7. B. N. Tarusov and V. A. Veselovskii, Superweak Luminescence of Plants and Their Applied Value [in Russian], Moscow (1978).
8. V. Ya. Shlyapintokh, et al., Chemiluminescence Methods of Investigating Slow Chemical Processes [in Russian], Moscow (1966).
9. G. F. Zakis, The Synthesis of Compounds Modeling Lignin [in Russian], Riga (1980), p. 19, 51.

SYNTHESIS OF BIOLOGICALLY ACTIVE ANALOGS OF LULIBERIN WITH SHORTENED AMINO ACID SEQUENCES

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Two new analogs of the releasing factor of the luteinizing hormone with shortened amino acid sequences have been synthesized by the methods of classical peptide chemistry. The influence of the preparations on the action of chorionic gonadotropin and on the course of processes of ovulation in experimental animals has been studied. The promising nature in this direction of the search for active analogs for luliberin has been shown.

In recent years, a large number of analogs of the releasing factor of the luteinizing hormone — luliberin — has been synthesized. The main efforts of research workers have been directed to obtaining effective inhibitors and compounds with a high agonistic activity. Such preparations can be used as contraceptive agents, and in the agricultural industry and clinical medicine in the treatment of certain endocrine and tumoral diseases [1, 2].

To obtain effective agonists, the glycine residue in the sixth position of the natural molecule is usually replaced by a D-amino acid residue and (or) the glycinamide residue in the tenth position by an ethylamide grouping [3]. The strategy for obtaining luliberin antagonists that has been developed amounts basically to the conversion into an inhibitor of a compound with a high agonistic activity by eliminating the histidine residue in the second position of the molecule or replacing it by a D-amino acid residue [4].

In our laboratory, certain new possible directions of the synthesis of active analogs of luliberin are being tested. One of such directions is the production of compounds with shortened amino acid sequences. The creation of active preparations of this type would make them more accessible for practical application and at the same time would permit new information to be obtained on the structure—activity interrelationships in the series of luliberin analogs.

The majority of the highly active analogs of the releasing hormone known at the present time contain from 8 to 11 amino acid residues in the molecule among which in many cases there are difficultly accessible unnatural amino acids such as, for example, substituted D-phenylalanine or amino acids causing certain complications in the course of peptide synthesis (such as histidine, tryptophan, and pyroglutamic acid) [5—8].

Repeated attempts to obtain luliberin analogs containing fewer than 8 amino acid residues have mainly led to inactive compounds as, for example, in the cases of H-Pro-

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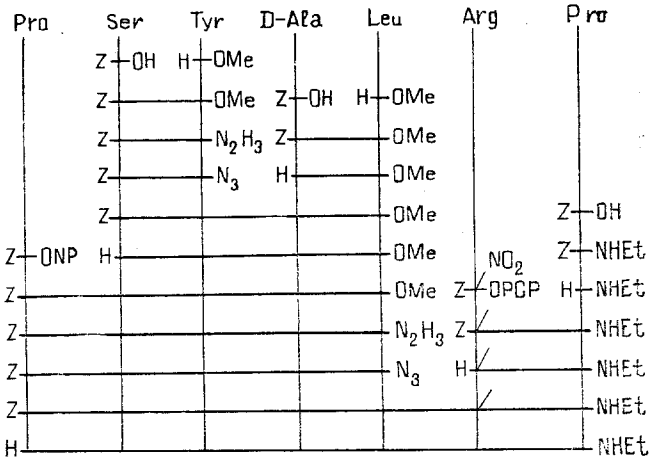
Gly-NH_2, H-Leu-Pro-Gly-NH_2, ..., pGlu-His-NH_2, pGlu-His-Trp-NH_2, ..., Ac-Ser-Tyr-Gly-Leu-Arg-Pro-NH_2, Ac-Gly-Leu-Arg-Pro-Gly-NH_2, and others.

Exceptions are the analogs obtained by König et al. on the basis of the highly active agonist [D-Ser(Bu^t)^6]-LH-RH EA, namely H-Trp-Ser-D-Ser(Bu^t)-Leu-Arg-Pro-NHEt and H-Ser-Tyr-D-Ser(Bu^t)-Leu-Arg-Pro-NHEt [9]. These compounds possess the capacity for inducing ovulation in rats previously treated with phenobarbital.

We have performed the synthesis of two new luliberin analogs created on the basis of the highly active agonist [D-Ala^6]-LH-RH EA, namely: H-Pro-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt (1) and Z-D-Phe-Pro-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt (2).

The analogs under consideration possess a comparatively high biological activity but at the same time are distinguished by simplicity of their synthesis.

In the preparation of compounds (1) and (2) we used the method of fragment condensation (see schemes 1 and 2). The guanidine group of arginine was protected by nitration, and the hydroxy groups of serine and tyrosine were left unprotected.



Scheme 1

In the case of the analog (1), after the elimination of the protective groups by catalytic hydrogenation over palladium on carbon, the product obtained was purified by ion-exchange chromatography on Sephadex CM-25. The fractions giving a positive reaction with the Sakaguchi reagent, with isatin, and with the reagent for tyrosine were collected, lyophilized, and subjected to gel filtration on Sephadex G-15 in 0.2 N acetic acid.

For the purification of compound (II) we used preparative thin-layer chromatography on silica gel plates in the following systems: tert-butanol—acetic acid—water (4:1:1) and chloroform—methanol (9:4), followed by gel filtration on Sephadex LH-20 in ethanol.

The purity of the intermediate compounds obtained in the course of the synthesis and of the final products was checked by thin-layer chromatography, electrophoresis, and elementary analysis, and the final products were also subjected to amino acid analysis.

In view of the possibility of the practical use of the analogs and also of literature information on tests of this type of compounds, in the experiments to determine biological activity we used the following tests: inhibition of ovulation in experimental animals, and influence on the action of chorionic gonadotropin on the uterus and ovary in sexually immature rats.

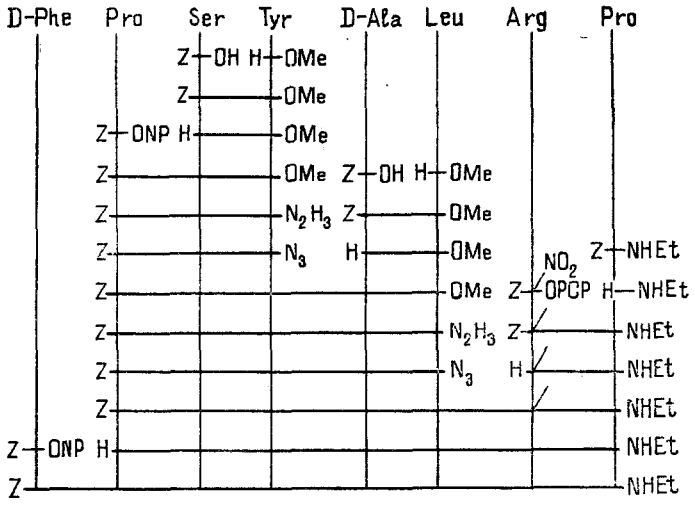
It was established that compound (1) possesses a pronounced inhibitory action on the course of the processes of ovulation in rats and rabbits on intraperitoneal injection in a dose of 1 mg/kg. Compound (2) exhibits a far smaller inhibiting activity under similar conditions.

A comparison of the results obtained with literature information indicates the comparatively high activity of compound (1) while it must be mentioned that it is structurally the

simplest luliberin analog possessing such a pronounced inhibiting action. Below we give information on the influence of luliberin antagonists on ovulation

Analog	Dose, mg	Suppression of ovulation, %
[D-Phe^2, D-Leu^6]-LH-RH	3×2	83
[D-Phe^2, Phe^3, D-Phe^6]-LH-RH	1,5	86
[Ac-D-Phe^1, D-p-Cl-Phe^2, D-Trp^{3,6}]-LH-RH	0,062	100
des-pGlu^1, des His^2,-[Pro^3, D-Ala^6]-LH-RH EA (1)	0,2	55
des His^2-[Z-D-Phe^1, Pro^3, D-Ala^6]-LH-RH EA (2)	0,2	8

A comparative investigation of the biological activities of the analogs synthesized with respect to the effects of chorionic gonadotropin on the rat ovary and uterus also showed a high activity of compound (1). This analog not only revealed a capacity for potentiating the action of chorionic gonadotropin but also possessed a considerable hormonal activity. The results of the tests showed that compound (II) does not possess hormonal activity but eliminates the action of chorionic gonadotropin on the ovary and weakens its influence on the uterus.



Scheme 2

EXPERIMENTAL

Melting points were determined in open capillaries without correction. The individuality of the compounds obtained was established by thin-layer chromatography on silica gel plates in the following solvent systems: 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 electrophoretograms were visualized with isatin, the Sakaguchi reagent, the tyrosine reagent, and chlorine, and the chromatograms in UV light and with iodine.

The results of the elementary and amino acid analyses of the compounds synthesized corresponded to the calculated C, H, and N contents and amino acid ratios. The amino acid analyses were performed on Jeol and LKB-3201 automatic analyzers. Optical rotations were measured on a Pepol-60 polarimeter (cell length 0.5 dm) at 22°C.

In all cases, unless otherwise mentioned, amino acids of the L series were used in the synthesis.

Solutions of substances in organic solvents (in ethyl acetate, after preliminary drying over Na_2SO_4) were evaporated in vacuum in a rotary evaporator at temperatures not exceeding 40°C. The following symbols have been adopted: CDI — N,N'-dicyclohexylcarbodiimide; Z — benzyloxycarbonyl group; TEA — triethylamine; NMM — N-methylmorpholine; HOBt — 1-hydroxybenzotriazole; DMF — dimethylformamide; EA — ethyl acetate; AcOH — acetic acid; TEA — trifluoroacetic acid.

1. Preparation of Z-Pro-NHEt. A solution of 9 g (0.036 mole) of Z-Pro-OH in absolute tetrahydrofuran was treated with 4 ml (0.036 mole) of NMM. The mixture was cooled to −30°C, and 5 ml (0.026 mole) of isobutyl chloroformate was added. The mixture was stirred at −30°C for 15 min and at a gradually rising bath temperature for 10 min, and then again cooled to −30°C and 4 ml (0.063 mole) of ethylamine was added dropwise. The reaction mixture was stirred at −30°C for 1 h and at −20°C for 1 h and was then left overnight in the refrigerator. The solvent was evaporated off, and the residue was distributed between EA and 1 N H_2SO_4. The organic phase was washed with water, and the solvent was evaporated off. This gave 9.8 g of product. Yield 98%. R_f 0.81 (1); 0.85 (2); 0.92 (3). mp 99.5–101°C; according to the literature: 104–105°C [11].

2. Preparation of Z-Arg(NO$_2$)-Pro-NHEt. A. Z-Pro-NHEt (2 g; 0.72 mmole) was treated with a solution of HBr in AcOH for 45 min. Then the solvent was evaporated off and the residue was dried in vacuum over KOH.

B. With stirring, 3.1 g (0.52 mmole) of Z-Arg(NO$_2$)-OPCP and 1 ml (0.72 mmole) of TEA were added to a solution of HBr·H-Pro-NHEt in DMF. The mixture was kept at room temperature for 4 days, and the solvent was evaporated off. The residue was dissolved in chloroform, and the solution was washed with 5% $NaHCO_3$ solution, with water, with 1 N HCl, and with water again and was dried over Na_2SO_4. The solvent was evaporated off and the residue was crystallized from ether. Where necessary, it was reprecipitated from ethanol–ether. This gave 1.41 g of product. Yield 57%. R_f 0.84 (1); 0.91 (2); 1(3). mp 143–144°C; according to the literature: 143–146°C [10].

3. Preparation of Z-Ser-Tyr-OMe. At room temperature, 2 g (0.86 mmole) of HCl·H-Tyr-OMe was treated with a saturated solution of NH_3 in chloroform for 10 min. The resulting precipitate was filtered off and the solvent was evaporated off. The residue was dried in vacuum for 1 h and was dissolved in EA–DMF. Then 4.2 g (0.86 mmole) of Z-Ser-OPCP was added 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 with EA. The organic phase was washed by the method of paragraph 2, and the solvent was evaporated off. This gave 3.6 g of product. Yield 100%. R_f 1(2); 1(3); 0.59 (4). mp 103–105°C; according to the literature: 108–115°C [11].

4. Preparation of Z-Pro-Ser-Tyr-OMe. A. A current of HBr was passed into a solution of 1 g (0.24 mmole) of Z-Ser-Tyr-OMe in TFA for 30 min. The reaction product was precipitated with absolute ether, filtered off, washed repeatedly with absolute ether, and dried in vacuum over KOH.

B. With stirring, 0.89 g (0.24 mmole) of Z-Pro-ONP and 0.26 ml (0.24 mmole) of NMM were added to a solution of HBr·H-Ser-Tyr-OMe in DMF. After 2 days at room temperature, the solvent was evaporated off and the residue was dissolved in EA. The resulting solution was treated by the method of paragraph 2. This gave 0.87 g of product. Yield 70%. R_f 0.93 (2); 0.93 (3); 0.75 (4). mp 176–178°C; $[\alpha]_D^{22}$ −54° (c 0.1; methanol).

5. Preparation of Z-Pro-Ser-Tyr-N_2H_3. A solution of 1.14 g (0.22 mmole) of Z-Pro-Ser-Tyr-OMe in boiling methanol was treated with 0.86 ml (2.7 mmole) of hydrazine. Then the mixture was kept in the refrigerator for a day, after which it was diluted with ether, and the resulting precipitate was filtered off and washed with a mixture of methanol and ether. It was recrystallized from methanol, giving 0.91 g of product. Yield 80%. R_f 0.71 (1); 0.62 (2); 0.84 (3). mp 204–205°C. $[\alpha]_D^{22}$ −31.3° (c 0.3; DMF).

6. Preparation of Z-D-Ala-Leu-OMe. The synthesis started from 20 g (0.09 mole) of Z-D-Ala-OH and 16.28 g (0.09 mole) of HCl·H-Leu-OMe, using a procedure similar to that of paragraph 1. This gave 25.12 g of product. Yield 80%. R_f 0.95 (1); 1(3). mp 66–69°C; according to the literature: 72–73°C [12].

7. Preparation of Z-Pro-Ser-Tyr-D-Ala-Leu-OMe. To a solution of 2.58 g (0.5 mmole) of Z-Pro-Ser-Tyr-N_2H_3 in DMF was added 2.9 ml (1.5 mmole) of a 5.2 N solution of HCl in tetrahydrofuran. Then the mixture was cooled to −30°C and, with stirring, 0.622 g (0.6 mmole) of tert-butyl nitrite was added. After 10 min, a cooled solution of HBr·H-D-Ala-Leu-OMe obtained from 3.52 g (1 mmole) of the benzyloxycarbonyl derivative by the method of paragraph 2A and 3.1 ml (2.5 mmole) of TEA were added. The pH of the reaction mixture was brought to 8.5 by the addition of TEA. Then it was stirred at −30°C for 1 h, at −10°C for 1 h, and at room temperature for 2 days. After this, it was acidified with 1 N HCl, and the solvent was evaporated off. The residue was dissolved in EA and the product was worked up by the method of paragraph 2, which gave 2.7 g of material. Yield 77%. R_f 0.91 (1); 0.88 (2); 0.96 (3). mp 137–140°C. $[\alpha]_D^{22}$ −64° (c 0.1; methanol).

8. Preparation of Z-Pro-Ser-Tyr-D-Ala-Leu-N_2H_3. Z-Pro-Ser-Tyr-D-Ala-Leu-OMe (3.1 g; 0.44 mmole) was treated by the method of paragraph 5. The product was recrystallized from dioxane. The yield was 2.67 g; 86%. R_f 0.81 (1); 0.82 (3). mp 173–174°C. $[\alpha]_D^{22}$ −30.4° (c 0.25; DMF).

9. Preparation of Z-Pro-Ser-Tyr-D-Ala-Leu-Arg(NO$_2$)-Pro-NHEt. Z-Pro-Ser-Tyr-D-Ala-Leu-N_2H_3 (0.6 g; 0.086 mmole) was treated by the method of paragraph 7. As the amino component was used a solution of HBr·H-Arg(NO$_2$)-Pro-NHEt in DMF obtained from 0.822 g (0.172 mmole) of the benzyloxycarbonyl derivative by the method of paragraph 2A. The reaction mixture was stirred at −30°C for 1 h, at −15°C for 1 h, at 4°C for two days and at room temperature for two days. Then it was acidified with 1 N HCl, and the solvent was evaporated off. The residue was dissolved in chloroform, and the solution was washed with water, 5% $NaHCO_3$ solution, and water again. The gel that had deposited was filtered off and it was dissolved in methanol and combined with the organic phase. The solvent was evaporated off and the residue was reprecipitated from aqueous methanol. The mother solution was evaporated and chromatographed on a column of silica gel (L 5/40) in a gradient formed from chloroform and a 10% solution of ethanol in chloroform. This gave 0.592 g of product in the form of an amorphous powder. Yield 68%.

10. Preparation of H-Pro-Ser-Thy-D-Ala-Leu-Arg(NO$_2$)-Pro-NHEt. A solution of 0.2 g (0.02 mmole) of Z-Pro-Ser-Tyr-D-Ala-Leu-Arg(NO$_2$)-Pro-NHEt in methanol with the addition of 10% AcOH was subjected to catalytic hydrogenation in the presence of palladium on carbon. The catalyst was filtered off and washed with 10% AcOH. The product was lyophilized, dissolved in 0.005 M NH OAc and chromatographed on a column of Sephadex CM-25 (9 × 300 mm) in a concentration gradient of NH_4OAc of from 0.005 to 0.5 M. The fractions containing the desired product were collected, lyophilized, and chromatographed on a column of Sephadex G-15 (22 × 1050 mm) in 0.2 N AcOH. The product was lyophilized.

11. Preparation of Z-Ser-Tyr-N_2H_3. Z-Ser-Tyr-OMe (2.24 g; 0.54 mmole) was treated by the method of paragraph 5. The product was recrystallized from aqueous methanol, and the yield was 1.9 g (85%). R_f 0.65 (1); 0.81 (2); 0.92 (3). mp 213.5–214.5°C; according to the literature: 213–214°C [13].

12. Preparation of Z-Ser-Tyr-D-Ala-Leu-OMe. Z-Ser-Tyr-N_2H_3 (1.4 g; 0.34 mmole) was treated by the method of paragraph 7. A solution of HBr·H-D-Ala-Leu-OMe obtained from 2.36 g (0.67 mmole) of the benzyloxycarbonyl derivative by the method of paragraph 2A was used as the amino component. The product was recrystallized from ether, filtered off, and washed on the filter with ether. Yield 1.48 g (73%). Rf 0.67 (1); 1(2); 1(3). mp 178–179°C. $[\alpha]_D^{22}$ −26° (c 1; methanol).

13. Preparation of Z-Pro-Ser-Tyr-D-Ala-Leu-OMe. The synthesis started from 3.28 g (0.55 mmole) of Z-Ser-Tyr-D-Ala-Leu-OMe by the method of paragraph 4, with the addition of 1.45 g (0.55 mole) of pentachlorophenol. This gave 3.11 g of product. Yield 82%. The constants of the compound obtained coincided with those given in paragraph 7.

14. Preparation of Z-D-Phe-Pro-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt.

NO_2
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Z-Pro-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt (0.2 g; 0.02 mmole) was hydrogenated by the method of paragraph 10. The solvent was evaporated off, and the residue was dissolved in DMF and treated with 0.2 ml (0.02 mmole) of 0.1 N HCl and 0.083 g (0.02 mmole) of Z-D-Phe-ONP. After 5 days, the solvent was evaporated off and the residue was crystallized from ether, and it was washed with EA and was then chromatographed on silica gel plates in systems 3 and 1. The product was eluted by methol and 10% AcOH and was purified on a column of Sephadex LH-20 (40 × 720 mm) in ethanol. The fractions containing the product were combined and lyophilized.

SUMMARY

The promising nature of the product of luliberin analogs with shortened amino acid sequences has been shown.

LITERATURE CITED

1. A. V. Schally, A. Arimura, A. J. Kastin, D. H. Coy, E. J. Coy, J. Vilchez-Martinez, T. W. Redding, R. Geiger, W. König, and H. Wissman, Pure Appl. Chem., 37, 315 (1974).
2. A. S. Dutta, B. J. A. Furr, M. B. Giles, B. Valcaccia, and A. L. Walpole, Biochem. Biophys. Res. Commun., 81, 382 (1978).
3. J. A. Vilchez-Martinez, D. H. Coy, A. Arimura, E. J. Coy, J. Hirotsu, and A. V. Schally, Biochem. Biophys. Res. Commun., 59, 1226 (1974).
4. J. P. Yardley, T. J. Foell, C. W. Beattie, and N. H. Grant, J. Med. Chem., 18, 1244 (1975).
5. T. Wasiak, J. Humphries, K. Folkers, and C. Y. Bowers, Biochem. Biophys. Res. Commun., 86, 843 (1979).
6. G. W. Moersch, M. C. Rebstock, E. L. Wittle, F. J. Tinney, E. D. Nicolaides, M. P. Hutt, T. F. Mich, and J. M. Vandenbelt, J. Med. Chem., 22, 935 (1979).
7. D. H. Coy, I. Mezo, E. Pedroza, M. V. Nekola, J. A. Vilchez-Martinez, P. Piyachurawatana, A. V. Schally, J. Seprodi, and I. Teplan, Proceedings of the 6th American Peptide Symposium (1979), p. 7759.
8. W. König, J. Sandow, and G. Geiger, Peptides. Chemistry, Structure and Biology, Ann Arbor Scientific Publishers, Ann Arbor, Michigan (1975), p. 883.
9. J. Sandow and W. König, J. Endocr., 81, 175 (1979).
10. S. Shinagawa and M. Fujino, Chem. Pharm. Bull., 23, 229 (1975).
11. R. F. Fischer and R. R. Whetstone, J. Am. Chem. Soc., 76, 5076 (1954).
12. W. J. Polglase and E. L. Smith, J. Am. Chem. Soc., 71, 3081 (1949).
13. K. Hofmann, A. Johl, A. E. Furlenmeier, and H. Kappeler, J. Am. Chem. Soc., 79, 1638 (1957).