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(71) Demandeur/Applicant:
OBSCHESTVO S ORGANICHENNOI OTVETSTVENNOSTIJU "KLINIKA INSTITUTA BIOREGUL YATTSII I GERONTOLOGII", RU

(72) Inventeur/Inventor:
KHAVINSON, VLADIMIR KHATSKELEVICH, RU

(74) Agent: KIRBY EADES GALE BAKER

(54) Titre : TETRAPEPTIDE AYANT UN EFFET GERONTO-PROTECTEUR, SUBSTANCE PHARMACOLOGIQUE A BASE DE CE DERNIER, ET PROCEDE D'APPLICATION

(54) Title: TETRAPEPTIDE REVEALING GEROPROTECTIVE EFFECT, PHARMACOLOGICAL SUBSTANCE ON ITS BASIS, AND THE METHOD OF ITS APPLICATION

(57) Abrégé/Abstract:
Tetrapeptide L-alanyl-L-gluthamyl-L-asparagyl-glycine of the general formula L-Ala-L-Glu-L-Asp-Gly is proposed as a biologically active compound revealing a geroprotective effect. The use of L-Ala-L-Glu-L-Asp-Gly tetrapeptide in medicine is proposed for preparing a substance revealing a geroprotective effect. There is proposed a pharmacological substance, which contains as its active base an effective amount of tetrapeptide of the formula L-alanyl-L-gluthamyl-L-asparagyl-glycine (L-Ala-L-Glu-L-Asp-Gly) or its salts of the amino group (acetate, hydrochloride, oxalate) and of carboxyl groups (the salts of metals - Natrium, Potassium, Calcium, Lithium, Zinc, Magnesium, and also the salts of organic and inorganic cations - ammonium and triethylammonium). The substance is proposed for parenteral, intranasal, oral administration, and local application. With respect to the invention, the method of premature ageing prevention implies prophylactic and/or therapeutic administration to a patient of the pharmacological substance in doses 0.01 to 100 µg/kg of the body weight at least once a day for a period necessary for the achievement of a therapeutic effect.

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(74) Agent: SPESIVTSEVA, Irina Y.; "Klinika Instituta Bioregulyatsii & Gerontologii",, Ltd., pr. Dinamo, 3, St.Petersburg, 197110 (RU).

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(71) Applicant *(for all designated States except US)*: **OBSCHESTVO S OGRANICHENNOI OTVETSTVENNOSTIJU "KLINIKA INSTITUTA BIOREGULYATSII I GERONTOLOGII"** [RU/RU]; pr. Dinamo, 3, St.Petersburg, 197110 (RU).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **KHAVINSON, Vladimir Khatskelevich** [RU/RU]; ul. Savushkina, 26-2-7, St.Petersburg, 197374 (RU).

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Tetrapeptide revealing geroprotective effect, pharmacological substance on its basis, and the method of its application

Field of the Invention

The invention is referred to the field of medicine and may be employed as a geroprotective substance for the prevention of premature ageing of the organism.

It is known that some of the dominating mechanisms of organism ageing are: an increase in molecular lesions caused by free radicals, functional disturbances of the anti-oxidation defence system, and disorder in physiological functions of the epiphysis (1, 2, 3).

Background of the Invention

Since the claimed tetrapeptide, according to the invention, reveals biological activity, and namely, geroprotective activity, a group of compounds possessing anti-oxidation properties must be referred to its analogues in application. Ionol food-supplement (2.6-di-tret-butyl-4-methylphenol), being a well-known inhibitor of radical processes, promoted an increase in the life span of LAF_1 strain in mice characterised by accelerated ageing (4). However, the pharmaceutical based on 2.6-di-tret-butyl-4-methylphenol (dibunol) is manufactured in the form of a liniment and is largely applied in urology practice for treatment of cancer patients (5). Addition of ethoxyhin anti-oxidant (santohin) to food extended the life span of mice of C3H strain (6). An increase in the life span of experimental animals is also promoted by 2-ethyl-6-methyl-3-oxypyridine chlorhydrate, a low-toxic water-soluble anti-oxidant, which is a structural analogue of vitamin B_6 (7, 8). An insignificant extension in the life span of experimental animals was facilitated by 2-merkaptoethanolamine, butyl hydroxytoluol, cystein, 3-hydroxypyridine, centrophenoxyn, lactic and gluconic acids, and glutathione (9, 10). Still, these compounds are not pharmaceutical preparations and they have found no employment in medicine as geroprotective substances. Administration of vitamins A, C, E resulted in an increase of the life span of experimental animals as well (11, 12, 13). Supersaturation of the organism with these vitamins, however, may unfavourably influence the functions of organs and systems and entail an intensive development of hypervitaminosis. β-catechol, a preparation whose composition is formed by vitamins and vegetable substances, is known to reveal anti-oxidation activity (14). Administration of this preparation to SAM-P8 strain mice showing accelerated ageing augmented the survival rate of the animals. However, the mechanisms of its geroprotective action have not been studied enough yet, thus limiting its integration with clinical practice. After the mice of SAM strain, disposed to accelerated ageing, had been kept on a diet with an increased amount of carnosine (β-Ala-His) for 7 months, their death rate decreased (15).

Still, carnosine is not a pharmaceutical preparation, its geroprotective properties being not studied enough yet. A minor increase in the average life span of mice was effected by the application of melatonin, an epiphyseal hormone (16). The impact of melatonin is associated with its anti-oxidation property (17). Nevertheless, the melatonin exposure of Drosophila melanogaster selected for a high embryonic mortality rate (HEM strain), failed to produce a geroprotective effect, though it was accompanied by an anti-oxidation one. Melatonin is not a pharmaceutical preparation and is manufactured in the form of a biologically active food additive.

Gerovital, a Novocain-containing drug, is used as a geroprotective substance. The detriments of this preparation consist in its possible negative influence on the functions of the cardiovascular system, its allergic impact, sometimes sleep impairment, the feeling of anxiety, muscular and articular pains.

Disclosing of the Invention

The claimed invention is aimed at obtaining a new biologically active compound of peptide origin capable of geroprotective activity.

The claimed peptide compound – tetrapeptide - has no structural analogues.

According to the invention, there is claimed tetrapeptide L-alanyl-L-gluthamyl-L-asparagyl-glycine of the general formula L-Ala-L-Glu-L-Asp-Gly.

With respect to the invention, the tetrapeptide L-alanyl-L-gluthamyl-L-asparagyl-glycine with the following amino acid sequence: L-Ala-L-Glu-L-Asp-Gly reveals biological activity, namely, geroprotective activity due to the stimulation of indices of the anti-oxidation defence system and due to the process of melatonin synthesis in structures of the diffusive neuroendocrine system.

The tetrapeptide is obtained by a classical method of peptide synthesis in a solution (19).

The geroprotective activity of L-Ala-L-Glu-L-Asp-Gly tetrapeptide was stated in experimental trials. The study of geroprotective activity was conducted in Drosophila melanogaster by analysing the indices of anti-oxidation defence, life span, and the length of reproduction period, as well as in rats by examining the synthesis of extra-pineal melatonin.

With respect to the invention, the pharmacological substance capable of geroprotective activity includes as its active base an effective amount of tetrapeptide of the formula L-alanyl-L-gluthamyl-L-asparagyl-glycine (L-Ala-L-Glu-L-Asp-Gly) or its salts.

With respect to the invention, the pharmacological substance capable of geroprotective activity may contain salts of the amino group (acetate, hydrochloride, and oxalate) or of carboxyl groups (the salts of metals – Sodium, Potassium, Calcium, Lithium, Zinc, Magnesium, and other organic and inorganic cations – ammonium, triethylammonium).

With respect to the invention, the pharmacological substance is meant for parenteral, intranasal, oral administration, or local application.

The claimed pharmacological substance revealing geroprotective activity is capable of stimulating the indices of the anti-oxidation defence system and the processes of melatonin synthesis in structures of the diffusive neuroendocrine system, which, in their turn, inhibit ageing processes and promote a life span increase.

The notion "geroprotective substance", used in this application, implies the substance, which inhibits ageing and prolongs life by means of preventing premature ageing, which needs stimulation of the anti-oxidation defence system and the regulatory influence on metabolic processes in structures of the diffusive neuroendocrine system.

The notion "pharmacological substance", used in this application, implies the employment of any drug form containing the tetrapeptide or its salts, which may be used for prophylactic and/or therapeutic purposes in medicine as a geroprotective substance in premature ageing.

The notion "effective amount", used in this application, implies the employment of such an amount of the active base, which, in compliance with its quantitative indices of activity and toxicity, as well as with respect to the knowledge available, must be effective in this drug form.

The notion "pharmaceutical composition", used in this application, implies various drug forms of the preparation.

In order to obtain pharmaceutical compositions, which meet the invention, the proposed tetrapeptide or its pharmaceutically applicable derivatives are stirred as an active ingredient and a pharmaceutical carrier in accordance with the methods of compounding accepted in pharmaceutics.

The carrier may have various forms, which depend on the drug form of the preparation desirable for administration, for example: parenteral, intranasal, or oral.

All known pharmacological components may be used for the preparation of compositions in doses preferable for oral application.

For parenteral (intranasal) administration, the carrier usually includes sterile water, although there may be included other ingredients instrumental for stability or maintaining sterility.

In accordance with the invention, the method embraces prophylactic or therapeutic exposure of patients to the claimed pharmacological substance in doses 0.01 to 100 mg/kg of the body weight, at least once a day during a period necessary for achieving a therapeutic effect – 10 to 40 days with respect to the character and severity of a pathologic process.

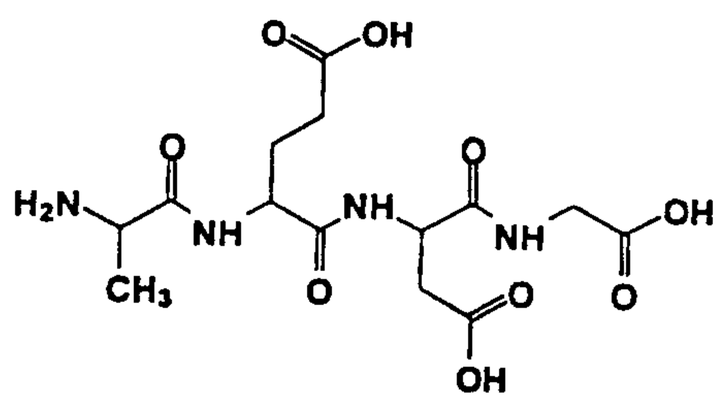
In accordance with the invention, the tetrapeptide is active in case it is administered in doses 0.01 to 100 mg/kg of the body weight, although lower (higher) doses may be used as well, with respect to the character and severity of a pathologic process.

Industrial application

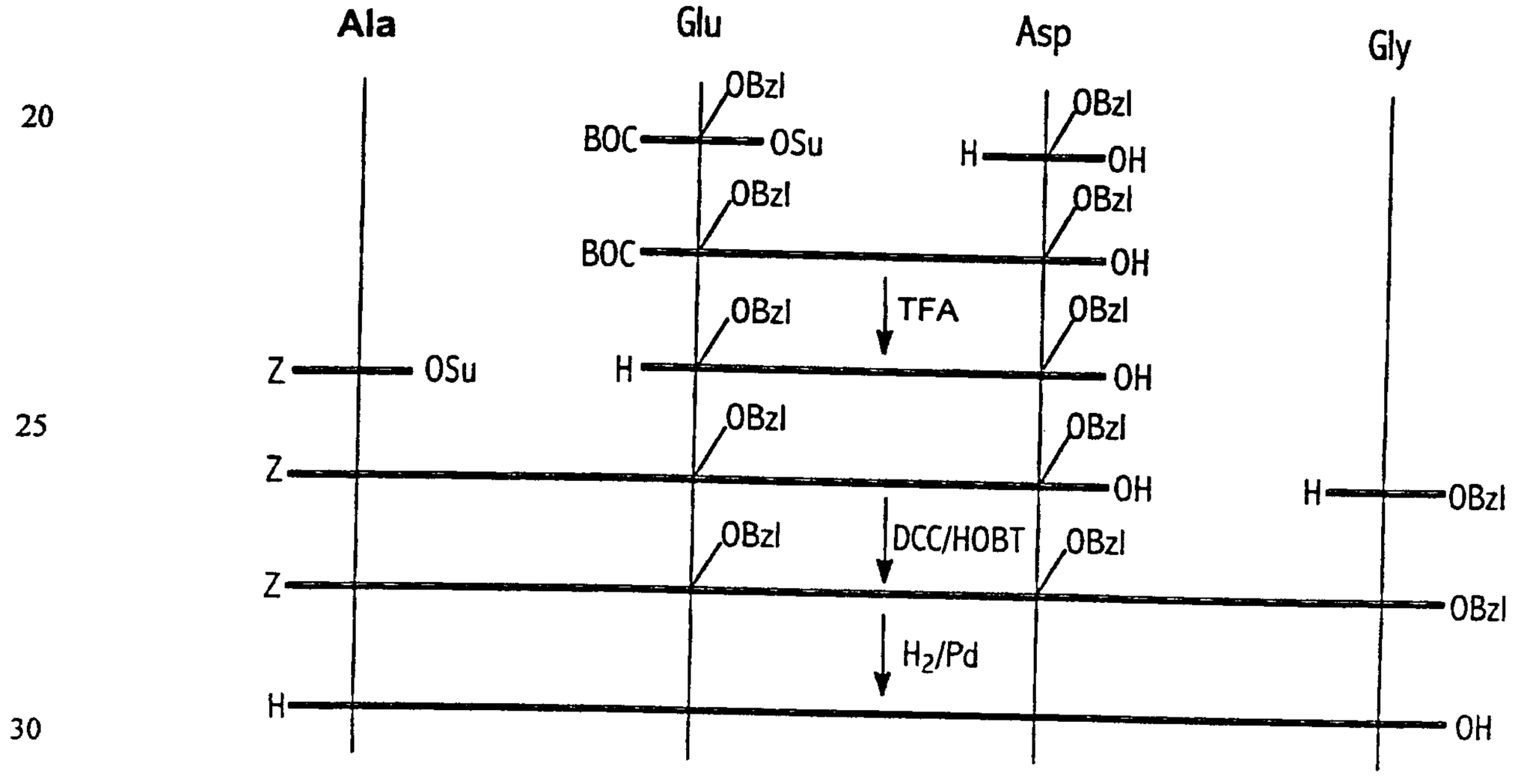
The invention is illustrated by an example of synthesis of the tetrapeptide, whose formula is L-alanyl-L-gluthamyl-L-asparagyl-glycine (L-Ala-L-Glu-L-Asp-Gly) (Example 1), by the examples of the tests for toxicity and biological activity of the tetrapeptide (Examples 2-8), and also by the examples, which show the results of clinical application of the tetrapeptide, thus demonstrating its pharmacological properties and attesting the possibility of achieving prophylactic and/or therapeutic effect (Examples 9, 10).

Example 1. Synthesis of L-Ala-L-Glu-L-Asp-Gly tetrapeptide

1. Product name: **L-alanyl-L-gluthamyl-L-asparagyl-glycine.**
2. Structural formula: **H-Ala-Glu-Asp-Gly-OH**



3. Molecular formula without ion pair: $C_4H_{22}N_4O_9$.
4. Molecular weight without ion pair: 390.35.
5. Ion pair: acetate.
6. Appearance: white amorphous powder without smell.
7. Method of synthesis: the peptide is obtained by a classical method of synthesis in a solution to the following scheme:



Z – benzyloxycarbonyl group;

BOC – tert.butyloxycarbonyl group;

OSu – N-oxysuccinimide ester;

OBzl – benzyl ester;

DCC – N, N′-dicyclohexylcarbodiimide;

HOBT – N-oxybenzotriazol.

N, N′-dimethylformamide was used as a solvent. By the addition of aspartic acid there was applied the defence of the α-COOH group by the salification method with the use of triethylamine. The removal of the BOC-protecting group was performed with the solution of trifluoracetic acid (TFA); the removal of the Z- protecting group was performed with catalytic hydrogenation. The extraction and purification of the product were conducted by the method of preparative high-performance liquid chromatography (HPLC) on the column with a reversed phase.

Properties of the ready product:

- amino acid analysis

Glu	**Asp**	**Ala**	**Gly;**
1.02	1.00	1.01	1.00;

- peptide content 98.4% (by HPLC, 220nm);
- thin layer chromatography (TLC) – individual, R_f=0.73 (acetonitrile - acetic acid - water 5:1:3);
- moisture content: 5%;
- pH of the 0.001%-solution: 4.37;
- specific rotary power: $[\alpha]_D^{22}$: -32° (c=1, H_2O).

Example of synthesis:

1. **BOC-Glu(OBzl)-Asp(OBzl)-OH(I), N-tert.butyloxycarbonyl-(γ-benzyl)gluthamyl-(β-benzyl)aspartate.**

4.3g (0.0100mol) of N-oxysuccinimide ester of N-tert.butyloxycarbonyl-(γ-benzyl)gluthamyl acid (BOC-Glu(OBzl)-OSu) are dissolved in 20ml of dimethylformamide and added 1.72ml (0.0125mol) of triethylamine and 2.80g (0.0125mol) of β-benzylaspartate. The mixture is stirred within 24 hours at room temperature. Afterwards the product is precipitated with 0.5N (150ml) of sulphuric acid, extracted by ethyl acetate (3x33ml), washed in 0.5N of sulphuric acid (2x20ml), water, 5%-solution of sodium bicarbonate (1x20ml), water, 0.5N of sulphuric acid (2x20ml), water, the solution is dried over anhydrous Na_2SO_4. Ethyl acetate is filtered and removed *in vacuo* under 40°C. The residue is dried *in vacuo* over P_2O_5. As a result, 5.68g (≈100%)

of oil is obtained. R_f=0.42 (benzol-acetone 2:1, Sorbfil plates, 8-12 μm of Silicagel, development by UV and chlorine/benzidine).

2. TFA·H-Glu(OBzl)-Asp(OBzl)-OH (II), (γ-benzyl)gluthamyl-(β-benzyl) aspartate trifluoracetate

5.68g (≈0.01mol) of N-tert.butyloxycarbonyl-(γ-benzyl)gluthamyl-(β-benzyl)aspartate (I) are dissolved in 20ml of dichlormethan-trifluoracetic acid mixture (3:1). In 2 hours the solvent is removed under 40°C, it is repeated with an addition of another portion of dichlormethan (2x10ml), the residue is dried *in vacuo* over NaOH. 5.80g (≈100%) of oil are obtained. R_f=0.63 (n-butanol-pyridine-acetic acid-water, 15:10:3:12).

3. Z-Ala-Glu(OBzl)-Asp(OBzl)-OH (III), N-carbobenzoxyalanyl-(γ-benzyl)gluthamyl-(β-benzyl)aspartate.

5.65g (0.01mol) of (γ-benzyl)gluthamyl-(β-benzyl)aspartate trifluoracetate (II) are dissolved in 10ml of dimethylformamide, added 2.80ml (0.02mol) of triethylamine and 4.14g (0.013mol) of the N-oxysuccinimide ester of N-carbobenzoxyalanyl. The reacting mixture is stirred within 24 hours at room temperature. The product is precipitated with 0.5N of sulphuric acid (150ml), extracted by ethyl acetate (3x30ml), washed in 0.5N of sulphuric acid (2x20ml), water, 5%-solution of sodium bicarbonate (1x20ml), water, 0.5N of sulphuric acid (2x20ml), water, the solution is dried over anhydrous Na_2SO_4. Ethyl acetate is filtered, removed *in vacuo* under 40°C, the residue is recrystallised in the ethyl acetate/hexane system. The product is filtered and dried *in vacuo* over P_2O_5. The yield is 4.10g (66%). The temperature of melting (T_{ml}) is 154°C. R_f=0.48 (benzol-aceton, 1:1), R_f=0.72 (n-butanol-pyridine-acetic acid-water, 15:10:3:12).

4. Z-Ala-Glu(OBzl)-Asp(OBzl)-Gly-OBzl (IV), N-carbobenzoxyalanyl (γ-benzyl)gluthamyl-(β-benzyl)aspartylglycine benzyl ester.

1.01g (3mmol) of benzyl ester of glycine tosilate TosOH·H-Gly-OBzl is suspended in 15ml of tetrahydrofuran and added 0.4ml (3mmol) of triethylamine while mixing, then, in 5 minutes, is added 1.28g (2mmol) of N-carbobenzoxyalanyl-(γ-benzyl)gluthamyl-(β-benzyl)aspartate (III), 0.27g (2mmol) of N-oxybenzotriazol. The mixture is cooled down to 0°C. Afterwards, the solution of 0.42g (2mmol) of the N,N′-dicyclohexylcarbodiimide in 5ml of tetrahydrofuran cooled down to 0°C is added. The mixture is stirred at this temperature within 2 hours and left for a night at room temperature. The residue of dicyclohexylurea is filtered out, the solvent is removed *in vacuo* and the residue is dissolved in 30ml of ethyl acetate. The solution is washed in 1N of sulphuric acid, water, 5%-solution of sodium bicarbonate, water, 1N of sulphuric acid, water, and dried over

anhydrous Na_2SO_4. The solvent is removed *in vacuo* and the product is recrystallised in the ethyl acetate/hexane system. The yield is 1.30g (82%). T_{ml}=146-148°C. R_f=0.75 (benzol-aceton, 2:1).

5. H-Ala-Glu-Asp-Gly-OH (V), alanyl-gluthamyl-aspartyl-glycine.

1.25g of N-carbobenzoxyalanyl-(γ-benzyl)gluthamyl(β-benzyl)aspartylglycine benzyl ester (III) are hydrogenated in the methanol-water-acetic acid system (3:1:1) over Pd/C. Progress of the reaction is monitored by TLC method in the benzol-aceton (2:1) and acetonitryl-acetic acid-water (5:1:3) systems. After the end of the reaction the catalyst is filtered, the filtrate is removed *in vacuo*, and the residue is recrystallised in the water-methanol system. The product is dried *in vacuo* over KOH. The yield is 520mg (95%). R_f=0.73 (acetonitryl-acetic acid-water, 5:1:3). For purification, 390mg of the product is dissolved in 4ml of 0.01%-triflouracetic acid and subject to HPLC on the column with a reversed phase 50x250mm Diasorb-130-C16T, 7μm. The chromatograph used is Beckman System Gold, 126 Solvent Module, 168 Diode Array Detector Module. The conditions of chromatography A: 0.1% TFA; B: 50% MeCN/0.1% TFA, gradient B 0 → 5% during 80 minutes. Sample volume constitutes 5ml, detection is conducted by 215nm, scanning - by 190-600nm, the flow speed constitutes 10ml/min. The fraction is selected within 54.0-66.0 min. The solvent is removed *in vacuo* at a temperature not exceeding 40°C, it is multiply repeated (5 times) with 10ml of 10%-solution of acetic acid. Finally the residue is dissolved in 20ml of deionised water and lyophilised. As a result there is obtained 290mg of purified product in the form of amorphous white powder without smell. The obtained peptide in the form of acetate is transferred to a free form by processing it with IRA anionite or with an analogous substance in the (OH^-)-form. Afterwards, salts of the amino group are obtained with the subsequent addition of an equivalent of a corresponding acid (hydrochloride or oxalic). The obtained water solution is lyophilised and analysed as a ready product.

In order to obtain corresponding salts of carboxyl groups, the free tetrapeptide is added a calculated amount of the water solution of hydroxide of a corresponding metal (NaOH, KOH, $Zn(OH)_2$, LiOH, $Ca(OH)_2$, $Mg(OH)_2$, NH_4OH). To obtain triethylammonium salt, the processing is carried out similarly, with the use of triethylamine as a base.

6. Analysis of the ready product.

- Peptide content is defined by the HPLC method on the Surelco LC-18-DB column, 4.6x250mm, grad. LC-18-DB. A: 0.1% of TFA; B: 50% of MeCN/0.1% of TFA; grad. B 0 →

20% during 30 min. The flow speed constitutes 1ml/min. Detection by 220nm, scanning - by 190-600nm, the sample volume is 20µl. Peptide content - 98.45%.

- The amino acid analysis is carried out on the tester AAA"T-339" Prague. Hydrolysis is conducted in 6N of HCl at 125°C within 24 hour.

Glu	Asp	Ala	Gly
1.02	1.00	1.01	1.00

- TLC: individual, R_f=0.73 (acetonitryl-acetic acid-water, 5:1:3). Sorbfil plates, 8-12µm Silicagel, developing in chlorine/benzydine.
- Moisture content: 5% (gravimetrically according to the mass loss by drying, - 20mg at 100°C).
- pH of 0.001%-solution: 4.37 (potentiometrically).
- Specific rotary power: $[\alpha]_D^{22}$: -32° (c=1, H_2O), "Polamat A", Carl Zeiβ Jena.

Example 2. Study of L-Ala-L-Glu-L-Asp-Gly tetrapeptide for toxicity.

The study of general toxicity of L-Ala-L-Glu-L-Asp-Gly tetrapeptide was conducted in accordance with "The rules of pre-clinical estimation of safety of pharmacological substances" (GLP).

The purpose of study consisted in defining the tolerable toxic doses of the preparation, in estimating the stage and character of pathologic alterations in various organs and systems of the organism, and in identifying the correlation between the toxic dose-related effect and the duration of drug application.

Determination of acute toxicity of L-Ala-L-Glu-L-Asp-Gly tetrapeptide was conducted according to Kerber. The investigation was carried out on 66 white outbred male mice weighing 20 to 23g, which were kept under standard regimen and fed upon standard rations in vivarium conditions. The animals were randomly divided into 6 equal groups by 11 mice in each. The animals were exposed to a single administration of the drug intramuscularly, 0.25ml in doses 1mg/kg, 2mg/kg, 3mg/kg, 4mg/kg, 5mg/kg (several thousand times exceeding the therapeutic dose, recommended for clinical trial). Control animals were administered the same amount of sodium chloride.

Within 72 hours and later on in 14 days none of the animals in either of the groups died. No alterations in the general state, behaviour, locomotory activity, hairy and skin integument, or physiological discharges of the animals were registered.

Thus, L-Ala-L-Glu-L-Asp-Gly tetrapeptide in doses several thousand times exceeding the therapeutic one, recommended for clinical trials, does not induce acute toxic reactions, which confirms a wide therapeutic applicability of the preparation.

The study of subacute toxicity of L-Ala-L-Glu-L-Asp-Gly tetrapeptide was carried out on 60 white outbred rats with body weight 150 to 250mg. Experimental animals were exposed daily to a

single administration of the drug intramuscularly for 90 days in doses 1μg/kg, 0.3mg/kg, 3mg/kg in 0.5ml of sodium chloride solution. Animals of the control group were administered the same amount of sodium chloride.

Within the whole period of study the animals were under daily supervision. Behaviour of the animals, their rations and water consumption, the state of hairy integument and mucous membranes were registered. The animals were weighed weekly. Prior to and on the 30th, 60th, and 90th day of the drug administration the morphological composition and properties of the peripheral blood were examined. Upon the experiment completion biochemical and coagulologic indices of blood were investigated.

Chronic toxicity of L-Ala-L-Glu-L-Asp-Gly tetrapeptide, obtained by the claimed method, was studied by its long-time administration to rats with body weight 150 to 250mg. The animals were exposed daily to a single administration of the drug intramuscularly in doses 1μg/kg, 0.1mg/kg, 1mg/kg in 0.5ml of sodium chloride solution during 6 months. Behaviour of the animals, their rations and water consumption, the state of hairy integument and mucous membranes were registered. The animals were weighed daily during the first 3 months of the experiment and then once a month. Haematological and biochemical studies were carried out in 3 months after the onset of the drug administration and on the experiment completion. Functions of the cardiovascular system, liver, pancreas, kidney, and adrenal gland were estimated. Upon the end of the drug administration some animals were subject to pathomorphological examination with the purpose to estimate the condition of various sections of the brain and spinal marrow, heart, aorta, lungs, liver, kidney, organs of the endocrine and immune systems.

Estimation of the general state of the animals, the morphological and biochemical indices of the peripheral blood, the morphological state of the intrinsic organs, the state of the cardiovascular and respiratory systems, liver and kidney functions showed no pathologic alterations.

The study of subacute and chronic toxicity of L-Ala-L-Glu-L-Asp-Gly tetrapeptide confirms the absence of side effects in case of long-time application of the drug in doses, which 100-1000 times exceeded the therapeutic one.

Example 3. ***Impact of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the ageing rate and free radical processes in Drosophila melanogaster of HEM strain selected for high embryonic mortality rate.***

L-Ala-L-Glu-L-Asp-Gly tetrapeptide was applied in Drosophila melanogaster larvae of the 2nd age of HEM strain selected for a high ageing rate (20). HEM strain is characterised by the unique dynamics of embryonic mortality, which consists in an increased frequency of early

embryonic lethal outcomes from 65% on the 1st day of egg-laying up to 95% on the 4th day, in the reduced average life span (29 days), and in the raised intensity of lipid peroxide oxidation.

L-Ala-L-Glu-L-Asp-Gly tetrapeptide was added in proportion 0.00001% of the culture medium weight, which is an extremely low dose. The doses stated in literature on the influence of various substances on Drosophila melanogaster usually ranged from 0.001 to 0.01%. Lower doses do not normally produce any effect.

Biochemical parameters were studied on 14-day old flies. Among them the activity of catalase and the intensity of tissue chemoluminescence were analysed (21, 22, 23). Catalase is the basic enzyme of anti-oxidation defence. The degree of its activity suggests the ability of the organism to withstand oxidative stress. Luminol-dependent chemoluminescence, induced by hydrogen peroxide, reflects the stage of active oxygen forms in tissues. Thus, a decrease in the chemoluminescence intensity exhibits an increase in the organism ability to resist oxidative stress.

The obtained results are displayed in Table 1. Since significant intersexual distinctions on the studied indices have been revealed the data for females and males are given separately.

The presented data evidence that L-Ala-L-Glu-L-Asp-Gly tetrapeptide promotes a significant rise in catalase activity in experimental male and female flies in comparison with the controls.

General anti-oxidation activity of tissues, characterised by the degree of chemoluminescence, increased under the influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide only in females.

Table 1

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on catalase activity and tissue chemoluminescence in Drosophila melanogaster of HEM strain

Variant of the experiment		Catalase activity (mmol H_2O_2/mg of protein per min.)	Chemoluminescence (conv. unit/mg of protein)
FEMALES	Control	85.5±2.78	14.7±1.44
	L-Ala-L-Glu-L-Asp-Gly tetrapeptide	98.8±1.62*	7.9±0.91*
MALES	Control	115.4±9.88	1.59±0.121
	L-Ala-L-Glu-L-Asp-Gly tetrapeptide	132.3±3.68*	2.03±0.182

* - P<0.05 in comparison with the control indices.

Table 2 displays the results of the life span analysis in different experimental groups.

Table 2

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the life span parameters in Drosophila melanogaster of HEM strain

Variant of the experiment		ALS (days)	Me (days)	90% (days)	R•10^{-2} ($days^{-1}$)	MRDT
FEMALES	Control	38+1.6	43.5	67	6.7+0.11	3.4
	L-Ala-L-Glu-L-Asp-Gly tetrapeptide	48+1.5**	48.0	76	4.0+0.29**	3.9
MALES	Control	40+1.5	43.5	64	5.5+0.27	3.6
	L-Ala-L-Glu-L-Asp-Gly tetrapeptide	42+1.3	38.0	62	4.5+0.37*	3.8

Note: ALS – average life span, Me – median life span, 90% - the age which 90% of the individuals reach, R•10^{-2} – the parameter of Gomperz equation, MRDT – mortality-rate doubling time.

* - P<0.05 in comparison with the control indices;

** - P<0.001 in comparison with the control indices.

As it is demonstrated in Table 2, L-Ala-L-Glu-L-Asp-Gly tetrapeptide produces a pronounced geroprotective effect. In females it significantly extends the average life span (P<0.001) and lowers the ageing rate (R) (P<0.001). In males it slightly decreases the ageing rate (P<0.05).

Thus, L-Ala-L-Glu-L-Asp-Gly tetrapeptide increases the average life span by 26% and reduces the population real ageing rate, estimated by the quantity of Gomperz parameter, by 40%.

Example 4. ***Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the reproductive functions in Drosophila melanogaster of HEM strain, selected for high embryonic mortality rate.***

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide upon Drosophila melanogaster was exerted at the stage of larvae of the 2^{nd}-3^{rd} age, by means of adding the preparation to culture

medium in dose 0.00001% of the medium weight, which approximately made 0.015mg/100ml. The flies, hatched on the 1st day, were placed into test tubes, 5 couples in each, and transferred to fresh medium every 3 to 6 days.

There have been considered the following factors:

- The share of the test tubes (of their initial amount), which contained live females (in Table 3 – the share of live cultures).
- The share of the test tubes (of the number of live cultures), in which posterity was given (in Table 3 – the share of fertile cultures).

In each experimental variant 75 parental couples were analysed. The distinctions between fertility indices were defined by means of Fischer exact criterion for 2x2-adjustment tables (24).

The results of the experiment are displayed in Table 3. For each experimental variant the shares of live and fertile cultures are presented. As it is shown in the Table, starting from the 19th day the share of tetrapeptide-processed fertile cultures exceeds the analogous share for the control. It is demonstrated that the share of fertile culture in L-Ala-L-Glu-L-Asp-Gly tetrapeptide variant is notably higher ($P<0.05$) than in controls in the age of 32 days.

The coefficients of lineal regression are presented in Table 4. As it is shown in the Table, the coefficients of the control regression line differ significantly from the experimental one. The "a"-coefficient (reflecting the slope of the straight line) in the control is 1.8 times higher than the regression coefficient of the experimental variant.

Thus, it is demonstrated that the share of fertile cultures in the control variant reduces twice faster in comparison with the L-Ala-L-Glu-L-Asp-Gly tetrapeptide variant. Duration of the reproductive period amounted to 30 days for the cultures, not exposed to the tetrapeptide, and to 47 days for the cultures processed with the preparation. It is stated that L-Ala-L-Glu-L-Asp-Gly tetrapeptide 1.5-fold extends the reproduction period.

The data obtained on the life span were analysed correspondingly. The data on the regression analysis are displayed in Table 5.

As it follows from the Table, the share of live cultures (i.e. the cultures, in which live females are present) decreases on average 1.5 times faster than in controls. The calculated median life span (i.e. the age, by which 50% individuals die) and the one that is really attained (i.e. the maximal life span in different variants of the experiment) are displayed in Table 6.

As it is demonstrated in the Table, alongside with an increase in the median life span, in the variant processed with L-Ala-L-Glu-L-Asp-Gly tetrapeptide, the maximal record-breaking rise in the life span was achieved (in the lines of Drosophila melanogaster with high ageing rate) – 100 days.

Table 3

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the dynamics of live and fertile cultures share in Drosophila melanogaster of HEM strain.

Days	Control		L-Ala-L-Glu-L-Asp-Gly tetrapeptide	
	Share of cultures			
	Live	Fertile	Live	Fertile
0	100-0.8	100-0.8	100-0.8	100+0.8
6	100-0.8	100-0.8	100-0.8	100+0.8
10	100-0.8	86±9.3	100-0.8	93±6.9
13	100-0.8	79±11.0	100-0.8	93±6.9
19	100-0.8	50±13.4	100-0.8	86±9.4*
23	100-0.8	29±12.1	100-0.8	43±13.2
27	93±6.9	15±10.0	93±6.9	31±12.9
32	93±6.9	0+0.8	85±10.0	36±14.5**
38	93±6.9	0+0.8	85±10.0	9±8.7
44	71±12.0	0+0.8	85±10.0	9±8.7
50	7+6.9	0+0.8	36±12.8	9±8.7
54	7+6.9	0+0.8	36±12.8	0+0.9
60	7+6.9	0+0.8	29±12.1	0+0.9
66	7+6.9	0+0.8	29±12.1	0+0.9
69	7+6.9	0+0.8	21±10.9	0+0.9
73	7+6.9	0+0.8	7±6.9	0+0.9
76	0+0.8	0+0.8	7±6.9	0+0.9
84	0+0.8	0+0.8	7±6.9	0+0.9
87	0+0.8	0+0.8	7±6.9	0+0.9
100	0+0.8	0+0.8	0+0.8	0+0.9

* - P<0.06 in comparison with the control indices;

** - P<0.05 in comparison with the control indices.

Table 4

Coefficients of lineal regression for the dependence of fertile culture share on age (y=B+Ax)

Coefficients	Control	L-Ala-L-Glu-L-Asp-Gly tetrapeptide
B	133±4.2	115±11.4
A	-4.4±0.22	-2.5±0.37*

* - P<0.001 in comparison with the control indices.

Table 5

Coefficients of lineal regression for the dependence of live culture shares on age (y=B+Ax).

Coefficients	Control	L-Ala-L-Glu-L-Asp-Gly tetrapeptide
B	160±22.8	137±10.6
A	-2.4±0.43	-1.6±0.17*

* - P<0.001 in comparison with the control indices.

Table 6

Parameters of the life span in different experimental variants (in days).

Indices	Control	L-Ala-L-Glu-L-Asp-Gly tetrapeptide
Average life span	46	54
Median life span	75	100

Example 5. ***Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the ageing rate and free radical processes in Drosophila melanogaster of low activity (LA) strain selected for a low sexual activity of males.***

The impact produced by L-Ala-L-Glu-L-Asp-Gly tetrapeptide on catalase activity, the content of the products of lipid peroxide oxidation (LPO), the life span, and the ageing rate were studied on Drosophila melanogaster of LA strain.

One-day old flies were placed in test tubes, 10 individuals of the same sex in each. Every 2 days they were transferred to a fresh medium, taking into consideration the number of deceased individuals. The average and maximal life spans were defined. The ageing rate was calculated by

L-Ala-L-Glu-L-Asp-Gly tetrapeptide was applied in the larvae of the 3rd age in dose 0.00001% of the culture medium weight, which constituted approximately 0.015mg/100ml. Catalase activity was estimated by generally accepted methods in the homogenates of adult 14 days old flies. The experiment was carried out with a subsequent repetition.

Regression and dispersal analyses served as the basic technique of statistic processing. The authenticity of the differences was confirmed by the method of the minimal relevant diversity.

It was stated that L-Ala-L-Glu-L-Asp-Gly tetrapeptide in females did not induce any alterations in the average life span, but did increase the maximal life span (LS_{max}). At the same time, the substance significantly extended the ALS in males. The analysis of the survival rate curves and Gomperz charts showed that L-Ala-L-Glu-L-Asp-Gly tetrapeptide in females served as a geroprotector of the 1st type (ALS and LS_{max} were increased, though the ageing rate did not change in comparison with the control). In males, however, it acted as a geroprotector of the 3rd type (ALS was increased, LS_{max} did not change, in addition to which a tendency to the ageing rate increase was observed). At the same time, the analysis of survival rate in flies without taking into consideration their intersexual distinctions evidenced that LS_{max} notably extended under the influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide.

The application of L-Ala-L-Glu-L-Asp-Gly tetrapeptide in males significantly increased catalase activity, while in females the content of LPO products went down considerably. Suppression of intersexual distinctions confirmed a high anti-oxidation activity of L-Ala-L-Glu-L-Asp-Gly tetrapeptide.

Table 7

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the life span parameters in female Drosophila melanogaster of LA strain.

Variant of the experiment	ALS (days)	Me (days)	LS_{max}	LnR_0 ($days^{-1}$)	G 10^2 ($days^{-1}$)	MRDT
Control	22±1.0	28.5	36±1.9	-4.322±0.4334	6.7±1.78	3.4
L-Ala-L-Glu-L-Asp-Gly tetrapeptide	20±1.3	17	44±1.9*	-3.806±0.2580	4.3±0.90	3.8

Note: LS_{max} – maximal life span, LnR_0 and G 10^2 – the parameters of Gomperz equation.

* - $P<0.05$ in comparison with the control indices.

Table 8

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the life span parameters in male Drosophila melanogaster of LA strain.

Variant of the experiment	ALS (days)	Me (days)	LS_{max}	LnR_0 ($days^{-1}$)	G 10^2 ($days^{-1}$)	MRDT
Control	19±1.2	28.5	31±0.9	-4.579±0.840	7.5±4.62	3.3
L-Ala-L-Glu-L-Asp-Gly tetrapeptide	24±0.96*	28.5	33±0.9	-5.496±0.711	10.9±3.45	2.9

- - $P<0.01$ in comparison with the control indices.

Thus, the application of L-Ala-L-Glu-L-Asp-Gly tetrapeptide in flies displayed the effect of ageing inhibition.

Table 9

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the biochemical indices of free radical processes in Drosophila melangaster of LA strain.

Variant of the experiment	Specific activity of catalase (μmol H_2O_2/mg of protein per min.)	Content of conjugated hydro-peroxides (nmol/g of tissue)
Control (females)	43.31	1.469
Control (males)	*49.27*	*1.247*
L-Ala-L-Glu-L-Asp-Gly tetrapeptide (females)	42.57	1.290*
L-Ala-L-Glu-L-Asp-Gly tetrapeptide (males)	*57.44**	*1.202*

* - $P<0.05$ in comparison with the control indices.

Example 6. ***Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the ageing rate and free radical processes in Drosophila melanogaster of environmental adaptation (EA) strain selected for a low adaptability to the environment.***

Drosophila melanogaster of EA strain served as a model for studying the influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on catalase activity, tissue chemoluminescence, and life span.

One-day old flies were placed in test tubes, 10 individuals of the same sex in each. Every 5 to 7 days they were transferred to a fresh medium, taking into account the number of deceased individuals. The average and maximal life spans were defined. The ageing rate was calculated by the parameters of Gomperz equation.

L-Ala-L-Glu-L-Asp-Gly tetrapeptide was applied in the larvae of the 3rd age in dose 0.00001% of the culture medium weight, which constituted approximately 0.015mg/100ml. Catalase activity and the intensity of chemoluminescence were estimated in the homogenates of adult 7-day old flies by generally accepted methods. The experiment was conducted with a subsequent repetition.

Regression and dispersal analyses served as the basic technique of statistic processing. The authenticity of the differences was confirmed by the method of the minimal relevant diversity.

The results of the investigation are displayed in Tables 10-13.

Table 10

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the life span parameters in female Drosophila melanogaster of EA strain (Experiment 1).

Variant	n	ALS (days)	LS_{max} (days)	LnR_0 ($days^{-1}$)	G 10^2 ($days^{-1}$)
Control	84	10.5±0.76	26	-2.6±0.18	7.7±1.57
L-Ala-L-Glu-L-Asp-Gly tetrapeptide	72	16.4±1.68**	39	-3.1±0.21	3.6±0.61*

* - P<0.05 in comparison with the control indices;

** - P<0.01 in comparison with the control indices.

It is stated that L-Ala-L-Glu-L-Asp-Gly tetrapeptide produces an effect on luminol-dependent chemoluminescence (Table 14). The reduction of this index evidences the activation of the anti-oxidation defence system in flies. The raise of intensity in the tissue anti-oxidation defence in this case is presumably associated with the activation of low-molecular endogenous anti-oxidants, such as glutathione, tocopherol, and the like.

Extension in the average and maximal life spans was observed only in cases of diminished viability and accelerated ageing in the control. As the displayed data show, it is the very case when the investigated tetrapeptide exerts a precise geroprotective effect.

Table 11

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the life span parameters in male Drosophila melanogaster of EA strain (Experiment 1).

Variant	n	ALS (days)	LS_{max} (days)	R_0 ($days^{-1}$)	G 10^2 ($days^{-1}$)
Control	80	14,2±1,31	39	-4,9±0,44	9,2±1,97
L-Ala-L-Glu-L-Asp-Gly tetrapeptide	100	10,8±1,09	39	-2,3±0,26	3,3±2,25

Table 12

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the life span parameters in female Drosophila melanogaster of EA strain (Experiment 2).

Variant	n	ALS (days)	LS_{max} (days)	R_0 ($days^{-1}$)	G 10^2 ($days^{-1}$)
Control	132	18,4±0,62	41	-4,7±0,37	14,5±2,26
L-Ala-L-Glu-L-Asp-Gly tetrapeptide	115	16,7±0,70	41	-3,6±0,41	4,2±1,68

Table 13

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the life span parameters in male Drosophila melanogaster of EA strain (Experiment 2).

Variant	n	ALS (days)	LS_{max} (days)	R_0 ($days^{-1}$)	G 10^2 ($days^{-1}$)
Control	150	19,4±1,20	45	-4,2±0,15	7,6±0,58
L-Ala-L-Glu-L-Asp-Gly tetrapeptide	120	17,4±0,88	45	-3,0±0,28	4,7±0,94*

* - $P<0,01$ in comparison with the control indices.

Table 14

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the biochemical indices of free radical processes in Drosophila melanogaster of EA strain.

Variant of the experiment	Specific activity of catalase (μmol H_2O_2/mg of protein per min.)	Chemoluminescence intensity (conv. unit/mg of protein per min.)
Control (females)	83,65±1,355	4,04±0,135
Control (males)	*102,10±2,095*	*8,16±0,090*
L-Ala-L-Glu-L-Asp-Gly tetrapeptide (females)	82,78±2,957	4,61±0,395
L-Ala-L-Glu-L-Asp-Gly tetrapeptide (males)	*100,39±3,807*	*4,93±0,109**

* - P<0,05 in comparison with the control index.

Example 7. ***Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the life span of Drosophila melanogaster of "wild " Canton-S strain.***

In the research Drosophila melanogaster of "wild" Canton-S strain were employed.

L-Ala-L-Glu-L-Asp-Gly tetrapeptide in the solution form was introduced into the culture medium provided for the growth of insects, which was cooled down to 50-60°C, with the subsequent careful stirring and depositing it into test tubes.

To make the conditions of both the control and experimental population development identical, an amount of sodium chloride equal to that used for L-Ala-L-Glu-L-Asp-Gly tetrapeptide solution was introduced into the culture medium for the control. The concentration of all the substances employed was calculated by the ratio to the mass of the culture medium provided for reproduction.

4-day old female flies underwent a randomly selected mating with males for 72 hours, a couple per each test tube, after which parental couples were removed from the test tubes. To form every new generation the posterity of 50 to 80 couples was used.

Life span (LS) was investigated on laboratory populations, each of them consisting of 90 individuals of each sex. Immediately after hatching the flies were randomly placed in test tubes,

Average values (ALS) and standard errors calculated by the traditional method were applied as life span distribution parameters. Average values were compared to each other with the application of Student t-criterion.

Table 15 displays the aftermath of the life span distribution characteristics (average values and the standard errors of these average values) for all the experimental and control groups.

For experimental study 6 concentrations of L-Ala-L-Glu-L-Asp-Gly tetrapeptide were selected: 0.01x, 0.1x, 1x, 5x, 7.5x, and $12.5x10^{-6}$% of the culture medium weight.

L-Ala-L-Glu-L-Asp-Gly tetrapeptide administered to males in the 4 lowest concentrations (0.01x to $5x10^{-6}$%) exerted a significant geroprotective effect: ALS in the experimental groups showed a statistically relevant rise. Relative extension in the ALS of males made 3.3 to 10.8%.

In experiments conducted on females L-Ala-L-Glu-L-Asp-Gly tetrapeptide exerted a geroprotective effect only in concentrations 0.01x and $0.1x10^{-6}$% with a significant increase in ALS correspondingly by 13.4% ($P<0.004$) and 11.8% ($P<0.03$).

The efficacy of L-Ala-L-Glu-L-Asp-Gly tetrapeptide applied in super-low concentrations, as shown by the obtained data, is unprecedented. It should be admitted that the application of L-Ala-L-Glu-L-Asp-Gly tetrapeptide in Drosophila melanogaster did not alter the duration of their stage development, which, in general, reflected the lack of genotoxic effect produced by the studied substance.

Table 15

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the life span parameters in Drosophila melanogaster of "wild" Canton-S strain.

1. Males

№	Administered substance	Concentration $x10^{-6}$%	ALS±statistic errors	Alterations in ALS %	t-Stud. P<
1. 2.	Control L-Ala-L-Glu-L-Asp-Gly tetrapeptide	 0.01	32.55±1.14 36.47±1.09	 +12.0	 0.02
3. 4.	Control L-Ala-L-Glu-L-Asp-Gly tetrapeptide	 0.1	31.44±1.35 34.85±1.18	 +10.8	 0.05
5. 6.	Control L-Ala-L-Glu-L-Asp-Gly tetrapeptide	 1.0	32.45±1.41 36.77±1.44	 +13.3	 0.04
7. 8.	Control L-Ala-L-Glu-L-Asp-Gly tetrapeptide	 5.0	33.90±1.14 37.74±1.16	 +11.3	 0.02
9. 10.	Control L-Ala-L-Glu-L-Asp-Gly tetrapeptide	 7.5	39.99±0.97 39.98±0.99	 -0.03	 Statisticall irrelevant
11. 12.	Control L-Ala-L-Glu-L-Asp-Gly tetrapeptide	 12.5	37.32±0.83 33.96±1.01	 -9.0	 0.02

2. Females

№	Administered substance	Concentration $x10^{-6}$%	ALS±statistic errors	Alterations in ALS %	t-Stud. P<
13. 14.	Control L-Ala-L-Glu-L-Asp-Gly tetrapeptide	0.01	31.22±0.91 35.41±1.07	+13.4	0.004
15. 16.	Control L-Ala-L-Glu-L-Asp-Gly tetrapeptide	0.1	31.22±0.91 35.41±1.07	+11.8	0.03
17. 18.	Control L-Ala-L-Glu-L-Asp-Gly tetrapeptide	1.0	36.17±1.23 35.76±1.35	-1.1	Statisticall irrelevant
19. 20.	Control L-Ala-L-Glu-L-Asp-Gly tetrapeptide	5.0	36.87±1.40 38.04±1.56	+3.2	Statisticall Irrelevant
21. 22.	Control L-Ala-L-Glu-L-Asp-Gly tetrapeptide	7.5	33.74±1.23 31.16±1.21	-7.6	Statisticall irrelevant
23. 24.	Control L-Ala-L-Glu-L-Asp-Gly tetrapeptide	12.5	35.09±1.36 37.06±1.12	+5.6	Statisticall irrelevant

Example 8. Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the extra-pineal synthesis of melatonin in rats after epiphysectomy.

The research was carried out on male Wistar rats weighing 130-140g. The total number of the animals (23) was divided into 5 groups. The 1st group (control) consisted of intact animals. The animals of groups 2-5 underwent epiphysectomy (EE). In 3 weeks after the surgery (on the 21st day) the animals of the 2nd and 3rd groups were subcutaneously injected with sodium chloride in dose 0.5ml for the next 10 days. The animals of groups 4 and 5 were administered to L-Ala-L-Glu-L-Asp-Gly tetrapeptide in dose 0.5μg according to the same scheme.

Slaughtering of the animals and isolation of their organs were conducted from 10 a.m. to 12 noon by daylight with the use of Nembutal anaesthesia (50mg/kg). The animals of groups 1, 2 and 4 were slaughtered in 3 days upon the final drug administration (on the 33rd day after the operation and the onset of the experiment), while the animals of groups 3 and 5 – in 12 days (on the 42nd day after epiphysectomy). Examined were the main organs of the diffusive neuroendocrine system (DNES): stomach, thyroid gland, and pancreas.

The surgery – epiphysectomy – was conducted under the ether anaesthesia according to the devised technique. A 1-1.5cm long incision was made along the central line of the head. Upon

the uncovering of the scull cap an aperture was drilled over the sinus confluence point by means of a hollow 0.5cm-diameter auger, which was manufactured of a metal tube especially for the experiment. The epiphysis was extracted by means of ophthalmic forceps through the opening in the cranium. The trepanation aperture was covered with an osteal fragment. The skin incision was sewn with silk threads.

Fragments of the extracted organs were fixed for 24 hours with Buen sour liquid for optic microscopy examination and according to Karnovsky method - for electron microscopy. Material dehydration and filling it in with paraffin for optic microscopy, as well as epone mixture for ultra-structural examination were prepared according to generally accepted techniques. Paraffin sections (7μm) were placed on a microscope slide covered with a poly-L-lysine film (Sigma). Ultra-thin sections (100nm) prepared on LKB-7A microtome (LKB) were contrasted with uranyl acetate and plumbum citrate.

Histological and immunohistochemical examinations were carried out in Jenamed-2 microscope (Zeizz). Electron-microscopic examination was conducted in electronic JEM-100S microscope (JEOL).

For staining hematoxyline-eosin was applied. The total population of APUD-cytes in the pyloric and fundal stomach sections was revealed according to Grimelius' histochemical silvering method (25).

Immunohistochemical exposure of enterochromophine–cells (EC-cells) was conducted by applying uniclonic antibodies of mice to serotonin (Dako, 1:15 titre). The uniclonic antibodies were identified according to avidin-biotin-peroxidase (ABP) method (Vectastain kit) in order to reveal immune serum globulins of mice.

The quantitative studies were carried out by means of computer analysis system of microscopic imprints (Imstar) with the use of Morphostar and Colquant (Imstar) applied licensed computer programmes, in accordance with the basic principals of stereology and morphometry (26).

The quantitative density of enteroendocrine (END) ($N_{END}/1mm^2$) and serotonin-positive cells ($N_{ser}/1mm^2$) was estimated in 10 visual fields. The test area (S) constituted $5mm^2$.

For the statistic processing of the received data the non-parametric U-criterion by Mann/Whitney was applied.

Results of the DNES functional morphology studies conducted in epiphysectomised rats with L-Ala-L-Glu-L-Asp-Gly tetrapeptide application showed that the preparation stimulated tissue and cellular metabolism.

It was stated that L-Ala-L-Glu-L-Asp-Gly tetrapeptide administration exerted a compensatory effect upon the structural and functional organisation of DNES cells in animals exposed to epiphysectomy. Its effect was manifested within 3 days upon the end of the drug

administration through complete suppression of the epiphysectomy impact and maintained for 12 days, i.e. till the experiment termination with respect to all the examined organs.

The received data confirm that the main point of L-Ala-L-Glu-L-Asp-Gly tetrapeptide application is the gastric EC-cells, in which L-Ala-L-Glu-L-Asp-Gly tetrapeptide promotes the extra-pineal synthesis of serotonin and melatonin (Table 16), thus, in essence, providing a complete compensation for the extracted epiphysis.

So, results of the experimental investigation proved that L-Ala-L-Glu-L-Asp-Gly tetrapeptide revealed no toxicity, normalised anti-oxidation defence indices, and regulated metabolic processes in the melatonin-producing structures of various tissues.

Table 16

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the quantitative characteristics of the examined parameters in various stomach sections of epiphysectomised rats

Group of animals	$N_{END}/1mm^2$		$N_{ser}/1mm^2$
	Pyloric	Fundal	Pyloric
Intact animals	20±1	48±5	26±2
Control (epiphysectomised animals+ sodium chloride solution) The 3rd day upon the end of drug administration	30±1*	76±1*	32±3*
Control (epiphysectomised animals+ sodium chloride solution) The 12th day upon the end of drug administration	15±1*	41±4	19±1*
Control (epiphysectomised animals+ L-Ala-L-Glu-L-Asp-Gly tetrapeptide) The 3rd day upon the end of drug administration	21±1**	64±5**	26±2**
Control (epiphysectomised animals+ L-Ala-L-Glu-L-Asp-Gly tetrapeptide) The 12th day upon the end of drug administration	19±1**	47±9	26±3**

* - P < 0,05 in comparison with the control indices;

** - P < 0,05 in comparison with the control indices.

Note: $N_{END}/1mm^2$ – the density of entero-endocrine cells; $N_{ser}/1mm^2$ – the density of serotonin-positive cells.

L-Ala-L-Glu-L-Asp-Gly tetrapeptide properties revealed in the pre-clinical experiment provide its manifested prophylactic and/or therapeutic application as a geroprotective substance.

Examples of the results received in the clinical trials of the proposed tetrapeptide demonstrate its pharmacological properties and confirm that the invention can be integrated with medical practice.

Example 9. ***Efficacy of the application of the pharmacological substance containing L-Ala-L-Glu-L-Asp-Gly tetrapeptide in patients with disturbances of the anti-oxidation defence system for premature ageing prevention.***

The studied pharmacological substance was applied in 14 patients (34 to 69 years old) with age-related pathology (hypertension disease, ischemic heart disease, chronic gastritis, non-insulin-dependent diabetes mellitus) and reduction of the anti-oxidation defence indices in blood.

The substance containing L-Ala-L-Glu-L-Asp-Gly tetrapeptide was administered intramuscularly in the form of an injection solution in a single daily dose 10μg per an injection for 10 days.

In cases of the drug application a significant rise in superoxide dismutase activity and a decrease in the content of lipid peroxide oxidation products were observed (Table 17).

Table 17

Influence of L-Ala-L-Glu-L-Asp-Gly tetrapeptide on blood anti-oxidation defence indices in age-related pathology

Index	Before treatment	After treatment
Activity of superoxide dismutase, conv. unit/mg of protein	0,26	0,61*
Products of spontaneous lipid peroxide oxidation, μM/l:		
- conjugated hydroperoxides;	7,3±0,6	2,5±0,2*
- Schiff bases.	4,6±0,3	2,9±0,2*

* - P<0,05 in comparison with the indices before treatment.

Results of the therapy prove the restorative effect of the pharmacological substance containing L-Ala-L-Glu-L-Asp-Gly tetrapeptide on the anti-oxidation defence system of the organism.

Example 10. ***Efficacy of the pharmacological substance containing L-Ala-L-Glu-L-Asp-Gly tetrapeptide applied for premature ageing prevention in patients with disturbance of melatonin synthesis.***

The pharmacological substance was applied in 11 patients in the age of 39 to 63 years old suffering aspirin bronchial asthma. The main characteristic of this disease consists in association of asphyxia fits with intolerance of acetylsalicylic acid and other non-steroid anti-0inflammatory substances. N-acetyl-5-methoxykynurenamine (N-AMK), an analogue of acetylsalicylic acid in its chemical structure, is known to be formed in the organism in the process of metabolism of melatonin, an epiphyseal hormone. Melatonin synthesis and, consequently, the level of endogenous N-AMK are significantly decreased in patients with aspirin bronchial asthma, which is confirmed by the low excretion with urine of 6-sulfatoxymelatonin, the basic melatonin metabolite.

This substantiated a new pathogenic approach to the treatment of aspirin bronchial asthma by means of correcting melatonin content in the organism with the pharmacological substance containing L-Ala-L-Glu-L-Asp-Gly tetrapeptide.

The substance was administered intramuscularly in the form of an injection solution daily in the morning in dose 10μg for 10 days.

The patients were treated in the phase of fading exacerbation or disease remission accompanied by permanent anti-asthma therapy, which included inhalation and oral glucocorticoids.

Prior to, during the treatment, and later on monthly the patients subjectively estimated their health state by such symptoms as coughing, sputum discharge, hard breathing, breast stuffing, whistling rale, fits of asphyxia, intolerance of physical activity, cold, and smells. Clinical effect produced by the preparation was estimated immediately after the treatment and within the next 7 months on the basis of analyses reported by the patients, which included the data on their health state dynamics, capacity for work, psychological condition, sleep impairments, and daily doses of the anti-asthmatic drugs taken.

Before the onset of the treatment and right after the administration of the final dose of the drug external respiration function was estimated in all the patients with the reference to the following parameters: vital breathing capacity (VBC), tidal volume (TV), total lung capacity (TLC), forced vital breathing capacity (FVBC), maximum volumetric exhalation rate (MVexhR), maximum volumetric exhalation rate by 50% and 75% forced VBC (VBC_{50} and VBC_{75}), forced exhalation volume per second (FEV_1). These parameters were measured per cent of the initial values. Additionally, the specific conduction of the bronchial tree in cm of aquatic column was measured. The whole complex of tests was repeated within 15 min. after an inhalation with Berotek. Dynamics of MVR, VBC_{50}, and VBC_{75} after a Berotek inhalation was estimated per cent

of the initial values. The FEV_1 index in all the patients before treatment made less than 80% of the due value.

Content of 6-sulfatoxymelatonin, the basic melatonin metabolite, was measured before, immediately after, and within 10 days after the treatment in the urine taken in the daytime (from 9a.m. till 21p.m.) and at night (from 21p.m. till 9a.m.), according to the immunofermental method by means of "DRG Instrument GmbH" kits (Marburg, Germany).

Results of the investigations confirmed that improvement in clinical state by the end of the treatment was observed in the overwhelming majority of patients treated with the studied pharmacological substance. A reduced frequency of day asthma symptoms, the intolerance of physical work, strong smells, and cold air, as well as sleep recovery was registered as well. Objective examination of the patients revealed a decrease in or an absolute disappearance of whistling rale in the lungs - the symptoms of bronchial obstruction. Examination of the external respiration function immediately after the treatment termination did not reveal any essential alterations in VBC, MVexhR, FEV_1, TV/TLC. However, the patients, exposed to the tetrapeptide-containing pharmacological substance, revealed an improvement in the reaction to Berotek on the level of distal bronchi. Excretion of 6-sulfatoxymelatonin with urine increased, which evidenced of an increase in melatonin production not only at night, but also in the daytime.

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CLAIMS

1. Tetrapeptide L-alanyl-L-gluthamyl-L-asparagyl-glycine of the general formula L-Ala-L-Glu-L-Asp-Gly.

2. Tetrapeptide L-alanyl-L-gluthamyl-L-asparagyl-glycine of the general formula L-Ala-L-Glu-L-Asp-Gly, as a substance producing geroprotective effect.

3. Pharmacological substance producing geroprotective effect, containing an active base and a pharmacologically admissible carrier, which is distinguished by the fact that it contains as its active base an effective amount of L-Ala-L-Glu-L-Asp-Gly tetrapeptide or its salts.

4. The substance described in Item 3, distinguished by the fact that it contains salts of the amino group, such as acetate, hydrochloride, and oxalate.

5. The substance described in Item 3, distinguished by the fact that it contains salts of carboxyl groups, for example, the salts of Sodium, Potassium, Calcium, Lithium, Zinc, Magnesium, or the salts of organic and inorganic cations, for example, ammonium, triethylammonium.

6. The substance described in Item 3, distinguished by the fact that it is meant for parenteral administration.

7. The substance described in Item 3, distinguished by the fact that it is meant for intranasal administration.

8. The substance described in Item 3, distinguished by the fact that it is meant for oral administration.

9. The substance described in Item 3, distinguished by the fact that it is meant for local application.

10. The method of premature ageing prevention, which consists in administering to a patient of the pharmacological substance containing an effective amount of L-Ala-L-Glu-L-Asp-Gly tetrapeptide or its salts of the amino group (acetate, hydrochloride, oxalate), or its salts of carboxyl groups (the salts of Natrium,Potassium, Calcium, Lithium, Zinc, Magnesium, and also the salts of organic and inorganic cations—ammonium and triethylammonium) in doses 0.01 to 100 mg/kg of the body weight at least once a day for a period necessary for the achievement of a therapeutic effect.