Synthesis and Biological Evaluation of Two New Analogues of Gonadotropin Releasing Hormone (GnRH)D-alanine[8] and D-alanine[10]

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Abstract

So far synthesis of Gonadotropin Releasing Hormone (GnRH) analogues reported in the literature has clarified some aspects of structural activity of the naturally released GnRH. As a part of continuing efforts for further understanding of this relationship, the present investigation was undertaken which involved synthesis and biological evaluation of two GnRH analogues, firstly, by replacement of the amino acid L-Argenine in the 8th position at the backbone structure of the natural hormone by the amino acid D-Alanine; and secondly, by replacement of the amino acid L-Glycine in the 10th position by D-Alanine also at the backbone structure of the nature hormone, to obtain the following analogues respectively:

PGlu-His-Trp-Ser-Tyr-Gly-Leu-DAla-Pro-Gly-NH_2 (Analogue I: D-Alanine[8] GnRH),

PGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-DAla-NH_2 (Analogue II: D-Alanine[10] GnRH), which were synthesized by applying conventional solution method. Peptides were purified by several recrystallization using appropriate solvent and proved to be homogenous. Conformity of the synthetic procedure was achieved by applying different physico-chemical analyses including Melting Point (mp), Thin Layer Chromatography (Tlc.), Infrared Spectroscopy (IR), Elemental Analysis (CHN), Amino Acid Analysis (AAA), and Nuclear Magnetic Resonance (H^1NMR).Preliminary biological activity of the two analogues was determined by testing their effects of parenteral administration on ascorbic acid depletion from the ovary of pseudopregnant mice and compared with that of natural GnRH hormone. Analogue II showed significant ascorbic acid depletion as compared to the native hormone while the percentage in ascorbic acid depletion after administration of analogue I were not significant as compared to the native hormone.

Key Words: Gonadotropine releasing hormone, Peptide synthesis, Biological activity of GnRH

الخلاصة

المنشورة في الأدبيات العالميةGonadotropin Releasing Hormone (GnRH) لقد كشفت مماثلات هورمون بعضا من جوانب العلاقة للفعالية الحيوية والبنية الجزيئية للهورمون الطبيعية، وكجزء من مساعينا بهذا الاتجاه والرامية الى الاستزادة من التفهم الأعمق والأشمل لهده العلاقة فقد تم تناول هذه الدراسة والتي تشمل تحضير مماثلين للهورمون الطبيعي وذلك عن طريق:-

١- احلال الحامض الأميني D-Alanine في الموقع 8 بدلاً من الحامض الأميني L-Arginine

٢- احلال الحامض الأميني D-Alanine في الموقع 10بدلا من الحاض الأميني L-Glycine

على التوالي وكما يلي:-

pGlu His Trp Ser Tyr Gly Leu Dala Pro Gly-NH_2 (Analogue I: D-Alanine[8] GnRH)

pGlu His Trp Ser Tyr Gly Leu LArg Pro DAla-NH_2 (Analogue II: D-Alanine[10] GnRH)

والتي خلقت باستخدام طرق المحلول المعهودة وتم تنقية المماثلات المحضرة عن طريق اعادة البلورة باستخدام مذيبات ملائمة. أما التقنيات الفيزياوية والكيمياوية التي الستخدمت بهدف التوصل للخواص المميزة للمماثلات المحضرة فقد كانت كروماتوكرافيا الطبقة الرقيقة، قياس درجة الانصار، مطياف الأشعة تحت الحمراء، الاستدارة البصرية تحليل العناصر ومن ثم تحليل الحوامض الأمينية ومطياف الرنين النووي المغناطيسي. تم اجراء التقييم البايولوجي البدائي حيث استند على تقدير تأثير الهورمون المصنع على معدل انخفاض تركيز حامض الاسكوربيك في مبايض الفئران كاذبة الحمل. لقد أدى حقن المماثلين المحضرين الى حصول انخفاض معنوي للمماثل (II) في معدل تركيز حامض الأسكوربيك بينما أظهر المماثل الأول انخفاض في معدل تركيز حامض الأسكوربيك في المبايض نسبة للفعالية البايويولوجية القياسية للهورمون الطبيعي.

Introduction

The hypothalamus releases Gonadotropin Releasing Hormone (GnRH), a decapeptide that stimulates the anterior pituitary to secrete Leutinzing Hormone (LH), and Follicular Stimulating Hormone (FSH) respectively. This peptide controls and regulates both male and female reproduction system. GnRH is a modified decapeptide: PyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Figure1). The pyroglutamte at the N terminus and the C-terminal amide distinguish this peptide from unmodified decapeptides [(1-3)].

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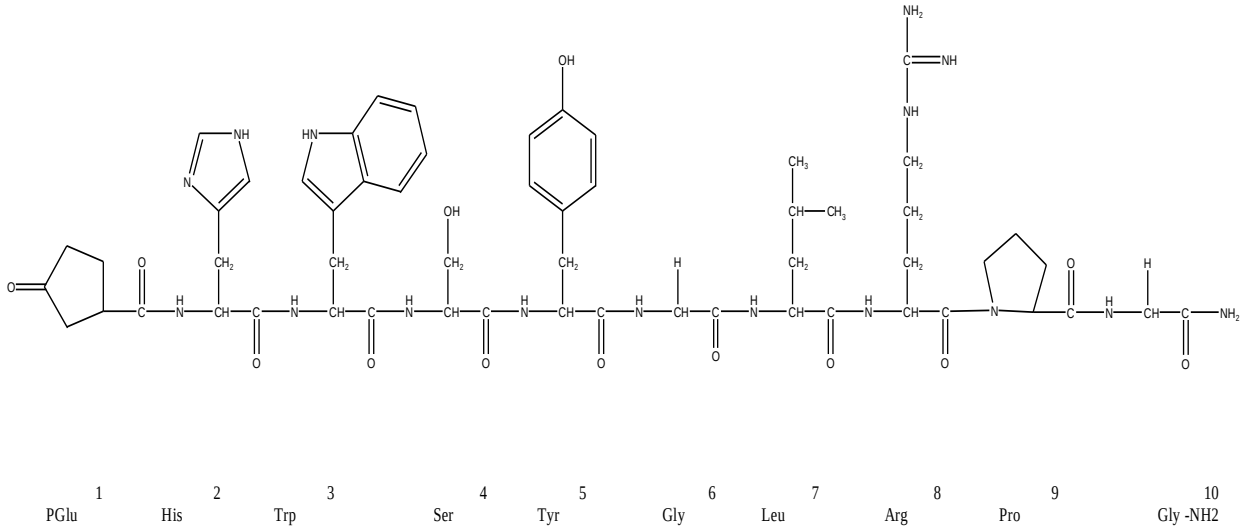


Figure1: Basic structure of Gn-RH hormone

The gonadotropins have a close functional relationship to estrogen, progesterone, and testosterone. They are called gonadotropins because of their action on the gonads. They control ovulation, spermatogenesis, and development of sex organs, and they maintain pregnancy. Included in this group GnRH, LH, FSH, Chorionic gonadotropin (CG; hCG is human gonadotropin), a glycoprotein produced by the placenta; its pharmacological actions are essentially the same as those of LH [4-9]. GnRH interacts with high-affinity receptors on the gonadotropes in the anterior pituitary, leading to the biosynthesis and release of the gonadotropins LH and FSH. The pulse-timing and concentration levels of GnRH are critical for the maintenance of gonadal steroidogenesis and for normal reproductive function [10-12].Because of its simple structure there has been an enormous amount of interest in development of analogues as medicinal agents. Over 3000 GnRH analogues have been synthesized and studied in an attempt to evaluate their structure activity relationship [3].The structure activity data reviewed in the previous works provide that Arg^8 was identified as being critical for high affinity binding to mammalian receptors alsoa number of early studies results showed that D-Arg, Gln, Leu, Ornithin, diaminobuteryl substitution for Arg^8 result in substantial decrease in activity while Lys retained most of the activity, it has been shown also that replacement of GlyNH2 at position10 by Ala resulted in mild reduction in activity, therefore we attempted to replace D-Ala at position8 and position10 respectively in the back bone structure of the native hormone in the hope of gaining more information of the structure activity relationship of the GnRH hormone[23]. This paper reports the synthesis and preliminary biological evaluation of $D\text{-}Ala^8$ and $D\text{-}Ala^{10}$ GnRH analogues using conventional solution method by stepwise elongation manner. The products obtained could provide enough materials for further chemical and physical characterization and for biological evaluation and for future work, especially for broad biological testing of the hormone, which is indicated because of its possibly far-reaching in clinical medicine[13].

Materials and Methods

All the chemicals used in this study were analar grade purchased from Sigma Company. Due to the presence of numerous complicated amino acids, some difficulties for the synthesis of the decapeptide should be expected. We therefore applied intermediates, which supposedly could be purified easily. Pyro Glutamic acid has been prepared according to Budavari [14]. C-terminal protecting esters of amino acids also prepared according to Huber and Brenner [15]. They are Histidine, Serine, Tyrosine, Leucine, Proline, Glycine, and D-Alanine ethyl esters by dissolving 2 gm (1 mol.) of the amino acid in 15 ml. ethanol. Then the mixture was cooled to -10^{o}C, thionyl chloride was added gradually with stirring. The mixture was left for one hour at this temperature, then left over night at 40^{o}C. The mixture was refluxed in water bath at 55-60^{o}C for three hours. Purification was done under vacuum using ether to obtain the precipitate

N-terminal protection

Benzyloxy carbonyl amino acids (Z) were prepared according to Zervas and Bergmann and Baily [16]. They are Z Tryptophan, ZD-Alanine, Z-Glycine, and Z-Arginine. Conventional solution method was applied as a coupling method between the protected amino acid for peptide bond formation using Dicyclohexyl carbodiimide DCC in the presence of 1-hydroxy benzotriazole HBT and N-methyl morpholine[25] NMM. Deprotection of C-terminal protecting groups (saponification) was performed using 1.5 equivalent of sodium hydroxide (NaOH) solution (1 N). Table 1 showed the physico-chemical properties of these esters. Deprotection of the N-terminal protecting groups was performed using hydrobromic acid (HBr) in glacial acetic acid (equimolar). Table 2 showed the physico-chemical properties of these Z protected groups [16]. The intermediates and segment peptides had been purified by repeating recrystalization several times (4 times) using different solvents like diethyl ether, petroleum ether (40-60^o), ethyl acetate, absolute ethanol, distilled water (D.W.), chloroform. Ascending thin layer chromatography was run on Kieslgel Gf 254 type (60) Merck, for checking the purity of the prepared compounds as well as monitoring the reaction process. Spots were revealed by reactivity with iodine vapour or irradiation with U.V. light or by ninhydrine [15] spraying reagent 2% in absolute ethanol chromatograms were eluted by the following systems:-

A	Chloroform	Methanol	Acetic acid 30%
	60	45	20
B	Chloroform	Methanol	Ammonia
	60	45	20
C	Butanol	Acetic acid	Water
	40	10	45

Table 1: Physico-chemical properties of the prepared amino acid esters

Name of amino acid ester		Rf_A	MP(C^o)	νcm^{-1}
1.	Histidine methyl ester	0.42	205-207	3180 for primary amine, 1750 for (C=O) of ester.
2.	Serine methyl ester	0.46	163-165	3400 for primary amine, 1750 for (C=O) of ester,1260 for (C-O)of ester.
3.	Leucine methyl ester	0.62	190-192	As in (2.)
4.	Proline methyl ester	0.49	150-153	As in (2.)
5.	Glycine methyl ester	0.58	69-72	As in (2.)
6.	D-Alanine methyl ester	0.66	172-174	As in (2.)

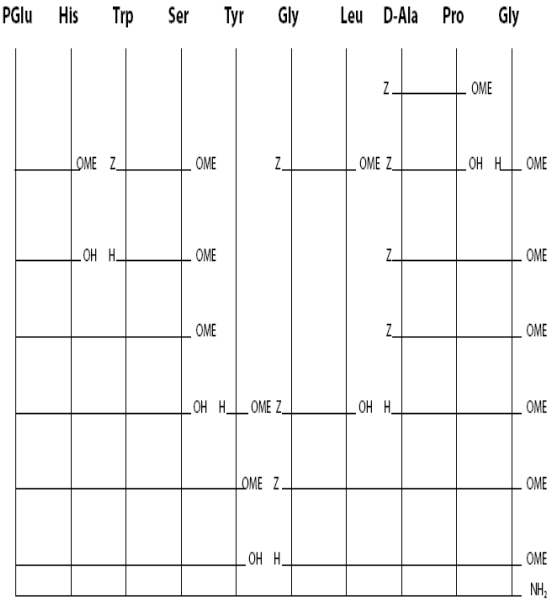
Benzyloxy carbonyl N-protected amino acids (Z) was produced as precipitate except for Z-Argenine which was produced as oily residue using 0.2 mol. and 0.2 mol. of benzyl chloroformate, 4 volumes of 1-N sodium hydroxide.

Table 2: Physico-chemical properties of the N- protected amino acids by benzyloxy carbonyl prepared (Z) in this work

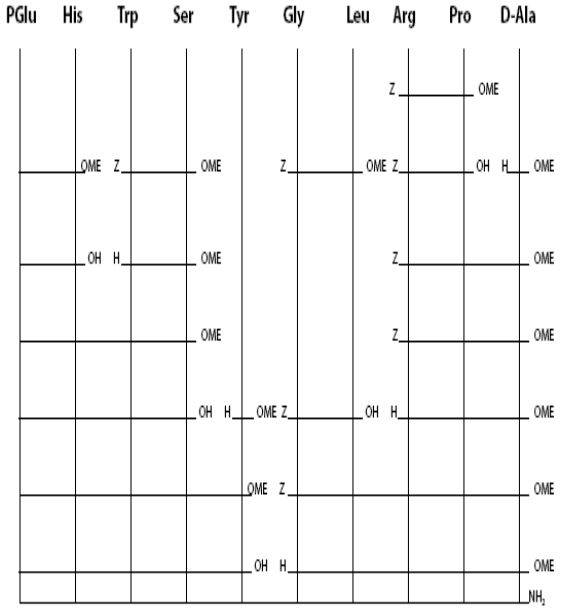
Name of Z-protected amino acid		Rf_A	MP(C^o)	cm^{-1}
1.	Z-tryptophan	0.84	114-121	3400 for secondary amine, 1500 secondary amide, 1680 for (C=O).
2.	Z-Glycine	0.82	124-129	3310 secondary amine, 1680 for (C=O).
3.	Z-Argenine	0.78	oily	As above
4.	Z-D-Alanine	0.69	118-122	As above

Scheme I and scheme II showed the pattern followed to obtain analogue I and analogue II respectively.

M.P., TLC., optical rotation, IR,CHN, and NMR were the analytical techniques utilized to the chemical evaluation of the different coupling procedures between amino acids and peptide intermediates and to confirm the success of the synthetic process for both analogues. All intermediates and peptides showed optical activity, and purity as revealed by TLC, and acceptable IR, CHN, H^1NMR, and Amino acid analysis as well.



Scheme I: Synthetic steps of Analogue I



Scheme II: synthetic steps of Analogue II.

Biological Activity of the prepared Analogues

The potency of each analogue was estimated by comparing their effect in treated mice with 0.5,1.0,2.0 mcg/0.1 ml of buffered plasma albumin (BpA)/mouse, on ascorbic acid depletion as compared with standard GnRH preparation according to Bogdauove and Gay [17] modified procedure published in British Pharmacopoeia (1988).Female rats approximately 21 days old has been chosen of approximately equal weights with the range 12-13 gm, and then randomly distributed into four groups 5each.group1 control group2-4 test group. The mice were hormonally treated as follows all pseudo pregnant mice were subcutaneously injected with 50 units of Human Chorionic Gonadotrophin (HCG) at the first ,third & fifth day of the starting time of the experiment and after 6 days 5 mice in the groups 2-4 were injected with 0.5,1.0,2.0 mcg of D-alanine[8] GnRH/0.1 ml BpA subcutaneously and respectively while the mice in the first group were injected with BpA for comparison after 3 hours all mice were killed, their ovaries are removed ,weighed and placed immediqately in ice bath to avoid losses and dryness ,the ascorbic acid concentration was measured by homogenizing the 2 ovaries of each mice in 10 ml of metaphosphoric acid allowing the homogenate to stand for 30 minutes & centrifuge ,then 7 ml of the clear supernatant liquid was added to a freshly prepared mixture of 7 ml of acetic acid (pH=7) 3 ml distilled water and 2 ml of the dye 2,6 dichlorophenol indophenol standard solution ,30 seconds after the mixing ,the absorbance of the resulting solution was measured at the maximum at about 520 nm [17].The result of the assay was calculated by standard statistical method using Complete Random Design (CRD) [18].The biological activity of D-Alanine[10] analogues has been estimated by same manner.Table 3and 4 show the percent of depletion of ascorbic acid in the ovaries of the treated group.

Table 3: Average weight of pseudo pregnant mice ovaries, concentration of ascorbic acid, and percent of depletion of ascorbic acid in treated groups by analogue (II):

Group number	Concentration of D-alanine 8GnRH analogue mcg/mouse	Average weight of mice* (gm)	Average weight of ovaries/mg	Concentration of ascorbic acid mcg/ovary	% of depletion of ascorbic acid
1	control	12.11	117.03	65.49±0.156	
2	0.5	12.34	117.38	61.109±0.195a	6.809
3	1.0	12.13	118.03	60.01±0.156b	8.37
4	2.0	12.17	117.52	59.18 ±0.372c	8.68

Table 4: Average weight of pseudo pregnant mice ovaries, concentration of ascorbic acid, and percent of depletion of ascorbic acid in the treated groups by analogue (I):

Group number	Concentration of D-alanine 8GnRH analogue mcg/mouse	Average weight of mice (gm)	Average weight of ovaries/mg	Concentration of ascorbic acid mcg/ovary	% of depletion of ascorbic acid
1	control	12.16	117.05	65.54 0.153	
2	0.5	12.32	117.36	62.86 0.147a	3.41
3	1.0	12.133	118.001	61.57 0.141b	4.17
4	2.0	12.175	117.63	60.59 0.389c	4.301

* Five mice in each group.
a, b, and c indicate significant difference at percentage of error 0.01.

Results and Discussion

The results of physico-chemical evaluation of intermediate peptides were good indication of the success of the synthetic methodology using M.P, TLC, and IR as shown in Table 5. Elemental analysis(CHN), optical rotation, and sometimes AAA have been applied to add conformity to the success of the synthetic procedure:

1. For the dipeptide P Glu-His-OH: $[\alpha]_D^{25}$ = -25.9 (C2 DMF) this indicates that this peptide is optically active. Elemental analysis (CHN); calculated: C 49.61, H 5.76 N 21.05, found: C 49.65 H 5.29 N 21.11.
2. For dipeptide Trp-Ser: $[\alpha]_D^{25}$ = -46.4 (C2 DMF) this indicates the optical purity of the peptide, CHN analysis; calculated: C 59.97 H 5.73 N 14.94, found: C 60.06 H 5.77 N 15.53.]
3. Tetrapeptide P Glu-His-Trp-Ser: $[\alpha]_D^{25}$ = -44 (C2 DMF), CHN analysis; calculated: C 55.64 H 5.42 N 18.18, found C 55.98 H 5.66 N 18.63.
4. Pentapeptide P Glu-His-Trp-Ser-Tyr: $[\alpha]_D^{25}$ = -39.1 (C2 DMF), CHN analysis; calculated: C 58.1 H 5.45 N 15.85, found: C 58.78 H 5.66 N 16.23.
5. Peptide Z-Gly-Leu: $[\alpha]_D^{25}$ = -40.6 (C1 DMF). CHN analysis; calculated C 51.3 H 8.61 N 14.96, found C 51.76 H 8.81 N 51.03.
6. For completion of Analogue I the following steps have been done
   Peptide D Ala-ProOCH$_3$: $[\alpha]_D^{25}$ = -14.0 (C1 DMF). CHN analysis; calculated: C 53.97 H 8.06 N 13.99, found: C 54.3 H 8.23 N 14.44.
7. Tripeptide D Ala-Pro-Gly-OCH$_3$: $[\alpha]_D^{25}$ = -12 (C1 DMF). Amino acid analysis was D-Ala 1.01, Pro 0.99, Gly 1.03.
8. Pentapeptide Z Gly-Leu-D Ala-Pro-Gly-OCH$_3$: C 57.73 H 7.00 N 12.47 O 22.80, found: C 58.32 H 7.38 N 12.86 O 23.11.
9. Z Deprotected pentapeptide Gly-Leu-D Ala-Pro-Gly-OCH$_3$: $[\alpha]_D^{25}$ = -33 (C=2 DMF). CHN analysis; calculated: C 53.37 H 7.78 N 16.39 O 22.46, found: C 53.91 H 7.99 N 16.83 O 22.71.
10. Decapeptide PGlu-His-Trp-Ser-Tyr-Gly-Leu-DAla-Pro-Gly-OCH$_3$: CHN analysis; calculated: C 57.22 H 6.26 N 16.38 O 20.15, found: C 57.49 H 6.57 N 14.01 O 20.65.
11. Aminolysis of the above decapeptide using ammonia and tri ethyl amine PGlu-His-Trp-Ser-Tyr-Gly-Leu-DAla-Pro-Gly-NH$_2$ (Analogue I).
12. For Completion of Analogue II the following steps have been done:
    Dipeptide ZArg-Pro-OCH$_3$: CHN analysis; calculated: C 57.25 H 6.97 N 16.70 O

19.09, found C 57.89 H 6.23 N 16.91 O 19.52.

13. Z Deprotected Arg-Pro-OCH_3: CHN, calculated: C 50.49 H 8.13 N 24.55 O 16.83, found: C 51.01 H 8.43 N 25.03 O 17.22.

14. Tripeptide ZArg-Pro-DAla-OCH_3: AAA Arg 0.89, Pro 0.93, D-Ala 1.09.

15. ZGly-Leu-Arg-Pro-DAla-OCH_3: CHN analysis; calculated: C 56.33 H 7.33 N 16.99 O 19.38, found: C 57.01 H 7.45 N 17.23 O 19.58.

16. Z Deprotected ZGly-Leu-Arg-Pro-DAla-OCH_3: CHN analysis; calculated: C 52.44 H 8.04 N 21.28 O 18.23, found: C 52.71 H 8.29 N 21.57 O 18.43.

17. Decapeptide Pglu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-DAla-OCH_3: CHN analysis; calculated, C 56.50 H 6.49 N 18.51 O 18.50, found: C 61.01 H 6.84 N 18.91 O 18.79.

18. Aminolysis of the above decapeptide using ammonia and tri ethyl amine Pglu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-DAla-NH_2.

Table 5: Some physico-chemical properties of the synthesized intermediate peptides (Percent yield, Melting points, Rf values, and Characteristic IR spectra).

Peptide Sequence	Amino Acid Residues	Percent Yield	Melting Point	Rf Value	Characteristic IR band (ν cm^{-1})
1-2	P Glu-His-OCH_3	55%	109-112	0.73_B	3320 stretching vibration of secondary amine, 1630 stretching vibration of amide band.
1-2	P Glu-His-OH	80%	214- 216	0.64_A	3320 stretching vibration of secondary amine disappearance of ester band at 1730, 1630 stretching vibration of amide band.
3-4	Trp-Ser-OCH_3	73%	128- 130	0.18_A	3220 stretching vibration of primary amine, 1660 stretching vibration of carbonyl of amide 1 band.
1-4	PGlu-His-Trp-Ser-OCH_3	66%	198- 200	0.89_B	3370 stretching vibration of secondary amine, 1640 stretching vibration of carbonyl of amide 1 band.
1-4	PGlu-His-Trp-Ser-OH	62%	172-177	0.81_A	1590 c-o vibration of carboxyl group.
1-5	PGlu-His-Trp-Ser- Tyr-OCH_3	64%	204- 206	6.78_C	3370 stretching vibration of secondary amine interfered with phenol stretching vibration at 3000, 1630 stretching vibration of carbonyl of amide 1 band.
1-5	PGlu-His-Trp-Ser- Tyr-OH	58%	221-226	0.78_C	3600-3300 (broad) of phenol group of tyrosine, C=C-H stretching vibration of aromatic ring 3100-3030, 760 out plane bend of aromatic C=C
6-7	Zgly-Leu-OCH_3	81%	101- 103	0.48_C	3380 stretching vibration of secondary amine, 1640 stretching vibration of amide 1 band
6-7	ZGly-Leu-OH	77%	99-103	0.53_A	2970-2920 and 2860-2830 stretching vibration of CH_3 and CH_2, disappearance of ester absorption.
Completion of analogue I D- Ala^8 GnRH					
8-9	ZD Ala- Pro-OCH_3	64%	181- 186	0.68_A	1630 stretching vibration of carbonyl of amide 1 band.
8-9	ZD Ala-Pr-OH	60%	162-165	0.33_C	3500 stretching vibration of carboxyl group interfered with secondary amine.
8-10	ZD Ala-Pro-Gly-OCH_3	72%	170- 174	0.55_C	1680 stretching vibration of carbonyl of amide 1.
8-10	D Ala-Pro-Gly-OCH_3	59%	126- 128	0.86_C	
6-10	Z Gly-Leu-D Ala-Pro-Gly-OCH_3	67%	152-156	0.72_B	1660 stretching vibration of carbonyl of amide 1 band, 1550 stretching vibration of secondary amine.
6-10	Gly-Leu-D Ala-Pro-Gly-OCH_3	55%	143-146	0.61_B	1650-1620 C=O stretching vibration of amide band.
1-10	PGlu-His-Trp-Ser-Tyr-Gly-Leu-DAla-Pro-Gly-OCH_3	75%	218-222	0.89_B	1650-1680 N-H bend, 1460, 1380 C-H bend of CH_3 and CH_2, 1240 O-H bend.
1-10	(Aminolysis of the decapeptide (Analogue I) was performed using ammonia in tri ethyl amine) PGlu-His-Trp-Ser-Tyr-Gly-Leu-DAla-Pro-Gly-NH_2. Yield 63%.				
Completion of analogue II D- Ala^{10} GnRH					
8-9	Z Arg-Pro-OCH_3	79%	181- 183	0.68_A	1675 stretching vibration of amide band.
8-9	Z Arg-Pro-OH	66%	173-176	0.55_A	1660-1630 C=N stretching vibration of Arginine, disappearance of ester band.
8-10	Z Arg-Pro-D Ala-OCH_3	71%	169- 172	0.48_A	3450 N-H stretching vibration of secondary amine, 1650 stretching vibration of carbonyl of amide 1, 1730 stretching vibration of carbonyl of ester group, 700 bending vibration of benzyl ring.
8-10	Arg- Pro- D Ala-OCH_3	53%	116- 118	0.76_B	
6-10	Z Gly- Leu- Arg- Pro- D Ala-OCH_3	82%	147- 150	0.72_A	3500 N-H stretching vibration of terminal primary amine, 1630 stretching vibration of carbonyl of amide 1 band.
6-10	Gly-Leu-Arg-Pro-D Ala-OCH_3	60%	140-146	0.59_A	
1-10	PGlu-His-Trp-Ser-Tyr-Gly-Leu-Leu-Arg-Pro-D Ala-OCH_3	72%	oily	0.87_A	
1-10	Aminolysis of the decapeptide (Analogue II) D Alanine[10] GnRH was performed using ammonia in tri ethyl amine. PGlu-His-Trp-Ser-Tyr-Gly-Leu-Leu-Arg-Pro-D Ala-NH_2. Yield 68%.				

Chemical evaluation of analogue I (D- Ala[8] GnRH):

Melting point (^{o}C) = 212- 214.

$R_f = 0.21_A, 0.65_B$.

C=1; -37 $[\alpha]_D^{25}$ Optical Rotation in dimethyl formamide

Elemental Analysis

	C	H	N
Calculated	56.91	6.25	17.88
Found	56.56	6.55	17.91

Amino acid composition of acid hydrolysates of the analogue was:-

Glu	His	Trp	Ser	Tyr
1.01	0.99	1.1	0.98	1.03
2Gly	D-ala	Pro		
2.03	0.89	0.9		

H^1NMR resolution at 300 MHz in deteriuarated dimethyl sulfoxide using tetramethyl silane as a standard showed the following characteristic chemical shifts represented in ppm starting from Glycine amide ending with pyroglutamine as follows:

NH_2 7.21 prim(s). amide, NH 9.04 sec(s). amide, CH_2 3.15;3.41 pyrrolidine (m), CH 4.40 pyrrolidine(m) , CH_2 2.34;2.09 pyrrolidine(m) , CH_2 2.02;1.92 pyrroline (m), NH 8.32 sec(s). amide, CH_2 1.75 methylene(d) , CH 4.53 methine (m), NH 8.32 sec. (s) amide, H 4.09 methylene (d), CH_2 4.09 methylene, OH 9.83 aromatic(s) C-OH, CH 6.95 1-benzene(s) , CH 6.68 1-benzene(s), CH 6.68 1-benzene(s) , CH 6.95 1-benzene(s) , NH 8.32 sec. amide(s) , CH_2 4.16;3.91 methylene(d) , CH 4.62 methine(m) , CH 7.58 3-indole(s), NH 8.32 sec. amide(s), CH 7.66 imidazole(d) , NH 13.4 imidazole (d), CH 8.73 imidazole (d), NH 8.32 sec. amide(s) ,NH 7.79 pyrrolidin-2-one(s) , CH_2 2.28;2.18 pyrrolidin-2-one(m) , CH_2 2.46;2.21 pyrrolidin-2-one(m) .

Chemical evaluation of analogue II D- Ala[10] GnRH

Melting point (^{o}C) = 218- 220

$R_f = 0.19_A, 0.36_B$

C=1; -28$[\alpha]_D^{25}$ Optical Rotation

Elemental Analysis

	C	H	N
Calculated	56.21	6.49	19.91
Found	56.29	6.71	19.99

Amino Acid Analysis

Glu	His	Trp	Ser	Tyr
1.08	0.98	0.89	1.11	1.09
Gly	Leu	Arg	Pro	D-ala
0.99	0.96	1.01	1.09	1.02

H^1NMR resolution at 300 MHz in deteriuarated dimethyl sulfoxide using tetramethyl silane as a standard showed the characteristic chemical shifts represented in ppm starting from D-alanine amide and ending with pyroglutamine as follows:-

NH_2 7.21 prim. amide(s), CH_3 1.48 methyl(d), CH 4.71 methine (m), NH 8.32 sec. amide (s) , CH_2 3.51;3.41 pyrrolidine (m), CH 4.40 pyrrolidine(m) , CH_2 2.34;2.09 pyrrolidine (m), CH_2 2.02;1.92 pyrrolidine (m), NH_2 6.63 amine, NH 2.0 amine(m), CH 4.53 methine(m) , NH 8.32 sec. amide(s) , CH_2 1.75 methylene(d) , CH 4.53 methine(m) , NH 8.32 sec. amide (s), H 4.09 methylene, CH_2 4.09 methylene, NH 9.04 sec. amide(s), OH 9.83 aromatic (s), (s) C-OH, CH 6.68 1-benzene, CH 6.68 1-benzene(s) , CH 6.95 1-benzene(s) , CH_2 3.17;2.92 methylene(d) , CH 4.92 methine (m), OH 4.78 alcohol(s) , CH_2 4.16;3.91 methylene (d), CH 4.62 methine (m), CH 7.34 3-indole (s),NH 10.85 3-indole(s) , CH 7.18 3-indole, CH 4.92 methine, NH 8.32 sec. amine(s), CH 7.66 imidazole (d), NH 13.4 imidazole (d),CH 8.73 imidazole (d), CH_2 3.17; 2.92 methylene, CH 4.92 methine, NH 8.32 sec. amide(s) , NH 7.79 pyrrolidin-2-one(s) , CH_2 2.28;2.18 pyrrolidon-2-one (m), CH_2 2.46;2.21 pyrrolidin-2-one (m). So, the synthetic approaches according to the step wise manner of this study were proved to be effective for the synthesis of homogeneous analogues as indicated from M.P., TLC., optical rotation, CHN, IR, and NMR. These analogues were preliminary estimated for GnRH activity, D- alanine[10]GnRH (Analogue II) was found to posses significant activity as shown in Table 3 while D-alanine[8] GnRH (Analogue I) showed lower activity as shown in table v. Chang et al[(22,23)] described the importance of basicaty of Arginine moiety at position (8) and the influence of it is multi structural function for biological activity there for injection of the analog D- alanine[10]GuRH in the mice lead to significant Depletion (1>0.01) in ascorbic acid concentration in the ovaries after 3hrs of the injection time as indicated in Table 3 the percent of decrease in ascorbic acid concentration was 6.089, 8.37, 8.68 % for the group. 2-4 respectively compared with the control. This is similar to that obtained by Gay and Bogudonve [(17)] who

mentioned that the percent of decrease in ascorbic acid concentration in the ovary was 10% after 3hrs of the injection time 1.35 mcg of standard GnRH preparation in the mice. This is a clear indication of the presence of significant GnRH activity of the prepared D-alanine[10] GnRH in stimulating the release of leutenizing hormone (LH) which in its turn affect the yellow body cells in the ovaries of pseudo pregnant mice (pretreated with HCG) which lead to the consume of ascorbic acid content in these cell will be to stimulate consumed peroxidase enzyme. This will lead to the oxidation of ascorbic acid and in this oxidation state ascorbic acid can be utilized for the production of very important steroid (progesterone) which is released from the yellow body cells, other probable mechanism of LH function in decreasing ascorbic acid content in the ovary is by increasing the excretion of ascorbic acid, as the venous blood exuded from the ovary [19, 21]. D-alanine[10]GnRH ascorbic acid depletion >D-alanine[8] GnRH. The result of this preliminary biological study may indicate that Gly at COOH terminal is not essential for biological activity since its replacement by D-Ala which has a reverse stereochemistry showed no reduction in the potency of the native hormone and may be the most important finding from this study is that Arg at position 8 seems to be critical for high affinity binding to mammalian receptors because the substitution of Arg8 by D-Aala cause a marked reduction in the biological activity and this could be based from the suggestion that Arg8 of GnRG may interact with acidic moieties on the receptors[24].

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References

1. Fink .G Gonadotropin secretion and its control.In : Knobil E , Neill .J (eds) The Phsyiology of Reproduction. Raven press, New York, 1988. pp 1349-1377.
2. Casper RF Clinical uses of gonadotropin-releasing hormone analogues . can Med Assoc J, 1991, 144:153-158.
3. Schneider J.S. and Rissman E.F.: Gonadotropin-releasing hormone II :a multi-purpose neuropeptide. Integr.comp.biol., November 1,2008; 48 (5) : 588-595.
4. Conn PM, Crowley Jr WF Gonadotropin-releasing hormone and its analogues. N Enggl J Med, 1994, 324:93-103.
5. Moghissi KS Clinical applications of gonadotropin-releasing hormone in reproductive disorders. Endocrinol Metab Clin North Am, 1992, 21:125-140
6. Barbieri RL Clinical applications of GnRH and its analogues. Trends Endocrinol Metab, 1992, 3:30-34.
7. EMons G, Schally AV The Leuteinzing hormone releasing hormone agonists and antagonists in gynaecological cancers. Hum Reprod, 1994 , 9:1364-1379.
8. DeRijk, R.H., Schaaf, M., and de Kloet, E.R.: J. Steroid Biochem.Mol. Biol., 2002 81:103-122.
9. Burns, K.H., and Matzuk, M.M.: Endocrinology 2002 143:2823-2835.
10. Conn, P.M., Janovick, J.A., Stanislaus, D., Kuphal, D., Jennes, L.: Molecular and cellular bases of gonadotropin-releasing hormone action in the pituitary and central nervous system. Vitam Horm 1995 50: 151–214.
11. Levavi-Sivan B.,Biran J.,and Fireman E. : Sex Steroid Are Involved in the Regulation of Gonadotropin-Releasing Hormone and Dopamine D2 Receptors in Female Tilapia Pituitary . Biol Repord, October 1,2006;75(4):642-650.
12. Karten, M.J., Rivier, J.E.: Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonist and antagonists: rationale and perspective. Endocr. Rev. 1986, 7: 44–66.
13. Sedgley K.R,Finch A.R,Caunt C.J , and McArdleC.A: Intracellular gonadotropin-releasing hormone receptors in breast cancer and gonadotrope lineage cells. J Endocrinol., December 1,2006;191(3):625-636.
14. Budavari,S.;Mecrk index.An encyclopedia of chemicals,drugs and biological.Merck and Co. Inc. USA, 1989.
15. Brenner, M and Huber,W.Herstullung Von alpha-aminosaureestern duch Alkoholyse der Methyl Ester. Helv.chem. Acta,1953,36,1109-1111.
16. Baily, L.:Techniques in protein chemistry, expanded ed. Elsevier publishing company, Amsterdam, 1967.
17. Bogadonve, E.M., and Gay, L.V.: Enhancement of ascorbic acid depletion response during estrogen-prolonged pseudopregnancy. Endocrinology 1967, 81: 1104-1117.

18. Steel, R., and Torrie, J.: Prinicples of procedures of statistics. McGraw-Hill Book Co., New York, 1960.

19. Niswender, G.D., and Nett, T.M.: Biological assay of gonadotropic and gonadal hormones. In reproduction indomestic animals, 3rd Ed. Cole. H.H. and P.T. Copps Academic press, Chicago. USA, 1976.

20. Agrawal, P. and Lalloraya, M.M.: Induction of peroxidase in corpora lutea of rat ovary by lutropin. Biochem. J. 1977 66:,205-208.

21. Yanaihara, N., Yanaihara, C., Sakagmi, M., Tsuji, K., and Hashimoto, T.: Synthesis and biological evaluation of LH and FSH releasing hormone and its analogs. J. Med. Chem1973,. 15: 373-377.

22. Chang, J.K., Sivertssam, H., Currir, B.L., Bogentoft, C., and Folkers, K.: Synthesis of LHRH of the hypothalamus and the 8-lysine analog. J. Med Chem. 1972,15:

23. Chang, J.K., Williams, R.H., and Humphries, A.J.: LHRH synthesis and Arg-analogs, and conformation-sequence-activity relationships. Biochem-biophys. Res. Commun. 1972,47: 727-732.

24. Fujino M, Kobayashi S, Obayashi M, Shinagawa S, Fukuda T structure activity relationship in the C-terminal part of the luteinizing hormone releasing hormone(LH-RH). Biophys Res Commun 1972 49:863-869 .

25. Rich,DH.Singh,j.The carbodiimide: The peptides, Analysis,Synthesis, Biology 2002,1st edition NewYourk Academic Center, Vol. 1,241-261