded 80 mg of **6**. FAB-MS $(M + H)^+$: calcd 1299.6, found 1300.0. Amino acid analysis: Asp 0.93 (1), Glu 0.95 (1), Gly 0.97 (1), His 1.85 (2), Lys 1.87 (2), Pro 0.91 (1).

[ϵ-*N*-Ac-Lys^4]GnRH-III (7). Imidazole (130 mg) and DIEA (100 μL) were added to a solution of crude peptide **5** preparation (210 mg) in DMF (30 mL). The solution was cooled to 0 °C. A solution of acetic anhydride (200 μL) in DCM (5 mL) was added, dropwise, with stirring which was continued for 1 h. Following evaporation of solvent and precipitation of synthetic product by diethyl ether, the Fmoc group was removed using piperidine as for **6**. The peptide was gel filtered in a solution of acetic acid (33% v/v) in water as for **6**. The solvent gradient for MPLC was formed by adding 300 mL of a mixture (3:2 v/v) of a solution of acetic acid (10% v/v) in water and methanol to 300 mL of a solution of acetic acid (10% v/v) in water. MPLC was repeated, during which the gradient was formed by adding 300 mL of a mixture (7:3 v/v) of a solution of acetic acid (10% v/v) in water and methanol to 300 mL of a solution of acetic acid (10% v/v) in water. Lyophilization of the peak fraction yielded 71 mg of **7**. FAB-MS $(M + H)^+$ calcd 1343.5, found 1344. Amino acid analysis: Asp 0.92 (1), Glu 0.94 (1), Gly 0.97 (1), His 1.82 (2), Lys 1.86 (2), Pro 0.92 (1).

[Glu^6]GnRH-III (8). The peptide was synthesized in 0.5 mmol scale. After gel filtration, the synthetic product was subjected to MPLC during which the solvent gradient was formed by adding 300 mL of a mixture (1:1 v/v) of 0.05 M ammonium acetate buffer, pH 4.0, and methanol to 300 mL of a mixture (7:3 v/v) of 0.05 M ammonium acetate buffer, pH 4.0, and methanol. The residue in the peak fractions was desalted by further MPLC with the solvent gradient formed by adding 300 mL of a mixture (4:1 v/v) of a solution of acetic acid (10% v/v) in water and methanol to 300 mL of a solution of acetic acid (10% v/v) in water. The yield was 51 mg of **8**. FAB-MS $(M + H)^+$: calcd 1273.6, found 1273.7. Amino acid analysis: Glu 0.90 (1), Gly 0.96 (1), His 1.84 (2), Lys 0.93 (1), Pro 0.93 (1), Ser 0.81 (1).

[Phe^7]GnRH-III (9). The peptide was synthesized in 0.2 mmol scale using the Fmoc-^{t}Bu strategy on 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin with substitution of 0.4 mmol g^{-1} in the ABI 432 peptide synthesizer. Amino acids were coupled as their 1-hydroxybenzotriazole active ester in *N*-methylpyrrolidine. Amino acid side chain protections were as follows: His, N^{im}-Trt; Trp, N^{in}-Boc; Ser, *t*-Bu; Asp, O-*t*-Bu; and Lys, Boc. The crude peptide was cleaved from the resin by a mixture of TFA, thioanisole, and ethanedithiol (90:5:5 v/v/v). Preparative HPLC was performed on a Vydac 218TP510 C18 column (250 mm × 10 mm) at a flow rate of 4 mL/min, using a linear gradient of 0−33% (v/v) CH_3CN added to 50 mM NH_4OAc, pH 4.5. Analytical HPLC was performed on a Vydac 218TP54 C18 column (250 mm × 4.6 mm) washed with a 3−35% (v/v) solvent gradient formed by adding a solution of TFA (0.09%) and acetonitrile (60% v/v) in water to 0.1% TFA, during 35 min at a flow rate of 1 mL/min. Peptide recovered from the preparative HPLC was eluted as a single peak from the analytical column. The yield was 60 mg. FAB-MS $(M + H)^+$: calcd 1220.5, found 1220.5. Amino acid analysis: Asp 0.95 (1), Glu 0.98 (1), Gly 0.97 (1), His 1.85 (2), Lys 0.90 (1), Pro 0.80 (1), Ser 0.75 (1).

[N^{in}-For-Trp^{3,7}, $\Delta^{3,4}$-Pro^9]GnRH-III (10). Peptide was synthesized in 3 mmol scale. Crude peptide preparation (380 mg) obtained after HF cleavage was subjected to HPLC using a Vydac 218TP1010 C18 semipreparative column. The solvent gradient was formed by adding 400 mL of a mixture (1:3 v/v) of acetonitrile and 0.1% TFA to 400 mL of a mixture (9:1 v/v) of acetonitrile and 0.1% TFA. The yield was 95 mg. ES-MS *m*/*z*: calcd 1313.0, found 1312.9.

GnRH-III(1−9)-EA (11). Boc-Pro-Merrifield resin (substitution 0.58 mmol g^{-1}) was prepared by the cesium salt method as described previously.[26] Peptide was synthesized in 1 mmol scale. Side chain protection was as described above, except that Boc-Asp(OFm) was used. Peptide ethylamide was cleaved from the resin by incubation in ethylamine (40 mL) at 0 °C for 8 h in a tightly stopped flask. Ethylamine was evaporated by stirring the reaction mixture at 25 °C overnight. The peptide was dissolved in DMF (50 mL). The solution was evaporated to dryness and after trituration of the residue with ether, a faint yellow powder (500 mg) was obtained. The side chain protecting group was removed by HF. The crude peptide preparation was gel filtered on Sephadex G-25 (2.5 cm × 100 cm) equilibrated with a solution of acetic acid (10% v/v) in water.

Final purification was by MPLC with the solvent gradient formed by adding 400 mL of a mixture (4:1 v/v) of a solution of acetic acid (10% v/v) in water and 2-propanol to 400 mL of a solution of acetic acid (10% v/v) in water. A total of 82 mg of pure peptide was obtained. FAB-MS $(M + H)^+$: calcd 1230.6, found 1230.5. Amino acid analysis: Asp 0.93 (1), Glu 0.94 (1), His 1.80 (2), Lys 0.94 (1), Pro 0.90 (1), Ser 0.82 (1).

[D-Ala^{10}]GnRH-III (12). The peptide was synthesized in 0.5 mmol scale. Following gel filtration the peptide preparation was subjected to MPLC during which the solvent gradient was formed by adding 400 mL of a mixture (1:1 v/v) of 0.05 M ammonium acetate buffer, pH 4.0, and methanol to 400 mL of a mixture (17:3 v/v) of 0.05 M ammonium acetate buffer, pH 4.0, and methanol. This was followed by further MPLC during which the gradient was formed by adding 400 mL of a mixture (3:1 v/v) of a solution of acetic acid (10% v/v) in water and 2-propanol to 400 mL of a solution of acetic acid (10% v/v) in water. A total of 81 mg of pure peptide was obtained. FAB-MS $(M + H)^+$: calcd 1274.4, found 1275.0. Amino acid analysis: Asp 0.94 (1), Glu 1.00 (1), Ala 0.96 (1), His 1.97 (2), Lys 1.03 (1), Pro 0.94 (1), Ser 0.96 (1).

[Ac-D-Trp^1,D-Ala^{10}]GnRH-III (13). The peptide was synthesized in 0.5 mmol scale and acetylated while on the resin with acetic acid anhydride (5 mmol) in the presence of imidazole (5 mmol) and DIEA (5 mmol). Following gel filtration of crude peptide product, **13** was purified by MPLC during which the solvent gradient was formed as for **12**. The yield was 86 mg. FAB-MS $(M + H)^+$: calcd 1389.6, found 1390.3. Amino acid analysis: Asp 0.92 (1), Ala 1.00 (1), His 1.95 (2), Lys 0.93 (1), Pro 0.92 (1), Ser 0.94 (1).

[Asu^6]GnRH-III (14). The peptide was synthesized as previously described.[27]

[$\Delta^{3,4}$-Pro^9]GnRH-III (15). The peptide was synthesized in 0.025 mmol scale by solid phase continuous-flow methodology using the Fmoc-^{t}Bu strategy on the ABI 432A peptide synthesizer. Polystyrene-based resin with the Knorr linker (Fmoc amide resin, ABI) and a substitution of 0.65 mmol g^{-1} was used. TBTU/HOBt activation was used for the coupling and the coupling time was optimized on the basis of the deprotection period which was followed by conductivity. Amino acid side chain protections were as follows: His, N^{im}-Trt; Trp, N^{in}-Boc; Ser, *t*-Bu; Asp, O-*t*-Bu; Lys, Boc. The crude peptide was cleaved from the resin by the mixture of TFA, thioanisole, and ethanedithiol (90:5:5 v/v/v) at room temperature. Preparative HPLC was performed on a Vydac 208TP510 C18 column (250 mm × 10 mm) at a flow rate of 4 mL/min forming gradients of 0−5% (v/v) acetonitrile in 5 min and 5−32% (v/v) acetonitrile in 100 min by adding a solution of TFA (0.09% (v/v)) in acetonitrile to a solution of TFA (0.1% (v/v)) in water. The main peak fraction had one peak when subjected to analytical HPLC performed on a Vydac 218TP54 C18 column (250 mm × 4.6 mm) at a flow rate of 1 mL/min forming gradients of 3−60% (v/v) acetonitrile in 35 min by adding 0.09% (v/v) TFA in acetonitrile to 0.1% (v/v) TFA in water. The yield was 17.1 mg. ES-MS *m/z*: calcd 1257.5, found 1257. Amino acid analysis: Asp 0.92 (1), Glu 0.95 (1), Gly 0.98 (1), His 1.90 (2), Lys 0.85 (1), Pro 0.44 (1), Ser 0.53 (1).

[N^{in}-For-Trp^{3,7},(^{3}H)Pro^9]GnRH-III (16). The peptide was prepared by catalytic saturation of the double bond in $\Delta^{3,4}$-Pro^9 of **10** (3.08 mg) using 10.3 mg of catalyst (Pd/$BaSO_4$, 10% Pd, Merck Art. 807105) in 1 mL of DMF. The reaction vessel was connected to a specially designed manifold for tritiation, cooled by liquid nitrogen, and evacuated. Tritium gas (Technobexport, Russia) liberated from uranium tritide by heating, expanded into the reaction vessel. The reaction mixture was stirred at room temperature for 50 min. The reaction was terminated by freezing and by adsorption of the unreacted tritium gas onto pyrophoric uranium. The catalyst was removed by filtration through Whatman GF/C filters and washed several times with ethanol/water (1:1 v/v). The residual labile tritium was removed by repeatedly dissolving the radiolabeled product in ethanol/water (1:1 v/v) followed by evaporation to dryness. The crude tritiated peptide had one spot with $R_f = 0.41$ and $= 0.21$, respectively, when examined by TLC on Fertigplatten Kieselgel 60 F_{254} (Merck) with the solvent systems pyridine/acetic acid/water/butanol (12:3:10: 13 v/v/v/v) and 2-propanol (water) ammonia (3:1:1 v/v/v). The radioactivity of the product (91.9 mCi) was determined by liquid scintillation counting. The specific radioactivity of the labeled peptide, based on UV spectrophotometric analysis, was 82 Ci/mmol.

[(^{3}H)Pro^9]GnRH-III (17). The peptide was prepared by catalytic saturation of the double bond in $\Delta^{3,4}$-Pro^9 of **15** (3.0 mg) in the presence of 10 mg of catalyst as for peptide **16**, except that residual labile tritium was removed by repeated dissolving of the radiolabeled product in a solution of acetic acid (1% v/v) in water followed by evaporation to dryness, three times. The tritiated peptide was found to be pure when analyzed by HPLC on a LiChrosper 100 RP-18 column (particle size 5 μm; Jasco, Merck 50943 LiChroCART 125-4) washed at a flow rate of 1 mL/min with a solution of acetonitrile (15% v/v) and 0.1% (v/v) TFA in water for 3 min and then with a solution in which acetonitrile content was increased to 20 and 50% (v/v) respectively, during the subsequent 22 and 5 min periods. A_{280} and radioactivity (Packard Radiomatic 500TR Series Flow Scintillation Analyzer 3H) of the effluent were recorded. The peptide emerged in a single peak and no impurities were detected. The total radioactivity of the product was 96.98 mCi and the specific radioactivity was 57.06 Ci/mmol.

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(28) Abbreviations: These are in accordance with the recommendations of the IUPAC−IUB Commission of Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9−37 and *J. Biol. Chem.* **1989**, *264*, 633−673. Other abbreviations: Asu, aminosuccinyl; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; TEA, triethylamine; DIEA, *N*-ethyldiisopropylamine; EA, ethylamine; TFA, trifluoroacetic acid; DIC, diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazole; BOP, benzotriazol-1-yloxy tris(dimethylamino)phosphonium hexafluorophosphate; HF, hydrogen fluoride; TBTU reagent, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; EtOAc, ethyl acetate; DMSO, methyl sulfoxide.

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