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SYNTHESIS OF A NEW CYCLIC ANALOGUE OF LULIBERIN

S. V. Nikolaev, S. V. Burov,
V. D. Bakharev, V. P. Makusheva,
and V. V. Korkhov

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A new cyclic analogue of luliberin possessing the capacity for stimulating ovulation in sexually mature and infantile rats and also exhibiting a pronounced prolongation of its influence on a number of behavioral reactions of animals has been synthesized.

Among the numerous directions of the synthesis of active analogues of luliberin, considerable interest is caused by the preparation of cyclic peptides. As early as 1977, a hypothesis was expressed on the existence of a hydrogen bond between the pyroglutamic acid and glycinamide residues [1]. Conformational investigations of the luliberin molecule have confirmed the energetic advantageousness of a quasi-cyclic structure – for example, [2]. The first cyclic analogues of luliberin, cyclo(βAla^1, D-Ala^6)LH-RH and cyclo(6-aminohexanoyl^1, D-Ala^6) LH-RH had 1.2% and 0.65% of its releasing activity, respectively [3]. The low activity of these peptides was apparently due to the considerable modification of the first position. It is known that this usually leads to a pronounced fall in activity while at the same time weakly influencing the capacity of the compound for binding with the receptor. The linear precursors of these peptides were inactive.

The analogue cyclo(4-7)(Glu^4,D-Ala^6,Orn^7)LH-RH also proved to be inactive [4]. According to spectroscopic results, such cyclization leads to pronounced conformational changes in the molecule.

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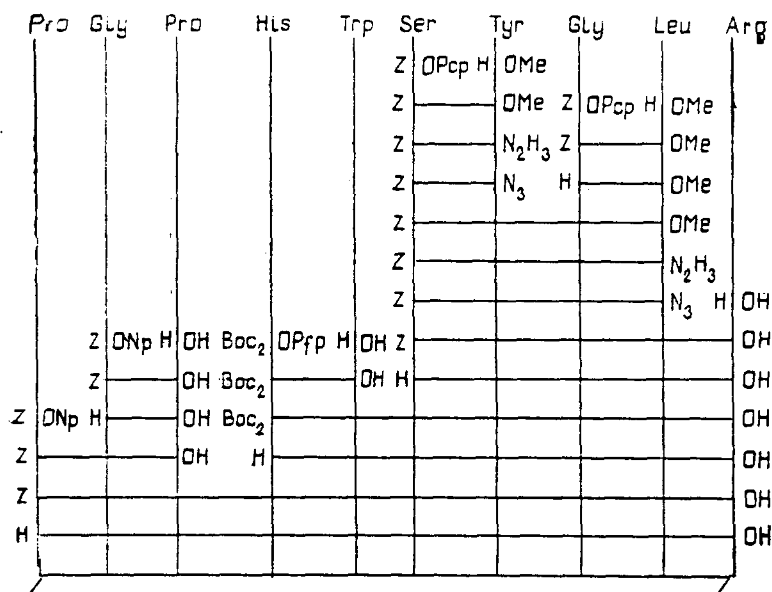
It has been possible to obtain significant results in the synthesis of cyclic antagonists. Thus, cyclo(Δ3, 4Pro[1], D-Phe(4Cl)[2], D-Trp[3,6], N-MeLeu[7], β-Ala[10])LH-RH [5] and similar analogues modified in positions 1, 2, 3, 6, and 10 [6] exhibit considerable antiovulatory activity. Analogues with the ring between positions 4 and 10 [7] also possess high antiovulatory activity. The best in the series of analogues proved to cyclo(4-10)(Ac-3-Nal(2)[1], D-Phe(4Cl)[2], 3-Pal(3)[3,6], Cys[4], Arg[5], Cys[10])LH-RH, [where Nal = naphthylalanine and Pal = pyridylalanine] which exhibited and antiovulatory action in a dose of 10 μg per 1 kg weight of the animal.

An interesting result was obtained in an investigation of the peptide cyclo(Pro[1,3], D-Phe[2], D-Ala[6])LH-RH [8]. This analogue raised the level of lutropin in the blood plasma and plasma and stimulated ovulation in sexually immature animals. At the same time, it inhibited ovulation in sexually mature animals. This duality of the action of a peptide from which it was difficult to expect agonistic properties, since the modification of the second position by D-amino acids, as a rule, leads to antagonist compounds binding with the receptor but not causing the generation of the "secondary signal" served as the impetus for the synthesis of a cyclic analogue, a potential agonist, with a minimal modification of the natural sequence. Such a minimal, but necessary, change is the replacement of the pyroglutamic acid residue in the first position by the structurally close proline, since the inclusion of pyroglutamic acid in a ring is extremely problematical.

In the choice of a scheme for the synthesis of the analogue, we based ourselves on the principal of minimal protection in association with fragment condensation. In view of the convenience of the synthesis and isolation of peptides with C-terminal arginine, and also of a series of communications on the successful performance of cyclization at an arginine residue, we decided, in the synthesis of a linear precursor, to introduce the C-terminal dipeptide (prolyglycine) into the N-terminal part of the molecule (scheme).



It was proposed to carry out the initial fragment condensation by a (3 + 2) scheme, but an attempt to condense ZProGlyProOH with histidyltryptophan using complex F or dicyclohexylcarbodiimide with 1-hydroxybenzotriazole gave unsatisfactory results. We therefore used a 3 + (2 + 5) scheme for the condensation of the fragments. The deblocking of the decapeptide was carried out by catalytic hydrogenation in methanol with acetic acid. Without the addition of acetic acid, deblocking took place slowly and an appreciable amount of a by-product was formed.

To close the ring we used the method of cyclization with an insoluble base. As the condensing agent we used diphenylphophoryl azide, and as the insoluble base potassium hydrogen phosphate. The use of this method permits cyclopeptides to be obtained in high yield and without the appreciable formation of dimers.

The purification of the cyclopeptide was carried out by ion-exchange chromatography on a column of sulfopropyl-Sephadex C-25 in a gradient of pyridine acetate buffer containing 50% of dioxane.

We investigated the capacity of the analogue for stimulating ovulation in sexually mature and infantile rats (detailed results of the biological tests and the methods for their performance have been given in [9]). The maximum effect for the sexually mature animals was shown in a dose of 200 μg per 1 kg weight, and for the infantile animals in a dose of 25 μg/kg. The substance was administered intramuscularly in the form of an aqueous solution. With an increase in the doses the effect fell because of the paradoxical effect of the inhibition of ovulation.

We investigated the change in the spectrum of behavioral reactions under the action of the peptide. In a dose of 150 μg/kg weight, the analogue improved primary learning and long-term memory in rats. In comparison with luliberin, the cyclic analogue possessed a prolonged action. The action of luliberin falls sharply 60-90 min after injection, while the analogue exhibited a substantial effect even 150 min after administration.

An investigation of the influence on the threshold of pain sensitivity showed that the analogue raised the threshold and decreased the emotional affective behavior of the animals in response to pain.

A study of anorexigenic activity showed that the analogue in a dose of 50 mg/kg reduced appetite without affecting the consumption of water.

In a study of influence on artificially induced aggression, it was established that the analogue increased aggressiveness in comparison with a control.

In experiments on the habituation of rats alcohol, the daily administration of the analogue in a dose of 100 μg/kg decreased the number of alcoholic rats from 45% in the control to 20%.

The results obtained indicate a pronounced psychostimulating action of the peptide. Its effects are possibly due to a modeling action on the noradrenergic and dopaminergic mediatory transmissions of the brain.

EXPERIMENTAL

Melting points were determined in open capillaries without correction. The individuality of the compounds obtained was checked with the aid of thin-layer chromatography on Silufol UV-254 (S) and Merck Fertigplatten K.60F (M) plates in the following solvent systems: 1) butan-2-ol–1% ammonia (3:1); 2) chloroform–methanol (5:1); 3) butan-1-ol–acetic acid–water–pyridine (15:3:6:10); 4) ethyl acetate–pyridine–acetic acid–water (60:20:6:11).

High-voltage electrophoresis was conducted on Filtrak FN-12 paper (GDR) in 2% acetic acid at 1500 V for an hour. Electrophoretic mobilities were determined relative to glycine. Elementary analysis was carried out on a Hewlett–Packard automatic C, H, N analyzer. The results of elementary analysis found corresponded to the calculated values. Amino acid analysis was performed on a AAA-339 automatic analyzer. High-performance liquid chromatography was conducted on a Altex-332 chromatograph with an Ultrasphere ODS 5 μm column having dimensions of 4.6 × 150 mm. Optical rotations were measured on a Pepol-60 polarimeter at 18°C.

Solutions of the substances in organic solvents were dried with anhydrous sodium sulfate and were evaporated in a rotary evaporator at a temperature not exceeding 40°C.

Abbreviations adopted: EA) ethyl acetate; DMFA) dimethylformamide; THF) tetrahydrofuran; TEA) triethylamine.

1. ZSerTyrOMe. A solution of 4.17 g (18 mmole) of the hydrochloride of tyrosine methyl ester was treated with 2.5 ml (18 mmole) of TEA, and the resulting precipitate was filtered off. The filtrate was treated with 5.85 g (12 mmole) of the pentachlorophenyl ester of benzyloxycarbonylserine, and TEA was added to pH 8. The mixture was stirred for a day and was then acidified with acetic acid to pH 7 and evaporated. The residue was dissolved in EA and the solution was washed with 5% sodium bicarbonate, 1 N hydrochloric acid, and water. Then it was dried and evaporated. The residue was recrystallized from EA–hexane. Yield 4.99 g (100%). Mp 109-110°C. R_f 0.73 (2 M). $[\alpha]_D^{20}$ + 6.7° (c 1; DMFA).

2. ZSerTyrN_2H_3. With heating, 9.55 g (22.9 mmole) of compound (1) was dissolved in 20 ml of methanol, and 5.74 ml of hydrazine hydrate was added. The mixture was kept overnight in the refrigerator and was then treated with water and the precipitate was filtered off. Yield 9.56 g (100%) mp 209-211.5°C, R_f 0.67 (2 M). $[\alpha]_D^{20}$ + 7.1° (c 1; DMFA).

3. ZGlyLeuOMe. The hydrochloride of leucine methyl ester (4.96 g; 27.32 mmole) was treated for 15 min with a saturated solution of ammonia in chloroform, the solid matter was filtered off, the solution was evaporated, and the residue was dried in a dessicator. It was then dissolved in EA, and 10 g (21.86 mmole) of the pentachlorophenyl ester of benzyloxycarbonylglycine was added. The mixture was stirred at room temperature for 18 h. Then it was diluted with EA and was washed successively with 5% sodium bicarbonate, 1 N hydrochloric acid, and water, and was dried and evaporated. The residue was an oil. Yield 6.73 g (92%). R_f 0.86 (1 S). $[\alpha]_D^{18}$ − 19.4° (c 1; methanol)

4. ZSerTyrGlyLeuOMe. A. Compound (3) (5.66 g) was treated for an hour with a 4 N solution of HBr in acetic acid. Then absolute ether was added and the precipitate was filtered off. It was reprecipitated from methanol with ether. Yield 76%. E_{Gly} = 15.

B. A solution of 4.215 g (10.12 mmole) of $ZSerTyrN_2H_3$ in DMFA was cooled to −30°C and 11.2 ml of 2.7 N HCl/THF (30.36 mmole) and 1.15 g (11.13 mmole) of tert-butyl nitrite were added. The mixture was stirred for 30 min and was then cooled to −45°C, and 4.24 ml (30.36 mmole) of TEA and 4.3 g (15.18 mmole) of the deblocked peptide (3) were added. The pH was brought to 8 by the addition of TEA. 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 precipitate was filtered off, and the filtrate was evaporated. The residue was dissolved in EA and the solution was washed with 5% sodium bicarbonate, 1 N hydrochloric acid, and water. It was dried and evaporated, and the residue was triturated with ether and was then reprecipitated from methanol with hexane. Yield 88%. mp 162-163°C. R_f 0.74 (2 M). $[\alpha]_D^{18}$ − 16.4° (c 1; methanol).

5. $ZSerTyrGlyLeuN_2H_3$. With heating, 3.23 g (5.5 mmole) of compound (4) was dissolved in 10 ml of methanol, and 10 equivalents of hydrazine hydrate was added. The mixture was kept overnight in the refrigerator and was then treated with water and the precipitate was filtered off and was washed with methanol–ether (1:1). It was recrystallized from methanol. Yield 95%. mp 217°C. R_f 0.6 (2 S). $[\alpha]_D^{18}$ −17.6° (c 1; DMFA).

6. ZSerTyrGlyLeuArg(HCl)OH. This was obtained in a similar way to compound (4) from 1.5 g (2.56 mmole of compound (5) and 1.47 g (7.67 mmole) of arginine. The arginine was introduced into the reaction in the form of a suspension in water so that the DMFA water ratio was approximately 9:1. After the completion of the reaction, the precipitate was filtered off, the filtrate was evaporated, the residue was dissolved in aqueous butan-1-ol, and the solution was washed with water. The butanol was evaporated off and the residue was dissolved in 10% acetic acid and was extracted six times with EA. The aqueous phase was evaporated, and the residue waa triturated with ether. Yield 1.79 g (92%). Amorphous. E_{Gly} = 0.8. R_f 0.38 (1M), 0.78, (3M). $[\alpha]_D^{18}$ −23.2° (c 0.5; DMFA).

7. BocHis(Boc)TrpOH. A suspension of 0.62 g (3.05 mmole) of tryptophan in DMFA was treated with 1.28 ml (9.15 mmole) of TEA and 1.16 ml (9.15 mmole) of chlorotrimethylsilane. The mixture was stirred for 10 min. The solid matter was filtered off and the filtrate was added to a solution of 1.59 g (3.05 mmole) of the pentafluorophenyl ester of di-tert-butoxycarbonylhistidine in DMFA. The solvent was evaporated off after 10 h. The residue was dissolved in EA and the solution was washed with 10% citric acid and with water. Then it was evaporated and the residue was reprecipitated from ether with hexane. Yield 1.12 g (68%). Amorphous. R_f 0.57 (2S). $[\alpha]_D^{18}$ −10.3 (c 0.3; methanol).

8. ZGlyProOH. A solution in water of 2.93 g (25.43 mmole) of proline and 1.02 g of caustic soda was treated with 7 g (21.19 mmole) of the para-nitrophenyl ester of benzyloxycarbonylglycine in dioxane. More dioxane was added until a clear solution had been obtained. This was stirred for a day, and then the dioxane was evaporated off and the residue was diluted with water and was extracted four times with ether. The aqueous solution was acidified with hydrochloric acid to pH 3 and was extracted four times with EA. The organic extracts were combined, washed with water, dried, and evaporated, and the residue was triturated with ether. Yield 80%. mp 152-153°C. R_f 0.55 (1S). $[\alpha]_D^{18}$ −54° (c 1; methanol).

9. ZProGlyProOH. Catalytic hydrogenolysis was used to deblock 2.5 g (8.16 mmole) of compound (8). E_{Gly} = 0.97. The product was dissolved in a small amount of water, and this solution was treated with 0.326 g (8.16 mmole) of caustic soda and with a solution of 3.325 g (8.98 mmole) of the para-nitrophenyl ester of benzyloxycarbonylproline in dioxane. The mixture was stirred for a day. Then 0.7 g (4.8 mmole) of arginine was added to bind the excess of activated ester. After an hour, the dioxane was evaporated off and the reaction mixture was worked up in a similar way to experiment 8. Yield 2.65 g (81%). Amorphous. R_f 0.33, (1M). $[\alpha]_D^{18}$ −87° (c 1; ethanol).

10. BocHis(Boc)TrpSerTyrGlyLeuArg(HCl)OH. The hydrogenation of 0.7 g (0.92 mmole) of compound (6) was performed over palladium black in methanol. E_{Gly} = 1.19. Compound (7) (0.545 g; 1.01 mmole) was dissolved in DMFA, the solution was cooled to 0°C, and 0.763 g (1.01 mmole) of complex F was added. An hour later, a solution of the deblocked compound (6) in DMFA was added to the reaction mixture. After 48 h, the DMFA was evaporated off, the residue was dissolved in aqueous butan-1-ol, and the solution was washed six times with water. The butanol was evaporated off, and the residue was reprecipitated from methanol with EA. Yield 0.59 g (56%). E_{Gly} = 1.03. R_f 0.74 (3M), 0.37 (4M).

11. ZProGlyProHisTrpSerTyrGlyLeuArgOH. Compound (10) (0.47 g; 0.41 mmole) was deblocked with 3 N methanesulfonic acid in acetic acid. E_{Gly} = 1.5. At 0°C, 0.34 g (0.45 mmole) of complex F was added to a solution of 0.18 g (0.45 mmole) of compound (9) in DMFA. After 2 h, the precipitate of dicyclohexylurea was filtered off, and the deblocked heptapeptide (10) and TEA to pH 8 were added to the filtrate. After 20 h, the solvent was evaporated off, and the residue was triturated with EA. The prcipitate was filtered off and was dissolved in aqueous butan-1-ol, and the solution was extracted three times with water. The butanol was evaporated off and the residue was chromatographed on a column of sulfopropyl-Sephadex C-25 in a gradient, from 0.005 to 0.5 M, of pyridine acetate buffer containing 50% of dioxane. The fractions containing the peptide were evaporated. Yield 0.29 g (59%). E_{Gly} = 1.0. R_f 0.64 (3M). HPLC: k' = 3.5 (eluent: 0.02 N triethylammonium formate in water—acetonitrile (70:30).

12. Cyclo(ProGlyProHisTrpSerTyrGlyArg). Compound (11) (80 mg; 0.06 mmole) was hydrogenated in methanol with the addition of 0.4 ml of 50% acetic acid over palladium black. 11 R_f 0.32 (4M). E_{Gly} = 1.56. The product was dissolved in 1.5 ml of DMFA, and 0.11 ml (0.06 mmole) of a 0.56 N HCl/THF solution was added. The mixture was cooled to 0°C, and 53 mg (0.31 mmole) of K_2HPO_4 and 34 mg of diphenylphosphoryl azide were added. The mixture was stirred at +5°c for 25 h. The solid matter was filtered off, and the filtrate was treated with ether. The product was chromatographed on a column of sulfopropyl-Sephadex C-25 under conditions analogous to those of experiment 11. Then it was rechromatographed on a column containing Toyopearl HW-40 in the 2 N acetic acid—dioxane (2:1) system. The fractions containing the analogue were lyophilized. Yield 39 mg (56%). E_{Gly} = 1.19. R_f 0.42 (3M). HPLC: k' = 3.25. Amino acid analysis: Pro 1.73(2), His 1.00(1), Trp (+), Ser 0.89(1), Tyr 0.99 (1), Gly 1.82(2), Leu 1.00(1), Arg 0.82(1).

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