# SYNTHESIS AND INVESTIGATION OF THE BIOLOGICAL ACTIVITY OF SHORTENED ANALOGUES OF LULIBERIN

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Five new luliberin analogues with shortened amino acid sequences have been synthesized. The different variants for obtaining the compounds under consideration are discussed. In an investigation of the biological activities of the analogues it was shown that they possess a pronounced action of the course of the ovulation processes in experimental animals.

The synthesis of shortened analogues of luliberin (LH-RH) is of great interest in the study of structure–activity interaction in the molecule of releasing hormones and at the same time may serve as a basis for the creation of effective pharmacological preparations. However, the possibilities of obtaining this type of active compound have been studied inadequately at the present time.

The basis for the present work was a study of fragments of the superactive luliberin agonist [D-Ser($Bu^t$)$^6$]-LH-RH EA conducted by König et al. [1], as a result of which shortened analogues (6-7 amino acid residues) were obtained with activities close to that of the natural releasing hormone. On the basis of these facts and the results of an investigation of the biological activities of the analogues modified in positions 6 and 10 [2], as the initial compound we selected [D-Ala$^6$]–LH-RH EA. In this series of preparations, the first to be obtained were the peptides (1) and (2) [3]:

H-Pro-Ser-Tyr-*D*-Ala-Leu-Arg-Pro-NHEt (1)

*Z*-*D*-Phe-Pro-Ser-Tyr-*D*-Ala-Leu-Arg-Pro-NHEt (2)

The proline residue in position I was introduced for reasons of structural similarity to pyroglutamic acid, which will permit the influence of γ-carbonyl group on the biological activity of an analogue to be evaluated. Benzyloxycarbonyl-D-phenylalanine was used on the basis of literature information that the presence of a free amino group at the N-end of the peptide chain lowers its biological activity [4] and that the introduction of acylated D-amino acids (including D-phenylalanine) into this position leads to an increase in activity [5].

The interesting results obtained in an investigation of the biological activities of peptides (1) and (2) indicated the promising nature of this direction and the necessity for further investigations. With this aim, we have synthesized compounds (3)-(7):

H-Ser-Tyr-*D*-Ala-Leu-Arg-Pro-NHEt (3)

H-*D*-Pro-Ser-Tyr-*D*-Ala-Leu-Arg-Pro-NHEt (4)

BOC-Phe-Ser-Tyr-*D*-Ala-Leu-Arg-Pro-NHEt (5)

pGlu-Ser-Tyr-*D*-Ala-Leu-Arg-Pro-NHEt (6)

H-Pro-Gly-Ser-Tyr-*D*-Ala-Leu-Arg-Pro-NHEt (7)

In the preparation of the compounds under consideration, two schemes of synthesis were used in the main scheme. The hydroxy groups of serine and tyrosine were left unprotected, and the guanidine group of arginine was protected by nitration. In the performance of the condensation of the fragments, the azide method was used, the amino component being taken in excess. The latter feature raised the yield of the reaction and considerably simplified the purification of the final product. The protection was eliminated from the guanidine group of arginine either at the final deblocking stage or immediately before the addition

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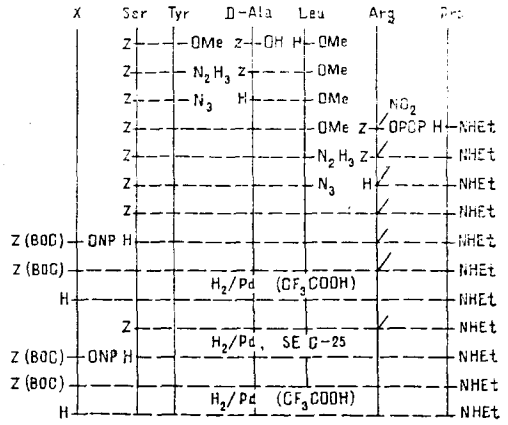
of the N-terminal amino acid residue. In the latter case, as a rule, it was possible to achieve a higher yield. In the preparation of the analogue (7), benzyloxycarbonylprolylglycine was added to the ethylamide of the hexapeptide with the aid of complex F (synthesis with preactivation).




At the stage of final deblocking we used catalytic hydrogenolysis in the presence of palladium black followed by treatment with trifluoroacetic acid (when a tert-butoxycarbonyl protective group was present). The purification of the analogues obtained was performed with the aid of ion-exchange chromatography on Sephadex SE C-25 in pyridine–acetate buffer followed by gel filtration on Sephadex G-15 in 0.2 N acetic acid. In the selection of the fractions containing the final product we used the results of qualitative reactions for tyrosine, arginine, and N-terminal proline.

The biological activities of the analogues were investigated on sexually mature and sexually immature female rats with a dose of 1 mg/kg body weight. In the first compound peptide (3) brought about a sharp reduction in the number of ovulated eggshells, compound (4) turned out to be inactive, and the remaining compounds exhibited a stimulating effect. In the experiments on sexually immature animals, analogues (3), (4), and (6) exhibited a stimulating action. Peptide (5) greatly increased the number of egg cells washed out, while analogue (7) proved to be inactive.

Because of the contradictory nature of the action of compound (3), we investigated its influence on the course of the estrous cycle in the rat. It was found that the estrous cycle passed through all its phases in 3-10 h as compared with 4-5 days in the control. This unusual result was confirmed by the presence of ovulated egg cells in the oviducts of the experimental animals in the estrous stage.

## EXPERIMENTAL

Melting points were determined in open capillaries without correction. The individuality of the compounds obtained was confirmed with the aid of thin-layer chromatography on Silufol UV-254 plates and Kieselgel Merck plates in the following systems: 1) sec butyl alcohol–1% ammonia (3:7); 2) chloroform–methanol (9:4); and 3) n-butyl alcohol–acetic acid–water–pyridine (15:3:6:10).

Electrophoresis was performed on Filtrak FN-12 paper in 2% acetic acid, electrophoretic mobilities being determined in relation to glycine. The results of the elementary and amino acid analyses of the compounds synthesized corresponded to the calculated C, H, and N contents and ratios of amino acids. Amino acid analysis was performed on Jeol JLC-6H and LKB-3201 automatic analyzers. Specific rotations were determined on a Pepol-60 polarimeter in a cell 5 cm long at 18-22°C.

In all cases where not otherwise stated, amino acids of the L-series were used in the synthesis.

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

Abbreviations adopted: DMFA, dimethylformamide; THF, tetrahydrofuran; complex F, complex of N,N'-dicyclohexylcarbodiimide with three molecules of pentafluorophenol.

1. Preparation of Z-Ser-Tyr-$N_2H_3$. A solution of 19.55 g (46.9 mmole) of Z-Ser-Tyr-OMe in 110 ml of hot methanol was treated with 36.6 ml (0.7 mole) of hydrazine hydrate. The reaction mixture was kept in the refrigerator for a day. The precipitate that had deposited was filtered off and was triturated with ether. The product was recrystallized from methanol. Yield 19.4 g (99%). $R_f$ 0.68 (system 1), 0.81 (system 2). mp 209-211.5°C; according to the literature: 213-214°C [7].

2. Preparation of Z-Ser-Tyr-D-Ala-Leu-OMe. The solution of 4 g (9.6 mmole) of Z-Ser-Try-$N_2H_3$ in DMFA was cooled to −30°C, and 6.7 ml (28.8 mmole) of 4.3 N HCl in THF and 1.18 g (11.5 mmole) of tert-butyl nitrite were added. The mixture was stirred at −30°C for 10 min and was then cooled to −45°C and 4 ml (28.8 mmole) of triethylamine was added. After this, a solution of H-D-Ala-Leu-OMe (14.4 mmole) obtained by the catalytic hydrogenolysis of the corresponding benzyloxycarbonyl derivative [6] was added to the reaction mixture, and this was stirred at −30°C for 1 h and at −15°C for 1 h and was kept in the refrigerator for 2 days. The resulting precipitate was filtered off and the solvent was evaporated off. The residue was dissolved in ethyl acetate and the solution was washed with 5% sodium bicarbonate, with 1 N sulfuric acid, and with water. The solvent was evaporated off and the residue was crystallized from ether. Yield 4.5 g (78%), $R_f$ 0.86 (system 1), 0.87 (system 2). mp 179-183°C (see also [6]).

3. Preparation of Z-Ser-Try-D-Ala-Leu $N_2H_3$. The synthesis was performed by the procedure of paragraph 1. Yield 85%, $R_f$ 0.9 (system 1), 0.83 (system 2). mp 205-206°C, $[\alpha]_D^{20}$ −22.8° (c 1.0; DMFA).

4. Preparation of Z-Ser-Try-D-Ala-Leu-Arg($NO_2$)-Pro-NHEt. A solution of 2.4 g (3.99 mmole) of Z-Ser-Try-D-Ala-Leu-$N_2H_3$ in DMFA cooled to −30°C was treated with 1.3 ml (11.97 mmole) of 9.2 N HCl in THF and 0.49 g (4.78 mmole) of tert-butyl nitrite. After 10 min, the reaction mixture was cooled to −45°C, and 1.67 ml (11.97 mmole) of triethylamine and a solution of HBr·H-Arg($NO_2$)-Pro-NHEt obtained from 3.8 g (7.98 mmole) of the benzyloxycarbonyl derivative that had previously been treated with 1.1 ml (7.98 mmole) of triethylamine were added. The mixture was stirred at −30°C for 1 h and at −15°C for 1 h and was left in the refrigerator for 2 days. The solvent was evaporated off and the residue was dissolved in water-saturated N-butyl alcohol. The solution was washed with 5% sodium bicarbonate solution, 1 N HCl, and water. The solvent was evaporated off and the residue was crystallized from ether. Yield 2.55 g (70%). $R_f$ 0.75 (system 1), 0.86 (system 2). mp 136-139°C.

5. Preparation of H-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt. A solution of 0.193 g of Z-Ser-Tyr-D-Ala-Leu-Arg($NO_2$)-Pro-NHEt in a mixture of methanol and 30% acetic acid (1:1) was hydrogenated over palladium black as catalyst. The catalyst was filtered off and the solvent was evaporated off. The residue was chromatographed on a column (15 × 120 mm) containing SE-Sephadex C-25 in gradient of pyridine–acetate buffer of 0.005 to 0.5 M. The fractions giving positive qualitative reactions for arginine and tyrosine were evaporated, dissolved in 0.2 N acetic acid, and subjected to gel filtration on Sephadex G-15 (8 × 975 mm column). The fractions containing the end-product were lyophilized. Yield 49 mg (32%), $E_{Gly}$ 1.34; $R_f$ 0.02 (system 1), 0.65 (system 3).

6. Preparation of BOC-pGlu-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt. To a solution of 0.15 g (0.2 mmole) of H-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt in DMFA were added 2 ml (0.2 mole) of a 0.1 N Solution of HCl and, after a few minutes, 0.084 g (0.24 mole) of BOC-pGlu-ONP. The reaction mixture was stirred for 5 days. The solvent was evaporated off, the residue was dissolved in water-saturated n-butyl alcohol, and the solution was washed with 5% sodium bicarbonate solution in water. The solvent was evaporated off and the residue was recrystallized from ether. Yield 0.136 g (72%). $R_f$ 0.27 (system 1). mp 171-195°C. $[\alpha]_D^{20}$ −19.6° (c 0.5; methanol).

7. Preparation of pGlu-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt. A solution of 0.11 g (0.12 mmole) of BOC-pGlu-Ser-Try-D-Ala-Leu-Arg-Pro-NHEt in trifluoroacetic acid was treated with 0.5 ml of anisole and the mixture was stirred for 30 min. The product was precipitated with absolute ether and was chromatographed on a column (9 × 130 mm) of Sephadex SE C-25 as in paragraph 5. The desired product was lyophilized. Yield 0.1 g. $E_{Gly}$ 0.8. $R_f$ 0.72 (system 3).

8. Preparation of Z-D-Pro-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt. A solution of 0.15 g (0.2 mmole) of H-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt in DMFA was treated with 2 ml (0.2 mmole) of a 0.1 N solution of HCl and, after a few minutes, with 0.089 g (0.24 mmole) of Z-D-Pro-ONP.

The reaction mixture was stirred for 4 days. The solvent was evaporated off and the residue was dissolved in water-saturated n-butyl alcohol. The solution was washed with 5% sodium bicarbonate solution and with water. The solvent was evaporated off and the residue was crystallized from ether. Yield 0.17 g (90%), $E_{Gly}$ 0.89. $R_f$ 0.38 (system 1). mp 120-137°C.

9. Preparation of H-D-Pro-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt. In solution in 6 ml of methanol containing 2 ml of 20% acetic acid, 0.17 g (0.18 mmole) of Z-D-Pro-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt was hydrogenated over palladium black as catalyst. The catalyst was filtered off and the solvent was evaporated off. The residue was chromatographed on a column (15 × 240 mm) of Sephadex SE C-25 as in paragraph 5. The fractions containing the desired product were evaporated and the residue was chromatographed on a column (8 × 1090 mm) of Sephadex G-10 in 0.2 N acetic acid. The product was rechromatographed on a column of Sephadex SE C-25 and was lyophilized. Yield 40 mg. $E_{Gly}$ 1.97. $R_f$ 0.53 (system 3).

10. Preparation of BOC-Phe-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt. The synthesis was carried out by the precedure of paragraph 8, starting from 0.15 g (0.2 mmole) of H-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt. Yield 0.16 g (82%). $E_{Gly}$ 0.81. $R_f$ 0.44 (system 1), 0.74 (system 2), 0.83 (system 3). $[\alpha]_D^{20}$ −19.8° (c 0.5; methanol).

11. Preparation of Z-Pro-Gly-Ser-Tyr-D-Ala-Leu-Arg($NO_2$)-Pro-NHEt. A solution of 0.043 g (0.14 mmole) of Z-Pro-Gly-OH in DMFA cooled to 0°C was treated with 0.106 g (0.14 mmole) of complex F. The reaction mixture was stirred at 0°C for 1 h and the precipitate that had deposited was filtered off. The filtrate was treated with a solution of 0.1 g (0.12 mmole) of HBr·H-Ser-Tyr-D-Ala-Leu-Arg($NO_2$)-Pro-NHEt in DMFA and 0.012 g (0.12 mmole) of triethylamine. Then the reaction mixture was stirred for 3 days. The solvent was evaporated off and the residue was dissolved in water-saturated n-butyl alcohol. The solution was washed with 5% sodium bicarbonate solution, with 1 N HCl, and with water. The solvent was evaporated off and the residue was crystallized from ether. Yield 0.073 g (77%). mp 170-197°C. $R_f$ 0.6 (system 1), 0.88 (system 2).

12. Preparation of H-Pro-Gly-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt. The hydrogenation of 0.07 g (0.07 mmole) of Z-Pro-Gly-Ser-Tyr-D-Ala-Leu-Arg($NO_2$)-Pro-NHEt dissolved in a mixture of methanol, acetic acid, and water (3:1:1) was carried out over palladium black as catalyst. The catalyst was filtered off and the solution was evaporated. The residue was chromatographed on a column (9 × 130 mm) of SE-Sephadex C-25 as described in paragraph 5. The fractions containing the desired product were lyophilized. Yield 0.027 g (47%). $E_{Gly}$ 1.1. $R_f$ 0.65 (system 3).

## CONCLUSION

Liliberin analogues with shortened amino acid sequences have been obtained which exert a pronounced action on the course of the processes of ovulation in experimental animals.

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