MODELING, SYNTHESIS AND *IN VITRO* TESTING OF PEPTIDES BASED ON UNUSUAL AMINO ACIDS AS POTENTIAL ANTIBACTERIAL AGENTS

A.S. Sargsyan[1]*, L.T. Karapetyan[1], A.V. Mkhitaryan[1], L.A. Stepanyan[1], T.H. Sargsyan[1,2], Yu.M. Danghyan[1], A.V. Sargsyan[1], G.G. Oganezova[1], N.A. Hovhannisyan[1,2]

[1]Scientific and Production Center "Armbiotechnology" NAS RA,
14 Gyurjyan str., Yerevan, 0056 Armenia;*e-mail: armenssargsyan@gmail.com
[2]Yerevan State University, 1 Alex Manoogian, Yerevan, 0025 Armenia

Currently non-protein amino acids and synthetic peptides are widely used as blocks in drug design. Many proteases are of great interest for pharmacology due to their key role in various pathologies. Bacterial collagenase (EC 3.4.24.3) is quite an attractive target for drug development as the inhibitors of bacterial collagenolytic protease may stop propagation of diseases caused by infections. The interaction of peptides containing unusual amino acids with *Clostridium histolyticum* collagenase has been evaluated by molecular docking followed by the measurement of enzyme inhibition by selected compounds. According to the docking analysis, 4 compounds were selected and synthesized for further research. Measurement of enzyme activity revealed that all tested compounds inhibited collagenase activity with IC_{50} values ranging within 1.45–2.08 μM. The antibacterial activity of synthesized compounds against some resistant strains was characterized by MICs values ranging within 4.6–9.2 μg/ml.

Key words: unusual amino acid; docking; collagenase; inhibitor; antibacterial activity

DOI: 10.18097/PBMC20247006413

INTRODUCTION

Today the emergence of multidrug-resistant (MDR) bacterial strains is a global problem for public health. Therapeutic treatment of bacterial infections with antibiotics is becoming increasingly difficult as bacteria develop resistance to the antibiotics used against them or even to antibiotics they have never been exposed to before [1]. The demand for new antimicrobials is growing with the spread of antibiotic resistant pathogenic bacterial strains. Bacterial extracellular proteases, acting as virulence factors are suitable targets to combat bacterial infections. Protease inhibitors and inhibitors of bacterial growth used in combination may increase the effectiveness of infection treatment.

Non-proteinogenic amino acids represent a class of compounds, which are widely used in drug design, particularly for developing peptide-based drug candidates [2].

Optically active non-protein α-amino acids have been screened for their ability to interact with collagenase of *Clostridium histolyticum*. Structure-based drug design approach (modeling) and the assay of enzyme activity in the presence of amino acids have been used to identify low molecular weight inhibitors of collagenase. According to the docking analysis, a number of non-protein amino acids have demonstrated ability to form bonds with collagenase. Following the docking analysis, enzyme activity has been determined in the presence of investigated amino acids. The results have shown that (*S*)-β-[4-allyl-3-butyl-5-thioxo-1,2,4-triazol-1-yl]-α-alanine, (*S*)-β-[4-allyl-3-(pyridin-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine and (*S*)-β-[4-allyl-3-(pyridin-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine inhibited collagenase activity. It was suggested that inhibitors of collagenases had distinct substituted moieties such as -3-butyl, 3-(pyridin-4′-yl), and -3-(pyridin-3′-yl) [3].

In order to increase the potency of collagenase inhibition the modeling of peptides, containing (*S*)-β-[4-allyl-3-(pyridin-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine and (*S*)-β-[4-allyl-3-(pyridin-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine in their structure was carried out, following the synthesis of selected peptides, study of their inhibitory impact on collagenase activity and bacterial growth.

Gram-negative bacteria *Enterobacteriaceae* are common infections threatening community. The most strains isolated from clinics demonstrate multidrug resistance (MDR), then they could be used to study antibiotic resistance. One of the approaches for MDR overcoming is design and synthesis of novel antimicrobials, preferably affecting more than one target in a bacterial cell [4]. During past decades, the non-protein amino acids and peptides were synthesized and screened for biological activities. This resulted in the finding of inhibitors of serine proteases and metalloproteases, the inhibitors of MDR *Pseudomonas* and *Stenotrophomonas* strains' growth [5]. The aim of the present study was to investigate the antibacterial activity of non-proteinogenic amino acids against antibiotic-resistant strains of *P. aeruginosa*, *E. coli*, *K. pneumonia*.

METHODS

Molecular docking

Structures of compounds were built by ChemBioOffice 2010 (ChemBio3D Ultra12.0). Ligand free energy was minimized using the MM2 force field and truncated Newton-Raphson method. The crystallographic structure of collagenase G was taken from the Protein Data Bank of Research Collaboratory for Structural Bioinformatics (PDB ID: 2Y50). Water molecules were removed and polar hydrogens were added. Docking of ligands to the enzyme was done using AutoGrid 4 and AutoDock Vina softwares [6]. The ligands were ranked using an energy-based scoring function and a grid-based protein-ligand interaction was used to speed up the score calculation. The most promising interaction models were chosen. For them the mode of interaction, as well as type and lengths of bonds were determined.

Chemical Materials

All reagents were obtained from commercial sources and used without further purification. Thin layer chromatography (TLC) was carried out on aluminium foil-backed sheets pre-coated with 0.2 mm Kieselgel 60 F254 (Merck, USA). Melting points (mp) were determined using an Elektrothermal melting point apparatus (Elektrothermal Engineering, UK). ^^{1}H and ^^{13}C NMR spectra were recorded on a Varian Mercury 300 MHz spectrometer (Varian Inc., USA) using TMS as an internal standard. Elemental analysis was performed using an Euro EA3000 analyzer (Eurovector, Italy). The chemical purity of the newly synthesized amino acid was assessed using HPLC system (Waters Separation Module e2695, Waters, USA) equipped with a Waters 2487 Dual λ Absorbance UV Detector (Waters). The chromatographic column used was an Alltima C18 (250 mm × 4.6 mm, 5 μm particle size; Hichrom Ltd., Germany). The mobile phase consisted of acetonitrile and 2 mM/L ammonium persulfate (15:85 v/v). The sample (10 mg in 1 ml water) was injected in a volume of 5 μl.

Synthesis of 9-Fluorenylmethoxycarbonyl-Amino Acids

A mixture of 0.0043 mol of amino acids (**2**, **3**) and 0.456 g (0.0043 mol) of Na_2CO_3 was added to a round-bottom flask. The mixture was stirred at room temperature with a magnetic stirrer until a clear solution was formed. Then, 1.955 g (0.0058 mol) of N-(9-fluorenylmethoxycarbonyl)succinimide (**1**), dissolved in 2 ml of 1,4-dioxane, was added to the reaction mixture. The reaction mixture was stirred at room temperature for 3 h.

The progress of the reaction was monitored using thin layer chromatography (TLC) on silica gel (SiO_2), with the mobile phase consisting of $CHCl_3$/ethyl acetate/MeOH (4:2:1) and developed with chlorotoluidine.

To remove unreacted starting materials, the reaction mixture was first treated twice with diethyl ether. Then, 20 ml of distilled ethyl acetate was added, and the mixture was acidified to pH 2 with 2 M hydrochloric acid. Subsequently, 10 ml of ethyl acetate was added and extracted twice. The combined organic fractions were dried over anhydrous sodium sulfate.

After decanting, the organic solvents were removed by vacuum evaporation at 50–60°C. The target product was recrystallized from a 1:3 ethyl acetate-hexane mixture, filtered, and dried under vacuum conditions at 50–60°C, resulting in a white crystalline mass.

*9-fluorenylmethoxycarbonyl-(S)-β-[4-allyl-3-(pyridinyl-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine (**4**)*

The yield of the final product was 65%. Anal. Calcd for $C_{28}H_{25}N_5O_4S$ (527.60) C, 63.74; H, 4.78; N, 13.27. Found: C, 63.81; H, 4.79; N, 13.31.

^{1}H NMR spectrum (DMSO-d_6/CCl_4 1/3, δ, p.p.m, Hz): 4.08–4.24 m (3H, OCH_2CH); 4.48 dd (1H, J=13.0, 8.4, NCH_2CH); 4.72 ddd (1H, J=8.4, 8.2, 4.6, NHCH); 4.81 dd (1H, J=13.0, 4,6, NCH_2CH); 4.72–4.86 m (2H, CH_2 All); 5.05 br.d (1H, J=17.3, $=CH_2$); 5.18 br. d (1H, J=10.6, $=CH_2$); 5.89 ddt (1H, J=17.3, 10.6, 4.6, =CH); 7.18–7.26 m (2H, Ar), 7.30–7.37 m (2H, Ar); 7.51–7.55 m (2H, Py); 7.54 b.d (1H, J=8,2, NH); 7.59–7.65 m (2H, Ar); 7.70–7.74 m (2H, Ar): 8.52–8.56 m (2H, Py); 12.5 v.b (1H, COOH): 13C: 46.5 (CH); 47.0 (CH_2); 49.4 (CH_2); 51.8 (NCH); 65.9 (OCH_2); 117.3; 119.29; 119.31; 121.5; 124.9; 125.0; 126.5; 126.96; 127.0; 130.8; 132.6; 140.4; 140.5; 143.4, 143.5; 147.5; 149.7; 155.3; 168.2; 170.4.

*9-fluorenylmethoxycarbonyl-(S)-β-[4-allyl-3-(pyridinyl-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine (**5**)*

The yield of the final product was 67%. Anal. Calcd for $C_{28}H_{25}N_5O_4S$ (527.60) C, 63.74; H, 4.78; N, 13.27. Found: C, 63.80; H, 4.80; N, 13.32.

^{1}H NMR spectrum (DMSO-d_6/CCl_4 1/3, δ, p.p.m, Hz): 4.08–4.26 m (3H, OCH_2CH); 4.50 dd (1H, J=12.9, 8.0, NCH_2CH); 4.73–4.77 m (2H, CH_2 All); 4.70 ddd (1H, J=8.2, 8.0, 4,8, NHCH); 4.79 dd (1H, J=12.9, 4.8, NCH_2CHNH); 5.03 br.d (1H, J=17.1); 5.18 br. d (1H, J=10.5, $=CH_2$); 5.88 ddt (1H, J=17.1, 10.5, 4.7, =CH All); 7.19–7.26 m (2H, Ar), 7.30–7.38 m (3H, Ar); 7.50 b.d (1H, J=8.2, NH); 7.60–7.65 m (2H, Ar); 7.70–7.74 m (2H, Ar): 7.97 ddd (1H, J=8.0, 2.0, 1.5, 6-H Py); 8.63 dd (1H, J=4.8, 1.5, 4-H Py); 8.80 d (1H, J=2.0, 2-H Py): 13C: 46.5 (CH); 46.9 (NCH_2); 49.2 (NCH_2); 51.9 (NCH); 65.9 (OCH_2); 117.3 (CH); 119.22 (CH); 119.24 (CH); 121.8; 122.9 (CH); 124.9 (CH); 125.0 (CH); 126.5 (2.CH); 126.91 (CH); 126.94 (CH); 130.9 (CH); 135.2 (CH); 140.4; 140.5; 143.3, 143.5; 147.3; 148.4 (CH); 150.8 (CH); 155.3; 167.9; 170.4.

*Synthesis of N-hydroxysuccinimide esters 9-fluorenylmethoxycarbonyl-(S)-β-[4-allyl-3-(pyridinyl-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine (7) and 9-fluorenylmethoxycarbonyl-(S)-β-[4-allyl-3-(pyridinyl-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine (**8**)*

0.291 g (0.0025 mol) of N-hydroxysuccinimide (**2**), dissolved in a mixture of 6 ml of 1,4-dioxane and 3 ml of methylene chloride, was added to 0.0023 mol of 9-fluorenylmethoxycarbonyl-amino acids (**4**, **5**). Then 0.495 g (0.0024 mol) of dicyclohexylcarbodiimide (DCC) dissolved in 3 ml of 1,4-dioxane was added to the reaction mixture. The mixture was stirred for approximately 2 h at 0°C and for 1 h at room temperature.

The formed precipitate was filtered off, and the solvent was removed by distillation using a rotary evaporator. The residue was crystallized from a mixture of ethyl acetate and hexane (1:2). The yield of product **7** was 75%, and product **8** yielded 70%.

Synthesis of Dipeptides I

0.425 g (0.0019 mol) of glycine was placed in a flat-bottom flask and 6.75 ml of 0.5 M NaOH and 0.053 g (0.00063 mol) of $NaHCO_3$ were added to glycine. After 15 min, 0.0017 mol of 9-fluorenylmethoxy-carbonyl-(*S*)-β-[4-allyl-3-(pyridinyl-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine and 9-fluorenylmethoxy-carbonyl-(*S*)-β-[4-allyl-3-(pyridinyl-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine N-hydroxysuccinimide esters, dissolved in 2 ml of 1,4-dioxane, were added separately to the reaction mixture.

The reaction mixtures were stirred for 3 h at room temperature and then kept at 5°C overnight. On the next day, 5 ml of ethyl acetate, 3 ml of 10% citric acid solution, and 0.2 g of NaCl were added to each reaction mixture and stirred for 15 min. The organic layer was separated, dried over Na_2SO_4, and the solvent was removed under vacuum at 50°C. The residue was recrystallized from an ethyl acetate-hexane mixture (1:3). The reaction was monitored by thin-layer chromatography using a system of chloroform:methanol:ethyl acetate (3:2:1).

*9-fluorenylmethoxycarbonyl-(S)-β-[4-allyl-3-(pyridinyl-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanylglycine (**9**)*

The yield of the final product was 60%. Anal. Calcd for $C_{30}H_{28}N_6O_5S$ (584.65) C, 61.63; H, 4.83; N, 14.37. Found: C, 61.69; H, 4.85; N, 14.41.

^{1}H NMR spectrum (DMSO-d_6/CCl_4 1/3, δ, p.p.m, Hz): 3.81 dd (1H, J=17.7, 5.6, CH_2NH); 3.84 dd (1H, J=17.7, 5.6, CH_2NH); 4.04–4.12 m (2H); 4.20 dd (1H, J=8.6, 6.0); 4.45 dd (1H, J=13.5, 8.8); 4.68–4.85 m (4H); 5.05 dtd (1H, J=17.2, 1.1, 0.7, $=CH_2$); 5.16 br. d (1H, J=10.5, $=CH_2$); 5.87 ddt (1H, J=17.2, 10.5, 4.8, =CH); 7.20–7.27 m (2H), 7.30–7.37 m (2H); 7.50 br.d (1H, J=6.5, NHCH), 7.50–7.54 m (2H, H-2,2' Py); 7.60–7.66 m (2H, Ar); 7.70–7.75 m (2H, Ar): 8.17 br.t (1H, J=5.6, $NHCH_2$), 8.48–8.54 m (2H, H-3-3' Py); 12.30 br (1H, COOH): 13C: 40.6 (CH_2); 46.4 (CH); 46.9 (NCH_2); 50.0 (NCH_2); 52.9 (NCH); 66.0 (OCH_2); 117.2; 119.20; 119.25; 121.5; 124.9; 125.1; 126.4; 126.90; 126.95; 130.8; 132.5; 140.4; 140.5; 143.2, 143.5; 147.5; 149.6; 155.3; 168.2; 168.3; 170.3.

*9-fluorenylmethoxycarbonyl-(S)-β-[4-allyl-3-(pyridinyl-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanylglycine (**10**)*

The yield of the final product was 60%. Anal. Calcd for $C_{30}H_{28}N_6O_5S$ (584.65) C, 61.63; H, 4.83; N, 14.37. Found: C, 61.71; H, 4.84; N, 14.40.

^{1}H NMR spectrum (DMSO-d_6/CCl_4 1/3, δ, p.p.m, Hz): 3.80 dd (1H, J=17.5, 5.6, CH_2NH); 3.84 dd (1H, J=17.7, 5.6, CH_2NH); 4.04–4.14 m (2H); 4.17–4.26 m (1H, OCH_2CH); 4.47 dd (1H, J=13.6, 8.9, NCH_2); 4.65–4.75 m (3H, NCH_2 and CH_2 All); 4.81 td (1H, J=8.9, 4.6, NCH); 5.02 dtd (1H, J=17.2, 1.7, 0.9, $=CH_2$); 5.15 br. d (1H, J=10.5, $=CH_2$); 5.86 ddt (1H, J=17.2, 10.5, 4.8, =CH); 7.20–7.27 m (2H), 7.30–7.37 m (2H); 7.30–7.37 (1H, 5-H Py); 7.49 b.d (1H, J=8.9, NHCH) 7.61–7.65 m (2H); 7.70–7.74 m (2H, 8H); 7.96 ddd (1H, J=8.0, 2.2, 1.4, 6-H Py); 8.16 br.t (1H, J=5.6, $NHCH_2$), 8.61 dd (1H, J=4.8, 1.4, 4-H, Py); 8.79 b.d (1H, J=2.2, 2-H, Py); 12.18 v. B. (1H, COOH): 13C: 40.7 (CH_2); 46.5 (CH); 46.9 (CH_2); 49.9 (CH_2); 53.1 (NCH); 66.1 (OCH_2); 117.3; 119.27; 119.30; 121.8; 123.0; 125.0; 125.1; 126.54; 127.00; 127.03; 131.0; 135.3; 140.4; 140.5; 143.3, 143.5; 147.5; 148.4; 150.8; 155.3; 168.2; 168.0.

*Synthesis protected 9-fluorenylmethoxycarbonyl potentially biologically active non-protein amino acids (**4**, **5**)*

The synthesis was optimized by varying the solvent and base combinations. The following solvent/base combinations were tested: methanol/NaOH, methanol/KOH, methanol/Na_2CO_3, methanol/K_2CO_3, acetone/NaOH, acetone/KOH, acetone/Na_2CO_3, 1,4-dioxane/NaOH, 1,4-dioxane/KOH, 1,4-dioxane/Na_2CO_3, 1,4-dioxane/K_2CO_3. The reaction was carried out according to the previously developed method [7]. The optimal reaction conditions were determined to be 1,4-dioxane as the solvent with Na_2CO_3 as the base at room temperature; under these conditions the reaction proceeded faster and with high yields. The schematic diagram of the reaction is given in Figure 1.

Rationale for Non-Protein Amino Acids

The selection of non-protein amino acids was based on several key properties of heterocyclic amino acids. Amino acids and peptides containing heterocycles are widely used in drug development due to their enhanced stability and therapeutic potential. Such compounds generally exhibit low toxicity, high bioavailability, and favorable metabolic and

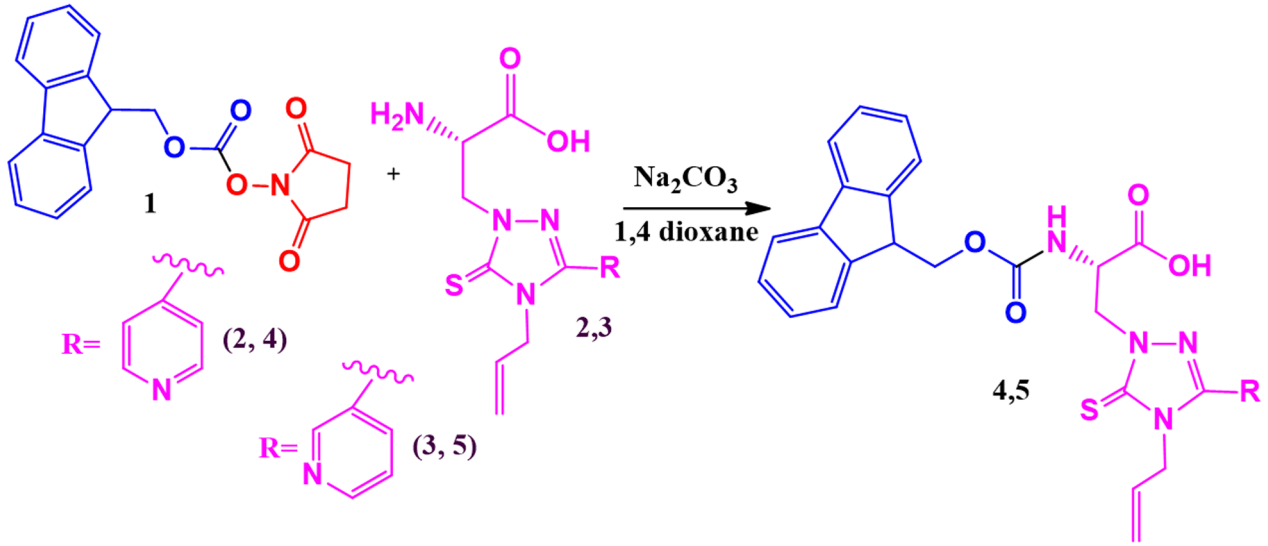

Figure 1. The protecting reaction for obtaining 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridinyl-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine and 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridinyl-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine (**4**, **5**) amino acids.

pharmacokinetic properties. Synthetic heterocyclic substituted amino acids and peptides offer a promising route for developing new, less toxic, and safer drugs with multiple beneficial properties [8–10]. Nitrogen-containing heterocyclic compounds are of considerable interest due to their diverse applications. They have been found to exhibit positive activities in various therapeutic areas, including: anti-inflammatory, antioxidant, anti-tumor, anti-ulcer, antidepressant, anti-malarial, anti-tuberculosis, antiviral, anti-hypertensive, anti-diabetic, cholinesterase inhibition [11, 12].

Synthesis of Dipeptides II

For the synthesis of dipeptides 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridinyl-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanylglycine (**9**) and 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridinyl-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanylglycine (**10**) in the first stage the conversion of the to succinimide esters were done according to Figure 2.

Condensation Reaction

In the final step, the activated esters of 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridinyl-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine and 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridinyl-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine (**7**, **8**) reacted with glycine in 0.5 M aqueous NaOH in 1,4-dioxane at room temperature. This reaction resulted in the synthesis of 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridinyl-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanylglycine (**9**) and 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridinyl-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanylglycine (**10**) dipeptides (Fig. 2).

Determination of collagenase activity

Collagenase activity was measured by the method based on determining free amino groups released as a result of substrate hydrolysis [13]. The reaction mixture contained 0.05 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (Merck), pH 7.2, 10 mg/ml gelatin (Merck) and 0.025 mg/ml collagenase (Alfa Aesar, USA). The reaction proceeded at 37°C. The concentration of amino groups in the reaction mixture was determined by *ortho*-phthalaldehyde (OPA) reagent containing 0.2 M borate buffer, pH 9.7, 0.1667 mg/ml OPA (Alfa Aesar) and 1.18 mM mercaptoethanol (Alfa Aesar). The aliquots (50 µl) are taken up every 30 min. The reaction was stopped by addition of 6 µl of 30% trichloroacetic acid. OPA reagent (1.5 ml) and H_2O (1.5 ml) were added to aliqoit and A_{340} was recorded after 5 min incubation at 27°C.

Antibacterial activity

The following strains were used as the test cultures: *P. aeruginosa* MDC 5249 resistant to kanamycin (kan), streptomycin (str), augmentin (amc), amoxicillin (amx), cefixime (cfx), ampicillin (amp), chloramphenicol (cam), ciprofloxacin (cip) (MDC, SPC "Armbiotechnology" NAS RA); and the strains, clinical isolates, were obtained from NIH RA: *P. aeruginosa* 80 (urine) resistant to imipenem (imi), piperacillin/tazobactam (pip/tab), aztreonam (azt), ciprofloxacin (cip), cefepime (cfp), *E. coli* ESBL 64 (wound discharge) resistant to imipenem (imi), aztreonam (azt), gentamicin (gen), tobramycin (tob), amikacin (amk), ciprofloxacin (cip); *K. pneumonia* 63 (sputum) resistant to ampicillin (amp), cefazolin (cfz), ciprofloxacin (cip), cefuroxime (Rx),

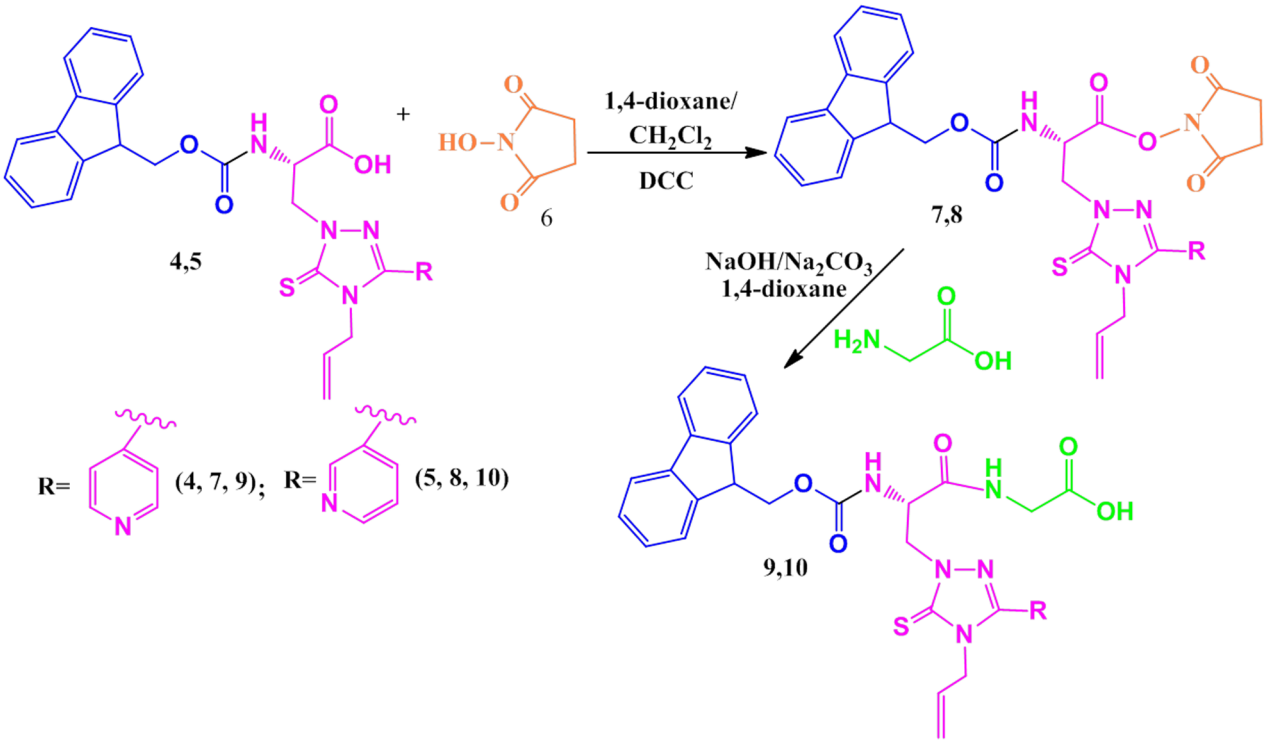

Figure 2. The reaction for obtaining 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridinyl-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanylglycine and 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridinyl-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanylglycine (**9**, **10**) peptides.

levofloxacin (lfx). The antibiotic resistance of strains was identified according to EUCAST standard definitions.

Antibacterial activity was assessed by serial dilution technique. An inoculum of 10^8 CFU/ml was used for agar (Nutrient Agar, HIMedia Laboratories Pvt.Ltd., India) and LB microdilution methods. The antibacterial activity was tested against MDR resistant strains *P. aeruginosa* 5249, *P. aeruginosa* 80, *K. pneumoniae* 63, *E. coli* ESBL 64. Susceptibility of the strains was assessed by serial dilutions in the presence of a studied compound at final concentrations in broth ranging from 0.005 mM to 0.5 mM. 96-well plates were inoculated with 10 µl culture, 10 µl non-protein amino acid and 200 µl broth. The inoculated plates were subsequently incubated for 16–18 h at 37°C (*S. maltophilia* was incubated at 30°C). The optical density of bacterial cultures was measured by Multiskan FC (Thermo Scientific, USA) microplate Photometer. Each assay was performed at least twice on separate days. The MIC was determined as the lowest concentration of the compound that prevented visible bacterial growth after incubation.

RESULTS AND DISCUSSION

Based on our previous results demonstrating inhibition of collagenase activity by (*S*)-β-[4-allyl-3-(pyridin-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine and (*S*)-β-[4-allyl-3-(pyridin-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine we decided to create some peptides based on them [3]. Random different structures with inclusion of mentioned unusual amino acids were generated and their synthesizability was checked. In the second stage we have performed docking analysis using AutoDock Vina. Lead compounds were chosen based on their ΔG (< -8 kcal/mol) values. After cut off by ΔG value we selected 4 leads. All these 4 compounds bound collagenase at the same site/pocket of the catalytic subdomain (Fig. 1) [14]. The number, position, and length of bonds in enzyme-ligand complexes have been calculated. Calculation results suggest that the *p-p* bonds play the main role in this interaction. So, according to the calculation results 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridine-4′-yl)-5-thioxo-1,2,4-triazol-1yl]-α-alanylglycine (**9**) formed *p-p* a bond with triazole ring of Tyr^{198} (10.438 Å) and a double *p-p* bond with Tyr^{201} (4.325 Å and 3.918 Å) (Fig. 3a). 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridine-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine (**4**) formed a double *p-p* bond with Tyr^{201} (4.231 Å and 4.295 Å) (Fig. 3b). 9-fluorenylmethoxy-carbonyl-(*S*)-β-[4-allyl-3-(pyridine-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanyl-glycine (**10**) interacted with collagenase by forming 5 *p-p* bonds: a double *p-p* with Tyr^{201} (4.859 Å and 3.798 Å), a double *p-p* with Tyr^{198} (6.116 Å and 9.926 Å), and *p-p* interaction involving three aromatic rings (with Tyr^{302}, its fluorenyl and pyridine groups of **10**) (13.224 Å) (Fig. 3c). 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridine-

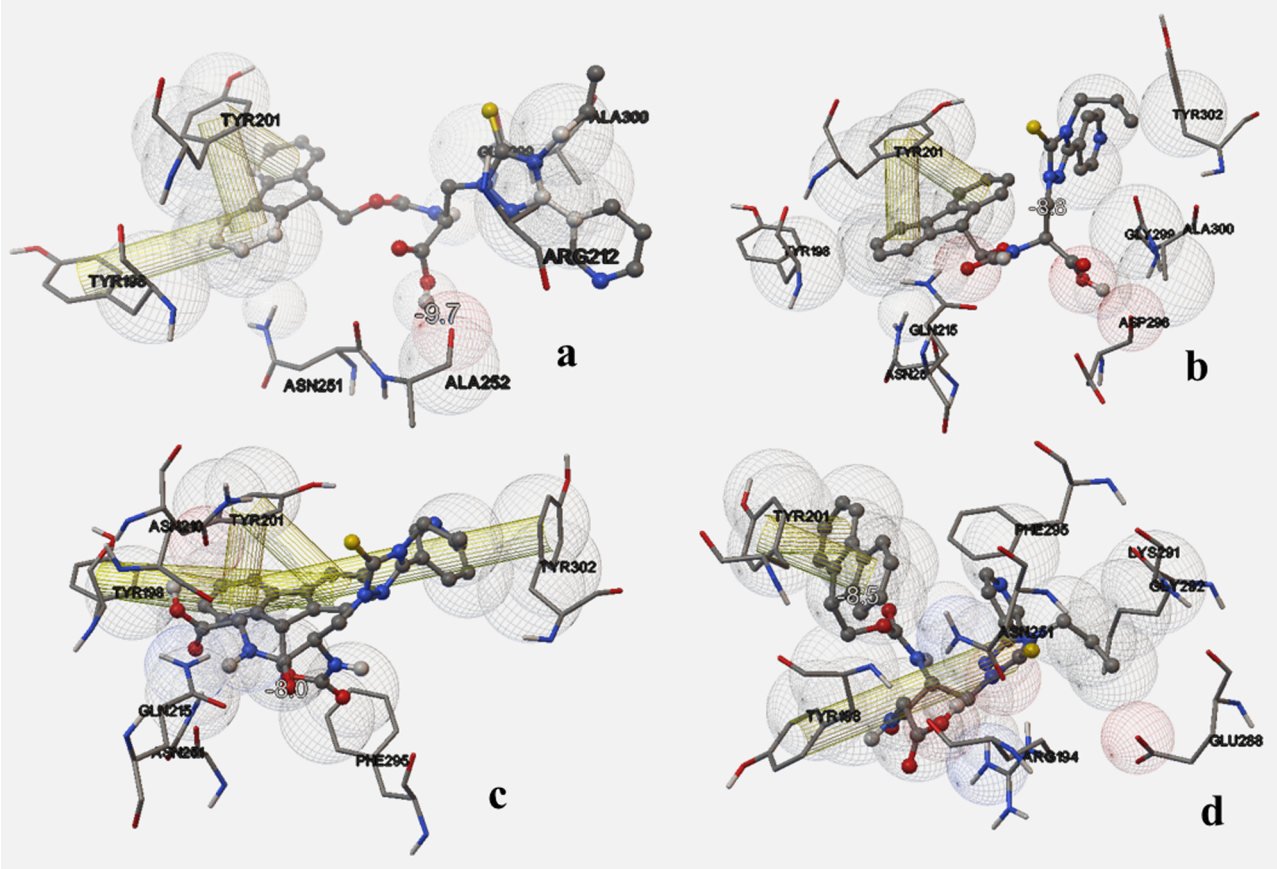

Figure 3. Interaction of bacterial collagenase with **a.** 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridine-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanylglycine (**9**), **b.** 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridine-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine (**4**), **c.** 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridine-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanylglycine (**10**), and **d.** 9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridine-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine (**5**). The color version of the figure is available in the electronic version of the article.

Table 1. Interaction and Inhibition effect of synthetic peptides on collagenase activity

Name	ΔG (kcal/mol)	IC_{50} (μM)
9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridine-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine (**4**)	-8.8	1.45
9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridine-4′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanylglycine (**9**)	-8.5	2.08
9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridine-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine (**5**)	-9.7	1.56
9-fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridine-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanylglycine (**10**)	-8.0	1.75

3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine (**5**) formed a double *p-p* bond with Tyr^{201} (4.277 Å and 4.224 Å) and one more with Tyr^{198} (6.492 Å) (Fig. 3d). These 4 compounds were chosen for synthesis (see above). After synthesis the influence of novel compounds on the activity of *C. histolyticum* collagenase was determined by measuring free amino groups according to *o*-phthalaldehyde (OPA) method. All of them inhibited collagenase activity and IC_{50} values shown in Table 1.

The antibacterial activities of the aforementioned non-proteinogenic amino acids and peptides based thereon were tested against *P. aeruginosa* MDC 5249, and clinical isolates *P. aeruginosa* 80, *E. coli ESBL* 64, and *K. pneumoniae* 63 by the microdilution method. 96-well plates were inoculated with 10 μl of a diluted culture. Various concentrations of studied compounds and 200 μl broth were added to the inoculum. Susceptibility of the strains to aforementioned compounds at final concentrations ranging from 0.001 mM to 0.5 mM was determined. Inoculated plates were subsequently incubated for 18 h at 37°C.

According to the obtained results, all compounds possess antibacterial activity towards tested strains. 9-Fluorenylmethoxycarbonyl-(*S*)-β-[4-allyl-3-(pyridinyl-3′-yl)-5-thioxo-1,2,4-triazol-1-yl]-α-alanine

Table 2. The antibacterial activity of compounds **4**, **5**, **9**, and **10**

Compounds / Strains*	(**5**) (μg/ml)	(**10**) (μg/ml)	(**4**) (μg/ml)	(**9**) (μg/ml)
P. aeruginosa MDC 5249	27	30	55	60
P. aeruginosa 80 (urine)	27	30	55	60
E. coli ESBL 64 (wound discharge)	55	60	>55	>60
K. pneumonia 63 (sputum)	55	30	55	>60

* Inoculum – 10^4 CFU/ml.

demonstrated the highest antibacterial activity with MIC 55 μg/ml. The *E. coli ESBL* 64 was seemed to be more resistant in respect of studied compounds with the MIC >60 μg/ml (Table 2).

CONCLUSIONS

The compounds **4**, **5**, **9**, **10** were chosen as potential inhibitors of collagenase according to the modeling. The inhibition of collagenase activity by these synthesized compounds have confirmed the prediction done by the *in silico* methods. In addition, these compounds demonstrated antibacterial activity against MDR test cultures. The revealed features of compounds **4**, **5**, **9**, and **10** allow us to consider these compounds as putative antibacterial agents.

FUNDING

This study was funded by the High Education and Science Committee of RA, in the frames of the research project No. 23T/AA-006 and the NAS RA within the framework of the "Young Scientists' Support Program" under the code 22YSSPD-015.

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any research involving humans or the use of animals as objects.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

Supplementary materials are available in the electronic version at the journal site (pbmc.ibmc.msk.ru).

REFERENCES

1. *Giacomini E., Perrone V., Alessandrini D., Paoli D., Nappi C., Degli Esposti L.* (2021) Evidence of antibiotic resistance from population-based studies: A narrative review. Infect. Drug Resist., **14**, 849–858. DOI: 10.2147/IDR.S289741
2. *Du Y., Li L., Zheng Y., Liu J., Gong J., Qiu Z., Li Y., Qiao J., Huo Y.X.* (2022) Incorporation of non-canonical amino acids into antimicrobial peptides: Advances, challenges, and perspectives. Appl. Environ. Microbiol., **88**(23), e0161722. DOI: 10.1128/aem.01617-22
3. *Sargsyan A.S., Babayan B.G, Avetisyan N.S., Mkrtchyan A.G, Hovhannisyan A.M., Hovhannisyan N.A.* (2018) Heterocyclic substituted non-protein amino acids as inhibitors of *Clostridium histolyticum* collagenase. Armenian Biological J., **70**(1), 12–15.
4. *Karaman R., Jubeh B., Breijyeh Z.* (2020) Resistance of gram-positive bacteria to current antibacterial agents and overcoming approaches. Molecules, **25**(12), 2888. DOI: 10.3390/molecules25122888.
5. *Hovhannisyan N., Harutyunyan Sh., Hovhannisyan A., Hambardzumyan A., Chitchyan M., Melkumyan M., Oganezova G., Avetisyan N.* (2023) The novel inhibitors of serine proteases. Amino Acids, **37**(3), 531–536. DOI: 10.1007/s00726-009-0257-4.X11
6. *Trott O., Olson A.J.* (2010) AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Comput. Chem., **31**(2), 455–461. DOI: 10.1002/jcc.21334
7. *Sargsyan T., Stepanyan L., Israyelyan M., Hakobyan H., Jamgaryan S., Gasparyan A., Saghyan A.* (2024) The synthesis and *in vitro* study of 9-fluorenylmethoxycarbonyl protected non-protein amino acids antimicrobial activity. Eurasian Chem.-Technol. J., **25**(4), 235–240. DOI: 10.18321/ectj1546
8. *Jolene L.L., Dunn M.K.* (2018) Therapeutic peptides: Historical perspectives, current development trends, and future directions. Bioorg. Med. Chem., **26**(10), 2700–2707. DOI: 10.1016/j. bmc.2017.06.052
9. *Bellotto O., Semeraro S., Bandiera A., Tramer F., Pavan N., Marchesan S.* (2022) Polymer conjugates of antimicrobial peptides (AMPs) with d-amino acids (d-aa): State of the art and future opportunities. Pharmaceutics, **14**(2), 446–465. DOI: 10.3390/pharmaceutics14020446
10. *Wang G., Li X., Wang Z.* (2016) APD3: The antimicrobial peptide database as a tool for research and education. Nucleic Acids Res., **44**(D1), D1087–D1093. DOI: 10.1093/nar/ gkv1278
11. *Kerru N., Gummidi L., Maddila S., Gangu K.K., Sreekantha B., Jonnalagadda A.* (2020) A review on recent advances in nitrogen-containing molecules and their biological applications. Molecules, **25**(8), 1909–1951. DOI: 10.3390/molecules25081909
12. *Kumar A., Singh A.K., Singh H., Vijayan V., Kumar D., Naik J., Thareja S., Yadav J.P., Pathak P., Grishina M., Verma A., Khalilullah H., Jaremko M., Emwas A.H., Kumar P.* (2023) Nitrogen containing heterocycles as anticancer agents: A medicinal chemistry perspective. Pharmaceuticals, **16**(2), 299–367. DOI: 10.3390/ph16020299

13. *Sargsyan A., Hakobyan H., Mardiyan Z., Jamharyan S., Dadayan A., Sargsyan T., Hovhannisyan N.* (2023) Modeling, synthesis and *in vitro* screening of unusual amino acids and as protease inhibitors. J. Chem. Technol. Metall., **58**(3), 615–620. DOI: 10.59957/jctm.v58i3.93

14. *Eckhard U., Schönauer E., Brandstetter H.* (2013) Structural basis for activity regulation and substrate preference of clostridial collagenases G, H, and T. J. Biol. Chem., **288**(28), 20184–20194. DOI: 10.1074/jbc.M112.448548

Received: 29. 09. 2024.
Revised: 06. 11. 2024.
Accepted: 11. 11. 2024.

МОДЕЛИРОВАНИЕ, СИНТЕЗ И *IN VITRO* ТЕСТИРОВАНИЕ ПЕПТИДОВ НА ОСНОВЕ НЕБЕЛКОВЫХ АМИНОКИСЛОТ В КАЧЕСТВЕ ПОТЕНЦИАЛЬНЫХ АНТИБАКТЕРИАЛЬНЫХ СОЕДИНЕНИЙ

А.С. Саргсян[1]*, Л.Т. Карапетян[1], А.В. Мхитарян[1], Л.А. Степанян[1], Т.О. Саргсян[1,2], Ю.М. Дангян[1], А.В. Саргсян[1], Г.Г. Оганезова[1], Н.А. Оганесян[1,2]

[1]Научно-производственный центр "Армбиотехнология" НАН РА,
Армения, 0056, Ереван, Гюрджян, 14; *эл. почта: armenssargsyan@gmail.com
[2]Ереванский Государственный Университет, Армения, 0025, Ереван, Алек Манукян, 1

В настоящее время небелковые аминокислоты и синтетические пептиды широко используются в качестве блоков при создании лекарственных препаратов. Многие протеазы представляют большой интерес для фармакологии в связи с их ключевой ролью в различных патологиях. Бактериальная коллагеназа (КФ 3.4.24.3) является весьма привлекательной мишенью для разработки лекарственных препаратов, поскольку ингибиторы бактериальной коллагенолитической протеазы могут остановить распространение заболеваний, вызванных инфекциями. В данной работе исследовали взаимодействие пептидов, содержащих необычные аминокислоты, с коллагеназой *Clostridium histolyticum*. По результатам докинг-анализа для дальнейших исследований были отобраны 4 пептида, и был проведён их синтез. Все протестированные соединения ингибировали активность коллагеназы с диапазоном значений IC_{50} 1,45–2,08 мкМ. Выявлена антибактериальная активность синтезированных соединений в отношении штаммов, устойчивых к антибиотикам. Минимальные ингибирующие концентрации (МИК) были определены в диапазоне 4,6–9,2 мкг/мл.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: небелковая аминокислота; докинг; коллагеназа; ингибитор; антибактериальная активность

Финансирование. Исследование выполнено при финансовой поддержке Комитета по высшему образованию и науке РА в рамках научного проекта № 23T/AA-006 и НАН РА в рамках "Программы поддержки молодых ученых" по коду 22YSSPD-015.

Поступила в редакцию: 29.09.2024; после доработки: 06.11.2024; принята к печати: 11.11.2024.