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TARGETED SYNTHESIS OF N-TERTBUTOXYCARBONYLGLYCYL-(*S*)-α-ALLYLGLYCYL-(*S*)-ALANINE TRIPEPTIDE AND STUDY OF ITS EFFECT ON COLLAGENASE ACTIVITY

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The 10 new peptides have been constructed on the basis of (*S*)-α-allyl-glycine (2-aminopent-4-enovic acid) non-protein amino acid by ChemOffice software. To study their possible interaction with collagenase enzyme, molecular docking program – AutoDockVina software was used. Analyzing the obtained results, N-t-BOC-Gly-(*S*)-α-allylGly-(*S*)-Ala tripeptide was identified by maximum values of Gibbs free energy (*ΔG=-6.2* kcal/mol) and minimum values of dissociation constant (K_D=0,28532μmol) of ligand-macromolecular interaction.

The synthesis of a new undescribed in the literature N-t-BOC-Gly-(*S*)-α-allylGly-(*S*)-Ala tripeptide has been carried out by the activated ester method.

In vitro study of the synthesized tripeptide effect on the activity of collagenase enzyme at various peptide concentrations has been carried out, as a result of which it has been found out that the inhibition of the enzyme is 41.06% at 0.8989 *mmol/l* concentration of the synthesized tripeptide.

Fig. 1, tables 2, references 15.

For more than 70 years the research in the field of synthesis and study of peptides as well as the possibility of their introduction to the medical practice has been carried out [1].

Currently, there are around 60-70 approved peptide drugs in the global market, with 100-200 more in clinical trials, 400-600 more in pre-clinical studies and possibly hundreds to thousands more on the laboratory bench [2]. It should be mentioned that most of them contain non-proteinogenic amino acids moieties [3]. There are well-known medicinal preparations obtained on the basis of synthetic peptides that are used in the following diseases: hypertension, type 2 diabetes, postmenopausal osteoporosis, paget's disease,

hypercalcaemia, advanced prostate cancer, acromegaly, carcinoid syndrome, central diabetes insipidus.

It is established that the moiety of non-protein amino acid extends the process of enzyme-substrate recognition that in turn leads to retardation of the peptide bond destruction. These and other properties of peptides, containing a fragment of non-protein amino acid, enable to create on their basis physiologically and pharmacologically active drugs [4].

Matrix metalloproteases (MMPs) are a major group of enzymes that regulates cell-matrix composition. Matrix metalloproteases (MMPs) play an important role in degradation of extracellular matrix in both norm and various pathologies [5]. Metalloproteases are targets for a wide range of medications, including antitumor and anti-inflammatory drugs [6, 7]. Matrix metalloproteases are responsible for many proteolytic processes that lead to tumor development. Involvement of gelatinases (MMP-9 and MMP-2) in the process of metastases and angiogenesis formation stimulated creation of synthetic gelatinase inhibitors able to stop the development of tumors [6, 7]. MMP-1 is also validated as a cancer target [8].

Unfortunately, clinical trials of gelatinase inhibitors on oncological patients so far have not revealed therapeutic effect; moreover undesirable side effects were registered. The majority of inhibitors are zinc-chelating compounds of a wide spectrum of action that do not have a specific effect. For example, calprotectin inhibits MMP by blocking zinc binding [9]. The search for new highly specific compounds able to inhibit metalloproteases is one of the directions in creation of drugs preventing spread of metastases [10]. It has been shown that some low molecular weight compounds are able to inhibit MMPs [11].

Taking into account the above mentioned, the research was aimed at constructing a new undescribed in the literature dipeptide on the basis of (*S*)-α-allylGly non-protein amino acid, implementing software study of the mentioned peptides, selecting possible active peptides to carry out their targeted synthesis and study the biological effect of the synthesized peptides.

At the first stage the structure-based drug design approach was used to identify potential inhibitors of enzyme. For this aim docking analysis was done for the identification of substances capable of interacting with collagenase.

Peptides structures were built by ChemBioOffice 2010 (ChemBio3D Ultra12.0). Ligand free energy was minimized using MM2 force field and truncated Newton–Raphson method. Crystallographic structure of collagenase was taken from http://www.rcsb.org website (PDB-ID: 1NQJ). Docking of ligand to enzyme has been done by AutoGrid 4, AutoDock Vina software [12]. AutoDock uses the Lamarckian genetic algorithm by alternating local search with selection and crossover [13].

The ligands are ranked using an energy-based scoring function and a grid-based protein-ligand interaction is used to speed up the score calculation. Dissociation constant was calculated by using the following formula:

$K_D = \exp((\Delta G \times 1000)/(Rcal \times TK))$

Rcal=1.98719 *cal* / (*mol* × *K*) (gas constant)

TK = 298.15 *K* (room temperature by Kelvin)

The data of enzyme-peptide interaction are presented in Table 1.

Table 1

Data of molecular modeling

Experimental dipeptides	Gibbs free energy (ΔG) *kcal*/*mol*	Dissociation constant (K_D) *μmol*
N-t-BOC-Gly-(*S*)-α-allylGly-(*S*)-Ala	-6.2	0.28532
Gly-(*S*)-α-allylGly-(*S*)-Ala	-6.0	0.24101
N-t-BOC-Gly-(*S*)-α-allylGly-Gly-Gly	-6.1	0.33778
Gly-(*S*)-α-allylGly-Gly-Gly	-5.9	0.47341
N-t-BOC-(*S*)-Ala-(*S*)-α-allylGly -Gly	-5.7	0.41258
(*S*)-Ala-(*S*)-α-allylGly -Gly	-5.6	0.45312
N-t-BOC-(*S*)-Ala-Gly-(*S*)-α-allylGly	-5.0	0.87528
(*S*)-Ala-Gly-(*S*)-α-allylGly	-4.8	0.52835
N-t-BOC-(*S*)-Ala-(*S*)-Ala -(*S*)-α-allylGly	-4.3	0.49756
(*S*)-Ala-(*S*)-Ala -(*S*)-α-allylGly	-3.9	0.33781

The negative value of ΔG proves that the complex was generated. According to Table 1, the value of ΔG is negative for all compounds, which proves that all dipeptides are interacting with the enzyme. Based on the results obtained, it was aimed at performing further research on a compound with a maximum value of the Gibbs free energy, which is N-t-BOC-Gly-(*S*)-α-allylGly-(*S*)-Ala.

The docking data are presented in Figure, where the fragments of ligand-collagenase interaction are shown.

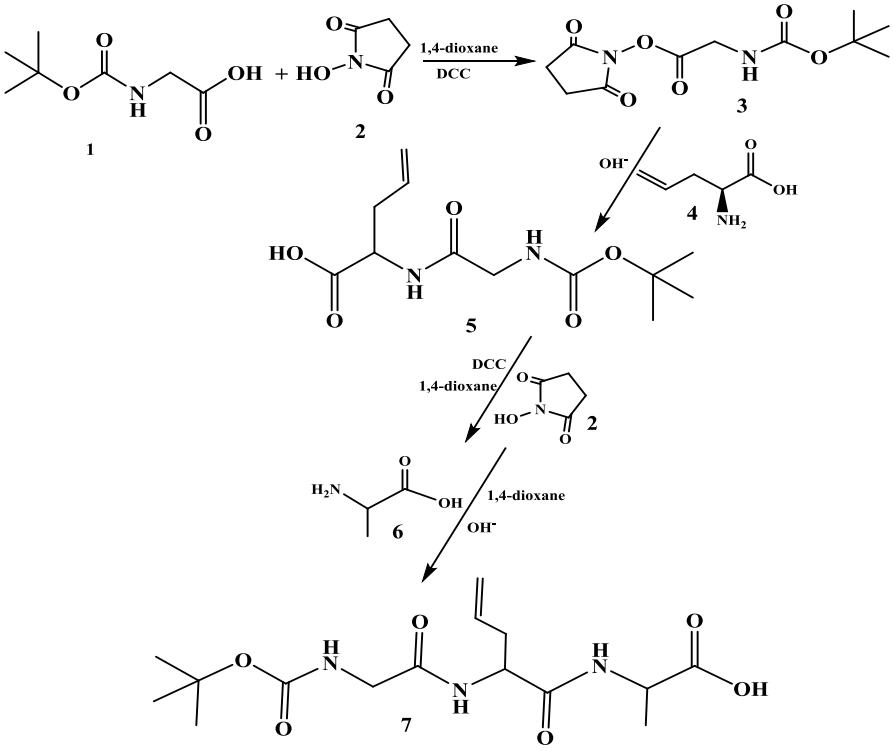
Taking into account the data of software modeling it was aimed at selecting N-t-BOC-Gly-(*S*)-α-allylGly-(*S*)-Ala tripeptide from the mentioned

range for further research, that is to carry out the peptide synthesis and study the synthesized peptide effect on the activity of collagenase enzyme.

The synthesis of peptide was carried out by the method of activated esters in a solution. The method is distinguished by its simplicity and allows to obtain final products in good yields and high purity [14].

At the first stage with the help of dicyclohexylcarbodiimide from N-tert-butyloxycarbonylglycine (1) its succinimide ether **(2)** was obtained, transformed by condensation with (*S*)-α-allylGly non-protein amino acid in alkaline aqueous-organic medium into the corresponding dipeptide – N-t-BOC-Gly-(*S*)-α-allylGly (4), then from the dipeptidem its succinimide ester (**5**) was obtained, transformed by condensation with Gly in alkaline aqueous-organic medium into the corresponding tripeptide – N-t-BOC-Gly-(*S*)-α-allylGly-(*S*)-Ala (Scheme).

Scheme



Experimental part

^{1}H NMR spectra were recorded on a "Varian Mercury 300VX" device with an operating frequency of 300.08 *MHz* in a solution of DMSO-D_6/CCl_4 1/3 using the method of double resonance. TLC was conducted on "Silufol UV-254" plates in a mixture of chloroform-ethyl acetate-methanol (4:4:1), developer - chlorotoluidine. Elemental analysis was performed on elemental analyzer CNS-O "Euro EA3000".

Synthesis of N-t-tertbutoxycarbonylglycine succinimide ester (3). 0.218 *g* (1.058 *mmol*) of dicyclohexylcarbodiimide, preliminary dissolved in 3 *ml* of dioxane was added at 0^oC to 0.175 *g* (1.0 *mmol*) of N-tert-butyloxycarbonylglycine **(1)** and 0.127 *g* (1.104 *mmol*) of N-hydroxysuccinimide (**2**) in a mixture of 6 *ml* of dioxane and 3 *ml* of methylene chloride. The reaction mixture was stirred for ~ 2 *h* at 0^oC and left overnight in a refrigerator.

The analysis was performed by TLC [SiO_2, $CHCl_3$/ethyl acetate/CH_3OH (4:2:1), developer – chlorotoluidine]. The precipitate formed was filtered off, the solvent distilled off on a rotary evaporator, and the precipitate crystallized from a mixture of ethyl acetate hexane (1:2). Yield: 0.25 *g* (75%).

N-t-Tertbutoxycarbonylglycyl-(S)-α-allylglycine (5). The resulting succinimide ether **3** was used at the next stage of dipeptide synthesis. In a flat-bottomed flask with a magnetic stirrer, 0.078 *g* (0.675 *mmol*) of (*S*)-α-allyl-Gly (**4**), 1.25 *ml* (0.63 *mmol*) of 0.5 *M* sodium hydroxide solution and 0.016 (0.19 *mmol*) of baking soda were placed. At room temperature, 0.2 *g* (0.735 *mmol*) of N-t-BOC-Gly-OSu (**3**) was added to 2 *ml* of dioxane, and the reaction mixture was stirred for 3 *h*. The next day, 5 *ml* of ethyl acetate and 1.45 *ml* of 10% citric acid were added to the flask contents. After vigorous stirring, the organic layer was separated, and the aqueous layer was extracted twice with ethyl acetate (5 *ml* each). The organic layer was dried with anhydrous sodium sulfate, then the solvent was evaporated to dryness.

The product was isolated by column chromatography using SiO_2 L-40/100 silica gel. The analysis was performed by TLC [SiO_2, $CHCl_3$/ethyl acetate/CH_3OH (4:2:1), the developer is chlorotoluidine]. The product yield per succinimide ester was 75%, Mp – 95-97^oC.

Synthesis of N-tertbutoxycarbonylglycyl-(S)-α-allylglycyl-(S)-alanine tripeptide (7). To synthesize tripeptide, the activation of N-t-BOC-glycyl-(S)-allylglycine (**5**) dipeptide was carried out in the same sequence at the primary phase; the activation was carried out by the method of N-t-BOC-glycyl-activated ester. Then the condensation reaction of N-tertbutoxycarbonylglycyl-(*S*)-allylglycyl-succinimide ester with (*S*)-alanine (**6**) was performed. The course of the reaction corresponds to the process of the synthesis of dipeptide (Scheme 4).

The process of reactions was controlled by the method of thin-layer chromatography, and as solvents chloroform: ethyl acetate:methanol were used at the ratio of 4:2:1. The yield was 68%. Mp – 135-137^oC. ^1H NMR (DMSO,δ, ppm, Hz): 1.32 (3H, d, J=7.3, CH_3); 1.41 (9H, s, $C(CH_3)_3$); 2.26-2.37 (m, 1H) and 2.39-2.49 (m, 1H, m, CH_2All); 3.49-3.65 (2H, m, $NHCH_2$); 4.21 (1H, k, J=7.3, $CHCH_3$,); 4.39 (1H, td, J=7.9, 5.4, $CHCH_2$); 4.99 (1H, ddt, J_1=10.1, 2.0, 1.0, =CH_2); 5.05 (1H, ddt, J_1=17.1, 2.0, 1.4, =CH_2) ; 5.72 (1H, ddt, J_1=17.1, 10.1, 7.1 =CH); 6.00 (1H, brs, COOH), 6.52 (1H, brt,

J=5.4, $NHCH_2$); 7.59 (1H, brd, J=7.9, NHCH); 7.97 (1H, brd, J=7.3, NHCH). Found, %: C 52.85; H 7.73; N 12.42 $C_{15}H_{25}N_3O_6$; Calculated, %: C 52.47; H 7.34; N 12.24. The chemical purity of N-tertbutoxycarbonyl-glycyl-(*S*)-α-allylglycyl-(*S*)-alanine tripeptide was 94% according to HPLC analysis.

***Collagenase activity*.** Collagenase activity was determined by measuring free amino groups according to o-phthalaldehyde (OPA) method [15].

The reaction mixture contained 0.05 M HEPES buffer, pH 7.2, 10 *mg*/*ml* gelatin and 0.025 *mg*/*ml* collagenase (activated by 0.36 *M* $CaCl_2$). Investigated compounds were added to the reaction mixture from 0.5 to 5 *mM* final concentration. The aliquot (50 *ul*) was taken and the remaining mixture was incubated at 37^{o}C. Every 30 *min* aliquot was taken. The reaction was stopped by adding 10 *ul* of 30% trichloroacetic acid. The concentration of free amino groups in the reaction mixture was determined by OPA reagent containing 0.2 *M* borate buffer, pH 9.7, 0.1667 *mg*/*ml* OPA and 1.25 *mM* mercaptoethanol. The reaction mixture (50 *ul*) was added to OPA reagent (1.5 *ml*) and H_2O (1.5 *ml*). A340 was recorded after 5 *min* incubation at RT. Peptide was tested at different concentrations to make it possible to correlate the concentration with the effect. The numerical data of the experiment are presented in Table 2.

Table 2

Effect of N-tertbutoxycarbonylglycyl-(*S*)-allylglycyl-(*S*)-alanine tripeptide on the activity of collagenase enzyme

Concentration, *mmol*/*l*	Enzyme activity, % N-tertbutoxycarbonylglycyl-(*S*)-allylglycyl-(*S*)-alanine
Control	100
0.4494	67.63
0.8989	58.94
1.7978	59.74

As can be seen from the Table, N-tertbutoxycarbonylglycyl-(*S*)-allylglycyl-(*S*)-alanine tripeptide inhibits the enzyme activity by 32.37% in the case of 0.4494 *mmol*/*l* concentration, but with increasing concentration the inhibition percentage does not proportionally increase. At concentrations of 0.9 *mmol*/*l* and higher, the effect remains at the same level. The latter may be due to saturation (maximum possible inhibition with N-tertbutoxycarbonylglycyl-(*S*)-allylglycyl-(*S*)-alanine).

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N-ՏՐԵՏԲՈՒՏԻԼՕՔՍԻԿԱՐԲՈՆԻԼԳԼԻՑԻԼ-(S)-α-ԱԼԻԼԳԼԻՑԻԼ-(S)-ԱԼԱՆԻՆ ՏՐԻՊԵՊՏԻԴԻ ՆՊԱՏԱԿԱԿԱՅԻՆ ՍԻՆԹԵԶԸ ԵՎ ԿՈԼԱԳԵՆԱԶ ՖԵՐՄԵՆՏԻ ՎՐԱ ԱԶԴԵՑՈՒԹՅԱՆ ՀԵՏԱԶՈՏՈՒՄԸ

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(S)-α-Allyl-glycine ոչ սպիտակուցային ամինաթթվի հենքի վրա ChemOffice software ծրագրի օգնությամբ կառուցվել են 10 նոր տրիպեպտիդներ:

AutoDockVina software համակարգչային ծրագրի կիրառմամբ իրականացվել է կառուցված պեպտիդների և կոլագենազ ֆերմենտի հնարավոր փոխազդեցության մոդելավորում: Ստացված տվյալների վերլուծության արդյունքում ընտրվել է՝ N-t-BOC-Gly-(S)-α-allylGly-(S)-Ala տրիպեպտիդը, որը ունեցել է Գիբսի ազատ էներգիայի (ΔG=-6.2 կկալ/մոլ) առավելագույն և դիսոցման հաստատունի (KD=0.28532 մկմոլ) նվազագույն արժեքներ:

Նոր դրականության մեջ չնկարագրված N-t-BOC-Gly-(S)-α-allylGly-(S)-Ala տրիպեպտիդի սինթեզը իրականացվել է՝ ակտիվացված էսթերների մեթոդի կիրառմամբ: Կատարվել է սինթեզված տրիպեպտիդի ազդեցության in vitro հետազոտում կոլագենազ ֆերմենտի ակտիվության վրա՝ պեպտիդի տարբեր կոնցենտրացիաների կիրառմամբ, որի արդյունքում բացահայտվել է, որ սինթեզված տրիպեպտիդի 0.8989 մմոլ/լ կոնցենտրացիայի դեպքում ֆերմենտի արգելակումը կազմում է 41.06%:

ЦЕЛЕНАПРАВЛЕННЫЙ СИНТЕЗ ТРИПЕПТИДА N-ТРЕТБУТИЛОКСИКАРБОНИЛГЛИЦИЛ-(S)-α-АЛЛИЛГЛИЦИЛ-(S)-АЛАНИНА И ИССЛЕДОВАНИЕ ЕГО ДЕЙСТВИЯ НА АКТИВНОСТЬ КОЛЛАГЕНАЗЫ

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С помощью программы ChemOffice software были построены структуры 10 новых не описанных в литературе трипептидов, содержащих небелковую аминокислоту (*S*)-α-аллилглицин. С использованием программы AutoDockVina software было проведено моделирование вероятного взаимодействия трипептидов с ферментом колагеназ. На основании полученных данных был выбран трипептид N-t-BOC-Gly-(*S*)-α-allylGly-(*S*)-Ala, который имеет наибольшую свободную энергию (ΔG=6.2 *ккал/мол*) и минимальное значение константы диссоцации (KD=0.28532 *мкмол*). Синтез N-t-BOC-Gly-(*S*)-α-allylGly-(*S*)-Ala осуществлен методом активированных эфиров. Проведено *in vitro* исследование влияния синтезированого трипептида на активность фермента коллагеназ при различных концентрациях пептида.

В результате было обнаружено, что при концентрации 0.8989 *ммол/л* синтезированного трипептида ингибирование фермента составляло 41.06%.

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