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SYNTHESIS AND INVESTIGATION OF THE ANTITUMORAL ACTIVITY OF SHORTENED ANALOGS OF LULIBERIN

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With the aim of obtaining antitumoral drugs possessing a binary action mechanism, we have synthesized a series of shortened analogs of luliberin, including some containing a 1-carboxymethyl-5-fluorouracil residue. Their antitumoral and hormonal activities have been investigated.

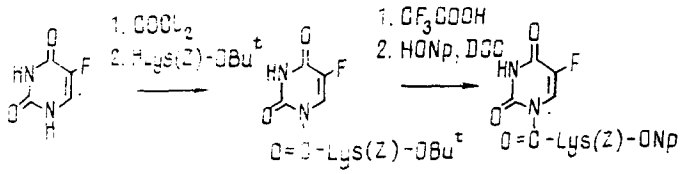
Synthetic luliberin analogs are widely used in oncology for the treatment of hormone-dependent tumors [1]. Here, two mechanisms of the antitumoral action of the compounds are possible — mediated, connected with a fall in the level of steroid sex hormones, and direct, through the action of the peptide on the tumor cells (it has been shown that, in a number of cases, cancer cells have receptors for luliberin-like peptides [2]).

In addition, because of the heterogeneity of tumor tissues they always contain cells insensitive to hormone treatment and, during treatment, a tumor is always transformed into the hormone-independent type. A high therapeutic effect is given by the use of various chemotherapeutic drugs, but a common defect of them is low selectivity and, as a consequence, high toxicity.

The addition of a cytotoxic grouping to the peptide chain will permit a simultaneous rise in the efficacy of a peptide drug and decrease in such an effect of the chemotherapeutic agent. Then aimed transport of compounds with a cytotoxic action directly to target cells having the appropriate receptors is possible. The use of such an approach in the synthesis of luliberin analogs has been described in [3].

With the aim of a more detailed study of the prospects of this direction, we have synthesized luliberin analogs covalently bound with 5-fluorouracil (5-FU). In this case great importance is attached to the choice of spacer grouping, which must, in the first place ensure stability of the bond beween the 5-FU and the peptide under the conditions of the conjugation reaction and subsequent purification, while, in the second place, the modification must not lead to an appreciable fall in biological activity of either component.

According to the literature, 5-FU derivatives of the carbamoyl type possess improved pharmacological properties as compared with the unmodified drug. Initially, therefore, in order to include the cytotoxic grouping in the peptide sequence we used 5-FU carbamoyl chloride [4]. In this way we obtained the *tert*-butyl ester of N^{α}-[(5-fluorouracil-1-yl)carbonyl]-N^{ε}-Z-lysine,



and then the corresponding *p*-nitrophenyl ester. However, in the selection of the conditions for conjugation with the peptide we came up against certain difficulties. Thus, it is possible to perform the reaction only in DMFA in the absence of contact

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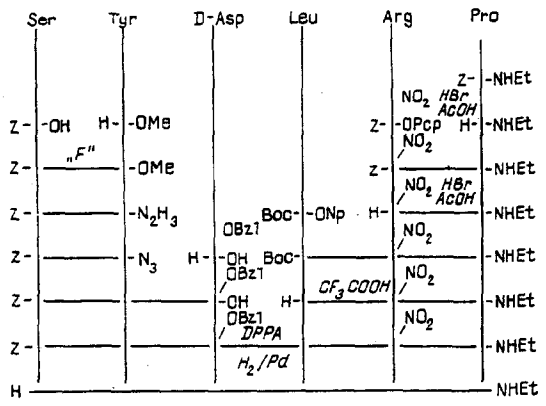
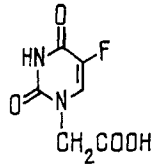


Fig. 1. Synthesis of the initial peptide. DPPA — diphenyl phosphorazidate; "F" — complex F.

with moisture; the use of a base is excluded; and, because of the instability of the compound in aqueous and alcoholic media, the subsequent purification and isolation of the product is difficult. In subsequent work, therefore, for the modification of the peptide we selected a considerably more stable alkyl derivative of 5-FU — 1-carboxymethyl-5-fluorouracil (CMFU):



It is known that this compound, when added to a polymer by an ester or amide bond, has a fairly high cytotoxic activity.

CMFU was obtained by condensing 5-FU in an aqueous solution of alkali with an excess of chloroacetic acid and was isolated by treatment with the ion-exchange resin Dowex 2×8. The *p*-nitrophenyl ester of CMFU (CMFU—ONp) can be used in peptide synthesis without serious limitations [6].

For modification by the cytotoxic agent we selectd luliberin analogs with shortened amino acid sequences. Investigations that we performed previously had shown that such compounds possess a considerable biological activity and, at the same time, are far more accessible synthetically than the releasing hormone itself and its superagonists and antagonists [7, 8].

The following analogs were synthesized in the course of this work:

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

CMFU-Ser-Tyr-*D*-Asp-Leu-Arg-Pro-NHEt (2)

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

CMFU-Pro-Ser-Tyr-*D*-Asp-Leu-Arg-Pro-NHEt (4)

The hexapeptide (1), the starting point for all the other compounds, was synthesized by the fragment condensation method, using a (2+1)+3 scheme (Fig. 1).

In the preparation of the C-terminal fragment, Z-Arg(NO_2)-Pro-NHEt, the deblocking of Z-Pro-NHEt by catalytic hydrogenolysis led to the formation of by-products, and the benzyloxycarbonyl protection was therefore eliminated by the action of HBr in acetic acid. The condensation of the hydrobromide so obtained with Z-Arg(NO_2)-OPcp in the presence of triethylamine took place slowly [7], apparently because of the high basicity of the imino group. To eliminate HBr from the reaction medium, the amino component was treated with Dowex 2×8 in methanol, which permitted a substantial increase in yield.

For the introduction of a *D*-Asp residue in position 6 of the natural sequence of the hormone we used a combination of the azide and the diphenyl phosphorazidate methods. This expedient is convenient in working with amino acids requiring a laborious multistage preparation of derivatives or imposing methodological limitations on the further course of synthesis.

After elimination of the protective groups by hydrogenolysis and purification on ion-exchange Sephadex, the initial hexapeptide (1) was used for obtaining the other analogs by the activated-ester method. In this process, the guanidine group of Arg was not protected.

TABLE 1. Antitumoral Effect of the Shortened Luliberin Analogs on a Transplantable Cancer of the Rat Prostate Gland Aci

Compound	Single dose, μg/kg	Inhibition (+, stimulation) of the growth of the tumor in relation to control, %				Level of testosterone (nmole/ml) 30 days after the beginning of treatment
		Days after the beginning of treatment				
		8	15	22	29	
(1)	100	+ 394	19.2	34	47	1.6 ± 0.1
	10	+ 552	+ 35	22	44	1.4 ± 0.5
	1	+ 920	+ 13	17	32	2.0 ± 0.2
	0.1	+ 529	+ 21	32	31	1.8 ± 0.4
(2)	100	+ 11	+ 19.6	+ 6.4	+ 22.8	3.8 ± 0.4
	10	+ 56	+ 134	38	28	2.8 ± 1.2
	1	4.2	7.7	14	+ 63	3.1 ± 0.7
	0.1	+ 69	+ 51	4.7	+ 2.8	3.4 ± 0.6
(3)	100	33	33	38	50	1.2 ± 0.8
	10	13	45	18	33	1.2 ± 0.6
	1	8	7	19	40	1.6 ± 0.4
	0.1	+ 66	+ 54	+ 66	1	1.8 ± 0.9
(4)	100	24	+ 17	41	46	1.7 ± 0.8
	10	+ 22	+ 28	15	7.9	3.8 ± 0.2
	1	+ 3	+ 4.9	+ 11	18	2.8 ± 1.2
	0.1	7.5	+ 26	+ 3.9	+ 81	2.9 ± 1.1
Control						3.5 ± 0.4

TABLE 2. Antitumoral Effect of Luliberin Analogs on the Transplantable Cancer of the Mouse Mammary Gland

Compound	Single dose, μg/kg	Inhibition (+, stimulation) of the growth of the tumor in relation to control, %			Level of estradiol (nmole/ml) 23 days after the beginning of treatment
		Days after the beginning of treatment			
		8	15	22	
(1)	100	+ 110	+ 20	15.7	0.17 ± 0.01
	10	+ 185	+ 110	+ 48.8	0.25 ± 0.06
	1	+ 74	+ 2.9	22.5	0.66 ± 0.06
	0.1	27.6	14	27.5	1.44 ± 0.70
(2)	100	+ 170	+ 47.8	+ 13.5	0.09 ± 0.03
	10	35.6	+ 51	+ 37	0.61 ± 0.19
	1	+ 61.7	+ 21	+ 3.8	0.28 ± 0.07
	0.1	+ 315	+ 200	+ 114	0.20 ± 0.08
(3)	100	+ 397	+ 26	29	—
	10	+ 15	33	26	—
	1	+ 112	7	19	—
	0.1	+ 220	+ 9	+ 24	—
(4)	100	+ 28	Tox.	Tox.	—
	10	+ 275	+ 135	+ 61	0.30 ± 0.19
	1	+ 338	+ 169	+ 78	0.65 ± 0.23
	0.1	+ 133	+ 64	+ 28	0.21 ± 0.03
Control					0.40 ± 0.17

The increased reactivity of CMFU—ONp — in comparison with activated amino acid esters — must be mentioned. Condensation with them in the production of analog (2) from compound (1) (without the use of a base) was complete after 1.5 h, and in the production of analog (4) using triethylamine, after 0.5 h. For comparison, the condensation of Boc-Pro-OPcp and peptide (1) in the presence of triethylamine took two days.

The peptides obtained were purified by ion-exchange chromatography on Sephadex SE C-25 in a molarity gradient of pyridine-acetate buffer. The final purification of the compounds was achieved with the aid of HPLC. Their structures were confirmed by the results of amino acid analysis and mass spectrometry.

For the purpose of demonstrating the possibility of the aimed transport of a cytotoxic grouping to target cells, we performed a series of experiments to study the stability of the bond between the CMFU and the peptide in biological media. It was established with the aid of HPLC that the concentration of peptide (4) in blood plasma and in gastric juice remained unchanged for a day. This part of the work will be considered in more detail in our next publication.

The investigation of the antitumoral and hormonal properties of this series of peptides was carried out in ONTs RAMN [Oncological Scientific Center, Russian Academy of Medical Sciences] on rats and mice with tumors of the prostate and mammary glands, respectively. The preparations were injected daily in doses of from 0.1 to 100 μg/kg, and the percentage inhibitions of the growth of tumors in relation to a control were measured after one, two, three, and four weeks. At the end of the experiment the level of steroid sex hormones in the blood was measured. The results are presented in Tables 1 and 2.

The compounds investigated had no appreciable inhibiting action on cancer of the mammary gland (Table 2). On the model of cancer of the prostate, appreciable activity was demonstrated by the unmodified analogs (1) and (3). Peptide (1) strongly stimulated the growth of the tumor in the initial stages of treatment, which is characteristic for luliberin analogs with agonistic activity, and then, after two weeks, it showed an inhibiting effect on the development of the tumor (Table 1).

The 5-FU-modified peptide (4) also exhibited an appreciable inhibiting action on cancer of the prostate in a dose of 100 μg/kg. It must be mentioned that, on the model of mammary gland cancer, compound (4) was toxic at this dose, which may serve as an independent proof of the retention of the cytotoxic properties of 5-FU.

In the experiment on rats, a correlation was traced between the level of testosterone in the blood and the antitumoral action of peptides (1) and (3) on cancer of the prostate gland.

Thus, a method has been developed for modifying peptides with a 1-carboxymethyl-5-fluorouracil residue using the corresponding *p*-nitrophenyl ester in the last stage of the synthesis; shortened analogs of luliberin, including some containing a 5-fluorouracil residue have been synthesized and their hormonal and antitumoral activities on models of cancer of the prostate and mammary glands have been studied.

EXPERIMENTAL

In this work we used amino acids and amino acid derivatives produced by the firm Reanal (Hungary).

The melting points of the compounds obtained were determined on a Nagema (Germany) Boetius instrument. UV spectra were taken on a Beckman 24/25 spectrophotometer (USA). The optical rotations of the peptides were determined on a Bellingham and Stanley Pepol-60 spectropolarimeter (United Kingdom) at 22°C. The individuality of the compounds obtained was checked by thin-layer chromatography and electrophoresis. Thin-layer chromatography was conducted on Silufol plates (Kavalier, Czechoslovakia) (s) and also on Merck (Germany) plates (m). The following solvent systems were used: 1) 1% ammonia—*sec*-butyl alcohol (1:3); 2) *tert*-butyl alcohol—acetic acid—water (4:1:1); 3) benzene—ethanol—ethyl acetate (3:1:1); and 4) methanol—chloroform (1:7). Electrophoresis was conducted on a Laboratorium Felszeresek instrument (Hungary) in 2% acetic acid on Filtrak FN-12 paper (Germany) with a potential difference on the electrodes of 1500 V for 30 min. Electrophoretic mobilities were determined in relation to glycine (E_{Gly}).

For all the compounds synthesized, the results of elemental analyses corresponded to the calculated values.

Preparative high-performance liquid chromatography was conducted on Du Pont 830 (USA) and Waters 600E (USA) chromatographs using Delta Pak C_{18} — 300 Å, 15 μm, 19 × 300 mm, columns and the eluents: A) 10% of 0.025 M $CH_3CO_2NH_4$, pH 5.00-90% of CH_3N; and B) 50% of 0.025 M $CH_3CO_2NH_4$, pH 5.00-50% of CH_3N. According to the results of high-performance liquid chromatography, the purity of the final products was greater than 95%.

Amino acid analysis was conducted on Biotronik LC-2000 (Germany) and Michrotechnica T 339 (Czechslovakia) amino acid analyzers. The peptides were hydrolyzed with 6 N HCl at 110°C for 20 h. The results of the amino acid analysis of all the final products corresponded to the calculated values, the amount of the main substance in each case being 83-85%.

Mass spectra were recorded on a time-of-flight mass reflectron with an ion source of the electrospray type (FINÉPKhF RAN).

Synthesis of CMFU. A mixture of 0.100 g (0.77 mmole) of 5-FU, 0.130 g (1.38 mmole) of chloroacetic acid, and 0.12 g (2.15 mmole) of KOH in 10 ml of water was boiled under reflux for 30 min. The cooled solution was transferred to a column (2.5 × 10 cm) of the resin Dowex 2×8 (Serva, Germany) in the Cl^- form, this was washed with cold water until the 5-FU had issued completely, and the product was then eluted with 30% acetic acid. The solvent was evaporated off and the residue was recrystallized from a mixture of ethanol and ether. Yield 0.040 g (27% of theoretical), *m/z* 189 (MH^+); mp 190-200°C (λ_{max} 274 (pH 7), R_f 0.14 (1, s); 0 (2, s); 0 (3, s).

Synthesis of CMFU-ONp. With stirring and ice-bath cooling, 0.200 g (0.85 mmole) of *p*-nitrophenyl trifluoroacetate was added to a suspension of 0.080 g (0.43 mmole) of CMFU in pyridine. After 1 h, the cooling was removed and the mixture was stirred at room temperature until the solid matter had dissolved completely. The pyridine was evaporated off, and the residue was triturated with ether. Yield 0.250 g (80% of theoretical), mp 195°C, R_f 0.87 (2, s); 0.65 (3, s); 0.53 (4, s).

Synthesis of Z-Arg-Pro-NHEt. The deblocking of 3.08 g (11 mmole) of Z-Pro-NHEt [7] was achieved by the action of HBr in acetic acid for 45 min. The HBr·H-Pro-NHEt was precipitated with hexane, dissolved in methanol, and passed through a column of the anion-exchange resin Dowex 2×8 in the OH^- form (2 × 10 cm), after which the methanol was evaporated off and the residue was dissoved in DMFA.

The resulting solution of H-Pro-NHEt (E_{Gly} 1.70) was treated with a solution of 6.02 g (10 mmole) of Z-Arg(NO_2)-OH in DMFA, and the mixture was kept at room temperature for 1.5 days, after which the solvent was evaporated off. A solution of the residue in chloroform was washed with 5% $NaHCO_3$ solution, water, 1 N HCl, and water, and was dried over Na_2SO_4. The solvent was evaporated off, and the product was purified on a column (4 × 10 cm) of silica gel L 100/160 (Chemapol, Czechoslovakia) in the ethyl acetate—hexane (3:1) system; after impurities had emerged,the product was eluted with ethanol. Yield 3.34 g (66% of theoretical), mp 143-144°C, R_f 0.69 (1, s); 0.82 (2, s); 0.42 (3, s).

Synthesis of Boc-Leu-Arg(NO_2)-Pro-NHEt. The deblocking of 2.45 g (5.2 mmole) of Z-Arg(NO_2)-Pro-NHEt was achieved by the action of HBr in acetic acid for 45 min. The HBr·H-Arg(NO_2)-Pro-NHEt (E_{Gly} 1.20) was precipitated with ether, filtered off, dried in a desiccator over KOH, and dissolved in DMFA, and the excess HBr was eliminated with solid $NaHCO_3$, which was then filtered off. The solution of HBr·H-Arg(NO_2)-Pro-NHEt so obtained was treated with 0.57 ml (5.2 mmole) of N-methylmorpholine and 2.75 g (7.8 mmole) of BOC-Leu-ONp. The reaction mixture was stirred at room temperature for two days, the DMFA was distilled off, and the residue was triturated with ether and was then dissolved in chloroform; the solution was washed with 5% $NaHCO_3$ solution, water, 2 N H_2SO_4, and water, the solvent was evaporated off, and the product was crystallized from ethyl acetate. Yield 2.10 g (72% of theoretical), mp 118-120°C, $[\alpha]_D^{22}$ −44° (1%; DMFA), R_f 0.75 (1, s); 0.87 (2, s); 0.52 (3, s).

Synthesis of Z-Ser-Tyr-*D*-Asp(OBzl)-OH. With cooling to 0°C, 0.94 ml (7.2 mmole) of a 7.7 N solution of HCl in dioxane was added to a solution of 1.00 g (2.4 mmole) of Z-Ser-Tyr-N_2H_3 [7] in DMFA; the mixture was cooled to 0°C, and, with stirring, it was treated with 0.30 g (2.9 mmole) of *tert*-butyl nitrite. After 45 min, 1.7 ml (12.0 mmole) of triethylamine and 1.16 g (4.8 mmole) of H-Asp(OBzl)-OH were added. The reaction mixture was kept at −30°C for 1 h, at −15°C for 1 h, at 0°C for 1 day, and at room temperature for 1 day. The DMFA was evaporated off, the residue was diluted with ethanol and was filtered, and the alcoholic solution was diluted with water and acidified with 2 N H_2SO_4. The precipitate that deposited was filtered off, washed with ether, and dried in vacuum. Yield 1.2 g (83% of theoretical), mp 88-89°C, $[\alpha]_D^{22}$ −18° (0.55%, methanol). R_f 0.62 (1, s); 0.89 (2, m); 0.60 (3, s).

Synthesis of Z-Ser-Tyr-*D*-Asp(OBzl)-Leu-Arg(NO_2)-Pro-NHEt (5). BOC-Leu-Arg(NO_2)-Pro-NHEt (0.823 g; 1.48 mmole) was deblocked by trifluoroacetic acid for 30 min. The H-Leu-Arg(NO_2)-Pro-NHEt trifluoroacetate was precipitated with ether, filtered off, and dried in a desiccator over KOH (E_{Gly} 1.1). The amino component obtained and 0.900 g (1.48 mmole) of Z-Ser-Tyr-*D*-Asp(OBzl)-OH were dissolved in DMFA, and 0.4 ml (2.96 mmole) of triethylamine was added. With cooling to 0°C and stirring, 0.32 ml (1.48 mmole) of diphenyl phosphorazidate was added.

The mixture was stirred at 0°C for 1 h and at room temperature for two days. The DMFA was evaporated off, and the residue was distributed between ethyl acetate and water. The aqueous layer was extracted three times with ethyl acetate. The organic fractions were combined and washed with 5% $NaHCO_3$ solution, water, 1 N HCl, and water. The solvent was evaporated off, and the residue was crystallized from ether. Yield 1.123 g (73% of theoretical), mp 118-120°C, $[\alpha]_D^{22}$ −20.5°C (0.50%; methanol). R_f 0.86 (1, m); 0.92 (2, m); 0.47 (8, m).

Synthesis of H-Ser-Tyr-*D*-Asp-Leu-Arg-Pro-NHEt (1). A solution of 0.151 g (0.14 mmole) of peptide (5) in methanol with the addition of 10% of acetic acid was hydrogenated in the presence of palladium black, the catalyst was filtered off, the solvent was evaporated off, and the product was chromatographed on a column (1 × 20 cm) of Sephadex SE C-25 (Pharmacia,

Sweden) in a molarity gradient of pyridine-acetate buffer (0.005-0.5 M). Yield 0.106 g (94% of theoretical). Final purification was achieved with the aid of HPLC. The product was lyophilized three times. E_{Gly} 1.4; *m/z*: 778 (MH^+).

Synthesis of CMFU-Ser-Tyr-*D*-Asp-Leu-Arg-Pro-NHEt (2). A solution of 0.067 g (0.09 mmole) of peptide (1) in DMFA was treated with 0.040 g (0.13 mmole) of CMFU-ONp, and after 1.5 h the DMFA was evaporated off, the residue was triturated with ether and the product was purified by ion-exchange chromatography on a column of Sephadex SE C-25 (1 × 11 cm) in a molarity gradient of pyridine acetate buffer (0.005-0.25 M). Yield 0.037 g (45% of theoretical). Final purification was achieved by HPLC, and the product was lyophilized three times. E_{Gly} 0.85, *m/z* 948 (MH^+).

Synthesis of BOC-Pro-Ser-Tyr-*D*-Asp-Leu-Arg-Pro-NHEt (6). A solution of 0.211 g (0.27 mmole) of peptide (1) in DMFA was treated with 0.250 g (0.54 mmole) of Boc-Pro-OPcp, and the pH was brought to 8 with triethylamine; after two days the DMFA was evaporated off, the residue was triturated with ether, and the product was purified on a column of Sephadex SE C-25 (1 × 25 cm) in a molarity gradient of pyridine acetate buffer (0.005-0.25 M). Yield 0.192 g (73% of theoretical). E_{Gly} 0.94, R_f 0.45 (1, s); 0.27 (2, s); 0 (3, s).

Synthesis of H-Pro-Ser-Tyr-*D*-Asp-Leu-Arg-Pro-NHEt (3). Compound (6) (0.192 g; 0.20 mmole) was treated with trifluoroacetic acid for 30 min, and the mixture was then evaporated with benzene and the residue was dried in a desiccator over KOH. Part of the product was used in subsequent synthesis without purification. The final product was purified with the aid of HPLC and was lyophilized three times, *m/z* 875 (MH^+), E_{Gly} 1.32.

Synthesis of CMFU-Pro-Ser-Tyr-*D*-Asp-Leu-Arg-Pro-NHEt (4). The pH of a solution of 0.125 g (0.14 mmole) of peptide (3) and 0.067 g (0.22 mmole) of CMFU-ONp in DMFA was brought to 8 with triethylamine. After 30 min, the DMFA was evaporated off, and the residue was triturated with ether and chromatographed on a column of Sephadex SE C-25 (1 × 21 cm) in a molarity gradient of pyridine acetate buffer (0.005-0.25 M). Yield 0.126 g (86% of theoretical), E_{Gly} 0.90. Final purification was achieved with the aid of HPLC. The product was lyophilized three times, *m/z*: 1045 (MH^+).

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