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Improved solid phase synthesis of luteinizing hormone releasing hormone analogues using 9-fluorenylmethyloxycarbonyl amino acid active esters and catalytic transfer hydrogenation with minimal side-chain protection and their biological activities

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Abstract. Using mainly 9-fluorenylmethyloxycarbonyl amino acid 2, 4, 5-trichlorophenyl esters in the presence of 1-hydroxybenzotriazole and the solid support *p*-alkoxybenzyl alcohol resin, synthesis of luteinizing hormone releasing hormone analogues was carried out with minimal side-chain protection. Catalytic transfer hydrogenation was employed for removal of NO_2 and Z-groups from Arg and < Glu respectively avoiding the use of HF and this led to good yields. An aromatic, hydrophilic amino acid, D-(*p*-hydroxyphenyl) glycine was incorporated into luteinizing hormone releasing hormone molecule along with other modifications. The agonistic as well as antagonistic activities of all the peptides have been studied.

Keywords. Fmoc-amino acid trichlorophenyl esters; solid phase synthesis; minimal side-chain protection; LHRH analogues; biological studies.

Introduction

Syntheses of the hypothalamic hormone, luteinizing hormone releasing hormone (LHRH) and its agonists and antagonists using acid labile protecting groups like Boc, Z, etc., for α-amino or side-chain protection generally involves final treatment with anhydrous liquid hydrogen fluoride leading to contamination of the final product with closely related impurities thereby necessitating extensive purificaiton. In solid phase synthesis (SPS) of peptides, use of base labile 9-fluorenylmethyl-oxycarbonyl (Fmoc) group for N^{α}-protection would allow milder conditions to be employed during the synthesis in addition to the requirement of minimal side-chain protection and this strategy was followed for the synthesis of LHRH analogues.

A survey of literature on structure-activity studies of several LHRH analogues (Schally and Coy, 1977; Schally, 1978; Schally *et al.,* 1979, 1980), indicates that highly potent agonists result from introduction of either aliphatic D-amino acids with bulky side-chains or aromatic D-amino acids in the place of Gly^6 residue, and/or by replacement of Gly^{10} residue by alkylamide (particularly ethylamide). A combination of these two modifications has synergistic effects on biological activity. The modification that enabled antagonists to be potent enough to block ovulation

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Abbreviations used: LHRH, Luteinizing hormone releasing hormone; SPS, solid phase synthesis; Fmoc, 9-fluorenylmethyloxycarbonyl; Hpg, (*p*-hydroxyphenyl)glycine; CTH, catalytic transfer hydrogenation; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; OTcp, trichlorophenyl esters; DMF, dimethylformamide.

was the incorporation of aromatic D-amino acids such as D-Phe rather than alkyl D-amino acids in place of His^2 and/or Gly^6 residues. D-Trp in position 3 has been extensively used to increase the activity of antagonists. Replacing <Glu in position 1 by the D-isomer or aromatic D-amino acid causes increase in potency (Rivier and Vale, 1978). Acylated D-amino acids at this position give even better results (Channabasavaiah and Stewart, 1979). The effective dose of antagonists can further be lowered by replacement of Gly^{10} by D-Ala (Erchegui *et al.,* 1981). Taking into account all these observations, 17 LHRH analogues (table 1) involving single or multiple changes at positions 1, 2, 3, 6 and 7 were designed. In some of these modifications, an aromatic hydrophilic amino acid, D-(*p*-hydroxyphenyl) glycine (Hpg) was incorporated as this would enable us to understand the role of a phenolic group on biological activities.

Materials and methods

Synthesis

All the amino acids employed, except glycine, are of L-configuration unless otherwise specified. The purity of the final peptides was demonstrated by paper chromatography on Whatman No. 1 chromatography paper strips by ascending method using the following solvent systems:

A. *n*-BuOH-HOAc-H_2O (4:5:5, upper phase, v/v)
B. *n*-BuOH-HOAc-H_2O-pyridine (30:6:24:20, v/v)

and R_f values were expressed as R_f A and R_f B respectively. For amino acid analyses, peptides were hydrolysed with 6 N HCl at 100° for 24 h. Elemental analyses were carried out after drying the products for 12 h at 50°C under vacuum.

p-Benzyloxybenzyl alcohol resin was prepared by refluxing 4-hydroxybenzyl alcohol and Merrifield resin in the presence of NaOMe in dimethylacetamide at 100°C for 48 h (Wang, 1973; Gui-shen Lu *et al.,* 1981). Fmoc-Cl prepared according to Carpino and Han (1972, 1973) was used for the introduction of Fmoc group into all the amino acids except for Gly and D-Ala for which Fmoc-N_3 (Tessier *et al.,* 1983) was used. Fmoc-amino acids and their active esters were prepared according to Chang *et al.* (1980) and Sivanandaiah and Gurusiddappa (1984) respectively.

The protocol for the synthesis of LHRH analogues is outlined in figure 1. The first amino acid was anchored to *p*-benzyloxybenzyl alcohol resin as its Fmoc derivative using DCC in the presence of *p*-dimethylaminopyridine (Meienhofer *et al.,* 1979) and the amino acid content on the resulting resin was determined spectrophotometrically. Fmoc cleavage was effected by using the mild base 60% Et_2NH in DMF in about 3 h. The completion of deprotection and coupling was monitored by Kaiser's qualitative ninhydrin test (Kaiser *et al.,* 1970). The protected peptide was released from the resin by ammonolysis/ethylammonolysis and the peptide thus obtained was finally subjected to catalytic transfer hydrogenation (CTH) (Sivanandaiah and Gurusiddappa, 1979, 1982). The product was purified by ion-exchange chromatography using CM-Sephadex C-25 and gel filtration on Sephadex G-25 and the chromatographically pure peptides were obtained in 31–42% yield (based on the original amino acid esterified to the resin).

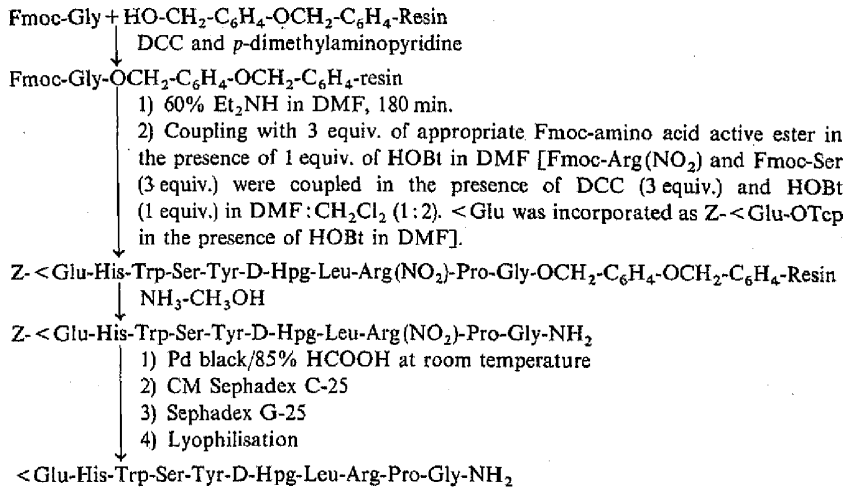

Figure 1. Outline of the synthesis of [D-Hpg^6] LHRH.

Experimental

Synthesis of [*D-Hpg*^{6}] *LHRH* (*analogue II*)

Fmoc-Gly-OCH$_2$*-C*$_6$*H*$_4$*-OCH*$_2$*-C*$_6$*H*$_4$*-Resin:* Fmoc-Gly (0·99 g, 3·3 mmol) was made to react with Hpg resin (2 g) at 0°C in the presence of dicyclohexylcarbodiimide (DCC) (0·68 g, 3·3 mmol) and p-dimethylaminopyridine (0·4 g, 3·3 mmol) for 30 min and at room temperature for 5 h. The reaction mixture was then chilled, treated with benzoyl chloride (1·14 ml) and pyridine (1·02 ml) and shaken for 30 min at 0° and 1 h at room temperature. The yield was found to be 2·26 g. The Fmoc-Gly content per gram of resin was estimated to be 0·41 mmol by spectrophotometric determination of N-(9-fluorenylmethyl)piperidine.

Z-<Glu-His-Trp-Ser-Tyr-D-Hpg-Leu-Arg(NO$_2$*)-Pro-Gly-OCH*$_2$*C*$_6$*H*$_4$*-OCH*$_2$*-C*$_6$*H*$_4$*-Resin:* Fmoc-Gly-OCH$_2$-C$_6$H$_4$-OCH$_2$-C$_6$H$_4$-Resin (2g, 0·7 mmol) was taken in a horizontal solid phase vessel and peptide synthesis was carried out by the manual procedure, similar to that employed for the synthesis of [Leu^5]enkephalin (Sivanandaiah and Gurusiddappa, 1981). Fmoc-amino acid active esters (3 equiv.) in the presence of 1-hydroxybenzotriazole (HOBt) (1 equiv.) was employed throughout except for the N-terminal residue when Z- < Glu-trichlorophenyl ester (OTcp) was used. The synthesis was carried out by a stepwise single addition of appropriate amino acids, until the assemblage of the target peptide was completed. The yield was found to be 2·74 g.

Z-<Glu-His-Trp-Ser-Tyr-D-Hpg-Leu-Arg(NO$_2$*)-Pro-Gly-NH*$_2$*:* The protected decapeptide resin, Z-<Glu-His-Trp-Ser-Tyr-D-Hpg-Leu-Arg(NO$_2$)-Pro-Gly-OCH$_2$-C$_6$H$_4$-OCH$_2$-G$_6$H$_4$-Resin was suspended in absolute methanol (75 ml/g) and subjected to ammonolysis for the release of protected peptide amide. The crude peptide thus obtained was dissolved in a minimum amount of methanol and precipitated

using ether and dimethylformamide (DMF)-ethyl acetate. The yield was found to be 0·740 g (68·7%).

<Glu-His-Trp-Ser-Tyr-D-Hpg-Leu-Arg-Pro-Gly-NH_2·3HOAc: To a solution of protected, peptide, Z- < Glu- His- Trp- Ser- Tyr-D- Hpg-Leu- Arg (NO_2)-Pro-Gly-NH_2 (0·20g, 0·15 mmol) in 85% HCOOH (2 ml) and methanol (1 ml), freshly prepared palladium black (0·15 g) was added and stirred for 2h at room temperature. The catalyst was filtered and the solution was evaporated *in vacuo.* The residue was dissolved in methanol (2 ml) and precipitated by adding ether. The isolated solid was reprecipitated using ethanol-ethyl acetate to yield 0·155 g of the product. This crude peptide was dissolved in a minimum amount of 0·005 M ammonium acetate buffer (pH 6·9) and subjected to ion-exchange chromatography on a column (2 × 11 cm) of CM-Sephadex C-25 (Pharmacia, Sweden) using a stepwise gradient of 0·005, 0·01, 0·05, 0·075, 0·1 and 0·2 M ammonium acetate buffer (pH 6·9) with a flow rate of 60 ml/h. The column fractions were monitored at 280 nm. The fractions corresponding to 0·075 and 0·1 M buffer containing the desired peptide were pooled and lyophilised. The resulting residue was dissolved in the minimum amount of 0·5 M HOAc and the solution was subjected to gel filtration on a column (1·5 × 160 cm) of Sephadex G-25 using the same solvent for elution. Material corresponding to the central portion of the main peak was recovered by lyophilisation as a white fluffy powder. Paper chromatography on Whatman No. 1 chromatography paper strips by ascending method with *n*-BuOH-HOAc-H_2O (4:1:1) gave a single spot after spraying with Pauli, Ehrlich, or Sakaguchi reagent and no spot with ninhydrin reagent. The yield was found to be 0·072 g (overall yield 41 % based on the amount of Fmoc-Gly esterified to the resin); R_f A, 0·60; R_f B, 0·69; $[\alpha]_D^{25}$ –37° (c, 1; 1% HOAc).

Analysis: C_{68} H_{89} O_{19} N_{15} Calcd. C, 57·50; H, 6·2; N, 14·79
Found. C, 57·12; H, 6·01; N, 14·58%.

Amino acid analysis: Gly(l), 1·05; Pro(l), -[a]; Arg(l), 0·87; Leu(l), 0·93; Hpg(l), 1·13; Tyr(l), 1·2; Ser(l), 0·91; Trp(l), -[b]; His(l), 0·91; Glu(l), 1·04.

[a] Pro was not estimated.

[b] Trp was destroyed during acid hydrolysis.

The other 16 analogues were synthesized by following the same procedure with appropriate modifications at each stage and the protected peptide resins were subjected to ammonolysis/ethylammonolysis by using CH_3OH-NH_3/CH_3OH-anhydrous ethylamine. The crude protected peptides, after isolation, were deprotected by 85% HCOOH/Pd black and purified by following the same method employed for the purification of [D-Hpg[6]] LHRH and yields of peptides are given in table 1.

Biological assay

The biological assays were performed at the Institute for Research in Reproduction, Bombay. The activities of these peptides were determined by *in vitro* anterior pituitary cell culture method. Following the method of Vale *et al.* (1972a, b), the ability to stimulate LH release (agonistic activity) and to inhibit LHRH stimulated LH release (antagonistic activity) of all the LHRH analogues were determined.

Table 1. List of LHRH analogues synthesized with yields and LH-releasing activity at 10 ng dose.

Peptide	Yield* (%)	LH-release in ng/ml
Control		1·4
		1·4
Standard LHRH		45·8
		38·2
[D-Hpg^2]LHRH (I)	40·0	1·8
		1·8
[D-Hpg^6]LHRH (II)	41·0	6·7
		9·8
[D-Hpg^7]LHRH (III)	36·7	1·7
		2·1
[D-Phe^2, D-Hpg^6]LHRH (IV)	36·7	1·9
		2·6
[Tyr^2, D-Hpg^6]LHRH (V)	39·2	1·7
		2·1
[Tyr^2, D-Phe^6]LHRH (VI)	36·2	2·3
		2·6
[D-Hpg^2, D-Phe^6]LHRH (VII)	37·2	1·9
		2·1
[D-Phe^1, D-Hpg^2, D-Trp^3, Trp^6]LHRH (VIII)	37·9	1·9
		1·4
[N-Ac-D-Trp^1, D-Hpg^2, D-Phe^6]LHRH (IX)	38·0	2·4
		1·3
[N-Ac-D-Phe^1, D-Hpg^2, D-Trp^3, Trp^6]LHRH (X)	38·8	2·4
		2·5
[N-Ac-D-Trp^1, D-Hpg^2, D-Trp^{3,6}]LHRH (XI)	36·5	1·8
		1·6
[Tyr^2, D-Trp^3, D-Ala^{6,10}]LHRH (XII)	36·2	1·7
		2·4
[D-Hpg^2, D-Trp^3, D-Ala^{6,10}]LHRH (XIII)	41·3	1·8
		1·5
[D-Hpg^7, Pro^9-NHEt]LHRH (XIV)	33·3	1·9
		1·4
[D-Trp^6, D-Hpg^7, Pro^9-NHEt]LHRH (XV)	32·1	3·2
		2·2
[D-Hpg^{2,3},(D-Trp-D-Hpg)^7, Pro^9-NHEt]LHRH (XVI)	34·5	2·3
		2·0
[D-Hpg^2, D-Trp^6, Pro^9-NHEt]LHRH (XVII)	32·1	1·6
		2·5

*Overall yield is based on the amount of amino acid esterified to the resin.

Results and discussion

Synthesis

The time required for the completion of coupling of each amino acid is about 1 to 2 h. The yields of the peptides are given in table 1, and the physical constants and analytical data in table 2. Amino acid analyses of all the peptides gave satisfactory results.

Only the guanidino group in the side-chain of Arg was protected by nitro group and all other side-chain functional groups of amino acids remained unprotected. The

Table 2. Physical constants and elemental analysis of peptides I, III to XVII.

Peptide	R_fA	$[\alpha]_D^{25}$ (c, l; 1% HOAc)	Molecular formula	Elemental analysis Calcd. (found) % C	H	N
I	0·62	−34	$C_{61}H_{83}O_{18}N_{15}$	55·72	6·33	15·98
				(55·38	6·15	15·61)
III	0·59	−34	$C_{67}H_{91}O_{20}N_{17}$	55·31	6·30	16·36
				(55·12	6·03	15·99)
IV	0·58	−34	$C_{68}H_{89}O_{18}N_{15}$	58·16	6·34	14·96
				(57·94	6·01	14·63)
V	0·62	−36	$C_{68}H_{89}O_{19}N_{15}$	57·50	6·27	14·79
				(57·09	6·05	14·49)
VI	0·64	−41	$C_{69}H_{91}O_{18}N_{15}$	58·43	6·42	14·82
				(57·98	6·12	14·70)
VII	0·59	−28	$C_{68}H_{89}O_{18}N_{15}$	58·16	6·34	14·96
				(57·89	6·09	14·69)
VIII	0·64	−36	$C_{74}H_{94}O_{17}N_{16}$	61·82	6·37	15·14
				(61·54	6·07	14·97)
IX	0·63	−30	$C_{74}H_{92}O_{16}N_{16}$	60·82	6·30	15·34
				(60·71	6·19	15·11)
X	0·69	−28	$C_{74}H_{92}O_{16}N_{16}$	60·82	6·30	15·34
				(61·02	6·25	15·15)
XI	0·71	−30	$C_{76}H_{93}O_{16}N_{17}$	60·82	6·25	15·86
				(60·23	6·24	15·69)
XII	0·56	−39	$C_{64}H_{87}O_{19}N_{14}$	56·63	6·41	14·45
				(56·43	6·18	14·24)
XIII	0·63	−33	$C_{63}H_{85}O_{19}N_{14}$	56·37	6·33	14·61
				(56·09	6·10	14·49)
XIV	0·68	−31	$C_{65}H_{89}O_{19}N_{16}$	55·80	6·41	16·02
				(55·53	6·32	15·77)
XV	0·65	−20	$C_{74}H_{96}O_{19}N_{17}$	58·17	6·33	15·58
				(57·86	6·23	15·34)
XVI	0·63	−29	$C_{73}H_{92}O_{20}N_{15}$	58·45	6·18	14·00
				(58·01	6·12	13·69)
XVII	0·67	−21	$C_{70}H_{91}O_{17}N_{15}$	59·44	6·44	14·86
				(58·97	6·24	14·68)

combination of Z for < Glu and NO_2 for Arg permitted the final deprotection of the protected peptide by CTH. As a result, the drastic treatment of the protected peptide with HF at the end of the synthesis was circumvented. Use of Fmoc group for N^{α}-protection and coupling by the active ester method necessitated minimal side-chain protection leading to better yields (35–40% based on the original amino acid esterified to the resin) of chromatographically pure peptides.

Biological assays

The LH-releasing activity of all the analogues (agonistic activity) *i. e.*, LH levels measured by radioimmunoassay is given in table 1. These assays indicate that these analogues possess very little LHRH activity, with [D-Hpg^6] LHRH (analogue II) having only 5% of the activity. Though, in general, replacement of the Gly^6 residue in LHRH by D-amino acids, particularly aromatic residues such as D-Phe and

D-Tyr makes the molecule agonistic, introduction of D-Hpg at this position leads to weak agonistic analogues only. The loss in activity is also observed when D-Hpg is substituted at position 2 or 7 (analogues I and III). Further, introduction of D-Hpg at position 2 or 7 in [D-Trp^6, Pro^9-NHEt] LHRH and at position 7 in [Pro9-NHEt] LHRH (analogues XVII, XV and XIV), leads to marked decrease in potency of these peptides, which are several times more active than LHRH.

The antagonistic properties were studied by their ability to inhibit LHRH stimulated LH release by adding 10 ng of standard LHRH and an analogue at 10 ng dose to duplicate cultures. These results indicate that, at a dosage of 10 ng, all these analogues possess no significant antagonistic activity. Though analogues like [D-Phe^2, D-Trp^6] LHRH, [D-Phe^{2,6}]LHRH, are known to be good antagonists, the introduction of D-Hpg residue at position 6 in [D-Phe^2]LHRH (analogue IV) leads to a marked decrease in potency. Further, introduction of Ac-D-Trp at position 1 in [D-Hpg^2, D-Phe^6] LHRH renders the molecule inactive (analogue IX). Tyr or D-Hpg at position 2 of [D-Trp^3, D-Ala^{6,10}] LHRH does not enhance activity at 10 ng dosage (analogues XII and XIII). These results suggest that introduction of D-Hpg into LHRH molecule generally leads to the inactivity.

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