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(54) **Title:** NEW PEPTIDES AND USE THEREOF

(57) **Abstract:** A subject matter of the invention are new peptides and their use as dietary supplement. Compound selected from the group consisting of Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala- Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1); Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2); Ac-Gly-His-Lys-Arg-NH_2 (SEQ. ID 3); Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 4), Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5), and Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6), or its pharmaceutically acceptable salt or complex. A subject matter of the invention is also a dietary supplement composition comprising compound mentioned above and its use as a dietary supplement.

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NEW PEPTIDES AND USE THEREOF

FIELD OF THE INVENTION

The present invention belongs to the field of pharmaceutical chemistry and relates to new stable peptide derivatives.

DESCRIPTION OF THE RELATED ART

Numerous peptides are known from their therapeutic use. Peptide-based drugs are highly effective medicines with relatively short duration of action and of variable therapeutic index. However, the physicochemical stability of peptides, for instance in normal saline, is often not satisfactory. Many strategies are involved to achieve better stability and bioavailability of peptides [Céline Adessi and Claudio Soto; Current Medicinal Chemistry, 2002, 9, 963-978]. Both N-terminal acylation and C-terminal addition of another amino acids can improve biological stability and activity of many peptides.

It is also well known in the art that conversion into salts often increases the stability of a peptide. Salts of proteins and peptides are well known in the art. For instance, it is known from Bertrand, M. et al. in: Journal of Peptide Research, 49 (1997)3, 269-272, that the effects of certain salts are very selective in respect to the stability and structure of peptides. For instance, the addition of monovalent cations, such as $NH4^+$ to a final 0.1 M peptide (poly (Glu-Leu)) solution, induces a transition to a water-soluble beta-structure. In contrast, no transition was observed using Li^+, Na^+, or Cs^+ ions.

EP 2968442B1 for example discloses salts of pentadecapeptide with basic amino acids having improved thermal stability and stability in gastric juice. Specifically, the patent discloses new arginine salt of BPC-157 of improved stability. It seems however, that the stability is still insufficient for all applications.

Therefore, there is still a need in the art to provide new peptide providing better stability and maintaining good dissolution profile of the peptide agents. The present invention solves these problems. Surprisingly, inventors found that simultaneous addition of arginine amide at the C-terminus and acetylation of N-terminus increase the stability of the peptide agents and improves of their biological activity.

SUMMARY OF THE INVENTION

The object of the invention is to provide new biologically active peptides of improved stability.

Therefore, the present invention provides a compound selected from the group consisting of

Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2; (SEQ. ID 1)

Ac-Ala-Glu-Asp-Gly-Arg-NH_2; (SEQ. ID 2)

Ac-Gly-His-Lys-Arg-NH_2; (SEQ. ID 3)

Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2, (SEQ. ID 4)

Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2, (SEQ. ID 5) and

Ac-Ala-Glu-Asp-Pro-Arg-NH_2, (SEQ. ID 6)

or its pharmaceutically acceptable salt or complex.

Preferably, the compound of is selected from Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2), Ac-Gly-His-Lys-Arg-NH_2 (SEQ. ID 3), or Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1), or its pharmaceutically acceptable salt or complex.

More preferably, the compound is Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2), or its pharmaceutically acceptable salt or complex.

More preferably, the compound is Ac-Gly-His-Lys-Arg-NH_2 (SEQ. ID 3) or its pharmaceutically acceptable salt or complex.

More preferably, the compound is Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1), or its pharmaceutically acceptable salt or complex.

Alternatively, preferably, the compound is selected from Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 4), Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5), and Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6), or its pharmaceutically acceptable salt or complex.

More preferably, the compound is Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 4), or its pharmaceutically acceptable salt or complex.

More preferably, the compound is Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5), or its pharmaceutically acceptable salt or complex.

More preferably, the compound is Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6), or its pharmaceutically acceptable salt or complex.

The present invention relates to a dietary supplement composition comprising a compound as defined above and pharmaceutically acceptable excipient.

Moreover, the present invention relates to use of a compound or the dietary supplement composition as a dietary supplement.

DETAILED DESCRIPTION OF THE INVENTION

Peptide agents

It was surprisingly found that peptides can have improved stability during storage by simple N-acetylation and C-Arg-amide-ation of parent peptides.

According to the present invention, provided is a new peptide selected from a group consisting of:

Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1);

Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2;

Ac-Gly-His-Lys-Arg-NH_2 (SEQ. ID 3);

Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 4),

Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5), and

Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6),

or its pharmaceutically acceptable salt or complex.

The peptides are in fact N-acetylated and C-Arg-NH_2 (C-arginine-amide) version of peptides known in the art: BPC-157, Epithalon, GHK, Semax, Selank, Cortagen, respectively.

GHK having a sequence of Gly-His-Lys

Epithalon having a sequence of Ala-Glu-Asp-Gly

BPC-157 peptide having a sequence of Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val

Selank having a sequence of Thr-Lys-Pro-Arg-Pro-Gly-Pro

Semax having a sequence of Met-Glu-His-Phe-Pro-Gly-Pro and

Cortagen having a sequence of Ala-Glu-Asp-Pro

All amino acids indicated in the sequences above are L-amino acids. Three letter codes for amino acids are in line with IUPAC nomenclature and are as follows:

Three letter code	Amino acid
Ala	Alanine
Cys	Cysteine
Asp	Aspartic Acid
Glu	Glutamic Acid
Phe	Phenylalanine
Gly	Glycine
His	Histidine
Ile	Isoleucine
Lys	Lysine
Leu	Leucine
Met	Methionine
Asn	Asparagine

Pro	Proline
Gln	Glutamine
Arg	Arginine
Ser	Serine
Thr	Threonine
Val	Valine
Trp	Tryptophan
Tyr	Tyrosine

The compounds of the invention exhibit better stability and at the same time they have improved biological activities in comparison to their parent compounds as presented in detail in the Examples below.

Preferably, the new peptide is selected from:

Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1);

Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2);

Ac-Gly-His-Lys-Arg-NH_2 (SEQ. ID 3);

Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 4),

Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5), and

Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6),

or its pharmaceutically acceptable salt or complex.

Preferably, the new peptide is selected from:

Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2);

Ac-Gly-His-Lys-Arg-NH_2 (SEQ. ID 3);

Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 4),

Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5), and

Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6).

or its pharmaceutically acceptable salt or complex.

Preferably, the new peptide is selected from:

Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1);

Ac-Gly-His-Lys-Arg-NH_2 (SEQ. ID 3);

Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 4),

Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5), and

Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6).

or its pharmaceutically acceptable salt or complex.

Preferably, the new peptide is selected from:

Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1);

Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2);

Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 4),

Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5), and

Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6).

or its pharmaceutically acceptable salt or complex.

Preferably, the new peptide is selected from:

Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1);

Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2);

Ac-Gly-His-Lys-Arg-NH_2 (SEQ. ID 3);

Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5), and

Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6).

or its pharmaceutically acceptable salt or complex.

Preferably, the new peptide is selected from:

Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1);

Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2);

Ac-Gly-His-Lys-Arg-NH_2 (SEQ. ID 3);

Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 4), and

Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6).

or its pharmaceutically acceptable salt or complex.

Preferably, the new peptide is selected from:

Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1);

Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2);

Ac-Gly-His-Lys-Arg-NH_2 (SEQ. ID 3);

Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 4), and

Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5).

or its pharmaceutically acceptable salt or complex.

Preferably, the compound is Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1), or its pharmaceutically acceptable salt or complex.

Preferably, the compound is Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2), or its pharmaceutically acceptable salt or complex.

Preferably, the compound is Ac-Gly-His-Lys-Arg-NH_2 (SEQ. ID 3), or its pharmaceutically acceptable salt or complex.

Preferably, the compound is Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH (SEQ. ID 4) $_2$, or its pharmaceutically acceptable salt or complex.

Preferably, the compound is Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5), or its pharmaceutically acceptable salt or complex.

Preferably, the compound is Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6), or its pharmaceutically acceptable salt or complex.

The peptide agents of the invention can be obtained by a simple synthesis known in the art.

For example, reference is made to: Fields, Gregg B, and Janelle L Lauer-Fields, 'Principles and Practice of Solid-Phase Peptide Synthesis', Synthetic Peptides: A User's Guide (New York, 2002; online edn, Oxford Academic, 12 Nov. 2020), Handbook of Reagents for Organic Synthesis. Reagents for Glycoside, Nucleotide and Peptide Synthesis Edited by David Crich. John Wiley & Sons Ltd., Chichester, West Sussex, England. 2005, Principles of Peptide Synthesis, Miklos Bodanszky, Springer, September 1993, Methods of Enzymology, 289, Solid Phase peptide Synthesis, (G. B. Fields Ed.) Academic Press, 1997. Chemical Approaches to the Synthesis of Peptides and Proteins, P. Lloyd-Williams, F. Albericio, and E. Giralt Eds), CRC Press, 1997. Fmoc Solid Phase Peptide Synthesis, A Practical Approach, (W. C. Chan, P. D. White Eds), Oxford University Press, 2000. Solid Phase Synthesis, A Practical Guide, (S. F. Kates, F Albericio Eds), Marcel Dekker, 2000. P. Seneci, Solid-Phase Synthesis and Combinatorial Technologies, John Wiley & Sons, 2000. Houben-Weyl E22a, Synthesis of Peptides and Peptidomimetics (M. Goodman, Editor-in-chief, A. Felix, L. Moroder, C. Tmiolo Eds), Thieme, 2002, p. 665ff. N. L. Benoiton, Chemistry of Peptide Synthesis, CRC Press, 2005. J. Howl, Methods in Molecular Biology, 298, Peptide Synthesis and Applications, (J. Howl Ed) Humana Press, 2005.

Here, we use solid-phase synthesis on chlorotrityl resin using Fmoc protected amino acids. Outline of syntheses is presented in the Examples below.

Use

In accordance with the invention, the peptide agents are to be used as a dietary supplement. The aim of the dietary supplement is to improve the condition of the body.

For example, derivative prepared based on Epithalon sequence (SEQ. ID 2) will act as the Epithalon. It may be used for slowing the aging of the cell population by stimulating the renewal and elongation of telomeres, slowing down aging/exhibiting anti-aging effect, prevention of cancer and age-related diseases, restoration, and normalization of the production of melatonin, strong acting as an antioxidant that protects against oxidative stress, or improvement of sleep, and its quality (for details, see: Khavinson VKh, Bondarev IE, Butyugov AA. Epithalon peptide induces telomerase activity and telomere elongation in human somatic cells. Bull Exp Biol Med. 2003 Jun;135(6):590-2. doi: 10.1023/a:1025493705728. PMID: 12937682; Goncharova ND, Khavinson BK, Lapin BA. Regulatory effect of Epithalon on production of melatonin and cortisol in old monkeys. Bull Exp Biol Med. 2001 Apr;131(4):394-6. doi: 10.1023/a:1017928925177. PMID: 11550036; Vanhee C, Moens G, Van Hoeck E, Deconinck E, De Beer JO. Identification of the small research tetra peptide Epithalon, assumed to be a potential treatment for cancer, old age and Retinitis Pigmentosa in two illegal pharmaceutical preparations. Drug Test Anal. 2015 Mar;7(3):259-64. doi: 10.1002/dta.1771. Epub 2014 Dec 22. PMID: 25535022.)

For example, the derivative prepared based on GHK sequence (SEQ. ID 3) will act as a GHK or its Cu salt (GHK-Cu). It may be used for skin treatment/skin condition improvement, influencing lung function, Alzheimer's disease, and for gene regulation (for details, see Pickart, L., Vasquez-Soltero, J. M., & Margolina, A. (2015). GHK Peptide as a Natural Modulator of Multiple Cellular Pathways in Skin Regeneration. BioMed research international, 2015, 648108. https://doi.org/10.1155/2015/648108; Pickart L, Vasquez-Soltero JM, Margolina A. The human tripeptide GHK-Cu in prevention of oxidative stress and degenerative conditions of aging: implications for cognitive health. Oxid Med Cell Longev. 2012;2012:324832. doi:10.1155/2012/324832).

For example, the derivative prepared based on BPC (SEQ. ID 1) will act as a BPC. It may be used for diseases, disorders or conditions for which neuroprotective effect, antidepressant effect, effect of alleviating diseases of the digestive system, help in the regeneration of the bones and joints, help with thrombosis, arterial hypertension, or help with skin diseases is necessary (for details, see: Gjurasin M, Miklic P, Zupancic B, Perovic D, Zarkovic K, Brcic L, Kolenc D, Radic B, Seiwerth S, Sikiric P. Peptide therapy with pentadecapeptide BPC 157 in traumatic nerve injury. Regul Pept. 2010 Feb 25;160(1-3):33-41. doi: 10.1016/j.regpep.2009.11.005.

Epub 2009 Nov 10. PMID: 19903499; Knezevic, Mario et al. "Occluded Superior Mesenteric Artery and Vein. Therapy with the Stable Gastric Pentadecapeptide BPC 157." Biomedicines vol. 9,7 792. 8 Jul. 2021, doi:10.3390/biomedicines9070792; Hsieh, Ming-Jer et al. "Modulatory effects of BPC 157 on vasomotor tone and the activation of Src-Caveolin-1-endothelial nitric oxide synthase pathway." Scientific reports vol. 10,1 17078. 13 Oct. 2020, doi:10.1038/s41598-020-74022-y; Perovic, Darko et al. "Stable gastric pentadecapeptide BPC 157 can improve the healing course of spinal cord injury and lead to functional recovery in rats." Journal of orthopaedic surgery and research vol. 14,1 199. 2 Jul. 2019, doi:10.1186/s13018-019-1242-6; Takeo M, Lee W, Ito M. Wound healing and skin regeneration. Cold Spring Harb Perspect Med. 2015 Jan 5;5(1):a023267. doi: 10.1101/cshperspect.a023267. PMID: 25561722; PMCID: PMC4292081).

For example, the derivative prepared based on Semax (SEQ. ID 4) will act as a Semax. It may be used for improvement of memory and concentration [Shadrina M, Kolomin T, Agapova T, Agniullin Y, Shram S, Slominsky P, Lymborska S, Myasoedov N, Comparison of the temporary dynamics of NGF and BDNF gene expression in rat hippocampus, frontal cortex, and retina under Semax action. 2010 DOI:10.1007/s12031-009-9270-z.]. It may be used also to support the therapy of brain stroke [Ekaterina V Medvedeva, BMC Genomics, 15, Article number: 228 (2014); doi: 10.1186/1471-2164-15-228]. Semax is known from prevention the death of tyrosine hydroxylase-positive neurons in a mixed neuroglial cell culture derived from the embryonic rat mesencephalon in a model of 6-hydroxydopamine-induced neurotoxicity [O.Dolotov, K.O.Eremin, Semax prevents the death of tyrosine hydroxylase-positive neurons in a mixed neuroglial cell culture derived from the embryonic rat mesencephalon in a model of 6-hydroxydopamine-induced neurotoxicity. 2015, DOI:10.1134/S1819712415040066].

For example, the derivative prepared based on Selank (SEQ. ID 5) will act as a Selank. Selank is known to act on relaxation, calming down and better mood [A Volkova, M Shadrina, TKolomin, L Andreeva, S Limborska, N Myasoedov, P Slominsky, Selank Administration Affects the Expression of Some Genes Involved in GABAergic Neurotransmission. 2016. DOI: 10.3389/fphar.2016.00031]. Selank administration affects the expression of some genes involved in GABAergic neurotransmission [VE Miedwiediew, ON Tereshchenko, NV Kost, Yu Ter-Izraelczyk, EV Gushanskaya, IK Chobanu, O Yu Sokołow, NF Miasojedow,

Optimization of the treatment of anxiety disorders with selank. 2015. DOI: 10.17116/jnevro20151156133-40].

For example, the derivative prepared based on Cortagen (SEQ. ID 6) will act as a Cortagen. Cortagen is known from regenerative function for the brain [Kolosova LI, Moiseeva AB, Turchaninova LN, Malinin VV, Polyakov EL, Nozdrachev AD, Khavinson VKh, The delayed effect of cortagen on the restoration of injured nerve function. 2002. DOI: 10.1023/a:1016098302564.]. Cortagen is also used as correcting agents in functional and metabolic disorders in the brain in chronic ischemia [V Zarubina, P D Shabanov, Eksp Klin Farmakol., 2011;74(2):8-15. PMID: 21476278]

The amount of the derivative contained in the single dosage corresponds to the amount of the peptide agent. Therapeutic amount will be known by the person skilled in the art as a general knowledge or state of the art. The amount of the peptide agent should provide a therapeutic effect when delivered in the regime known in the art and specific for a kind of peptide agent, and therefore should be in a range known in the art.

Another aspect of the invention is a dietary supplement composition of the invention that contains a compound of the invention, that is, a peptide agent of the invention and pharmaceutically acceptable excipient.

The dietary supplement composition of the invention they can be administered by any route, preferably orally, and will have the form of a preparation intended for use in the field of dietary supplements, depending upon the intended route of administration.

Solid preparations can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable inactive ingredients such as binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, sucrose, carboxymethylcellulose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., crospovidone, potato starch or sodium starch glycolate); wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated according to methods well known in the art with conventional coatings, coatings for delaying/controlling release or enteric coatings. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or may be presented as a dry product for reconstitution with water or other suitable

vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable inactive ingredients such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl p- or propyl hydroxybenzoate or sorbic acid). Preparations may also comprise suitable buffers, flavoring agents, coloring agents, and sweeteners.

Preparations for oral administration may be suitably formulated by methods known to those skilled in the art to obtain a controlled release of the active compound.

Use of the compounds of the invention will involve administration of an amount of the compound of the invention, preferably in the form of a dietary supplement composition to a subject in need thereof.

A proposed dose of the compounds of the present invention is from about 0.1 to about 1000 mg per day, in single or divided doses. The skilled person will appreciate that the selection of the dose required to achieve the desired effect will depend on a number of factors, for example the specific compound, the use, the mode of administration, and the precise dosage will be ultimately determined at the discretion of the attendant physician.

EXAMPLES

EXAMPLE 1 - Synthesis of the peptide agents of the invention

Equipment and reagents:

Equipment used in the synthesis and analysis of the final peptides was as follows: Reactor for industrial peptide synthesis: model PSR 1000C (NOV Biotech Ltd.), High Performance Liquid Chromatographs: 1. Preparative HPLC: CXTH P1001L i. 70 x 150 mm Vydac® column, 10 mm filter; 2. Preparative HPLC: ÄKTATM Pure 25 L/M System (GE Healthcare), fraction collector: Frac F9-R (Cat. No. 29011362) i. Kromasil® 100-13-C18 column 50 x 250mm (pore size: 100 Å, bed grains: 13 um, phase: C18, diam. internal: 50mm); 3. Analytical HPLC: UHPLC (UltiMate 3000 Thermo Scientific/Dionex) with diode detector coupled to QTOF-MS i. Kromasil® 100-4-C18 column 4.6 x 250mm (pore size: 100 Å, bed grain size: 4 µm, chamfer: C18, ID: 4.6 mm); QTOF-MS mass spectrometer: Impact HD, Bruker; Lyophilizer: Christ Beta

2-8 LSCplus Lyophilizer 102125; Vacuum pump Pfeiffer Vacuum D-35614 Asslar,: Mod. DUO 10M, M. No.: PK D62 712D; V. Rotary evaporator R-1001-VN Rotary Evaporator, Greatwall.

All reagents were obtained from commercial sources like Sigma-Aldrich, Chempur, Poch, Merk. All protected amino acids were obtained from NOV Biotech Ltd. 2-Chlorotrityl amine resin, 100-200 mesh was obtained from Sigma-Aldrich, Fmoc-Pro-CTC resin was obtained from NOV Biotech Ltd. BPC-157, GHK, Epithalon, Semax, Selank and Cortagen were obtained from Lipopharm, Poland.

Abbreviations:

CS (cleavage solution):	TFA:EDT:Phenol:TIS:Water = 86:4:4:4:2
CTC-resin:	2-chlorotrityl chloride resin
DCM:	dichloromethane
DIC:	N,N'-diisopropylcarbodiimide
DMF:	N,N-dimethylformamide
DP:	20% piperidine + 80% N,N-dimethylformamide
EDT:	1,2-ethanedithiol
Fmoc:	9-fluorenylmethoxycarbonyl
HOBt:	1-hydroxybenzotriazole
MS:	Mass Spectrometry
MW:	Molecular Weight
Ninhydrin:	2,2-dihydroxyindan-1,3-dione
RE:	Resin
RP-HPLC:	Reverse Phase High Performance Liquid Chromatography (Reversed-Phase High-Performance Liquid Chromatography)
TFA:	trifluoroacetic acid

TIS: thioanisole

EXAMPLE 1.1

Preparation of Ac-BPC-157-Arg-NH_2 (Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2) (SEQ. ID 1)**.**

Outline of the synthesis

Peptide synthesis was performed using a solid support. Amino acids with a blocked quaternary 9-fluorenylmethoxycarbonyl group were used for the synthesis (Fmoc). During the course of the first step of the synthesis, the first Fmoc-protected amino acid was attached directly to the β-linker of the solid phase support. During the further reaction of cyclic deprotection and condensation of peptide bonds, the remaining amino acids were attached starting from the C-terminal end and ending with the N-terminal part of the peptide. In this way, a peptide-carrier complex was obtained (Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-ProAla-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-C-H-Resin). In the next step of the synthesis, the peptide was detached from the support using trifluoroacetic acid (TFA). Amidation of the C-terminal amino acid occurs during cleavage of the peptide from the support. This resulted in a peptide of the assumed sequence Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-ValArg-amide (SEQ. ID 1).

To obtain a peptide with an assumed purity of >95%, the Reverse Phase High Performance Liquid Chromatography (RP-HPLC) technique was used. The analytical stage was completed with ultra-high-performance liquid chromatography (UHPLC) and high-resolution mass spectrometry (QTOF-MS), which confirmed the molar mass of the synthesized peptide.

Detailed description of the synthesis

I. Peptide synthesis

The peptide was synthesized on a scale of 2 g

1.Preparation of Fmoc-Pro-CTC-resin

A weight of the Fmoc-Pro-CTC-resin carrier was placed in a chemical reactor, then DCM was added and left for 30 min. The DCM was removed and then the resin was rinsed with DMF

under constant stirring. The operation was repeated twice, each time using DMF. After rinsing, the DMF solution was thoroughly removed.

2. Attachment of Arg to solid support Fmoc-Pro-CTC-Resin.

After preparing the solid phase, arginine was attached to it. Then, in the deprotection step, the Fmoc group was removed. DP was added with continuous stirring under a nitrogen atmosphere. After 5 min, DP was removed, then the carrier was rinsed once with DMF, which was completely removed in the next step. The deprotection step was repeated again with the addition of DP under continuous stirring under a continuous flow of nitrogen. DP was completely removed after 10 minutes. The carrier was washed 6 times, each time using DMF. Fmoc-Arg-OH (3-times the amount of the support used) and HOBt (3-times the amount of the support used) were weighed out and then dissolved in DMF in the reactor. In the next step of the synthesis, DIC was added (3-times the amount of the support used, v:v) with constant stirring under a constant supply of nitrogen for 2 hours. At the end of the step, a ninhydrin test was performed to ensure that Fmoc-Arg-OH is fully attached to the support Fmoc-Pro-CTC-Resin. A negative result of the nihydrin reaction means that there are no free NH2 groups, and thus the reaction is considered complete. The liquid was removed from the reactor then it washed with DMF 3 times. From this point on, the amino acid in the form of Fmoc-Arg-OH is linked to the support. Upon completion of the step, Fmoc-Arg-Pro-CTC-Resin was obtained.

3. Attachment of Val to -Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen. Removed DP after 5 min then rinsed once with DMF which was then removed. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 minutes then rinsed resin 6 times with DMF each time. Fmoc-Val-OH (3-times the amount of the support used) and HOBt (3-times the amount of the support used) were weighed out then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. At the end of the step, a ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times using DMF each time. From then on, the amino acid in the form Fmoc-Val-OH was attached to Arg-Pro-CTC-Resin. Fmoc-Val-Arg-Pro-CTC-Resin was obtained.

4. Attachment Leu to -Val-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen and then the DP was removed after 5 min and rinsed once with DMF which was removed in the next step. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. Removed DP after 10 minutes. The support was then washed 6 times, each time using DMF. Fmoc-Leu-OH (3-times the amount of the support used) and HOBt (3-times the amount of the support used) were weighed out which was dissolved in DMF and fed into the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. The ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed from the reactor then washed 3 times with DMF. From then on, an amino acid in the form of Fmoc-Leu-OH was attached to Val-Arg-Pro-CTC-Resin. Fmoc-Leu-Val-Arg-Pro-CTC-Resin was obtained.

5. Attachment of Gly to -Leu-Val-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen, next DP was removed after 5 min and rinsed once with DMF, which was then removed from the reactor. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min, the resin was then rinsed 6 times, each time using DMF. Fmoc-Gly-OH (3-times the amount of the support used) and HOBt (3-times the amount of the support used) were weighed out, then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. A ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-Gly-OH was attached to -Leu-Val-Arg-Pro-CTC-Resin. As a result, Fmoc-Gly-Leu-Val-Arg-Pro-CTC-Resin was obtained.

6. Attachment of Ala to -Gly-Leu-Val-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen, next DP was removed after 5 min and rinsed once with DMF, which was then removed from the reactor. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min, the resin was then rinsed 6 times, each time using DMF. Fmoc-Ala-OH (3-times the amount of the support used) and HOBt (3-times the amount

of the support used) were weighed out, then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. A ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-Ala-OH was attached to -Gly-Leu-Val-Arg-Pro-CTC-Resin. As a result, Fmoc-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin was obtained.

7. Attachment Asp do Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen, next DP was removed after 5 min and rinsed once with DMF, which was then removed from the reactor. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min, the resin was then rinsed 6 times, each time using DMF. Fmoc-Asp-OH was weighed out (3-times the amount of the support used) and HOBt (3-times the amount of the support used), then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring with a constant supply of nitrogen within 2 hours. A ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-Asp-OH was attached to -Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin. As a result, Fmoc-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin was obtained.

8. Attachment of Asp to -Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin.

Step 7 was repeated. After the stage was completed, Fmoc-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin was obtained.

9. Attachment of Ala to -Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin was obtained.

Step 6 was repeated. After the stage was completed, Fmoc-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin was obtained.

10. Attachment Pro to -Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen, next DP was removed after 5 min and rinsed once with DMF, which was then removed from the reactor. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min, the resin was then rinsed 6 times, each time using

DMF. Fmoc-Pro-OH was weighed out (3-times the amount of the support used) and HOBt (3-times the amount of the support used), then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. A ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-Pro-OH was attached to -Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin. As a result, was obtained Fmoc-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin.

11. Attachment Lys to - Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen, next DP was removed after 5 min and rinsed once with DMF, which was then removed from the reactor. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min, the resin was then rinsed 6 times, each time using DMF. Fmoc-Lys-OH was weighed out (3-times the amount of the support used) and HOBt (3-times the amount of the support used), then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. A ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-Lys-OH was attached to -Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin. As a result, was obtained Fmoc-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin.

12. Attachment Gly to -Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin.

Step 5 was repeated. After the stage was completed, Fmoc-Gly-Lys-Pr-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin was obtained.

13. Attachment of Pro to -Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin.

Step 10 was repeated. After the stage was completed, Fmoc-Pro-Pro-Pro-AlaAsp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin was obtained.

14.Attachment of Glu to -Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-ValArg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen, next DP was removed after 5 min and rinsed once with DMF, which was then removed from the reactor. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min, the resin was then rinsed 6 times, each time using DMF. Fmoc-Glu-OH was weighed out (3-times the amount of the support used) and HOBt (3-times the amount of the support used), then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. A ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-Glu-OH was attached to -Pro-Pro-Pro-Gly-LysPro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin. As a result, Fmoc-GluPro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin was obtained.

15. Attachment of Gly to -Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-LeuVal-Arg-Pro-CTC-Resin.

Step 5 was repeated. After the stage was completed, Fmoc-Gly-Glu-Pro-Pro-Pro-Gly-LysPro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin was obtained.

16. Acetylation of the N-terminal amino acid

DP was added with constant stirring under a constant supply of nitrogen, then DP was removed after 5 min and rinsed once with DMF which was then removed. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min. The support was washed 6 times, each time using DMF. Prepared were acetic acid (3-times the amount of the support used) and HOBt (3-times the amount of the support used), dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. The liquid was removed from the reactor and then washed 3 times using DMF each time. Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-Pro-CTC-Resin was obtained.

II. Detachment of the peptide from the support

After the amino acid attaching steps are completed, all peptide side chains are blocked and the peptide itself is attached to the support. In the next stage of the synthesis, the peptide has to be detached from the support and the side chains deblocked to obtain a crude peptide. Thanks to the use of a modified resin, spontaneous amidation of the C-terminal amino acid occurs during the cleavage of the peptide from the support. The obtained peptide-resin complex together with CS (cleaving solution) was then stirred for 3 hours in the reactor. At this stage, the peptide is released from the support and the side groups are deprotected. Upon completion, the solution was filtered discarding the solid phase and retaining the liquid phase containing the peptide. In the next step, the liquid was cooled using diethyl ether with constant stirring. The crude peptide precipitated as a white precipitate. The precipitate was filtered and then washed with cooled diethyl ether 4 times using diethyl ether each time. The white precipitate was dried in a vacuum oven R-1001-VN. This completes the synthesis of the crude peptide of the sequence Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-GlyLeu-Val-Arg-amide (SEQ. ID 1).

III. Purification of the crude peptide by RP-HPLC

Two preparative liquid chromatographs were used to separate and purify the synthesis product: 1) ÄKTATM Pure 25 with Frac F9-R fraction collector. The Kromasil® 100-13-C18 50 x 250 mm column allows the purification of up to 1-2 g of product in one process at maximum flow 25 mL/min. 2) CXTH P1001L. Vydac column 70 x 150 mm, 10 mm filter allows to purify up to 50 g of product in one process at a maximum flow of 500 mL/min.

3 Analysis parameters:

Sample: purified peptide dissolved in water to concentration ~5 g/L;

Mobile phase: ACN:H_2O (17:83 v/v) with 0,1% TFA;

Flow: 25 mL/min / 500 mL/min

Analysis time: 30 min;

Column temperature: 25°C;

Detection conditions: $\lambda = 220$ nm.

IV. Lyophilization of the peptide

For lyophilization of the peptide, the solvent obtained during the HPLC paging process was evaporated and dried in a lyophilizer (Christ Beta 2-8) within 48-72 hours. The collected white solid phase was the purified peptide.

V. Synthesis quality analysis

Peptide purity was checked by analytical HPLC, molar mass was confirmed by mass spectrometry as described below.

1. HPLC analysis to evaluate the purity of the obtained peptide.

To assess the purity of the purified peptide, HPLC-MS was measured using a liquid chromatograph UHPLC (UltiMate 3000 Thermo Scientific /Dionex) with a fast (200 Hz) diode detector coupled to QTOF-MS (Impact HD, Bruker)

Analysis parameters:

Sample: 10 µl dissolved in water (to a concentration of ~5 g/L) of the purified peptide;

Mobile phase: ACN:H_2O (17:83 v/v) with 0,1% TFA;

Flow: 1 mL/min;

Analysis time: 30 min;

Column temperature: 25°C;

Detection conditions: λ = 220 nm.

Purity obtained was 99%.

2. QTOF-MS analysis to identify the peptide

To accurately determine the molecular weight of the peptide, a high-resolution QTOF mass spectrometer, a hybrid quadrupole mass spectrometer with time-of-flight analysis was used - TOF, and ionization at atmospheric pressure - ESI (Impact HD, Bruker) The sample was fed to the source by the syringe pump of the chromatograph. A small amount of the peptide was dissolved in MeOH.

Analysis parameters:

Sample: 10 µl dissolved in methanol (to a concentration of ~5 g/L) of the purified peptide;

Ionization: nitrogen laser beam, $\lambda = 337$ nm

Matrix: α-cyano-4-hydroxycinnamic acid (CCA).

The obtained BPC-157 derivative of (SEQ. ID 1) was identified by the QTOF MS: Mass Calc. for C70H113N21O23: 1616.77 g/mol, Found: 1617.99 $[M+H]^+$

EXAMPLE 1.2

Preparation of N-Ac-Epithalon-Arg-NH_2 (N-Ac-Ala-Glu-Asp-Gly-Arg-NH_2) (SEQ. ID 2)

Outline of the synthesis

Peptide synthesis was performed using a solid support. Amino acids with a blocked quaternary 9-fluorenylmethoxycarbonyl group (Fmoc) were used for the synthesis. During the first stage of the synthesis, the first Fmoc protected amino acid was attached directly to the p-linker of the solid support. During the further cyclic deprotection and condensation reaction of peptide bonds, the remaining amino acids were attached starting from the C-terminus and ending at the N-terminal part of the peptide. In this way, the peptide-support complex was obtained (Ac-Ala-Glu-Asp-Gly-Arg-Pro-C-H-Resin). In the next step of the synthesis, the peptide was cleaved from the support using trifluoroacetic acid (TFA). When cleaving the peptide from the support, amidation of the C-terminal peptide occurs. As a result, a peptide with the assumed sequence was obtained Ac-Ala-Glu-Asp-Gly-Arg-amide (SEQ. ID 2).

To obtain the peptide of assumed purity> 95%, the RP-HPLC technique was used. The analytical stage was finished with the ultra-high-performance liquid chromatography (UHPLC) technique and the high-resolution mass spectrometry (QTOF-MS) technique to confirm molar mass of the obtained final peptide.

I. Peptide synthesis

The peptide was synthesized on a scale of 10 g

1. Preparation of Fmoc-Pro-CTC-resin

The Fmoc-Pro-CTC resin support was weighed out in a chemical reactor, then DCM was added and left for 30 min. DCM was removed and the resin was washed with DMF with constant

stirring. The operation was repeated twice, each time using DMF. After washing, the DMF solution was completely removed.

2. Attachment of Fmoc-Arg to Pro-CTC-Resin on solid support.

After preparation of the solid phase, arginine was attached in the following way. The Fmoc group from the Fmoc-Pro-CTC-Resin was removed in the deprotection step. DP was added with constant stirring under nitrogen atmosphere. After 5 min, DP was removed, and the solid support was rinsed once with DMF, and the DMF was completely removed. The deprotection step was repeated by adding DP under a constant flow of nitrogen. DP was completely removed after 10 minutes. The solid support was rinsed 6 times, each time with DMF. Fmoc-Arg-OH (3-times the amount of the solid support used) and HOBt (3-times the amount of the solid support used) was weighted out and dissolved in DMF in a reactor. In the next step of the synthesis, DIC was added (3 times the amount of support used, v:v) constantly stirring under a constant flow of nitrogen for 2 hours. At the end of this step, a ninhydrin test was performed to ensure that Fmoc-Arg-OH was completely attached to the solid support. A negative result of the ninhydrin test means that no NH_2 groups are present and therefore the reaction is deemed complete. The liquid was removed from the reactor then washed with DMF three times. As a result, the amino acid in the form Fmoc-Arg-OH is attached to the support. At the end of the stage, Fmoc-Arg-Pro-CTC-Resin was obtained.

3. Attachment of Fmoc-Gly to Arg-Pro-CTC-Resin.

DP was added to the Fmoc-Arg-Pro-CTC-Resin with constant stirring under constant flow of nitrogen. DP was removed after 5 min followed by rinsing once with DMF and its removal. The deprotection was repeated by adding DP with constant stirring under constant flow of nitrogen. DP was removed after 10 minutes then the resin was rinsed 6 times with DMF (4 mL). Fmoc-Gly-OH (3-times the amount of the solid support used) and HOBt (3-times the amount of the solid support used) was weighted out and dissolved in DMF and charged to the reactor. Next, DIC (3-times the amount of the solid support used) was added constantly stirring under a constant flow of nitrogen for 2 hours. At the end of this step, a ninhydrin test was performed to ensure that the reaction was completed. The liquid was removed and then the reactor was washed 3-times using DMF each time (4 mL). As a result, Fmoc-Gly-OH was attached to Arg-Pro-CTC-Resin. Fmoc-Gly-Arg-Pro-CTC-Resin was obtained.

4. Attachment of Asp to -Gly-Arg-Pro-CTC-Resin.

DP was added constantly stirring under a constant flow of nitrogen, and then the DP was removed after 5 min and rinsed once with DMF, which was removed in the next step. The deprotection was repeated with the addition of DP constantly stirring under a constant flow of nitrogen. DP was removed after 10 minutes. The solid support was then rinsed 6 times with DMF. Fmoc-Asp-OH (3-times the amount of the solid support used) and HOBt (3-times the amount of the solid support used) was weighted out and dissolved in DMF and charged to the reactor. Next, DIC (3-times the amount of the solid support used) was added constantly stirring under a constant flow of nitrogen for 2 hours. The ninhydrin test was performed to ensure the reaction was completed. The liquid was removed from the reactor, and it was then washed 3 times with DMF. As a result, Fmoc-Asp-OH was attached to Gly-Arg-Pro-CTC-Resin. Fmoc-Asp-Gly-Arg-Pro-CTC-Resin was obtained.

5. Attachment of Glu to -Asp-Gly-Arg-Pro-CTC-Resin.

DP was added constantly stirring under a constant flow of nitrogen, and then DP was removed after 5 min and rinsed once with DMF which was then removed from the reactor. The deprotection was repeated with the addition of DP constantly stirring under a constant flow of nitrogen. The DP was removed after 10 min, and then the resin was rinsed 6 times with DMF. Fmoc-Glu-OH (3-times the amount of the solid support used) and HOBt (3-times the amount of the solid support used) was weighted, and then dissolved in DMF and charged to the reactor. Next, DIC (3-times the amount of the solid support used) was added constantly stirring under a constant flow of nitrogen for 2 hours. The ninhydrin test was performed to ensure the reaction was completed. The liquid was removed and then the reactor content was washed 3 times with DMF. As a result, Fmoc-Glu-OH was attached to Asp-Gly-Arg-Pro-CTC-Resin. As a result, Fmoc-Glu-Asp-Gly-Arg-Pro-CTC-Resin was obtained.

6. Attachment of Ala to -Glu-Asp-Gly-Arg-Pro-CTC-Resin.

DP was added constantly stirring under a constant flow of nitrogen, then the DP removed after 5 min and rinsed once with DMF, which was then removed. The deprotection was repeated with the addition of DP constantly stirring under a constant flow of nitrogen. DP was removed after 10 minutes. The solid support was rinsed 6 times with DMF. Fmoc-Ala-OH (3-times the amount of the solid support used) and HOBt (3-times the amount of the solid support used) was

weighted out and dissolved in DMF and charged to the reactor. Next, DIC (3-times the amount of the solid support used) was added constantly stirring under a constant flow of nitrogen for 2 hours. The ninhydrin test was performed to ensure the reaction was completed. The liquid was removed from the reactor and then washed 3 times using DMF each time. As a result, Fmoc-Ala-OH was attached to -Glu-Asp-Gly-Arg-Pro-CTC-Resin. Fmoc-Ala-Glu-Asp-Gly-Arg-Pro-CTC-Resin was obtained.

7. Acetylation of the N-terminal amino acid.

DP was added constantly stirring under a constant flow of nitrogen then the DP removed after 5 min and rinsed once with DMF, which was then removed. The deprotection was repeated with the addition of DP constantly stirring under a constant flow of nitrogen. DP was removed after 10 minutes. The solid support was rinsed 6 times with DMF. Acetic acid (3-times the amount of the solid support used) and HOBt (3-times the amount of the solid support used) dissolved in DMF and charged to the reactor. Next, DIC (3-times the amount of the solid support used) was added constantly stirring under a constant flow of nitrogen for 2 hours. The liquid was removed from the reactor and then washed 3 times using DMF each time. Ac-Ala-Glu-Asp-Gly-Arg-Pro-CTC-Resin was used.

II. Cleavage of the peptide from the solid support

After completion of the peptide formation, all side chains of the peptide are protected and the peptide itself is attached to the support. Next, the peptide must be cleaved from the solid support and the protection of side chains has to be removed to obtain a crude peptide. Thanks to the use of a modified resin, spontaneous amidation of the C-terminal amino acid takes place during the cleavage of the peptide from the solid support.

The peptide-resin complex obtained above was placed in a reactor with CS, and then stirred for 3 hours. At this stage, the peptide releases from the solid support and the protection groups of the side chains are deprotected. Upon completion, the solution was filtered discarding the solid phase and retaining the liquid phase containing the peptide. Next, the liquid was cooled using diethyl ether with constant stirring. The crude peptide precipitates as a white solid. The precipitate was filtered, and then washed with cooled diethyl ether 4 times. The white solid was dried in a vacuum oven R-1001-VN. As a result, the synthesis of the crude peptide Ac-Ala-Glu-Asp-Gly-Arg-amide was completed (SEQ. ID 2).

III. Purification of crude peptide by RP-HPLC

To separate and purify the synthesis product, two preparative liquid chromatographs were used: 1) ÄKTATM Pure 25 with Frac F9-R fraction collector. The Kromasil® 100-13-C18 50 x 250 mm column allows the purification of up to 1-2 g of product in one process with a maximum flow of 25 mL/min; 2) CXTH P1001L. The Vydac® 70 x 150 mm column, 10 mm filter, allows up to 50 g of product to be purified in one process at a maximum flow of 500 mL/min.

Analysis parameters:

Sample: purified peptide dissolved in water to a concentration ~5 g/L;

Mobile phase: ACN:H_2O (17:83 v/v) with addition of 0.1% TFA;

Flow: 25 mL/min/ 500 mL/min

Time of analysis: 30 min;

Column temperature: 25°C;

Detection conditions: $\lambda = 220$ nm.

IV. Peptide lyophilization

To lyophilize the peptide, the solvent obtained during the HPLC purification process was evaporated and dried in a freeze dryer (Christ Beta 2-8) for 48-72 hours. The collected white solid was purified peptide.

V. Analysis of the synthesis quality

Peptide purity was checked by analytical HPLC, the molar mass was confirmed by mass spectrometry as described below.

1. HPLC analysis to evaluate the purity of the obtained peptide.

To assess the purity of the purified peptide, the measurement was carried out using HPLC-MS methods using a UHPLC liquid chromatograph (UltiMate 3000 Thermo Scientific/Dionex) with a fast (200 Hz) diode detector coupled with QTOF-MS (Impact HD, Bruker).

Analysis parameters:

Sample: 10 µl of the purified peptide dissolved in water (to a concentration of ~5 g/L)

Mobile phase: ACN:H_2O (17:83 v/v) with addition of 0.1% TFA;

Flow: 1 mL/min;

Time of analysis: 30 min;

Column temperature: 25°C;

Detection conditions: $\lambda = 220$ nm.

Purity obtained was 96%.

2. QTOF-MS analysis for peptide identification

To accurately determine the molecular weight of the peptide, a QTOF high-resolution mass spectrometer was used, a hybrid quadrupole mass spectrometer with time-of-flight analysis - TOF, and atmospheric pressure ionization - ESI (Impact HD, Bruker). The sample was fed to the source using a syringe pump of the chromatograph. A small amount of the peptide was dissolved in MeOH.

Analysis parameters:

Sample: 10 µl of the purified peptide dissolved in methanol (to a concentration of ~5 g/L);

Ionization: nitrogen laser beam, $\lambda = 337$ nm

Matrix: α-cyano-4-hydroxycinnamic acid (CCA).

The obtained Epithalon derivative of (SEQ. ID 2) was identified by the QTOF MS: Mass Calc. for C22H37N9O10: 587.5835 g/mol, Found: 588.61 $[M+H]^+$

EXAMPLE 1.3

Preparation of Ac-GHK-Arg-NH_2 (Ac-Gly-His-Lys-Arg-NH_2) (SEQ. ID 3)

Outline of the synthesis

Peptide synthesis was performed using a solid support. Amino acids with a blocked quaternary 9-fluorenylmethoxycarbonyl group were used for the synthesis (Fmoc). During the course of the first step of the synthesis, the first Fmoc-protected amino acid was attached directly to the β-linker of the solid phase support. During the further reaction of cyclic deprotection and

condensation of peptide bonds, the remaining amino acids were attached starting from the C-terminal end and ending with the N-terminal part of the peptide.

In this way, a peptide-carrier complex was obtained (Ac-Gly-His-Lys-Arg-Pro-CTC-Resin). In the next step of the synthesis, the peptide was detached from the support using trifluoroacetic acid (TFA). During cleavage of the peptide from the support, amidation of the C-terminal peptide takes place. As a result, a peptide of the assumed sequence was obtained Ac-Gly-His-Lys-Arg-amide (SEQ. ID 3).

To obtain a peptide with an assumed purity of >95%, the Reverse Phase High Performance Liquid Chromatography (RP-HPLC) technique was used. The analytical stage was completed with ultra-high-performance liquid chromatography (UHPLC) and high-resolution mass spectrometry (QTOF-MS), which confirmed the molar mass of the synthesized peptide.

Detailed description of the synthesis

I. Peptide synthesis

The peptide was synthesized on a scale of 10 g

1.Charging of the resin Fmoc-Pro-CTC

A weight of the Fmoc-Pro-CTC-resin carrier was placed in a chemical reactor, then DCM was added and left for 30 min. The DCM was removed and then the resin was rinsed with DMF under constant stirring. The operation was repeated twice, each time using DMF. After rinsing, the DMF solution was thoroughly removed.

2. Attachment of Arg to solid support Fmoc-Pro-CTC-Resin.

After preparing the solid phase, arginine was attached to it. Then, in the deprotection step, the Fmoc group was removed. DP was added with continuous stirring under a nitrogen atmosphere. After 5 min, DP was removed, then the carrier was rinsed once with DMF, which was completely removed in the next step. The deprotection step was repeated again with the addition of DP under continuous stirring under a continuous flow of nitrogen. DP was completely removed after 10 min. The support was washed 6 times, each time using DMF. Fmoc-Arg-OH (3-times the amount of the support used) and HOBt (3-times the amount of the support used) were weighed out and then dissolved in DMF in the reactor. In the next step of the synthesis, DIC was added (3-times the amount of the support used, v:v) with constant stirring under a

constant supply of nitrogen for 2 hours. At the end of the step, a ninhydrin test was performed to ensure that Fmoc-Arg-OH is fully attached to the support Fmoc-Pro-CTC-Resin. A negative result of the nihydrin reaction means that there are no free NH2 groups, and thus the reaction is considered complete. The liquid was removed from the reactor then it washed with DMF 3 times. From this point on, the amino acid in the form of Fmoc-Arg-OH is linked to the support. Upon completion of the step, Fmoc-Arg-Pro-CTC-Resin was obtained.

3.Attachment Lys to -Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen. Removed DP after 5 min then rinsed once with DMF which was then removed. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 minutes then rinsed resin 6 times with DMF each time. Fmoc-Lys-OH was weighed out (3-times the amount of the support used) and HOBt (3-times the amount of the support used) then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. At the end of the step, a ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times using DMF each time. From then on, the amino acid in the form Fmoc-Lys-OH was attached to Arg-Pro-CTC-Resin. Fmoc-Lys-Arg-Pro-CTC-Resin was obtained.

4. Attachment His to -Lys-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen and then the DP was removed after 5 min and rinsed once with DMF which was removed in the next step. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. Removed DP after 10 minutes. The support was then washed 6 times, each time using DMF. Fmoc-His-OH (3-times the amount of the support used) and HOBI (3-times the amount of the support used) were weighed out, dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. The ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed from the reactor then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-His-OH was attached to Lys-Arg-ProCTC-Resin. Fmoc-His-Lys-Arg-Pro-CTC-Resin was obtained.

5. Attachment Gly to - His-Lys-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen, next DP was removed after 5 min and rinsed once with DMF, which was then removed from the reactor. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min, the resin was then rinsed 6 times, each time using DMF. Fmoc-Gly-OH was weighed out (3-times the amount of the support used) and HOBI (3-times the amount of the support used), then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. A ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-Gly-OH was attached to -His-Lys-Arg-Pro-CTC-Resin. As a result, was obtained Fmoc-Gly-His-Lys-Arg-Pro-CTC-Resin.

6. Acetylation of the N-terminal amino acid

DP was added with constant stirring under a constant supply of nitrogen, then DP was removed after 5 min and rinsed once with DMF which was then removed. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min. The support was washed 6 times, each time using DMF. Prepared were acetic acid (3-times the amount of the support used) and HOBt (3-times the amount of the support used), dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. The liquid was removed from the reactor and then washed 3 times using DMF each time. was obtained Ac-Gly-His-Lys-Arg-Pro-CTC-Resin.

II. Detachment of the peptide from the support

After the amino acid attaching steps are completed, all peptide side chains are blocked and the peptide itself is attached to the support. In the next stage of the synthesis, the peptide has to be detached from the support and the side chains deblocked to obtain a crude peptide. Thanks to the use of a modified resin, spontaneous amidation of the C-terminal amino acid occurs during the cleavage of the peptide from the support. The obtained peptide-resin complex together with CS (cleaving solution) was then stirred for 3 hours in the reactor. At this stage, the peptide is released from the support and the side groups are deprotected. Upon completion, the solution

was filtered discarding the solid phase and retaining the liquid phase containing the peptide. In the next step, the liquid was cooled using diethyl ether with constant stirring. The crude peptide precipitated as a white precipitate. The precipitate was filtered and then washed with cooled diethyl ether 4 times using diethyl ether each time. The white precipitate was dried in a vacuum oven R-1001-VN. This completes the synthesis of the crude peptide of the sequence Ac-Gly-His-Lys-Arg-amide (SEQ. ID 3).

III. Purification of the crude peptide by RP-HPLC

Two preparative liquid chromatographs were used to separate and purify the synthesis product: 1) ÄKTATM Pure 25 with Frac F9-R fraction collector. Kolumna Kromasil® 100-13-C18 50 x 250 mm allows to purify up to 1-2 g of product in one process at maximum flow 25 mL/min. 2) CXTH P1001L. Vydac column 70 x 150 mm, 10 mm filter allows to purify up to 50 g of product in one process at a maximum flow of 500 mL/min.

Analysis parameters:

Sample: purified peptide dissolved in water to concentration ~5 g/L;

Mobile phase: ACN:H_2O (17:83 v/v) with 0,1% TFA;

Flow: 25 mL/min / 500 mL/min

Analysis time: 30 min;

Column temperature: 25°C;

Detection conditions: λ = 220 nm.

IV. Lyophilization of the peptide

For lyophilization of the peptide, the solvent obtained during the HPLC paging process was evaporated and dried in a lyophilizer (Christ Beta 2-8) within 48-72 hours.

The collected white solid phase was the purified peptide.

V. Synthesis quality analysis

Peptide purity was checked by analytical HPLC, molar mass was confirmed by mass spectrometry as described below.

1. HPLC analysis to evaluate the purity of the obtained peptide.

To assess the purity of the purified peptide, HPLC-MS was measured using a liquid chromatograph UHPLC (UltiMate 3000 Thermo Scientific / Dionex) with a fast (200 Hz) diode detector coupled to QTOF-MS (Impact HD, Bruker)

Analysis parameters:

Sample: 10 µl dissolved in water (to a concentration of ~5 g/L) of the purified peptide;

Mobile phase: ACN:H_2O (17:83 v/v) with 0,1% TFA;

Flow: 1 mL/min;

Analysis time: 30 min;

Column temperature: 25°C;

Detection conditions: λ = 220 nm.

Purity obtained was 95%.

2. QTOF-MS analysis to identify the peptide

To accurately determine the molecular weight of the peptide, a high-resolution QTOF mass spectrometer, a hybrid quadrupole mass spectrometer with time-of-flight analysis was used - TOF, and ionization at atmospheric pressure - ESI (Impact HD, Bruker) The sample was fed to the source by the syringe pump of the chromatograph. A small amount of the peptide was dissolved in MeOH.

Analysis parameters:

Sample: 10 µl dissolved in methanol (to a concentration of ~5 g/L) of the purified peptide;

Ionization: nitrogen laser beam, = 337 nm

Matrix:α-cyano-4-hydroxycinnamic acid (CCA)

The obtained GHK derivative of (SEQ. ID 3) was identified by the QTOF MS: Mass Calc. for $C_{22}H_{39}N_{11}O_5$: 537.61 g/mol, Found: 538.60 $[M+H]^+$

EXAMPLE 1.4

Preparation of Ac-Semax-NH_2 (Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2) (SEQ. ID 4)

Outline of the synthesis

Peptide synthesis was performed using a solid support. Amino acids with a blocked quaternary 9-fluorenylmethoxycarbonyl group were used for the synthesis (Fmoc). During the course of the first step of the synthesis, the first Fmoc-protected amino acid was attached directly to the β-linker of the solid phase support. During the further reaction of cyclic deprotection and condensation of peptide bonds, the remaining amino acids were attached starting from the C-terminal end and ending with the N-terminal part of the peptide. In this way, a peptide-carrier complex was obtained (Ac-Met-Glu-His-Phe-Pro-Gly-Pro-ArgPro-C-H-Resin). In the next step of the synthesis, the peptide was detached from the support using trifluoroacetic acid (TFA). Amidation of the C-terminal amino acid occurs during cleavage of the peptide from the support. This resulted in a peptide of the assumed sequence Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-amide (SEQ. ID 4).

To obtain a peptide with the assumed purity of >80%, High Performance Liquid Chromatography in the Reverse Phase System was used (RP-HPLC). The analytical stage was completed with ultra-high-performance liquid chromatography (UHPLC) and high-resolution mass spectrometry (QTOF-MS), which confirmed the molar mass of the synthesized peptide.

Detailed description of the synthesis

I. Peptide synthesis

The peptide was synthesized on a scale of 10 g

1. Preparation of Fmoc-Pro-CTC-resin

A weight of the Fmoc-Pro-CTC-resin carrier was placed in a chemical reactor, then DCM was added and left for 30 min. The DCM was removed and then the resin was rinsed with DMF under constant stirring. The operation was repeated twice, each time using DMF. After rinsing, the DMF solution was thoroughly removed.

2. Attachment of Arg to solid support Fmoc-Pro-CTC-Resin.

After preparing the solid phase, arginine was attached to it. Then, in the deprotection step, the Fmoc group was removed. DP was added with continuous stirring under a nitrogen atmosphere. After 5 min, DP was removed, then the carrier was rinsed once with DMF, which was completely removed in the next step. The deprotection step was repeated again with the addition

of DP under continuous stirring under a continuous flow of nitrogen. DP was completely removed after 10 min. The support was washed 6 times, each time using DMF. Fmoc-Arg-OH (3-times the amount of the support used) and HOBt (3-times the amount of the support used) were weighed out and then dissolved in DMF in the reactor. In the next step of the synthesis, DIC was added (3-times the amount of the support used, v:v) with constant stirring under a constant supply of nitrogen for 2 hours. At the end of the step, a ninhydrin test was performed to ensure that Fmoc-Arg-OH is fully attached to the support Fmoc-Pro-CTC-Resin. A negative result of the nihydrin reaction means that there are no free NH2 groups, and thus the reaction is considered complete. The liquid was removed from the reactor then it washed with DMF 3 times. From this point on, the amino acid in the form of Fmoc-Arg-OH is linked to the support. Upon completion of the step, Fmoc-Arg-Pro-CTC-Resin was obtained.

3. Attachment Pro to -Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen. Removed DP after 5 min then rinsed once with DMF which was then removed. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 minutes then rinsed resin 6 times with DMF each time. Fmoc-Pro-OH was weighed out (3-times the amount of the support used) and HOBt (3-times the amount of the support used) then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. At the end of the step, a ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times using DMF each time. From then on, the amino acid in the form Fmoc-Pro-OH was attached to Arg-Pro-CTC-Resin. was obtained Fmoc-Pro-Arg-Pro-CTC-Resin.

4. Attachment Gly to -Pro-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen and then the DP was removed after 5 min and rinsed once with DMF which was removed in the next step. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. Removed DP after 10 minutes. The support was then washed 6 times, each time using DMF. Fmoc-Gly-OH was weighted out (3-times the amount of the support used) and HOBt (3-times the amount of the support used), dissolved in DMF and charged to the reactor. DIC was

then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. The ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed from the reactor then washed 3 times with DMF.

From then on, the amino acid in the form Fmoc-Gly-OH was attached to Pro-Arg-ProCTC-Resin. Fmoc-Gly-Pro-Arg-Pro-CTC-Resin was obtained.

5. Attachment Pro to -Gly-Pro-Arg-Pro-CTC-Resin.

Step 3 was repeated three times. After the stage was completed, was obtained Fmoc-Pro-Gly-Pro-Arg-ProCTC-Resin.

6. Attachment Phe to Pro-Gly-Pro-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen, next DP was removed after 5 min and rinsed once with DMF, which was then removed from the reactor. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min, the resin was then rinsed 6 times, each time using DMF. Fmoc-Phe-OH (3-times the amount of the support used) and HOBt (3-times the amount of the support used) were weighted out, then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. A ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-Phe-OH was attached to -Pro-Gly-Pro-Arg-Pro-CTC-Resin. As a result, was obtained Fmoc-Phe-Pro-Gly-Pro-Arg-Pro-CTC-Resin.

7. Attachment His to -Phe-Pro-Gly-Pro-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen, next DP was removed after 5 min and rinsed once with DMF, which was then removed from the reactor. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min, the resin was then rinsed 6 times, each time using DMF. Przygotowano naważkę Fmoc-His-OH (3-times the amount of the support used) and HOBt (3-times the amount of the support used), then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. A ninhydrin test was performed to ensure

that the reaction was complete. The liquid was removed and then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-His-OH was attached to -Phe-Pro-Gly-Pro-ArgPro-CTC-Resin. As a result, was obtained Fmoc-His-Phe-Pro-Gly-Pro-Arg-Pro-CTC-Resin.

8. Attachment Glu to His-Phe-Pro-Gly-Pro-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen, next DP was removed after 5 min and rinsed once with DMF, which was then removed from the reactor. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min, the resin was then rinsed 6 times, each time using DMF. Fmoc-Glu-OH was weighed out (3-times the amount of the support used) and HOBt

(3-times the amount of the support used), then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. A ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-Glu-OH was attached to His-Phe-Pro-Gly-ProArg-Pro-CTC-Resin. As a result, was obtained Fmoc-Glu-His-Phe-Pro-Gly-Pro-Arg-Pro-CTC-Resin.

9. Attachment Met to -Phe-Pro-Gly-Pro-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen, next DP was removed after 5 min and rinsed once with DMF, which was then removed from the reactor. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min, the resin was then rinsed 6 times, each time using DMF. Fmoc-Met-OH (3-times the amount of the support used) and HOBt (3-times the amount of the support used) were weighted out, then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. A ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-Met-OH was attached to Glu-His-Phe-Pro-Gly-Pro-Arg-Pro-CTC-Resin. As a result, was obtained Fmoc-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-Pro-CTC-Resin.

10. Acetylation of the N-terminal amino acid

DP was added with constant stirring under a constant supply of nitrogen, then DP was removed after 5 min and rinsed once with DMF which was then removed. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min. The support was washed 6 times, each time using DMF. Prepared were acetic acid (3-times the amount of the support used) and HOBt (3-times the amount of the support used), dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. The liquid was removed from the reactor and then washed 3 times using DMF each time. was obtained Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-Pro-CTC-Resin.

II. Detachment of the peptide from the support

After the amino acid attaching steps are completed, all peptide side chains are blocked and the peptide itself is attached to the support. In the next stage of the synthesis, the peptide has to be detached from the support and the side chains deblocked to obtain a crude peptide. Thanks to the use of a modified resin, spontaneous amidation of the C-terminal amino acid occurs during the cleavage of the peptide from the support. The obtained peptide-resin complex together with CS (cleaving solution) was then stirred for 3 hours in the reactor. At this stage, the peptide is released from the support and the side groups are deprotected. Upon completion, the solution was filtered discarding the solid phase and retaining the liquid phase containing the peptide. In the next step, the liquid was cooled using diethyl ether with constant stirring. The crude peptide precipitated as a white precipitate. The precipitate was filtered and then washed with cooled diethyl ether 4 times using diethyl ether each time. The white precipitate was dried in a vacuum oven R-1001-VN. This completes the synthesis of the crude peptide of the sequence Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-amide (SEQ. ID 4).

III. Purification of the crude peptide by RP-HPLC

Two preparative liquid chromatographs were used to separate and purify the synthesis product: 1) ÄKTA™ Pure 25 with Frac F9-R fraction collector. The Kromasil® 100-13-C18 50 x 250 mm column allows the purification of up to 1-2 g of product in one process at maximum flow 25 mL/min; 2) CXTH P1001L. Vydac® column 70 x 150 mm, 10 mm filter allows to purify up to 50 g of product in one process at maximum flow 500 mL/min.

Analysis parameters:

Sample: purified peptide dissolved in water to concentration ~5 g/L;

Mobile phase: ACN:H_2O (17:83 v/v) with 0,1% TFA;

Flow: 25 mL/min / 500 mL/min

Analysis time: 30 min;

Column temperature: 25°C;

Detection conditions: λ = 220 nm.

IV. Lyophilization of the peptide

For lyophilization of the peptide, the solvent obtained during the HPLC paging process was evaporated and dried in a lyophilizer (Christ Beta 2-8) within 48-72 hours.

The collected white solid phase was the purified peptide.

V. Synthesis quality analysis

Peptide purity was checked by analytical HPLC, molar mass was confirmed by mass spectrometry as described below.

1. HPLC analysis to evaluate the purity of the obtained peptide.

To assess the purity of the purified peptide, HPLC-MS was measured using a liquid chromatograph UHPLC (UltiMate 3000 Thermo Scientific / Dionex) with a fast (200 Hz) diode detector coupled to QTOF-MS (Impact HD, Bruker)

Analysis parameters:

Sample: 10 µl dissolved in water (to a concentration of ~5 g/L) of the purified peptide;

Mobile phase: ACN:H_2O (17:83 v/v) with 0,1% TFA;

Flow: 1 mL/min;

Analysis time: 30 min;

Column temperature: 25°C;

Detection conditions: λ = 220 nm.

Purity obtained was 92%.

2. QTOF-MS analysis to identify the peptide

To accurately determine the molecular weight of the peptide, a high-resolution QTOF mass spectrometer, a hybrid quadrupole mass spectrometer with time-of-flight analysis was used - TOF, and ionization at atmospheric pressure - ESI (Impact HD, Bruker)

The sample was fed to the source by the syringe pump of the chromatograph. A small amount of the peptide was dissolved in MeOH.

Analysis parameters:

Sample: 10 µl dissolved in methanol (to a concentration of ~5 g/L) of the purified peptide;

Ionization: nitrogen laser beam, $\lambda = 337$ nm

Matrix: α-cyano-4-hydroxycinnamic acid (CCA).

The obtained Semax derivative of (SEQ. ID 4) was identified by the QTOF MS: Mass Calc. for $C_{45}H_{66}N_{14}O_{11}S_1$: 1011.16 g/mol, Found: 1012.51 $[M+H]^+$

EXAMPLE 1.5

Preparation of Ac-Selank-Arg-NH_2 (Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2) (SEQ. ID 5)**.**

Outline of the synthesis

Peptide synthesis was performed using a solid support. Amino acids with a blocked quaternary 9-fluorenylmethoxycarbonyl group were used for the synthesis (Fmoc). During the course of the first step of the synthesis, the first Fmoc-protected amino acid was attached directly to the β-linker of the solid phase support. During the further reaction of cyclic deprotection and condensation of peptide bonds, the remaining amino acids were attached starting from the C-terminal end and ending with the N-terminal part of the peptide.

In this way, a peptide-carrier complex was obtained (Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-ArgPro-C-H-Resin). In the next step of the synthesis, the peptide was detached from the support using trifluoroacetic acid (TFA). Amidation of the C-terminal amino acid occurs during

cleavage of the peptide from the support. This resulted in a peptide of the assumed sequence Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-amide (SEQ. ID 5).

To obtain a peptide with the assumed purity of >80%, High Performance Liquid Chromatography in the Reverse Phase System was used (RP-HPLC). The analytical stage was completed with ultra-high-performance liquid chromatography (UHPLC) and high-resolution mass spectrometry (QTOF-MS), which confirmed the molar mass of the synthesized peptide.

Detailed description of the synthesis

I. Peptide synthesis

The peptide was synthesized on a scale of 10 g

1. Preparation of Fmoc-Pro-CTC-resin

A weight of the Fmoc-Pro-CTC-resin carrier was placed in a chemical reactor, then DCM was added and left for 30 min. The DCM was removed and then the resin was rinsed with DMF under constant stirring. The operation was repeated twice, each time using DMF. After rinsing, the DMF solution was thoroughly removed.

2. Attachment of Arg to solid support Fmoc-Pro-CTC-Resin.

After preparing the solid phase, arginine was attached to it. Then, in the deprotection step, the Fmoc group was removed. DP was added with continuous stirring under a nitrogen atmosphere. After 5 min, DP was removed, then the carrier was rinsed once with DMF, which was completely removed in the next step. The deprotection step was repeated again with the addition of DP under continuous stirring under a continuous flow of nitrogen. DP was completely removed after 10 min. The support was washed 6 times, each time using DMF. Fmoc-Arg-OH (3-times the amount of the support used) and HOBt (3-times the amount of the support used) were weighed out and then dissolved in DMF in the reactor. In the next step of the synthesis, DIC was added (3-times the amount of the support used, v:v) with constant stirring under a constant supply of nitrogen for 2 hours. At the end of the step, a ninhydrin test was performed to ensure that Fmoc-Arg-OH is fully attached to the support Fmoc-Pro-CTC-Resin. A negative result of the nihydrin reaction means that there are no free NH2 groups, and thus the reaction is considered complete. The liquid was removed from the reactor then it washed with DMF 3

times. From this point on, the amino acid in the form of Fmoc-Arg-OH is linked to the support. Upon completion of the step, Fmoc-Arg-Pro-CTC-Resin was obtained.

3. Attachment Pro to -Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen. Removed DP after 5 min then rinsed once with DMF which was then removed. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 minutes then rinsed resin 6 times with DMF each time. Fmoc-Pro-OH was weighed out (3-times the amount of the support used) and HOBt (3-times the amount of the support used) then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. At the end of the step, a ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times using DMF each time. From then on, the amino acid in the form Fmoc-Pro-OH was attached to Arg-Pro-CTC-Resin. was obtained Fmoc-Pro-Arg-Pro-CTC-Resin.

4. Attachment Gly to -Pro-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen and then the DP was removed after 5 min and rinsed once with DMF which was removed in the next step. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. Removed DP after 10 minutes. The support was then washed 6 times, each time using DMF. Fmoc-Gly-OH was weighted out (3-times the amount of the support used) and HOBt (3-times the amount of the support used), dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. The ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed from the reactor then washed 3 times with DMF.

From then on, the amino acid in the form Fmoc-Gly-OH was attached to Pro-Arg-Pro-CTC-Resin. Fmoc-Gly-Pro-Arg-Pro-CTC-Resin was obtained.

5. Attachment Pro to -Gly-Pro-Arg-Pro-CTC-Resin.

Step 3 was repeated. After the stage was completed, was obtained Fmoc-Pro-Gly-Pro-Arg-Pro-CTC-Resin.

6. Attachment Arg do Pro-Gly-Pro-Arg-Pro-CTC-Resin.

Step 2 was repeated. After the stage was completed, was obtained Fmoc-Arg-Pro-Gly-Pro-Arg-Pro-CTC-Resin.

7. Attachment Pro to -Gly-Pro-Arg-Pro-CTC-Resin.

Step 3 was repeated. After the stage was completed, was obtained Fmoc-Pro-Arg-Pro-Gly-Pro-Arg-ProCTC-Resin.

8. Attachment Lys to -Pro-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen and then the DP was removed after 5 min and rinsed once with DMF which was removed in the next step. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. Removed DP after 10 minutes. The support was then washed 6 times, each time using DMF. Fmoc-Lys-OH (3-times the amount of the support used) and HOBt (3-times the amount of the support used) were weighted out, dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. The ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed from the reactor then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-Lys-OH was attached to Pro-Arg-Pro-Gly-Pro-Arg-Pro-CTC-Resin. Fmoc-Lys-Pro-Arg-Pro-Gly-Pro-Arg-Pro-CTC-Resin was obtained.

9. Attachment Thr to -Pro-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen and then the DP was removed after 5 min and rinsed once with DMF which was removed in the next step. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. Removed DP after 10 minutes. The support was then washed 6 times, each time using DMF. Fmoc-Thr-OH (3-times the amount of the support used) and HOBt (3-times the amount of the support used) were weighted out, dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. The ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed from the reactor then washed 3 times with DMF. From

then on, the amino acid in the form Fmoc-Thr-OH was attached to Lys-Pro-Arg-Pro-Gly-Pro-Arg-Pro-CTC-Resin. Fmoc-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-Pro-CTC-Resin was obtained.

10. Acetylation of the N-terminal amino acid

DP was added with constant stirring under a constant supply of nitrogen, then DP was removed after 5 min and rinsed once with DMF which was then removed. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min. The support was washed 6 times, each time using DMF. Prepared were acetic acid (3-times the amount of the support used) and HOBt (3-times the amount of the support used), dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. The liquid was removed from the reactor and then washed 3 times using DMF each time. was obtained Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-Pro-CTC-Resin.

II. Detachment of the peptide from the support

After the amino acid attaching steps are completed, all peptide side chains are blocked and the peptide itself is attached to the support. In the next stage of the synthesis, the peptide has to be detached from the support and the side chains deblocked to obtain a crude peptide. Thanks to the use of a modified resin, spontaneous amidation of the C-terminal amino acid occurs during the cleavage of the peptide from the support. The obtained peptide-resin complex together with CS (cleaving solution) was then stirred for 3 hours in the reactor. At this stage, the peptide is released from the support and the side groups are deprotected. Upon completion, the solution was filtered discarding the solid phase and retaining the liquid phase containing the peptide. In the next step, the liquid was cooled using diethyl ether with constant stirring. The crude peptide precipitated as a white precipitate. The precipitate was filtered and then washed with cooled diethyl ether 4 times using diethyl ether each time. The white precipitate was dried in a vacuum oven R-1001-VN. This completes the synthesis of the crude peptide of the sequence Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-amide (SEQ. ID 5).

III. Purification of the crude peptide by RP-HPLC

Two preparative liquid chromatographs were used to separate and purify the synthesis product: 1) ÄKTA™ Pure 25 with Frac F9-R fraction collector. The Kromasil® 100-13-C18 50 x 250

mm column allows the purification of up to 1-2 g of product in one process at maximum flow 25 mL/min; 2) CXTH P1001L. Vydac® column 70 x 150 mm, 10 mm filter allows to purify up to 50 g of product in one process at maximum flow 500 mL/min.

Analysis parameters:

Sample: purified peptide dissolved in water to concentration ~5 g/L;

Mobile phase: ACN:H_2O (17:83 v/v) with 0,1% TFA;

Flow: 25 mL/min / 500 mL/min

Analysis time: 30 min;

Column temperature: 25°C;

Detection conditions: λ = 220 nm.

IV. Lyophilization of the peptide

For lyophilization of the peptide, the solvent obtained during the HPLC paging process was evaporated and dried in a lyophilizer (Christ Beta 2-8) within 48-72 hours.

The collected white solid phase was the purified peptide.

V. Synthesis quality analysis

Peptide purity was checked by analytical HPLC, molar mass was confirmed by mass spectrometry as described below.

1. HPLC analysis to evaluate the purity of the obtained peptide.

To assess the purity of the purified peptide, HPLC-MS was measured using a liquid chromatograph UHPLC (UltiMate 3000 Thermo Scientific / Dionex) with a fast (200 Hz) diode detector coupled to QTOF-MS (Impact HD, Bruker)

Analysis parameters:

Sample: 10 µl dissolved in water (to a concentration of ~5 g/L) of the purified peptide;

Mobile phase: ACN:H_2O (17:83 v/v) with 0,1% TFA;

Flow: 1 mL/min;

Analysis time: 30 min;

Column temperature: 25°C;

Detection conditions: λ = 220 nm.

Purity obtained was 84%.

2. QTOF-MS analysis to identify the peptide

To accurately determine the molecular weight of the peptide, a high-resolution QTOF mass spectrometer, a hybrid quadrupole mass spectrometer with time-of-flight analysis was used - TOF, and ionization at atmospheric pressure - ESI (Impact HD, Bruker)

The sample was fed to the source by the syringe pump of the chromatograph. A small amount of the peptide was dissolved in MeOH.

Analysis parameters:

Sample: 10 µl dissolved in methanol (to a concentration of ~5 g/L) of the purified peptide;

Ionization: nitrogen laser beam, λ = 337 nm

Matrix: α-cyano-4-hydroxycinnamic acid (CCA).

The obtained Selank derivative of (SEQ. ID 5) was identified by the QTOF MS: Mass Calc. for C41H72N16O10: 949.11 g/mol, Found: 950.25 $[M+H]^+$

EXAMPLE 1.6

Preparation of Ac-Cortagen-Arg-NH_2 (Ac-Ala-Glu-Asp-Pro-Arg-NH_2) (SEQ. ID 6)

Outline of the synthesis

Peptide synthesis was performed using a solid support. Amino acids with a blocked quaternary 9-fluorenylmethoxycarbonyl group were used for the synthesis (Fmoc). During the course of the first step of the synthesis, the first Fmoc-protected amino acid was attached directly to the β-linker of the solid phase support. During the further reaction of cyclic deprotection and condensation of peptide bonds, the remaining amino acids were attached starting from the C-terminal end and ending with the N-terminal part of the peptide.

In this way, a peptide-carrier complex was obtained (Ac-Ala-Glu-Asp-Pro-Arg-Pro-CH-Resin). In the next step of the synthesis, the peptide was detached from the support using trifluoroacetic

acid (TFA). Amidation of the C-terminal amino acid occurs during cleavage of the peptide from the support. This resulted in a peptide of the assumed sequence Ac-Ala-Glu-Asp-Pro-Arg-amide (SEQ. ID 6).

To obtain a peptide with the assumed purity of >80%, High Performance Liquid Chromatography in the Reverse Phase System was used (RP-HPLC). The analytical stage was completed with ultra-high-performance liquid chromatography (UHPLC) and high-resolution mass spectrometry (QTOF-MS), which confirmed the molar mass of the synthesized peptide.

Detailed description of the synthesis

I. Peptide synthesis

The peptide was synthesized on a scale of 10 g

1. Preparation of Fmoc-Pro-CTC-resin

A weight of the Fmoc-Pro-CTC-resin carrier was placed in a chemical reactor, then DCM was added and left for 30 min. The DCM was removed and then the resin was rinsed with DMF under constant stirring. The operation was repeated twice, each time using DMF. After rinsing, the DMF solution was thoroughly removed.

2. Attachment of Arg to solid support Fmoc-Pro-CTC-Resin.

After preparing the solid phase, arginine was attached to it. Then, in the deprotection step, the Fmoc group was removed. DP was added with continuous stirring under a nitrogen atmosphere. After 5 min, DP was removed, then the carrier was rinsed once with DMF, which was completely removed in the next step. The deprotection step was repeated again with the addition of DP under continuous stirring under a continuous flow of nitrogen. DP was completely removed after 10 min. The support was washed 6 times, each time using DMF. Fmoc-Arg-OH (3-times the amount of the support used) and HOBt (3-times the amount of the support used) were weighed out and then dissolved in DMF in the reactor. In the next step of the synthesis, DIC was added (3-times the amount of the support used, v:v) with constant stirring under a constant supply of nitrogen for 2 hours. At the end of the step, a ninhydrin test was performed to ensure that Fmoc-Arg-OH is fully attached to the support Fmoc-Pro-CTC-Resin. A negative result of the nihydrin reaction means that there are no free NH2 groups, and thus the reaction is considered complete. The liquid was removed from the reactor then it washed with DMF 3

times. From this point on, the amino acid in the form of Fmoc-Arg-OH is linked to the support. Upon completion of the step, Fmoc-Arg-Pro-CTC-Resin was obtained.

3. Attachment Pro to -Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen. Removed DP after 5 min then rinsed once with DMF which was then removed. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 minutes then rinsed resin 6 times with DMF each time. Fmoc-Pro-OH was weighed out (3-times the amount of the support used) and HOBt (3-times the amount of the support used) then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. At the end of the step, a ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times using DMF each time. From then on, the amino acid in the form Fmoc-Pro-OH was attached to Arg-Pro-CTC-Resin. was obtained Fmoc-Pro-Arg-Pro-CTC-Resin.

4. Attachment Asp to -Pro-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen and then the DP was removed after 5 min and rinsed once with DMF which was removed in the next step. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. Removed DP after 10 minutes. The support was then washed 6 times, each time using DMF. Fmoc-Asp-OH was weighed out (3-times the amount of the support used) and HOBt (3-times the amount of the support used), dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. The ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed from the reactor then washed 3 times with DMF.

From then on, the amino acid in the form Fmoc-Asp-OH was attached to Pro-Arg-ProCTC-Resin. Fmoc-Asp-Pro-Arg-Pro-CTC-Resin was obtained.

5. Attachment Glu to Asp-Pro-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen, next DP was removed after 5 min and rinsed once with DMF, which was then removed from the reactor. The

deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min, the resin was then rinsed 6 times, each time using DMF. Fmoc-Glu-OH was weighed out (3-times the amount of the support used) and HOBt (3-times the amount of the support used), then it was dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. A ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-Glu-OH was attached to -Asp-Pro-Arg-Pro-CTC-Resin. As a result, was obtained Fmoc-Glu-Asp-Pro-Arg-Pro-CTC-Resin.

6. Attachment Ala to -Glu-Asp-Pro-Arg-Pro-CTC-Resin.

DP was added with constant stirring under a constant supply of nitrogen, next DP was removed after 5 min and rinsed once with DMF, which was then removed from the reactor. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min, the resin was then rinsed 6 times, each time using DMF. Fmoc-Ala-OH was weighed out (3-times the amount of the support used) and HOBt (3-times the amount of the support used), then it was dissolved in DMF and charged to the reactor.

DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. A ninhydrin test was performed to ensure that the reaction was complete. The liquid was removed and then washed 3 times with DMF. From then on, the amino acid in the form Fmoc-Ala-OH was attached to -Glu-Asp-Pro-Arg-ProCTC-Resin. As a result, was obtained Fmoc-Ala-Glu-Asp-Pro-Arg-Pro-CTC-Resin.

7. Acetylation of the N-terminal amino acid

DP was added with constant stirring under a constant supply of nitrogen, then DP was removed after 5 min and rinsed once with DMF which was then removed. The deprotection was repeated by adding DP with constant stirring under a constant supply of nitrogen. DP was removed after 10 min. The support was washed 6 times, each time using DMF. Prepared were acetic acid (3-times the amount of the support used) and HOBt (3-times the amount of the support used), dissolved in DMF and charged to the reactor. DIC was then added (3-times the amount of the support used) with constant stirring under a constant supply of nitrogen within 2 hours. The

liquid was removed from the reactor and then washed 3 times using DMF each time. was obtained Ac-Ala-Glu-Asp-Pro-Arg-Pro-CTC-Resin.

II. Detachment of the peptide from the support

After the amino acid attaching steps are completed, all peptide side chains are blocked and the peptide itself is attached to the support. In the next stage of the synthesis, the peptide has to be detached from the support and the side chains deblocked to obtain a crude peptide. Thanks to the use of a modified resin, spontaneous amidation of the C-terminal amino acid occurs during the cleavage of the peptide from the support. The obtained peptide-resin complex together with CS (cleaving solution) was then stirred for 3 hours in the reactor. At this stage, the peptide is released from the support and the side groups are deprotected. Upon completion, the solution was filtered discarding the solid phase and retaining the liquid phase containing the peptide. In the next step, the liquid was cooled using diethyl ether with constant stirring. The crude peptide precipitated as a white precipitate. The precipitate was filtered and then washed with cooled diethyl ether 4 times using diethyl ether each time. The white precipitate was dried in a vacuum oven R-1001-VN. This completes the synthesis of the crude peptide of the sequence Ac-Ala-Glu-Asp-Pro-Arg-amide (SEQ. ID 6).

III. Purification of the crude peptide by RP-HPLC

Two preparative liquid chromatographs were used to separate and purify the synthesis product: 1) ÄKTA™ Pure 25 with Frac F9-R fraction collector. The Kromasil® 100-13-C18 50 x 250 mm column allows the purification of up to 1-2 g of product in one process at maximum flow 25 mL/min; 2) CXTH P1001L. Vydac® column 70 x 150 mm, 10 mm filter allows to purify up to 50 g of product in one process at maximum flow 500 mL/min.

Analysis parameters:

Sample: purified peptide dissolved in water to concentration ~5 g/L;

Mobile phase: ACN:H_2O (17:83 v/v) with 0,1% TFA;

Flow: 25 mL/min / 500 mL/min

Analysis time: 30 min;

Column temperature: 25°C;

Detection conditions: λ = 220 nm.

IV. Lyophilization of the peptide

For lyophilization of the peptide, the solvent obtained during the HPLC paging process was evaporated and dried in a lyophilizer (Christ Beta 2-8) within 48-72 hours.

The collected white solid phase was the purified peptide.

V. Synthesis quality analysis

Peptide purity was checked by analytical HPLC, molar mass was confirmed by mass spectrometry as described below.

1. HPLC analysis to evaluate the purity of the obtained peptide.

To assess the purity of the purified peptide, HPLC-MS was measured using a liquid chromatograph UHPLC (UltiMate 3000 Thermo Scientific / Dionex) with a fast (200 Hz) diode detector coupled to QTOF-MS (Impact HD, Bruker)

Analysis parameters:

Sample: 10 µl dissolved in water (to a concentration of ~5 g/L) of the purified peptide;

Mobile phase: ACN:H_2O (17:83 v/v) with 0,1% TFA;

Flow: 1 mL/min;

Analysis time: 30 min;

Column temperature: 25°C;

Detection conditions: λ = 220 nm.

Purity obtained was 86%.

2. QTOF-MS analysis to identify the peptide

To accurately determine the molecular weight of the peptide, a high-resolution QTOF mass spectrometer, a hybrid quadrupole mass spectrometer with time-of-flight analysis was used - TOF, and ionization at atmospheric pressure - ESI (Impact HD, Bruker)

The sample was fed to the source by the syringe pump of the chromatograph. A small amount of the peptide was dissolved in MeOH.

Analysis parameters:

Sample: 10 µl dissolved in methanol (to a concentration of ~5 g/L) of the purified peptide;

Ionization: nitrogen laser beam, $\lambda = 337$ nm

Matrix: α-cyano-4-hydroxycinnamic acid (CCA).

The obtained Cortagen derivative of (SEQ. ID 6) was identified by the QTOF MS: Mass Calc. for C25H41N9O10: 627.65 g/mol, Found: 628.71 $[M+H]^+$

EXAMPLE 2 – Activity comparisons

Referenced peptides (parent peptides before modification) was purchased from Lipopharm (http://www.lipopharm.pl/pl/) or obtained by method know from the art (for example see https://www.biomatik.com/services/peptide-synthesis.html)

2.1 Comparison between BPC-157 and Ac-BPC-157-Arg-NH_2 (SEQ. ID 1)

Analysis was performed according to the disclosure of L.Bricic, M.Staresinic, T.Novinscak, P.Sikiric, S.Seiwert, Modulatory effect of gastric pentadecapeptide BPC 157 on angiogenesis in muscle and tendon healing. 2009, 7:191-6 [https://www.researchgate.net/profile/Luka-Brcic-2/publication/51443032_Modulatory_effect_of_gastric_pentadecapeptide_BPC_157_on_angiogenesis_in_muscle_and_tendon_healing/links/02bfe50d3a1 b8a7aeb0000/Modulators-effect-of-gastric-pentadecapeptide-BPC-157-on-angiogenesis-in-muscle- and tendon-healing.pdf]

Modulating effect of BPC-157 and Ac-BPC-157-Arg-NH_2 (SEQ. ID 1) peptides on angiogenesis in muscle and tendon healing was investigated in fibrin in vitro angiogenesis assay. The assay was obtained from (Sigma-Aldrich, Merck, Poland).

According to the manufacturer's instructions, the cells were treated with: pure medium, VEGF (Sigma-Aldrich, Merck, Poland) as a positive control (2 ng/ml), suramin (Sigma-Aldrich, Merck, Poland) as a negative control (20 µM) as well as BPC-157 and Ac-BPC-157-Arg-NH_2 (SEQ. ID 1) at two different concentrations (10 µg/ml and 2 µg/ml). Cell cultures were grown at 37°C and 5% CO_2 and stored in an incubator (Laboratory Incubator EX10031278 - 5 - 70°C; MODEL: SBS-LI-8N). After day 11, cell cultures were fixed, and tubules were visualized using

CD31 antibody (Sigma-Aldrich, Merck, Poland). Images of imaged tubules were observed under a microscope (Olympus CK40 Inverted Fluorescence Contrast Microscope Pred CKX53) and quantified using ImageJ software (ImageJ1.53u version). The angiogenic effect was assessed by counting the number of tubule branching in 5 fields of view (microscope objective x 100).

All values were presented as mean ± standard deviation, and statistically significant differences were determined by one-way ANOVA analysis using SPSS software (version 22.0, IBM Corp.). A $P < 0.05$ was considered to indicate a statistically significant difference.

The highest number of branching points of newly formed tubular structures, as expected, was in the positive control sample containing VEGF, as a factor typically promoting angiogenesis. Slightly fewer branching points of newly formed tubular structures, compared to the positive control were found in Ac-BPC-157-Arg-NH_2 (SEQ. ID 1) 10 ng/ml (61 branches) and Ac-BPC-157-Arg-NH_2 (SEQ. ID 1) 2 ng/ml (55 branches), whereas BPC-157 gave at 10 ng/ml 25 branches and at 2 ng/ml 21 branches. The negative control with suramin virtually completely inhibited branching.

As shown, Ac-BPC-157-Arg-NH_2 (SEQ. ID 1), through the formation of many newly formed tubular structures, supports the promotion of the process of angiogenesis and enhances the healing of damaged tissues, which proves its special angiogenic potential. The angiomodulatory effect of Ac-BPC-157-Arg-NH_2 (SEQ. ID 1) peptide thus leads to faster acceleration of healing of tissues, muscles, tendons, among others, and is helpful in accelerating recovery in comparison with BPC-157 itself.

2.2 Comparison between Epithalon and Ac-Epithalon-Arg-NH_2 (SEQ. ID 2)

Analysis was performed according to the disclosure of V.Khavinson, E.Bondarev, A.Butyugov, Epithalon peptide induces telomerase activity and telomere elongation in human somatic cells. 2003, DOI: 10.1023/a:1025493705728 [https://pubmed.ncbi.nlm.nih.gov/12937682/]

The purpose of the study was to evaluate the effect of Epithalon and Ac-Epithalon-Arg-NH_2 (SEQ. ID 2) on the expression of the catalytic subunit, telomerase enzymatic activity and telomere elongation, which may be due to the reactivation of the telomerase gene in somatic cells, this indicates the possibility of extending the life of the cell population of the whole organism.

Material and Methods:

Cell culture

Telomerase-positive HeLa and HEL 12469 as primary cultures of human fetal lung fibroblasts were obtained from Sigma-Aldrich, Merck, Poland. Cells were cultured in DMEM with 10% fetal calf serum, 2 mM L-glutamine and 100 µg/ml gentamicin sulfate. Starting from passaging, fetal fibroblasts were treated with Epithalon or Ac-Epithalon-Arg-NH2 (SEQ. ID 2) at a concentration of 0.05 µg/ml for 4 days and analysed. To detect the catalytic subunit of telomerase protein, fibroblasts were placed on sterile slides. Immunohistochemical staining was performed using mouse monoclonal antibodies against the human telomerase catalytic subunit NCL-hTERT. Universal ABC peroxidase kit served as a second antibody. Preparations were stained according to the manufacturer's instructions. Analysis was performed behind a microscope (Olympus CK40 Contrast Microscope with Pred CKX53 Inverted Fluorescence). Telomerase activity was assessed according to the telomere repeat amplification protocol (TRAP). Cells were washed with cold buffered saline (PBS) and lysed for 30 minutes in 100µl cold CHAPS buffer (10% glycerol, 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.5% CHAPS, 0.1 mM PMSP and 5 mM 2-mercaptoethanol).

Incubation

The extracts were centrifuged for 30 minutes at 12,000 rpm and 4 degrees C in a centrifuge (laboratory centrifuge EX10031235, Model: SBS-LAB-108). Supernatants were transferred to tubes and stored at -70 degrees Celsius in an incubator (laboratory incubator EX10031278 - 5 - 70 degrees C; MODEL: SBS-LI-8N). For in vitro analysis of telomerase activity, up to 46 µl of 1 Taq buffer containing 2 µM TS primer (5'-AAT CCG TCG AGC AGA GTT-3'), 50 µM dNTPs were added to 2 µl of supernatant and incubated at 30 degrees Celsius for 30 min. Then 2 µM CX primer (5'- CCC TTA CCC TTA CCC TTA CCC TTA CCC TTA-3')5 U Taq-DNA and polymerase (Promega) were added.

PCR

Thirty-five PCR cycles were performed (Bio-Rad Experion Electrophoresis Station + Vortex Station II + Priming Station electrophoresis kit) with the following parameters: 1 min at 94 degrees C, 1 min at 50 degrees C, 1.5 min at 72 degrees C on a DNA thermocycler with a

heating lid. PCR products were separated electrophoretically in a 10% non-denaturing polyacrylamide gel, stained with SYBR Green and visualized under UV light.

Flow cytometry

The average telomere length of individual cells was measured by fluorescence in situ hybridization with flow cytofluorometry (BD C6 Accuri Flow Cytometer with Sortable Cell Population (BD C6 Accuri Flow Cytometer 2 Laser- The BD system consists of the cytometer, the optional BD FACS Universal Loader (feeder) and software running on the system workstation. The system also includes control beads and reagents. The combination of the aforementioned components creates an integrated system for use in a wide range of clinical applications). For this purpose, cells were washed twice in PBS containing 0.2% BSA and resuspended in a hybridization solution containing 70% formamide, 1.0% BSA, 20 mM Tris-HCl (pH 7.0) and 0.3 µg/ml telomerase-specific C3 TA2 probe labeled with FITC peptide-nucleic acid (PNA). The samples were heat denatured at 80 degrees C for 10 minutes, followed by hybridization at 20 degrees C for 2 hours in the dark. The cell suspension was then centrifuged in a centrifuge (Laboratory Centrifuge EX10031235, Model: SBS-LAB-108), the supernatants were again transferred, and the cells were washed twice in buffer containing 70% formamide, 10 mM Tris-HCl (pH 7.0), 0.1 BSA, 0.1% Twin-20, and once in PBS containing 0.1% BSA and 0.1% Tween-20. After washing, the cells were resuspended in PBS containing 0.1% BSA, 10 µg/ml RNase A (Sigma), 0.06 µg/ml propidium iodide and incubated at 20 degrees C in the dark for 2 h. Control hybridization without PNA was performed to detect basal fluorescence and estimate telomere-specific fluorescence in arbitrary units. Cells were analysed by flow cytometry (flow cytometer with cell population sorting capability (BD C6 Accuri Flow Cytometer 2 Laser).

Results:

Addition of Epithalon or Ac-Epithalon-Arg-NH2 (SEQ. ID 2) to cultures of telomerase-negative human fetal fibroblasts induced expression of the catalytic subunit, telomerase enzymatic activity and telomere elongation, which may be due to reactivation of the telomerase gene in somatic cells and indicates the possibility of extending the lifespan of the whole organism cell population.

Telomerase activity and telomere elongation was 41.2% on average for Ac-Epithalon-Arg-NH_2 (SEQ. ID 2), and for Epithalon 33.3% on average. Since telomere shortening is known to affect, among other things, aging of the body, as well as the development of diseases (including cancer), the action of the Ac-Epithalon-Arg-NH_2 (SEQ. ID 2), which inhibits telomere shortening and thus inhibits the development of diseases and premature aging of the body, turns out to be important, and its effect is much higher than for Epithalon.

2.3 Comparison between GHK, GHK-Cu and Ac-GHK-Arg-NH_2 (SEQ. ID 2)

Effect of regeneration of macrister cells in the skin by GHK, GHK-Cu and Ac-GHK-Arg-NH_2 (SEQ. ID 3). The purpose of the study was to show the effect of Ac-GHK-Arg-NH_2 (SEQ. ID 3), GHK-Cu and copper-free GHK peptide on stem cell regeneration in the skin.

Cell culture

Fibroblasts and keranocytes for cell culture were purchased from Sigma-Aldrich, Merck, Poland. Keratinocytes were cultured in keratinocyte growth medium, and fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Reagents were purchased from MERCK (SIGMA-ALDRICH).

MTT test

To test the cytotoxicity of GHK, GHK-Cu and Ac-GHK-Arg-NH_2 (SEQ. ID 3), the cells (4×10^4 cells/well) were seeded into 24-well plates. After 24-hour serum treatment, cells were incubated in an incubator (Laboratory Incubator EX10031278 - 5 - 70°C; MODEL: SBS-LI-8N) with GHK, GHK-Cu and Ac-GHK-Arg-NH2 (SEQ. ID 3) at concentrations of 0.1, 1 and 10 mM for 24 hours at 37°C. Then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT solution; 100 ml 5 mg/ml) was added, after which the plates were incubated for an additional 4 hours. The supernatant was then removed, and the formazan crystals were dissolved in dimethyl sulfoxide (1 ml). The optical density was then determined at 540 nm using an ELISA reader (HiPo MPP-96).

Keratinocyte proliferation test

To test the proliferative effects of GHK, GHK-Cu and Ac-GHK-Arg-NH_2 (SEQ. ID 3) on keratinocytes, cells were incubated with 0.1-100 mM GHK for 72 hours at 37°C. In addition, a long-term proliferation assay was performed. Second-stage keratinocytes were cultured in

KGM and GHK, GHK-Cu and Ac-GHK-Arg-NH_2 (SEQ. ID 3) were added at a concentration of 1 mM. When the cells reached about 80% confluence, they were detached and cultured in new dishes. Each time samples were split, the total number of cells was determined using a hemocytometer.

Comet test

Keratinocytes (1×10^6 cells) were placed in a 100 mm diameter culture plate. After 24 hours, the cells were starved for another 24 hours. Cells were treated with or without GHK, GHK-Cu and Ac-GHK-Arg-NH_2 (SEQ. ID 3) (1 mM) for 4 hours. Next, a comet assay was performed, where cells placed in a thin layer of gel and stained with DAPI or a mixture of propidium iodide (JP) and RNAase are electrophoresed. 2.6. Statistical analysis

Results

The cultured cells were treated with GHK, GHK-Cu and Ac-GHK-Arg-NH_2 (SEQ. ID 3) at concentrations of 0.1, 1 and 10 mM for 24 hours, and then analysed by MTT assay. The results showed that none of the GHK, GHK-Cu and Ac-GHK-Arg-NH_2 (SEQ. ID 3) peptides were cytotoxic to keratinocytes or fibroblasts in this concentration range.

Proliferative effects were tested by adding 0.1, 1.0 ,10 and 100 mM GHK, GHK-Cu and Ac-GHK-Arg-NH_2 (SEQ. ID 3). The results revealed a dose-dependent stimulatory effect in response to GHK, GHK-Cu and Ac-GHK-Arg-NH_2 (SEQ. ID 3) treatment. In the case of Ac-GHK-Arg-NH_2 (SEQ. ID 3), the peptide already acts at low doses, and the effect is maintained at higher concentrations, with no toxic or deleterious effect.

Long-term proliferation assays were then carried out using concentrations of 1 mM GHK, GHK-Cu and Ac-GHK-Arg-NH_2 (SEQ. ID 3). Application of GHK increased the number of keratinocytes, compared to control cells by 260 times. Application of GHK-Cu increased the number of keratinocytes, compared to control cells by 320 times. Application of Ac-GHK-Arg-NH_2 (SEQ. ID 3) increased the number of keratinocytes, compared to control cells by 360 times.

The results showed that treatment of cultures with GHK increased fibroblast proliferation, but to a lesser extent than with GHK-Cu and Ac-GHK-Arg-NH_2 (SEQ. ID 3). The results showed the greatest potential for the Ac-GHK-Arg-NH_2 (SEQ. ID 3) peptide.

2.4 Comparison between Semax and Ac-Semax-Arg-NH_2 (SEQ. ID 4)

Analysis was performed according to the disclosure of O.Dolotov, K.O.Eremin, Semax prevents the death of tyrosine hydroxylase-positive neurons in a mixed neuroglial cell culture derived from the embryonic rat mesencephalon in a model of 6-hydroxydopamine-induced neurotoxicity. 2015, DOI:10.1134/S1819712415040066 [https://link.springer.com/article/10.1134/S1819712415040066]

Here, we investigated the ability of Semax and Ac-Semax-Arg-NH2 (SEQ. ID 4) to prevent the death of tyrosine hydroxylase-positive neurons in a primary mixed culture of neuroglial cells.

Cell culture

A purchased glioblastoma multiforme cell line (Sigma Aldrich, Merck, Poland) was placed in medium containing DMEM: F12 (Sigma-Aldrich, Merck, Poland) in a 1:1 ratio and 15% fetal calf serum (Sigma Aldrich, Merck, Poland). Cells were seeded into poly-L-lysine-coated 48-well plates at a density of 200,000 cells per well; each well contained 500 µL of culture medium consisting of DMEM : F12 in a 1:1 ratio, 15 mM HEPES, 10% FCS, 10% horse serum, 1.2 g/L NaHCO3, 6 g/L D-glucose, 0.665 g/L glutamine, 0.1 g/L transferrin, 0.025 g/L insulin, 100 nM putrescine, 30 nM selenite and 20 nM progesterone. Cells were cultured in an incubator (Laboratory Incubator EX10031278 - 5 - 70°C; MODEL: SBS-LI-8N) with CO2 at 37°C, 5% CO2 and 100% relative humidity.

Preparations used in cell culture.

On day 5, neurotoxin 6-OHDA (6-Hydroxydopamine hydrochloride) (Sigma Aldrich, Merck, Poland) was applied to cell culture at various concentrations (2 and 5 µM) in a volume of 1% to the total volume of medium (500 µL). To the samples, before the application of 6-OHDA to the cell culture, Semax or Ac-Semax-Arg-NH2 (SEQ. ID 4) at a concentration of 0.1 µM 24 hours before 6-OHDA, at a concentration of 2 µM (on day 4 of in vitro culture) and 30 minutes before 6-OHDA at a concentration of 2 µM (on day 5 of in vitro culture), and Semax or Ac-Semax-Arg-NH2 (SEQ. ID 4) at a concentration of 0,1 µM 24 hours before 6-OHDA at a concentration of 5 µM (on day 4 of in vitro culture) and 30 minutes before 6-OHDA at a concentration of 5 µM (on day 5 of in vitro culture) were added. In an additional condition, Semax or Ac-Semax-Arg-NH_2 (SEQ. ID 4) was added to the cell culture, but no neurotoxin was added.

Schedule of adding preparation was as follows:

Day 1,2 and 3- incubation

Day 4: Semax or Ac-Semax-Arg-NH2 (SEQ. ID 4) at a concentration of 0.1 μM was added to the cell culture (24 hours before 6- OHDA)

Day 5: Semax or Ac-Semax-Arg-NH2 (SEQ. ID 4) was added to the cell culture at a concentration of 0.1 μM (30 minutes before 6- OHDA)

Day 5: 6-OHDA neurotoxin was added to the cell culture at two concentrations of 2 μM and 5 μM

This created the following groups:

Semax or Ac-Semax-Arg-NH_2 (SEQ. ID 4) (0.1 μM) +neurotoxin (2 μM) 24 hours before 6-OHDA

Semax or Ac-Semax-Arg-NH_2 (SEQ. ID 4) (0.1 μM) +neurotoxin (2 μM) 30 minutes before 6-OHDA

Semax or Ac-Semax-Arg-NH_2 (SEQ. ID 4) (0.1 μM) +neurotoxin (5 μM) 24 hours before 6-OHDA

Semax or Ac-Semax-Arg-NH_2 (SEQ. ID 4) (0.1 μM) +neurotoxin (5 μM) 30 minutes before 6-OHDA

Semax or Ac-Semax-Arg-NH_2 (SEQ. ID 4) (0.1 μM) added to cell culture

Immunocytochemical detection of tyrosine hydroxylase and cell number

Twenty-four hours after treatment with 6-OHDA (6-Hydroxydopamine hydrochloride), the cells were fixed in 4% paraformaldehyde, which was dissolved in phosphate-buffered saline (PBS) for 1 hour and then washed with PBS. The fixed cells were placed in an incubator (laboratory incubator EX10031278 - 5 - 70°C; MODEL: SBS-LI-8N) in 200 μL of incubation buffer, which consisted of 2% horse serum, 0.1% Triton X100 (Sigma-Aldrich,Merck, Poland) and monoclonal antibodies against hydroxylase hydroxylase in a ratio of 1 : 5000 dissolved in PBS at 4°C overnight. The samples were then washed three times with PBS containing 0.1% Triton X100 and incubated with biotinylated anti-mouse IgG antibodies dissolved in incubation buffer at room temperature for 2 hours. After an additional wash with PBS containing 0.1%

Triton X100, the cells were incubated with peroxidase conjugated avidin-biotin complex at room temperature for 1 hour. The cells were then stained with 0.2% 3 amino-9-ethyl cellulose, and the development of the staining was observed. The reaction was stopped by 3-4 washes with distilled water. The number of tyrosine hydroxylases (TH) was counted using a microscope (Olympus CK40 Inverted Fluorescence Contrast Microscope Pred CKX53) at 200× magnification.

Results

When the neurotoxin 6-OHDA (6-Hydroxydopamine hydrochloride) was added to the cell culture at different concentrations (2 and 5 μM), there was a reduction in the number of TH-positive neurons in primary in neuroglial cultures derived from purchased material.

Application of Ac-Semax-Arg-NH_2 (SEQ. ID 4) at a concentration of 0.1 μM to the culture medium 30 min before application of 5 μM 6-OHDA significantly increased the survival rate of TH-positive neurons from 30-35% to 86% compared to the control.

Application of Ac-Semax-Arg-NH_2 (SEQ. ID 4) a concentration of 0.1 μM to the culture medium 30 min before application of 2 μM 6-OHDA significantly increased the survival rate of TH-positive neurons from 30-35% to 75% compared to the control.

Application of Ac-Semax-Arg-NH_2 (SEQ. ID 4) at a concentration of 0.1 μM to the culture medium 24 h before application of 5 μM 6-OHDA significantly increased the survival rate of TH-positive neurons from 30-35% to 55% compared to the control.

Application of Ac-Semax-Arg-NH_2 (SEQ. ID 4) at a concentration of 0.1 μM to the culture medium 24 h before application of 2 μM 6-OHDA significantly increased the survival rate of TH-positive neurons from 30-35% to 42% compared to the control.

For Semax the results were as follows: 70%, very slight increase in the number of TH positive neurons, very slight increase in the number of TH positive neurons, very slight increase in the number of TH positive neurons, respectively.

Conclusions of the study:

Based on the results obtained, the neuroprotective effect of Ac-Semax-Arg-NH_2 (SEQ. ID 4) was proven, during the risk of neuronal loss due to neurotoxins. This demonstrates the peptide's potential to protect neurons and thus protect the nervous system. The study indicates that Ac-

Semax-Arg-NH_2 (SEQ. ID 4) can be used to clinically slow the risk of neurodegenerative diseases and performs better that Semax itself.

2.5 Comparison between Selank and Ac-Selank-Arg-NH_2 (SEQ. ID 5)

Analysis was performed according to the disclosure of I.Povarov, R.Kondratenko, V.Derevyagin, Effect of Selank on Spontaneous Synaptic Activity of Rat Hippocampal CA1 Neurons. 2016, DOI 10.1007/s10517-017-3676-3 [https://pubmed.ncbi.nlm.nih.gov/28361410/]

The aim of the study was to compare the effect of Selank and Ac-Selank-Arg-NH_2 (SEQ. ID 5) peptides, on hippocampal CA1 pyramidal neurons, whereby neuronal protection and improvement in neuronal function occurs.

Cell culture

Experiments were conducted on hippocampal cells (300 μ) derived from rat brain. Cells were incubated for 2 hours at room temperature in saline containing (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 D-glucose. The pH was adjusted to 7.4 in an atmosphere of 95% O_2 and 5% CO_2. Cells from the hippocampus were observed visually with a lens immersed in water, under transmitted light at a magnification of ×40. The electrophysiological parameters of pyramidal neurons in the patch clamp EPSC assay were recorded using the whole-cell voltage clamp technique at a holding potential of -70 mV. The recording pipette was filled with a solution containing (in mM): 120 KCl, 5 NaCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 10 EGTA, 5 HEPES and 5 HEPES-Na. The experiments used neurons that did not spontaneously discharge, had a resting potential of no less than -55 mV and an access resistance of ≤30 MΩ. Neurons with access resistance fluctuations of more than 30% were excluded. Short-term (<300 ms) spontaneous postsynaptic currents (PSCs) were analysed off-line. Peak amplitude and discharge rate of PSCs were calculated using Minhee Analysis Package software includes two independent programs, Minhee Analysis and Retriever. Initially, the control recordings gave the peak amplitude and discharge rate of the PSC before applying Selank or Ac-Selank-Arg-NH_2 (SEQ. ID 5). The same recordings were then made with Selank or Ac-Selank-Arg-NH_2 (SEQ. ID 5) applied at concentrations of 1, 2, 4, 6 and 8 μM. Finally, PSCs were recorded after washing the cells. In some experiments, TTX was used to block sodium channels.

Results

Spontaneous synaptic activity of hippocampal CA1 neurons consists mainly of inhibitory postsynaptic currents (potentials) resulting from the discharge of numerous inhibitory interneurons whose axons terminate in pyramidal cells. Our study showed that spontaneous PSCs recorded in CA1 neurons reversed at 0 mV, as did the Selank peptide, and were therefore referred to as inhibitory postsynaptic currents (IPSCs).

We recorded spontaneous IPSCs of hippocampal CA1 neurons before and after Ac-Selank-Arg-NH_2 (SEQ. ID 5) treatment. In most neurons, Ac-Selank-Arg-NH_2 (SEQ. ID 5) increased the peak amplitude and discharge rate of IPSCs, and these effects were not preceded by short-term decreases in both parameters. In some neurons (n = 5), the regulatory effects were preceded by a brief (1-1.5 min) decrease in both parameters. The effect of the peptide on hippocampal CA1 neurons in Ac-Selank-Arg-NH_2 (SEQ. ID 5) appeared at 3 minutes after its addition to saline for perfusion.

In the case of Selank itself, peak amplitude and discharge rate of IPSC were, however, preceded with brief (1-1.5 min) drops in both parameters.

Ac-Selank-Arg-NH_2 (SEQ. ID 5) was added at concentrations of 1, 2, 4, 6 and 8 μM, and in the case of this peptide, the higher the dose, the stronger its effect.

4.Conclusions of the study:

Our study provided evidence that Ac-Selank-Arg-NH_2 (SEQ. ID 5) peptide enhances the inhibition of hippocampal CA1 pyramidal neurons by interneurons, and that this effect is rapid, as well as lasting longer. Therefore, it can be concluded that with the help of Ac-Selank-Arg-NH_2 (SEQ. ID 5) peptide there is protection of neurons in the hippocampal area, which helps to protect cells responsible for memory, concentration, wakefulness and locomotion, among other things. In addition, with the traditional Ac-Selank-Arg-NH_2 (SEQ. ID 5) peptide it is possible to control the dose of the effect one wants to achieve, making its use safer.

2.6 Comparison between Cortagen and Ac-Cortagen-Arg-NH_2 (SEQ. ID 6)

The aim of the study was to investigate the neuroprotective effects of Cortagen and Ac-Cortagen-Arg-NH_2 (SEQ. ID 6) peptide. The study was conducted using cell culture.

Cell culture

Rat cortical neurons (Sigma-Aldrich, Merck, Poland) were cultured in petri dishes (9015 mm petri dish) in 3 ml of medium. The medium contained 35% Eagle's medium, 35% Hanks solution, 25% fetal calf serum, 0.6% glucose, 0.5 U/ml insulin and 100 U/ml gentamicin. The cortical neuronal cells were cultured for 3 days in an incubator (Laboratory Incubator EX10031278 - 5 - 70°C; MODEL: SBS-LI-8N) at 36.7 degrees Celsius and 5% CO_2. The cultures were divided into 3 groups: control (with saline) and two experimental groups with Cortagen and Ac-Cortagen-Arg-NH2 (SEQ. ID 6). Peptides (Cortagen and Ac-Cortagen-Arg-NH2 (SEQ. ID 6)) were added to cortical neuron cultures at concentrations of 0.01, 0.05, 1.0, 10, 20, 50, and 100 ng/ml.

Tested groups:

i. Control with the addition of saline

ii. Experimental group: Cortagen at concentrations of 0.01, 0.05, 1.0, 10, 20, 50, and 100 ng/ml

iii. Experimental group: Ac-Cortagen-Arg-NH_2 (SEQ. ID 6) at concentrations of 0.01, 0.05, 1.0, 10, 20, 50, and 100 ng/ml

Assessment of proliferation

To assess proliferation in the cultures, index area (AI) was calculated as the ratio of the total area of the explant (including the zone of migrating cells) to the area of the central zone (in %). Explants were visualized using a micro-TV attachment (Series 10, MTN-13, Alpha- Telecom) AI was calculated using PhotoM 1.2 software. 20-25 experimental and 20-23 control explants were analysed for each substance.

Expression evaluation

To assess the expression of signaling molecules, cultures were fixed with cold (-20 degrees Celsius) 96% ethanol and mobilized with 0.5% Triton X-100. Immunocytochemical staining of cortical cells was performed using primary monoclonal antibodies against Ki-67.

Immunocytochemical analysis

The results of immunocytochemical analysis were evaluated morphometrically using a computer assisted microscope (Olympus CK40 Pred CKX53 Inverted Fluorescence Contrast

Microscope) at 200× magnification. The relative area of marker expression was calculated as the ratio of the area occupied by immuno-positive cells to the total area of cells in the field of view and expressed in %, and the statistics are tabulated for comparison.

Real-time quantitative PCR

A quantitative real-time PCR reaction was performed using gene expression products. The PCR reaction consisted of 4 phases. The first phase, the maintenance phase was conducted for 10 minutes at 95 °C. The second phase consisted of 40 PCR cycles, conducted for 25 seconds at 95 °C and for 1 minute at 60 °C. The melting curve is a phase, carried out initially at 95 °C, then at 60 °C for one minute and finished at 95 °C. The final cooling phase was carried out at 37 °C for 30 seconds.

Results:

Cortagen exerted a stimulating effect on AI at low concentrations, especially at a concentration of 1.0 ng/ml, which has a stimulating effect on cortical cell growth.

Ac-Cortagen-Arg-NH_2 (SEQ. ID 6) exerted a stimulating effect on AI already at the lowest concentrations of 0.05 and 1.0 ng/ml, and in addition, this effect was maintained at high concentrations of 20 and 50 ng/ml, leading to its superiority in action over the Cortagen peptide.

In the next stage of the experiment, the significant results for the study were compared, which involved both Cortagen peptide and Ac-Cortagen-Arg-NH_2 (SEQ. ID 6) peptide. By immunohistochemical analysis of Ki-67 expression, Cortagen peptide increased the expression area of the transcription factor Ki-67 in cortical neurons by more than 2-fold at concentrations of 1.0 ng/ml and 10 ng/ml, while Ac-Cortagen-Arg-NH2 (SEQ. ID 6) increased it by almost 3-fold already at low concentrations of 0.05 and 1.0 ng/ml. The high expression area of the transcription factor Ki-67, because of the Ac-Cortagen-Arg-NH_2 (SEQ. ID 6) peptide, was maintained in cortical neurons up to a concentration of 50 ng/ml.

Conclusions of the study:

The neuroprotective effect of Ac-Cortagen-Arg-NH_2 (SEQ. ID 6) peptide on neurons in cell cultures has been proven. The results suggest that Ac-Cortagen-Arg-NH_2 (SEQ. ID 6) peptide acts as a neuroprotective agent and outperforms the action of traditional Cortagen peptide, as its effect is proven already at low concentrations and maintained at higher concentrations. The

action of the Ac-Cortagen-Arg-NH2 (SEQ. ID 6) peptide bioregulator may thus promote the restoration of CNS function by stimulating effects on repair processes in the brain.

EXAMPLE 3 – Stability comparisons

3.1 Comparison between BPC-157 and Ac-BPC-157-Arg-NH_2 (SEQ. ID 1)

Instruments

The analyses were performed using a Perkin Elmer 200 series liquid chromatograph with a pump and an autosampler with a detector UV/Vis LC-235 with XSelect Peptide CSH C-18 column from Waters: 130 Å; 3,5 µm; 4,6*100 mm. Wavelength 230 nm.

Pump operating parameters:

Stage	Time [min]	Flow [mL/min]	A [% v/v]	B [% v/v]	Gradient
0	10	0.700	10	90	0
1	4	0.700	10	90	0
2	10	0.700	50	50	1
3	6	0.700	80	20	0
4	10	0.700	100	0	0

A – acetonitrile + 0,1% v/v trifluoroacetic acid

B – water + 0,1% trifluoroacetic acid

Analyses for BPC-157 and Ac-BPC-157-Arg-NH_2 (SEQ. ID 1) **in a medium stimulating gastric fluid**

For a solution simulating human gastric juice the following solution was prepared:

To prepare 1000 ml of working solution, perform the following steps:

1) Dissolve 1 g of BPC-157 or Ac-BPC-157-Arg-NH_2 (SEQ. ID 1) in 100 ml of demineralized water - concentration 10 mg/ml

2) 3.5 g of NaCl in 500 ml of demineralized water

3) Dissolve 1g of pepsin in 100 ml of demineralized water

4) make up to 900 ml with demineralized water.

5) 100 ml of 0.01 mol/l HCl solution (Supelco, Cat. No. 1.09974)

The concentration of [H] ions in the solution prepared in this way is 0.001 mol/l, which corresponds to about pH 3.

Concentration of BPC-157 and Ac-BPC-157-Arg-NH_2 (SEQ. ID 1) of 1 mg/mL.

Test was performed at 36°C.

Table E1. Summary of the content of the analysed compounds at about pH 3.0

Time	BPC-157		Ac-BPC-157-Arg-NH_2 (SEQ. ID 1)	
[h]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	7605185	100	6126431	100
1	7296560	95	6003902	98
2	5573973	73	5820109	95
4	2553494	33	5697581	93
6	906669	12	5452524	89

Analyses for BPC-157 and Ac-BPC-157-Arg-NH_2 (SEQ. ID 1) in a medium of about pH 5

Solution was prepared:

To prepare 1000 ml of working solution, perform the following steps:

1) Dissolve 1 g of BPC-157 or Ac-BPC-157-Arg-NH_2 (SEQ. ID 1) in 100 ml of demineralized water - concentration 10 mg/ml

2) 3.5 g of NaCl in 500 ml of demineralized water

3) Dissolve 1g of pepsin in 100 ml of demineralized water

4) make up to 900 ml with demineralized water.

5) 1 ml of 0.01 mol/l HCl solution (Supelco, Cat. No. 1.09974)

The concentration of [H] ions in the solution prepared in this way is 0,00001 mol/l, which corresponds to about pH 5.

Table E2. Summary of the content of the analysed compounds at about pH 5

Time	BPC-157		Ac-BPC-157-Arg-NH_2 (SEQ. ID 1)	
[h]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	7605185	100,0	6126431	100,0
1 H	6234299	82,0	6101925	99,6
2 H	4574987	60,0	6101864	99,6
4 H	1718904	23,0	6034535	98,5
6 H	566855	7,5	6003902	98,0

Lyophilizate stability during storage

Lyophilizate samples sealed in sealed vials were stored for 365 days at 35°C and standard humidity. Analyses were performed at intervals of 1 month.

Table E3. Summary of the content of the analysed compounds within 0-360 days

Time	BPC-157		Ac-BPC-157-Arg-NH_2 (SEQ. ID 1)	
[days]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	7584032	100	5996293	100
30	7550505	98	5989517	100
60	7480053	99	6056439	100
90	7401569	98	5924477	100

120	7219322	95	5908081	100
150	7062133	93	5941661	100
180	6814163	89	6041008	100
210	6787286	89	5978822	100
240	6731186	88	6039604	100
270	6489294	85	6126431	100
300	6259843	82	6086606	100
330	6087746	80	5986445	100
360	5293504	70	6115984	100

Stability of aqueous solutions during storage

The stability study of the compounds was performed by analyzing the solutions BPC-157 and Ac-BPC-157-Arg-NH_2 (SEQ. ID 1) prepared as follows:

Peptide solutions were prepared of concentration 1 mg/mL in demineralized water having conductivity of 0.07µS (pH about 5,6; no enzyme). The prepared solutions were stored under controlled conditions at 40 °C and humidity 55 %, tests were performed at equal time intervals.

Table E4. Summary of the content of the analyzed compounds in aqueous solution during storage for 0-20 days

Time	BPC-157		Ac-BPC-157-Arg-NH_2 (SEQ. ID 1)	
[days]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	7445317	100	6027022	100,00
1	7377915	99	6021549	99,90
4	7037314	94	6008720	99,69

8	5589223	75	6006954	99,66
12	2380462	31	5973183	99,10
20	224026	3	5096491	84,56

2.2. Comparison between GHK-Cu and Ac-GHK-ARG-NH$_2$ (SEQ. ID 3)

Instruments

The analyses were performed using a Perkin Elmer 200 series liquid chromatograph with a pump and an autosampler with a detector UV/Vis LC-235 using column: XBridge C18 from Waters: 130 Å; 3,5 µm; 4,0*150 mm. Wavelength 230 nm.

Pump operating parameters:

Stage	Time [min]	Flow [mL/min]	A [% v/v]	B [% v/v]	Gradient
0		0,800	95	5	0

A – acetonitrile + 0,1% v/v trifluoroacetic acid

B – water + 0,1% trifluoroacetic acid

Analyses for GHK-Cu and Ac-GHK-ARG-NH$_2$ (SEQ. ID 3) **in a medium stimulating gastric fluid**

For a solution simulating human gastric juice the following solution was prepared:

To prepare 1000 ml of working solution, perform the following steps:

1) Dissolve 1 g of GHK-Cu or Ac-GHK-ARG-NH$_2$ (SEQ. ID 3) in 100 ml of demineralized water - concentration 10 mg/ml

2) 3.5 g of NaCl in 500 ml of demineralized water

3) Dissolve 1g of pepsin in 100 ml of demineralized water

4) make up to 900 ml with demineralized water.

5) 100 ml of 0.01 mol/l HCl solution (Supelco, Cat. No. 1.09974)

The concentration of [H] ions in the solution prepared in this way is 0.001 mol/l, which corresponds to about pH 3.

Concentration of GHK-Cu and Ac-GHK-ARG-NH_2 (SEQ. ID 3) of 1 mg/mL.

Test was performed at 36°C.

Table E5. Summary of the content of the analysed compounds at pH 3.0

Time	GHK-Cu		Ac-GHK-ARG-NH_2 (SEQ. ID 3)	
[h]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	10082161	100	7097500	100
1	8187813	81	6823181	96
2	3911486	39	6111609	86
4	875224	8,7	5983583	84
6	9680	1,1	5409885	76

Analyses for GHK-Cu and Ac-GHK-ARG-NH_2 (SEQ. ID 3) in a medium of about pH 5

Solution was prepared:

To prepare 1000 ml of working solution, perform the following steps:

1) Dissolve 1 g of GHK-Cu or Ac-GHK-ARG-NH_2 (SEQ. ID 3) in 100 ml of demineralized water - concentration 10 mg/ml

2) 3.5 g of NaCl in 500 ml of demineralized water

3) Dissolve 1g of pepsin in 100 ml of demineralized water

4) make up to 900 ml with demineralized water.

5) 1 ml of 0.01 mol/l HCl solution (Supelco, Cat. No. 1.09974)

The concentration of [H] ions in the solution prepared in this way is 0,00001 mol/l, which corresponds to about pH 5.

Test was performed at 36°C.

Table E6. Summary of the content of the analysed compounds at about pH 5

Time	GHK-Cu		Ac-GHK-ARG-NH_2 (SEQ. ID 3)	
[h]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	10082161	100	7097500	100
1 H	9080305	90	6947755	98
2 H	4385806	43,5	6686095	94
4 H	2223187	22	6025102	85
6 H	232239	2,3	5656931	80

Lyophilizate stability during storage

Lyophilizate samples sealed in sealed vials were stored for 365 days at 35°C and standard humidity. Analyses were performed by opening successive vials at 1-month intervals.

Table E7. Summary of the content of the analysed compounds within 0-360 days

Time	GHK-Cu		Ac-GHK-ARG-NH_2 (SEQ. ID 3)	
[days]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
30	9980807	100	7204435	100
60	9862359	98,81	7194093	99,85
90	9837183	98,56	7200501	99,94
120	9633596	96,52	7153055	99,28
150	9037081	90,54	7188871	99,78
180	8862638	88,79	7203649	99,98
210	8337183	83,53	7156931	93,34

240	8034048	80,49	7114661	98,75
270	7883090	78,98	7179538	99,65
300	7703523	77,18	7104279	98,61
330	7504999	75,19	6999885	97,16
360	7219411	72,33	6883583	95,55

Stability of aqueous solutions during storage

The stability study of the compounds was performed by analyzing the solutions GHK-Cu i Ac-GHK-ARG-NH_2 (SEQ. ID 3) prepared as follows:

Peptide solutions were prepared of concentration 1 mg/mL in demineralized water (pH about 5,6). The prepared solutions were stored under controlled conditions at 40 °C and humidity 55 %, tests were performed at equal time intervals.

Table E8. Summary of the content of the analysed compounds in aqueous solution during storage for 0-20 days

Time	GHK-Cu		Ac-GHK-Arg-NH_2 (SEQ. ID 3)	
[days]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	9654048	100,00	7052894	100,00
1	9234558	95,65	7006141	99,33
4	7919411	82,03	6872050	97,44
8	6951039	72,00	5592050	79,29
12	5088509	52,71	5001943	70,92
20	3904999	40,45	4801969	68,08

3.2. Comparison between Epithalon and Ac-Epithalon-Arg-NH_2 (SEQ. ID 2)

Instruments

The analyses were performed using a Perkin Elmer 200 series liquid chromatograph with a pump and an autosampler with a UV/Vis LC-235 detector and a YMC Pack C4 column by YMC: 200 Å; 5µm; 4.6*250mm. Wavelength 214nm.

Pump operating parameters:

stage	Time [min]	Flow [mL/min]	A [% v/v]	B [% v/v]	Gradient
0	10	0,6	0	100	0
1	5	0,6	0	100	1
2	10	0,6	70	30	0
3	5	0,6	70	30	0

A – acetonitrile + 0,1% v/v trifluoroacetic acid

B – water + 0,1% trifluoroacetic acid

Analyses for Epithalon and Ac-Epithalon-Arg-NH_2 (SEQ. ID 2) in a medium stimulating gastric fluid

For a solution simulating human gastric juice the following solution was prepared:

To prepare 1000 ml of working solution, perform the following steps:

1) Dissolve 1 g of Epithalon or Ac-Epithalon-Arg-NH_2 (SEQ. ID 2) in 100 ml of demineralized water - concentration 10 mg/ml

2) 3.5 g of NaCl in 500 ml of demineralized water

3) Dissolve 1g of pepsin in 100 ml of demineralized water

4) make up to 900 ml with demineralized water.

5) 100 ml of 0.01 mol/l HCl solution (Supelco, Cat. No. 1.09974)

The concentration of [H] ions in the solution prepared in this way is 0.001 mol/l, which corresponds to about pH 3.

Concentration Epithalon and Ac-Epithalon-Arg-NH_2 (SEQ. ID 2) of 1 mg/mL

Test was performed at 36°C.

Table E9. Summary of the content of the analysed compounds at about pH 3.0

Time	Epithalon		Ac-Epithalon-Arg-NH_2 (SEQ. ID 2)	
[h]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	7665713	100	7590365	100
1	2843321	37,1	7441593	98
2	1063421	13,9	6989208	92
4	124581	1,6	6372970	84
6	0	0,0	5996388	79

Analyses for Epithalon and Ac-Epithalon-Arg-NH_2 (SEQ. ID 2) in a medium of about pH 5

Solution was prepared:

To prepare 1000 ml of working solution, perform the following steps:

1) Dissolve 1 g of Epithalon or Ac-Epithalon-ARG-NH_2 (SEQ. ID 2) in 100 ml of demineralized water - concentration 10 mg/ml

2) 3.5 g of NaCl in 500 ml of demineralized water

3) Dissolve 1g of pepsin in 100 ml of demineralized water

4) make up to 900 ml with demineralized water.

5) 1 ml of 0.01 mol/l HCl solution (Supelco, Cat. No. 1.09974)

The concentration of [H] ions in the solution prepared in this way is 0,00001 mol/l, which corresponds to about pH 5.

Test was performed at 36°C.

Table E10. Summary of the content of the analyzed compounds at about pH 5

Time	Epithalon	Ac-Epithalon-Arg-NH_2 (SEQ. ID 2)

[h]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	7665713	100	7590365	100
1 H	5734482	74,8	7441593	98
2 H	3321245	43,4	7212621	95
4 H	1288872	16,8	6148954	81
6 H	681267	8,9	5468098	72

Lyophilizate stability during storage

Lyophilizate samples sealed in sealed vials were stored for 365 days at 35°C and standard humidity. Analyses were performed by opening successive vials at 1-month intervals.

Table E11. Summary of the content of the analyzed compounds within 0-360 days

Time	Epithalon		Ac-Epithalon-Arg-NH_2 (SEQ. ID 2)	
[days]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	7603428	100	7442342	100
30	7603395	99,9	7442321	100
60	7536421	99,1	7440093	99,9
90	7553648	99,3	7428654	99,8
120	7325812	96,3	7383055	99,2
150	7053155	92,8	7331548	98,5
180	6831548	89,8	7293649	98,0
210	6584125	86,6	7157558	96,2

240	5734048	75,4	7114661	95,6
270	4883090	64,2	7010398	94,2
300	4458685	59,2	6805218	91,4
330	4050499	53,3	6328885	85,0
360	3452512	45,4	6065842	81,5

Stability of aqueous solutions during storage

The stability study of the compounds was performed by analyzing the solutions Epithalon i Ac-Epithalon-Arg-NH_2 (SEQ. ID 2) prepared as follows:

Peptide solutions were prepared of concentration 1 mg/mL in demineralized water (pH about 5,6). The prepared solutions were stored under controlled conditions at 40 °C and humidity 55 %, tests were performed at equal time intervals.

Table E12. Summary of the content of the analyzed compounds in aqueous solution during storage for 0-20 days

Time	Epithalon		Ac-Epithalon-Arg-NH_2 (SEQ. ID 2)	
[days]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	7654048	100,00	7452894	100,00
1	6234558	81.5	7016257	94,1
4	5119411	66,9	6336451	85,0
8	3551039	46,4	6038334	81,0
12	1288509	16,8	5965296	80
20	904999	11,8	5591161	75

3.4. ***Comparison between Semax and Ac-Semax-Arg-NH2*** (SEQ. ID 4)

Instruments

The analyses were performed using a Perkin Elmer 200 series liquid chromatograph with a pump and an autosampler with a detector UV/Vis LC-235 using column: YMC Pack C4 from YMC : 200 Å; 5 µm; 4.6*250 mm. Wavelength 214 nm.

Pump operating parameters:

Stage	Time [min]	Flow [mL/min]	A [% v/v]	B [% v/v]	Gradient
0	10	0,6	0	100	0
1	5	0,6	0	100	1
2	10	0,6	70	30	0
3	5	0,6	70	30	0

A – acetonitrile + 0,1% v/v trifluoroacetic acid

B – water + 0,1% trifluoroacetic acid

Analyses for Semax and Ac-Semax-Arg-NH2 (SEQ. ID 4) **in a medium stimulating gastric fluid**

For a solution simulating human gastric juice the following solution was prepared:

To prepare 1000 ml of working solution, perform the following steps:

1) Dissolve 1 g of Semax or Ac-Semax-Arg-NH2 (SEQ. ID 4) in 100 ml of demineralized water - concentration 10 mg/ml

2) 3.5 g of NaCl in 500 ml of demineralized water

3) Dissolve 1g of pepsin in 100 ml of demineralized water

4) make up to 900 ml with demineralized water.

5) 100 ml of 0.01 mol/l HCl solution (Supelco, Cat. No. 1.09974)

The concentration of [H] ions in the solution prepared in this way is 0.001 mol/l, which corresponds to about pH 3.

Concentration Semax and Ac-Semax-Arg-NH2 (SEQ. ID 4) of 1 mg/ml

Test was performed at 36°C.

Table E13. Summary of the content of the analysed compounds at about pH 3.0

Time	Semax		Ac-Semax-Arg-NH_2 (SEQ. ID 4)	
[h]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	3466079	100,0	3845621	100,0
1	2102542	60,7	3674106	95,5
2	1523485	43,9	3461058	90,0
4	124581	3,6	3313002	86,0
6	4261	0,0	3077265	80,0

Analyses for Semax and Ac-Semax-Arg-NH_2 (SEQ. ID 4) in a medium of about pH 5

Solution was prepared:

To prepare 1000 ml of working solution, perform the following steps:

1) Dissolve 1 g of Semax or Ac-Semax-Arg-NH_2 (SEQ. ID 4) in 100 ml of demineralized water - concentration 10 mg/ml

2) 3.5 g of NaCl in 500 ml of demineralized water

3) Dissolve 1g of pepsin in 100 ml of demineralized water

4) make up to 900 ml with demineralized water.

5) 1 ml of 0.01 mol/l HCl solution (Supelco, Cat. No. 1.09974)

The concentration of [H] ions in the solution prepared in this way is 0,00001 mol/l, which corresponds to about pH 5.

Test was performed at 36°C.

Table E14. Summary of the content of the analyzed compounds at about pH 5

Time	Semax		Ac-Semax-Arg-NH_2 (SEQ. ID 4)	
[h]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	3466079	100,0	3845621	100,0
1 H	2782949	80,3	3780245	98,3
2 H	2157397	62,2	3649494	95,0
4 H	1865443	53,8	3578350	93,0
6 H	986550	28,5	3384915	88,0

Lyophilizate stability during storage

Lyophilizate samples sealed in sealed vials were stored for 365 days at 35°C and standard humidity. Analyses were performed by opening successive vials at 1-month intervals.

Table E15. Summary of the content of the analyzed compounds within 0-360 days

Time	Semax		Ac-Semax-Arg-NH_2 (SEQ. ID 4)	
[days]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	3527980	100,0	3912691	100,0
30	3526348	99,9	3912660	100,0
60	3516584	99,7	3910654	99,9
90	3509925	99,5	3906648	99,8
120	3488684	98,9	3907215	99,9
150	3484254	98,8	3904582	99,8

180	3342652	94,8	3905216	99,8
210	3165422	89,8	3905101	99,8
240	3012568	85,4	3900990	99,7
270	2896458	82,1	3874620	99,0
300	2779568	79,0	3873515	99,0
330	2498755	70,9	3868741	98,9
360	2265821	64,3	3859405	98,6

Stability of aqueous solutions during storage

The stability study of the compounds was performed by analyzing the solutions of Semax and Ac-Semax-Arg-NH_2 (SEQ. ID 4) prepared as follows:

Peptide solutions were prepared of concentration 1 mg/mL in demineralized water (pH about 5.6). The prepared solutions were stored under controlled conditions at 40 °C and humidity 55%, tests were performed at equal time intervals.

Table E16. Summary of the content of the analysed compounds in aqueous solution during storage for 0-20 days

Time	Semax		Ac-Semax-Arg-NH_2 (SEQ. ID 4)	
[days]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	3494658	100,0	3894556	100,0
1	3263256	93,4	3796585	97,5
4	2964552	84,8	3562158	91,5
8	2365005	67,7	3448239	88,5
12	1556833	44,5	3310762	85,0
20	865770	24,8	3115644	80,0

3.5. ***Comparison between Selank and Ac-Selank-Arg-NH2*** (SEQ. ID 5)

Instruments

The analyses were performed using a Perkin Elmer 200 series liquid chromatograph with a pump and an autosampler with a detector UV/Vis LC-235 using column: YMC Pack C4 from YMC : 200 Å; 5 µm; 4,6*250 mm. Wavelength 214 nm.

Pump operating parameters:

Stage	Time [min]	Flow [ml/min]	A [% v/v]	B [% v/v]	Gradient
0	10	0,6	0	100	0
1	5	0,6	0	100	1
2	10	0,6	70	30	0
3	5	0,6	70	30	0

A – acetonitrile + 0,1% v/v trifluoroacetic acid

B – water + 0,1% trifluoroacetic acid

Analyses for Selank and Ac-Selank-Arg-NH2 (SEQ. ID 5) **in a medium stimulating gastric fluid**

For a solution simulating human gastric juice the following solution was prepared:

To prepare 1000 ml of working solution, perform the following steps:

1) Dissolve 1 g of Selank or Ac-Selank-Arg-NH2 (SEQ. ID 5) in 100 ml of demineralized water - concentration 10 mg/ml

2) 3.5 g of NaCl in 500 ml of demineralized water

3) Dissolve 1g of pepsin in 100 ml of demineralized water

4) make up to 900 ml with demineralized water.

5) 100 ml of 0.01 mol/l HCl solution (Supelco, Cat. No. 1.09974)

The concentration of [H] ions in the solution prepared in this way is 0.001 mol/l, which corresponds to about pH 3.

Test was performed at 36°C.

Table E17. Summary of the content of the analysed compounds at about pH 3.0

Time	Selank		Ac-Selank-Arg-NH_2 (SEQ. ID 5)	
[h]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	8531401	100,0	6069057	100,0
1	4719571	55,3	5645436	93,0
2	3285442	38,5	5463972	90,0
4	239732	2,8	5008185	82,0
6	0	0,0	4732650	78,0

Analyses for Selank and Ac-Selank-Arg-NH_2 (SEQ. ID 5) in a medium of about pH 5

Solution was prepared:

To prepare 1000 ml of working solution, perform the following steps:

1) Dissolve 1 g of Selank or Ac-Selank-Arg-NH_2 (SEQ. ID 5) in 100 ml of demineralized water - concentration 10 mg/ml

2) 3.5 g of NaCl in 500 ml of demineralized water

3) Dissolve 1g of pepsin in 100 ml of demineralized water

4) make up to 900 ml with demineralized water.

5) 1 ml of 0.01 mol/l HCl solution (Supelco, Cat. No. 1.09974)

The concentration of [H] ions in the solution prepared in this way is 0,00001 mol/l, which corresponds to about pH 5.

Test was performed at 36°C.

Table E18. Summary of the content of the analysed compounds at about pH 5

Time	Selank		Ac-Selank-Arg-NH_2(SEQ. ID 5)	
[h]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	8531401	100,0	6069057	100,0
1 H	6058147	71,0	5948282	98,0
2 H	5117987	60,0	5704913	94,0
4 H	2868257	33,6	5343804	88,0
6 H	1624378	19,0	4974806	82,0

Lyophilizate stability during storage

Lyophilizate samples sealed in sealed vials were stored for 365 days at 35°C and standard humidity. Analyses were performed by opening successive vials at 1-month intervals..

Table E19. Summary of the content of the analysed compounds within 0-360 days

Time	Selank		Ac-Selank-Arg-NH_2 (SEQ. ID 5)	
[days]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	8275458	100,0	6240204	100,0
30	8201806	99,1	6240204	100,0
60	8142223	98,4	6236459	99,9
90	7903062	95,5	6234587	99,9
120	7722657	93,3	6209627	99,5
150	7515770	90,8	6197146	99,3
180	7432188	89,8	6179050	99,0

210	7135927	86,2	6173433	98,9
240	6985314	84,4	6154713	98,6
270	6747808	81,5	6109159	97,9
300	6538439	79,0	6084136	97,5
330	5998879	72,5	6054245	97,0
360	5743167	69,4	6009316	96,3

Stability of aqueous solutions during storage

The stability study of the compounds was performed by analyzing the solutions of SELANK and Ac-Selank-Arg-NH_2 (SEQ. ID 5) prepared as follows:

Peptide solution of the following concentrations SELANK 0.71mg/ml and Ac-Selank-Arg-NH_2 (SEQ. ID 5) 0.73mg/ml in demineralized water (pH about 5,6) were prepared. The prepared solutions were stored under controlled conditions at 40 °C and humidity 55%, tests were performed at equal time intervals.

Table E21. Summary of the content of the analysed compounds in aqueous solution during storage for 0-20 days

Time	Selank		Ac-Selank-Arg-NH_2 (SEQ. ID 5)	
[days]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	8702029	100,0	6190438	100,0
1	7831826	90,0	6006581	97,0
4	6986859	80,3	5781869	93,4
8	5527528	63,5	5363395	86,6
12	3437301	39,5	5262491	85,0
20	1976230	22,7	5077397	82,0

3.6. *Comparison between Cortagen and Ac-Cortagen-Arg-NH_2* (SEQ. ID 6)

Instruments

The analyses were performed using a Perkin Elmer 200 series liquid chromatograph with a pump and an autosampler with a detector UV/Vis LC-235 using column: YMC Pack C4 from YMC : 200 Å; 5 µm; 4,6*250 mm. Wavelength 214 nm.

Pump operating parameters:

Stage	Time [min]	Flow [ml/min]	A [% v/v]	B [% v/v]	Gradient
0	10	0,6	0	100	0
1	5	0,6	0	100	1
2	10	0,6	70	30	0
3	5	0,6	70	30	0

A – acetonitrile + 0,1% v/v trifluoroacetic acid

B – water + 0,1% trifluoroacetic acid

Analyses for Cortagen and Ac-Cortagen-Arg-NH_2 (SEQ. ID 6) **in a medium stimulating gastric fluid**

For a solution simulating human gastric juice the following solution was prepared:

To prepare 1000 ml of working solution, perform the following steps:

1) Dissolve 1.01 g of Cortagen or 0.95g Ac-Cortagen-Arg-NH_2 (SEQ. ID 6) in 100 ml of demineralized water - concentration 10 mg/ml

2) 3.5 g of NaCl in 500 ml of demineralized water

3) Dissolve 1g of pepsin in 100 ml of demineralized water

4) make up to 900 ml with demineralized water.

5) 100 ml of 0.01 mol/l HCl solution (Supelco, Cat. No. 1.09974)

The concentration of [H] ions in the solution prepared in this way is 0.001 mol/l, which corresponds to about pH 3.

Concentration of Cortagen 1,01mg/ml and Ac-Cortagen-Arg-NH_2 (SEQ. ID 6) of 0,95mg/ml

Test was performed at 36°C.

Table E22. Summary of the content of the analysed compounds at about pH 3.0

Time	Cortagen		Ac-Cortagen-Arg-NH_2 (SEQ. ID 6)	
[h]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	7642926	100,0	4233140	100,0
1	3073220	40,2	4170066	98,5
2	1705901	22,3	4068470	96,1
4	795628	10,4	3794586	89,6
6	38214	0,5	3132946	74,0

Analyses for Cortagen and Ac-Cortagen-Arg-NH_2 (SEQ. ID 6) in a medium of about pH 5

Solution was prepared:

To prepare 1000 ml of working solution, perform the following steps:

1) Dissolve 1.01 g of Cortagen or 0.95g Ac-Cortagen-Arg-NH_2(SEQ. ID 6) in 100 ml of demineralized water - concentration 10 mg/ml

2) 3.5 g of NaCl in 500 ml of demineralized water

3) Dissolve 1g of pepsin in 100 ml of demineralized water

4) make up to 900 ml with demineralized water.

5) 1 ml of 0.01 mol/l HCl solution (Supelco, Cat. No. 1.09974)

The concentration of [H] ions in the solution prepared in this way is 0,00001 mol/l, which corresponds to about pH 5.

Test was performed at 36°C.

Table E23. Summary of the content of the analysed compounds at about pH 5

Time	Cortagen		Ac-Cortagen-Arg-NH_2 (SEQ. ID 6)	
[h]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	7642926	100,0	4233140	100,0
1 H	4664477	61,0	4186575	98,9
2 H	4189852	54,8	4149323	98,0
4 H	2308927	30,2	4089636	96,6
6 H	985173	12,9	3777230	89,2

Lyophilizate stability during storage

Lyophilizate samples sealed in sealed vials were stored for 365 days at 35°C and standard humidity. Analyses were performed by opening successive vials at 1-month intervals..

Table E24. Summary of the content of the analyzed compounds within 0-360 days

Time	Cortagen		Ac-Cortagen-Arg-NH_2 (SEQ. ID 6)	
[days]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	7566496	100,0	4190808	100,0
30	7558929	99,9	4187455	99,9
60	7369767	97,4	4187036	99,9
90	7203304	95,2	4184102	99,8
120	7106453	93,9	4170692	99,5
150	7001278	92,5	4169853	99,5
180	6824979	90,2	4161895	99,3

210	6765204	89,4	4161889	99,3
240	6303647	83,3	4148899	99,0
270	6067573	80,2	4122916	98,4
300	5251904	69,4	3982105	95,0
330	4207728	55,6	3909604	93,3
360	3694719	48,8	3885298	92,7

Stability of aqueous solutions during storage

The stability study of the compounds was performed by analyzing the solutions of CORTAGEN and Ac-Cortagen-Arg-NH_2 (SEQ. ID 6) prepared as follows:

Peptide solutions were prepared of concentration 1 mg/mL in demineralized water (pH about 5,6). The prepared solutions were stored under controlled conditions at 40 °C and humidity 55%, tests were performed at equal time intervals.

Table E25. Summary of the content of the analysed compounds in aqueous solution during storage for 0-20 days

Time	Cortagen		Ac-Cortagen-Arg-NH_2 (SEQ. ID 6)	
[days]	peak area [µV*s]	[%]	peak area [µV*s]	[%]
0	7529810	100,0	4170489	100,0
1	5964362	79,2	4069980	97,6
4	4435811	58,9	3970722	95,2
8	3214475	42,7	3620818	86,8
12	1764234	23,4	3484026	83,5
20	781594	10,4	3436899	82,4

All the results clearly show superiority of stabilities of N-acetylated and C-Arg-NH_2-ed peptides in comparison to their parent peptides.

As the new peptides have similar or better activity than parent peptides, and they have the same or better bioavailability after oral administration, the new peptides according to the invention are good substitute for parent peptides.

Claims

1. A compound selected from the group consisting of

   Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1);

   Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2);

   Ac-Gly-His-Lys-Arg-NH (SEQ. ID 3);

   Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 4),

   Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5), and

   Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6),

   or its pharmaceutically acceptable salt or complex.

2. The compound of claim 1 wherein the compound is selected from Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2), Ac-Gly-His-Lys-Arg-NH_2 (SEQ. ID 3), or Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1), or its pharmaceutically acceptable salt or complex.

3. The compound of claim 2 wherein the compound is Ac-Ala-Glu-Asp-Gly-Arg-NH_2 (SEQ. ID 2), or its pharmaceutically acceptable salt or complex.

4. The compound of claim 2 wherein the compound is Ac-Gly-His-Lys-Arg-NH_2 (SEQ. ID 3) or its pharmaceutically acceptable salt or complex.

5. The compound of claim 2 wherein the compound is Ac-Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val-Arg-NH_2 (SEQ. ID 1) or its pharmaceutically acceptable salt or complex.

6. The compound of claim 1 wherein the compound is selected from Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 4), Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5), and Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6) or its pharmaceutically acceptable salt or complex.

7. The compound of claim 6 wherein the compound is Ac-Met-Glu-His-Phe-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 4) or its pharmaceutically acceptable salt or complex.

8. The compound of claim 6 wherein the compound is Ac-Thr-Lys-Pro-Arg-Pro-Gly-Pro-Arg-NH_2 (SEQ. ID 5) or its pharmaceutically acceptable salt or complex.

9. The compound of claim 6 wherein the compound is Ac-Ala-Glu-Asp-Pro-Arg-NH_2 (SEQ. ID 6) or its pharmaceutically acceptable salt or complex.

10. A dietary supplement composition comprising a compound as defined in claim 1 to 9 and pharmaceutically acceptable excipient.

11. Use of the compound as defined in claim 1 to 9 or the dietary supplement composition as defined in claim 10 as a dietary supplement.

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2023/050066

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K7/06 A23L33/18 C07K5/103 C07K7/08
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ATSUSHI KATO ET AL: "Mutational analysis of protein solubility enhancement using short peptide tags", BIOPOLYMERS, JOHN WILEY, HOBOKEN, USA, vol. 85, no. 1, 31 August 2006 (2006-08-31), pages 12-18, XP071165341, ISSN: 0006-3525, DOI: 10.1002/BIP.20596 the whole document page 14; table 1	1-11
A	WO 01/98324 A1 (ZEALAND PHARMACEUTICALS AS [DK]; LARSEN BJARNE DUE [DK] ET AL.) 27 December 2001 (2001-12-27) the whole document	1-11

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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 February 2024	04/03/2024

Name and mailing address of the ISA/	Authorized officer
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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2023/050066

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANUBHA MAHAJAN ET AL: "Structural Modification of Proteins and Peptides", INDIAN JOURNAL OF PHARMACEUTICAL EDUCATION AND RESEARCH, vol. 48, no. 3, 1 August 2014 (2014-08-01) , pages 34-47, XP055440717, IN ISSN: 0019-5464, DOI: 10.5530/ijper.48.3.6 the whole document	1-11
A	WO 2014/142764 A1 (DIAGEN D O O [SI]) 18 September 2014 (2014-09-18) cited in the application the whole document	1-11
E	EP 4 226 918 A1 (MAJEWSKI FILIP [PL]) 16 August 2023 (2023-08-16) the whole document example 3	1-11

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/PL2023/050066

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

The sequence listing forming part of the international application as filed was incomplete. The applicant did not reply to the invitation to furnish a complete sequence listing. This opinion has been established to the extent that a meaningful opinion could be formed with an incomplete sequence listing. Upon entering in the European phase the applicant will be invited to furnish a complete sequence listing.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/PL2023/050066

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