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SYNTHESIS OF [D-Phe^3(NH_2); Pro^3; D-Ala^6]- and [D-Phe^2(NO_2); Pro^3; D-Ala^6]LULIBERINS

S. V. Burov, O. A. Kaurov, V. F. Martynov, and M. P. Smirnova

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In order to study the influence of substituents of the aromatic ring of D-phenylalanine on the inhibiting capacity of luliberian analogs, we have synthesized two new analogs: [D-Phe(NH_2)^2; Pro^3; D-Ala^6]- and [Phe(NO_2)^2; Pro^3; D-Ala^6]luliberin. The synthesis was performed by the fragmentary condensation method using 2 + (3 + 5) and 2 + (5 + 3) schemes. A new and convenient method of obtaining the amide of the C-terminal tetrapeptide of the luliberin sequence has been developed. In the condensation of the fragments, both the azide and the carbodimide method of synthesis with the addition of 1-hydroxybenzotriazole were used. The guanidino group of arginine was protected by nitration, while the hydroxy groups of serine and of tyrosine were not protected. The complete elimination of the protective groups from the decapeptides was performed by catalytic hydrogenation over Pd on carbon and by anhydrous HF with the addition of anisole at 0°C. The protected octa- and decapeptides were purified by gel filtration on Sephadex LH-20 in ethanol or by preparative thin-layer chromatography on silica gel plates. The final peptides were purified by ion-exchange chromatography on Sephadex CM-25.

The releasing factor of the luteinizing hormone — luliberin — was first isolated in 1971 from extracts of porcine hypothalamus [1]. As was found, it consists of the amide of a linear decapeptide having the sequence:

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2.
1 2 3 4 5 6 7 8 9 10

By controlling the secretion of luteinizing and follicle-stimulating hormones, luliberin exerts a decisive influence on the course of ovulation processes.

The possibility of using both the releasing hormone itself and its analogs as pharmacological preparations is leading to great interest in the search for luliberin agonists and antagonists.

In the present paper we consider the synthesis of two new luliberin analogs that are potential inhibitors of the releasing hormone. The synthesis was carried out by using the classical method by a scheme permitting the building up of analogs modified in positions 2, 3, and 6 in large amounts and with good yields.

As is known, the introduction of D-(amino acid)s into position 2 of the luliberin molecule leads to compounds with a high antagonistic activity [2]. At the same time, in Yardley's

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investigations on the synthesis of [D-Phe(F)^2; D-Ala^6]- and [D-Phe(Cl)^2; D-Ala^6]luliberins it has been established that only the first of these analogs is an effective inhibitor, while the second analog does not possess the activity under consideration [3].

With the aim of a more detailed study of the influence of substituents in the aromatic ring of D-phenylalanine on the inhibiting capacity of luliberin analogs we decided to introduce p-amino- and p-nitro-D-phenylalanine into the second position of the hormone molecule. Since, according to the literature, the introduction of proline into position 3 [4] and of D-alanine into position 6 [5] intensifies the inhibiting activity, we also used these modifications.

To synthesize the analogs under consideration we used the method of fragment condensation by 2 + (3 + 5) and 2 + (5 + 3) schemes (see below), which enabled us to vary the nature of the amino acid residues in positions 2, 3, and 6.

As a rule, the synthesis of the C-terminal tetrapeptide of the luliberin sequence causes particular difficulties because of the solubility of this peptide and of all the intermediate compounds in water due to the hydrophilic properties of the guanidino group of arginine and the glycinamide grouping [6]. The protection of the guanidino group of arginine and the glycinamide grouping [6]. The protection of the guanidino group of arginine by nitration that is usually used does not ensure the complete suppression of the hydrophilic properties of the latter and, in view of this, it imposes certain limitations on the method of activating the carboxy group of this amino acid in connection with the formation of a lactam. Furthermore, beginning the synthesis with glycinamide is undesirable because of the difficulties connected with its introduction into the reaction, while the amidation of esters of tri- and tetrapeptides and higher peptides leads to degradation of the nitroguanidino group, and in the case of a dipeptide it may give rise to urea derivatives and hydantoins as byproducts [7].

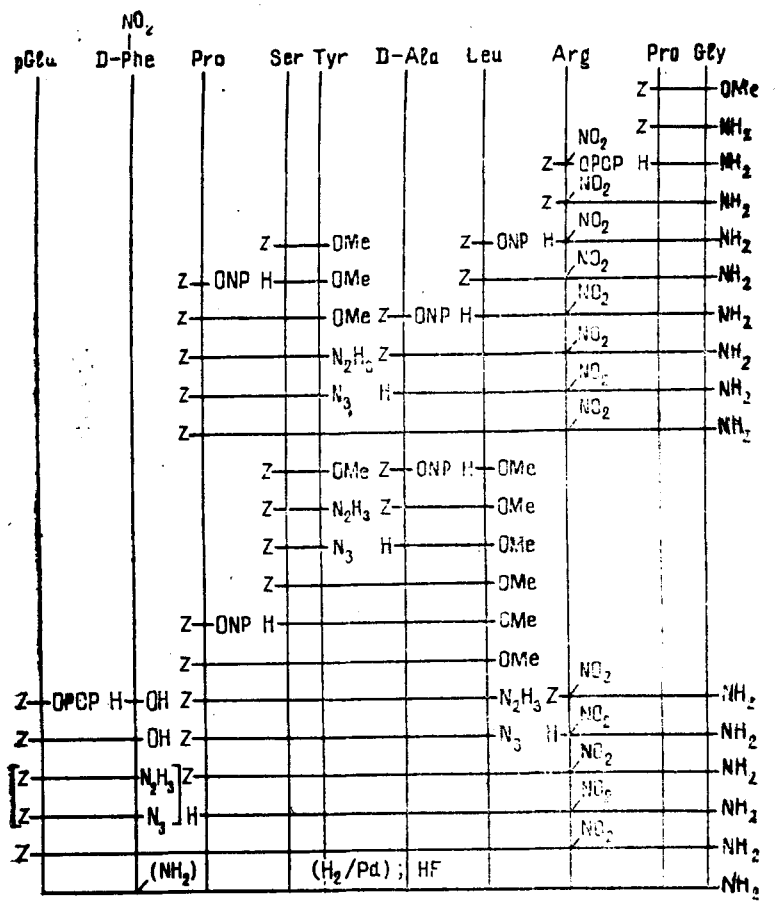
We attempted to overcome some of the difficulties mentioned with the aid of a number of known methodological approaches to peptide synthesis which have been used in the production of peptides with different sequences. Thus, having tested several variants of the amidation conditions and of protective groupings in proline, we obtained the amide of the C-terminal dipeptide without using glycinamide. A number of variants of the synthesis of the C-terminal tetrapeptide and the results obtained by their use have been published previously [8].

The introduction of benzyloxycarbonylnitroarginine in the form of the pentachlorophenyl ester that has been described in the literature [9] also proved to be successful. To eliminate the benzyloxycarbonyl protection we used hydrogen bromide in trifluoroacetic acid. This permitted us to avoid the incomplete deblockage at the stage of the tetra- and pentapeptides that has been reported in the literature [10] and also the acetylation of the free hydroxy group of serine and tyrosine taking place when hydrogen bromide in acetic acid is used.

In all the condensation reactions by the p-nitrophenyl ester method (with the exception of the synthesis of peptides containing serine and tyrosine, because of the formation of byproducts) we used 1,2,4-triazole as catalyst. Such a catalyst permits a considerable shortening of the reaction time and some increase in yield. The intermediate protected peptides were condensed by the azide method or by the carbodiimide method with the addition of 1-hydroxybenzotriazole.

Hitherto (in the literature), the azide synthesis has been used only in the case of peptides with free pyroglutamic acid because of the instability of the corresponding derivatives protected at the nitrogen atom under the conditions of hydrazinolysis. However, in recent years a new method of synthesizing hydrazides has been proposed which uses a carbodiimide with the addition of 1-hydroxybenzotriazole, and is particularly valuable in cases where hydrazinolysis is undesirable [11]. Using this method we have obtained for the first time and characterized the hydrazide of benzyloxycarbonylpyroglutaminyl-D-nitrophenylalanine and have shown the possibility of using it in the azide synthesis.

The protective groups were eliminated completely from the protected decapeptide by catalytic hydrogenation over palladium on carbon and by anhydrous hydrogen fluoride with the addition of anisole at 0°C.



Scheme of Synthesis

The protected octa- and decapeptides were subjected to gel filtration on Sephadex LH-20 in ethanol or to preparative thin-layer chromatography on silica gel plates. The final peptides were purified by ion-exchange chromatography on Sephadex CM-25. The purity of the intermediate compounds and of the final product was checked by thin-layer chromatography, electrophoresis, and elementary and amino acid analyses.

EXPERIMENTAL

Melting points were determined in open capillaries without correction.

The individuality of the compounds obtained was established by thin-layer chromatography on plates coated with silica gel fixed with gypsum in the following system: 1) chloroform—methanol (9:4); 2) sec-butanol—1% NH_4OH (3:1); 3) tert-butanol—acetic acid—water (4:1:1); and 4) benzene—ethanol—ethyl acetate (3:1:1), and also by paper electrophoresis in 2% acetic acid. The zones on the electrophoretograms were revealed with isatin, the Sakaguchi reagent, and chlorine, and those on the chromatograms with iodine.

The elementary and amino acid analyses of the compounds synthesized corresponded to the calculated C, H, N, contents and amino acid ratios. The amino acid analyses were performed on Beckman and Philips automatic analyzers.

Solutions of the substances in organic solvents (in ethyl acetate, previously dried over Na_2SO_4) were evaporated in vacuum in a rotary evaporator at a temperature not exceeding 40°C.

The following abbreviations have been used: CDI — N,N'-dicyclohexylcarbodiimide; Z — the benzyloxycarbonyl group; TEA — triethylamine; NMM — N-methylmorpholine; HOBt — 1-hydroxybenzotriazole; DMFA — dimethylformamide; and EA — ethyl acetate.

1. Preparation of Z-Pro-Gly-OMe. The hydrochloride of the methyl ester of glycine (2.44 g; 1.9 mmole) was treated with a saturated solution of ammonia in chloroform at room temperature for 10 min. The precipitate was filtered off and the solvent was evaporated off.

The oil obtained was dried in vacuum for 1 h and was dissolved in EA, after which 1.12 g (1.6 mmole) of 1,2,4-triazole and then, with stirring, a solution of 6 g (1.6 mmole) of the p-nitrophenyl ester of benzyloxycarbonyl-L-proline in EA were added. After 1 h at room temperature, the solution was washed with 5% $NaHCO_3$, with water, with 1 N HCl, and with water again. The solvent was evaporated off and the resulting oil was dried in vacuum. Yield 100% of theory. The product was chromatographically homogeneous (R_f 0.84 in system 2; 0.89 in system 3).

2. Preparation of Z-Pro-Gly-NH_2. The methyl ester of the dipeptide obtained by the procedure of paragraph 1 was treated with saturated solution of ammonia in methanol at room temperature for a day with continuous chromatographic monitoring in systems 2 and 4. The solvent was driven off in vacuum and the product was crystallized from ether. This gave 4.64 g of product in the form of a white crystalline powder. Yield 94% of theory. The product was chromatographically homogeneous (R_f 0.56 in system 4), mp 114-116°C; according to the literature, mp 120°C [12].

3. Elimination of the Z- Protective Group from Z-Pro-Gly-NH_2. A current of hydrogen bromide was passed through a solution of 1 g (0.3 mmole) of benzyloxycarbonyl-L-prolylglycinamide in trifluoroacetic acid at room temperature for 20 min. The product was precipitated with ether, filtered off, and dried in vacuum over KOH. The benzyloxycarbonyl protective group was eliminated from the other peptides in a similar manner.

NO_2
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4. Preparation of Z-Arg-Pro-Gly-NH_2. The hydrobromide of the dipeptide amide obtained by the method of paragraph 3 was dissolved in DMFA and, with stirring, the resulting solution was treated with 2.36 (0.39 mmole) of the pentachlorophenyl ester of benzyloxycarbonyl-L-nitroarginine and 0.9 ml (0.67 mmole) of TEA. After 2 days at room temperature, the reaction mixture was acidified and the solvent was evaporated off. The residue was dissolved in water-saturated butan-1-ol. The solution was washed with 5% $NaHCO_3$, water, 1 N HCl, and water again. The solvent was evaporated off and the product was crystallized from ether. It was reprecipitated from a mixture of ethanol with ether. This gave 1.44 g of product in the form of a light yellow amorphous powder. Yield 87% of theory; chromatographically homogeneous (R_f 0.54 in system 2; 0.7 in system 3; 0.18 in system 4).

NO_2
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5. Preparation of Z-Leu-Arg-Pro-Gly-NH_2. The hydrobromide of the tripeptide amide obtained from 2 g (0.39 mmole) of the benzyloxycarbonyl derivative was dissolved in DMFA and to the resulting solution were added with stirring 0.27 g (0.39 mmole) of 1,2,4-triazole, 3.05 g (0.78 mmole) of the p-nitrophenyl ester of benzyloxycarbonyl-L-leucine, and 0.43 ml (0.39 mmole) of NMM. After 2 days at room temperature, the solvent was evaporated off and the subsequent working up was carried out by the method of paragraph 4. The product was crystallized from ether, filtered off, and washed on the filter with ether. This gave 1.77 g of a white amorphous substance. Yield 72% of theory. The product was chromatographically homogenous (R_f 0.55 in system 2, 0.73 in system 3, and 0.17 in system 4).

NO_2
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6. Preparation of Z-D-Ala-Leu-Arg-Pro-Gly-NH_2. The synthesis was performed by the method of paragraph 5, starting with 1.7 g (0.27 mmole) of the benzyloxycarbonyltetrapeptide amide. This gave 1.63 g of a white amorphous product. Yield 85% of theory. The product was chromatographically homogeneous (R_f 0.61 in system 2, 0.79 in system 3, and 0.19 in system 4).

7. Preparation of Z-Ser-Tyr-OMe. A. By the procedure of paragraph 1, 2 g (0.86 mmole) of the hydrochloride of L-tyrosine methyl ester was treated with a saturated solution of ammonia in chloroform. The resulting white crystalline substance was dissolved in EA with the addition of a small amount of DMFA. The resulting solution was treated with 4.2 g (0.86 mmole) of the pentachlorophenyl ester of benzyloxycarbonyl-L-serine and the pH of the reaction mixture was brought to 8 by the addition of NMM. After 3 days at room temperature, the reaction mixture was acidified and the solvent was evaporated off. The residue was diluted with water and extracted three times with EA. The organic phase was washed with 5% $NaHCO_3$,

water, 1 N HCl, and water again, and the solvent was evaporated off. This gave 3.6 g of a white crystalline powder. Yield 100% of theory. The product was chromatographically homogeneous (R_f 1 in system 2, 1 in system 3, and 0.59 in system 4), mp 103-105°C; according to the literature, mp 108-115°C [13].

B. The hydrochloride of L-tyrosine ethyl ester (2 g; 0.86 mmole) was treated by the method of paragraph A. With cooling to 0°C, a solution of 1.96 g (0.95 mmole) of CDI in EA was added to a solution of 2.1 g (0.86 mmole) of Z-L-serine and 1.2 g (0.86 mmole) of HOBt in EA cooled to 0°C. The mixture was stirred at 0°C for 1 h and at room temperature for some time, after which the previously prepared solution of the amino component was added to the reaction mixture. After a day at room temperature the solution was acidified with acetic acid, the N,N'-dicyclohexylurea was filtered off, and the product was worked up by the method of paragraph A. This gave 3.6 g of product in the form of white crystalline powder. Yield 100% of theory. The product was chromatographically homogeneous; mp 100-104°C.

8. Preparation of Z-Pro-Ser-Tyr-OMe. The synthesis was performed from 1 g (0.24 mmole) of the methyl ester of the benzyloxycarbonyldipeptide by a method analogous to that of paragraph 5 but without the addition of the 1,2,4,-triazole. This gave 0.7 g of a white crystalline substance. Yield 70% of theory. The product was chromatographically homogenous (R_f 0.93 in system 2, 0.93 in system 3, and 0.75 in system 4); mp 176-178°C.

9. Preparation of Z-Pro-Ser-Tyr-$NHNH_2$. The tripeptide methyl ester (1.14 g; 0.22 mmole) was dissolved in boiling methanol and the resulting solution was treated with 0.86 ml (2.7 mmole) of hydrazine. The reaction mixture was kept in the refrigerator for a day. The product was precipitated with ether, filtered off, and washed with a mixture of ethanol and ether. It was recrystallized from methanol. This gave 0.88 g of product in the form of a light gray crystalline powder. Yield 77% of theory. The product was chromatographically homogeneous (R_f 0.71 in system 1, 0.62 in system 2, and 0.84 in system 3); mp 204-205°C,

10. Preparation of Z-D-Ala-Leu-OMe. The synthesis was performed from 3 g (1.7 mmole) of the hydrochloride of L-leucine methyl ester by a method analogous to that of paragraph 1. This gave 4.82 g or product in the form of an oil. Yield 100% of theory. The product was chromatographically homogeneous (R_f 0.95 in system 1, and 1 in system 3); according to the literature, mp 72-73°C [14].

11. Preparation of Z-Ser-Tyr-$NHNH_2$. The methyl ester of the dipeptide (2.24 g; 0.54 mmole) was treated by the method of paragraph 9. The product was recrystallized from dioxane and was then worked up by the procedure of paragraph 4. This gave 1.4 g of product in the form of a white crystalline powder. Yield 63% of theory. It was chromatographically homogeneous (R_f 0.65 in system 1, 0.81 in system 2, and 0.92 in system 3); mp 190-192°C; according to the literature, mp 213-214°C [15].

12. Preparation of Z-Ser-Tyr-D-Ala-Leu-OMe. The hydrobromide of the dipeptide methyl ester obtained from 2.36 g (0.67 mmole) of the benzyloxycarbonyl derivative was dissolved in DMFA. With cooling, 3.8 ml (1.02 mmole) of a 2.65 N solution of HCl in tetrahydrofuran was added to a solution of 1.4 g (0.34 mmole) of the dipeptide hydrazide in DMFA. The reaction mixture was cooled to −30°C and, with stirring, 0.42 g (0.4 mmole) of tert-butyl nitrite was added. After 10 min, a cooled solution of the amino component and 2.4 ml (1.7 mmole) of TEA were added. The pH of the reaction mixture was brought to 8.5 by the addition of TEA. The solution was stirred at −30°C for 1 h, at −10°C for 1 h, and at room temperature for 2 days. Then it was acidified with 1 N HCl and the solvent was evaporated off. The residue was dissolved in EA and was worked up by the method of paragraph 1, after which the product was washed on the filter with ether. This gave 1.48 g of a white crystalline powder. Yield 73% of theory. The product was chromatographically homogenous (R_f 0.67 in system 1, 1 in system 2, and 1 in system 3); mp 178-189°C.

13. Preparation of Z-Pro-Ser-Tyr-D-Ala-Leu-OMe. The synthesis was carried out from 3.28 g (0.55 mmole) of the benzyloxycarbonyltetrapeptide methyl ester by a procedure analogous to that of paragraph 8, with the addition of 1.45 g (0.55 mmole) of pentachlorophenol. This gave 3.11 g of product in the form of a white crystalline substance. Yield 82% of theory. The product was chromatographically homogeneous (R_f 0.91 in system 1, 0.88 in system 2, and 0.96 in system 3); mp 121-123°C.

14. Preparation of Z-Pro-Ser-Tyr-D-Ala-Leu-$NHNH_2$. The synthesis was performed from 3.1 g (0.44 mmole) of the pentapeptide methyl ester by the method of paragraph 9. The prod-

uct was recrystallized from dioxane, which gave 1.63 g of a white crystalline powder. Yield 53% of theory. The product was chromatographically homogeneous (R_f 0.81 in system 1, and 0.82 in system 3); mp 173-174°C.

NO_2
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15. Preparation of Z-Pro-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly-NH_2. A. The hydrazide of benzyloxoycarbonyl-L-prolyl-L-seryl-L-tyrosine (0.26 g; 0.05 mmole) was treated by a method analogous to that of paragraph 12, using n-butyl nitrite. A solution of the hydrobromide of D-alanyl-L-leucyl-L-nitroarginyl-L-propylglycinamide in DMFA obtained from 0.35 g (0.05 mmole) of the benzyloxycarbonyl derivative was used as the amino component. The reaction mixture was kept at −30°C for 1 h and at room temperature for 3 days. Then it was worked up by the method of paragraph 4. After the solvent had been evaporated off, the product was either purified by gel filtration on a column of Sephadex LH-20 (72 × 4 cm) in ethanol, or it was dissolved in a mixture of EA and water, the solution was extracted three times with the EA, the aqueous layer was evaporated, and the product was crystallized from a mixture of EA and ether (fraction 0.25 g). The organic phase was evaporated and the residue was crystallized from a mixture of EA and ether and was reprecipitated several times from a mixture of methanol and ether (fraction 0.05 g). This gave 0.3 g of product in the form of a white amorphous substance. Yield 55% of theory. The product was chromatographically homogeneous (R_f 0.71 in system 1, 0.53 in system 2, and 0.75 in system 3).

B. The synthesis was performed from 1 g (0.14 mmole) of the hydrazide of benzyloxycarbonyl-L-prolyl-L-seryl-L-tyrosyl-D-alanyl-L-leucine using tert-butyl nitrite and the hydrobromide of L-nitroarginyl-L-prolylglycinamide obtained from 1.45 g (0.29 mmole) of the benzyloxycarbonyl derivative by a method analogous to that of paragraph A. For purification, the product, in 0.2-portions, was subjected to separation on silica gel plates for preparative thin-layer chromatography in system 1. The product was eluted with methanol and the solvent was evaporated off. This gave 0.7 g of a white amorphous powder. Yield 47% of theory. In its physicochemical characteristics, the compound synthesized was identical with that obtained by method A.

NO_2
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16. Preparation of Z-p-Glu-D-Phe-OH. With stirring, a solution of 2.44 g (0.5 mmole) of the pentachlorophenyl ester of benzyloxycarbonyl-L-pyroglutamic acid in dioxane was added to a solution of 1 g (0.5 mmole) of nitro-D-phenylalanine [16] in 4.8 ml (0.5 mmole) of 1 N NaOH. Then 0.5 ml (0.5 mmole) of NMM was added to the reaction mixture and it was heated to 30°C for 1 h. After a day at room temperature, the precipitate that had been deposited was filtered off and was washed with water and ether. The dioxane was evaporated off on the filtrate, and the residue was treated with 5% $NaHCO_3$ to pH 7.5 and was extracted with EA. The aqueous layer was acidified with 1 N HCl to pH 2, and the precipitate that deposited was filtered off. The total amount of product obtained was 1.52 g; light yellow powder. Yield 70% of theory. The product was chromatographically homogeneous (R_f 1 in system 2 and 0.67 in system 4); mp 176-178°C.

NO_2
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17. Preparation of Z-pGlu-D-Phe-$NHNH_2$. A solution of 0.46 g (0.1 mmole) of the dipeptide in DMFA was treated with 0.27 g (0.2 mmole) of HOBt and, with cooling to 0°C, with a solution of 0.2 g (0.1 mmole) of CDI and, dropwise, a solution of 0.03 g (0.01 mmole) of hydrazine in DMFA. The mixture was stirred at 0°C for 1 h and at room temperature for a day. Then it was diluted with water and the precipitate was filtered off. This gave 0.33 g of product in the form of white crystalline substance. Yield 70% of theory. The product was chromatographically homogeneous (R_f 0.47 in system 2, and 0.91 in system 3).

NO_2 NO_2
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18. Preparation of Z-pGlu-D-Phe-Pro-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly-NH_2. The hydrobromide of the octapeptide amide obtained from 0.3 g (0.03 mmole) of the benzyloxycarbonyl derivative was dissolved in DMFA, and 0.13 g (0.03 mmole) of benzyloxycarbonyl-L-pyroglutaminyl-D-nitrophenylalanine, 0.04 ml (0.03 mmole) of TEA, and 0.08 g (0.06 mmole) of HOBt and, with cooling to 0°C, 0.09 g (0.045 mmole) of CDI were added. The mixture was stirred at 0°C for 1 h and at room temperature for a day. The solvent was evaporated off and then the product was puri-

fied either by gel filtration on a column on Sephadex LH-20 (72 × 4 cm) in ethanol or by the method of paragraph 4 (the pH of the solution used was brought to 7.5 with $NaHCO_3$). This gave 0.38 g of product in the form of a light yellow crystalline powder. Yield 96% of theory. It was chromatographically homogeneous (R_f 0.66 in system 1, 0.50 in system 2, and 0.70 in system 3); mp 121-124°C.

NH_2
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19. Preparation of pGlu-D-Phe-Pro-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly-NH_2. The protective groups were removed from the decapeptide amide by catalytic hydrogenation of a solution in methanol with the addition of 10% acetic acid in the presence of Pd on carbon as catalyst. The course of the reaction was monitored by electrophoresis. The catalyst was filtered off and was washed with 10% acetic acid. The methanol was evaporated off from the solution and the residue was lyophilized. The lyophilizate was dissolved in 0.005 M NH_4OAc and was purified on a column of ion-exchange Sephadex CM-25 (9 × 150 mm) in a gradient of from 0.005 M to 0.3 M NH_4OAc. The fractions giving a positive reaction with the Sakaguchi reagent were lyophilized and desalted on Sephadex G-25 in 50% acetic acid.

NO_2
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20. Preparation of pGlu-D-Phe-Pro-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly-NH_2. The protective groups were removed from the decapeptide amide by the action of anhydrous HF with the addition of anisole at 0°C for 1 h. The residue was dried in vacuum over KOH, dissolved in water, and lyophilized. It was treated further by the method of paragraph 19.

SUMMARY

1. New analogs of the luteinizing hormone releasing factor have been synthesized:
NH_2 NO_2
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[D-Phe^2; Pro^3; D-Ala^6]- and [D-Phe^2; Pro^3; D-Ala^6]luliberin.

2. A scheme of synthesis which is convenient for obtaining luliberin analogs modified in positions 2, 3, and 6 has been developed.

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