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The Synthesis and *in vitro* Study of 9-fluorenylmethoxycarbonyl Protected Non-Protein Amino Acids Antimicrobial Activity

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Abstract

Using the 9-fluorenylmethoxycarbonyl protecting group, 9-fluorenylmethoxycarbonyl-*(S)*-β-(N-imidazolyl)-α-alanine protected non-protein amino acid, not described in the literature, were developed. Then 9-fluorenylmethoxycarbonyl-*(S)*-α-methylphenylalanine, 9-fluorenylmethoxycarbonyl-*(S)*-α-allylglycine, 9-fluorenylmethoxycarbonyl-*(S)*-α-propargylglycine were synthesized by the same method. It was shown, that the 9-fluorenylmethoxycarbonyl-*(S)*-β-(N-imidazolyl)-α-alanine (3) inhibited the growth of Gram-negative *Salmonella typhimurium G-38* and 9-fluorenyl-methoxycarbonyl-*(S)*-α-methyl-phenylalanine (4) inhibited the growth Gram-positive *Bacillus subtilis 17-89* bacteria.

1. Introduction

Despite the fact that amino acids, amino acid derivatives, and peptides have been studied in various fields of chemistry and medicine for decades, interest in peptides remains topical today. Peptides are pharmacologically active compounds used in the treatment of various diseases from diabetes to tumors [1–3].

Nowadays, among amino acid derivatives, protected amino acids are being successfully studied, as the introduction of a protective group can strongly change the properties of the amino acid. One such protecting group is the 9-fluorenylmethoxycarbonyl group, which is considered the best protecting group in the peptide synthesis as it can be introduced into the amino acid structure with high yield and can be selectively removed at the end when obtaining free peptide. Moreover, it has anti-inflammatory, and antimicrobial properties, according to a number of publications in the last decade. For example, 9-fluorenylmethoxycarbonyl-phenylalanyl-phenylalanine dipeptide possesses antimicrobial activity against Gram-positive and Gram-negative bacteria.

Among the cholinesterase inhibitors, the 9-fluorenylmethoxycarbonyl leucine, 9-fluorenylmethoxycarbonyl tryptophan have high cholinesterase inhibition properties, etc. [4–7].

2. Experimental part

2.1. Chemistry Materials

All reagents were obtained from commercial sources and used without further purification. Thin layer chromatography (TLC) was carried out on Merck aluminium foil backed sheets pre-coated with 0.2 mm Kielselgel 60 F254. Melting points (mp) were determined by "Elektrothermal". ^{1}H and ^{13}C NMR spectra were recorded on Varian Mercury 300 MHz spectrometer using TMS as an internal standard. Elemental analysis was done by Euro EA3000.

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The synthesis of 9-fluorenylmethoxycarbonyl-(S)-β-(N- imidazolyl)-α-alanine: The mixture of g (0.0043 mol) of *(S)*-β-(N-imidazolyl)-α-alanine (2), 0.456 g (0.0043 mol) of Na_2CO_3 was added in a round-bottomed flask. Then, the mixture was stirred at room temperature with a magnetic stirrer until a clear solution was formed, after which 1.955 g (0.0058 mol) of 9-fluorenylmethoxycarbonyl-N-oxysuccinimide (1) ester 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 reaction was monitored by the TLC method [SiO_2, $CHCl_3$/ethyl acetate/MeOH (4:2:1); developer: chlorotoluidine]. To remove the unreacted starting material, first, the reaction mixture was extracted twice with diethyl ether, then 20 ml of distilled ethyl acetate was added to the reaction mixture and acidified with 2 N hydrochloric acid to pH 2, and finally, 10 ml of ethyl acetate was added and extracted two times.

Then the organic fractions were combined and 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 ethyl acetate-hexane 1:3, filtered and dried under vacuum conditions (50-60 °C). As a result, a white crystalline mass was obtained. The yield of the final product was 70%. Anal.Calcd for $C_{21}H_{19}N_3O_4$ (377.40) C, 66.83; H, 5.07; N, 11.13. Found: C, 66.95; H, 5.27; N, 11.28. ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm, J/Hz): δ=4.21 brt (1H, J=6.5, CH); 4.40 brd (2H, J=6.5, OCH_2); 4.54 brdd (1H, J=12.8, 8.3, NCH_2); 4.66-4.84 m (2H, $CHCH_2N$); 7.29-7.36 m (2H); 7.37-7.44 m (2H); 7.61-7.65 m (2H); 7.79-7.83 m (2H, 8 Ar-H); 7.53 brs (2H 2 imid-H); 8.88 brs (1H 2 imid-H).

^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 48.4 (CH), 50.8 (NCH_2), 55.2 (NCH), 68.0 (OCH_2), 121.0 (2CH), 124.1 (CH), 126.1 (2CH), 128.2 (2CH), 128.9 (2CH), 137.2 (CH), 142.6 (2C), 145.1 (CH), 158.3 (CO), 171.3 (CO).

The syntheses of 9-fluorenylmethoxycarbonyl-*(S)*-α-methylphenylalanine, 9-fluorenylmethoxycarbonyl-*(S)*-α-allylglycine, 9-fluorenylmethoxycarbonyl-*(S)*-α-propargyl-glycine, acids were carried out by the above mentioned method, the physicochemical parameters of the synthesized amino acid derivatives were investigated and compared with the literature data.

Chemical purity of 9-fluorenylmethoxycarbonyl-*(S)*-β-(N-imidazolyl)-α-alanine amino acids were carried out by the HPLC method.

The chemical purity of newly synthesized amino acid was carried out using the HPLC system "Waters Separation module e2695" (USA) equipped with a "Waters 2487" Dual λ Absorbance UV Detector (Milford, MA, USA). As a chromatographic column an Alltima C18, 250 X4.6 mm with 5 µm particle size was used. The mobile phase was acetonitrile and 2 mmol/L Ammonium persulfate (15:85 v/v). Sample weight 10 mg in 1 ml water, injection volume 5 µl (Table 1).

Table 1. Chromatography conditions for the chemical purity of amino acids

Chromatographic column	Alltima C18, 250 x 4.6 mm, 5 µm
Detector wavelength	210 nm, UV
Flow rate	0.5 ml/min
Column temperature	40 °C
Mobile phase	A: 15% CH_3CN B: 85% $(NH_4)_2S_2O_8$

Chemical purity of the synthesized amino acids: 9-fluorenyl-methoxycarbonyl-*(S)*-phenylalanine, 9-fluorenylmethoxycarbonyl-*(S)*-α-allylglycine, 9-fluorenylmethoxycarbon-yl-*(S)*-α-propargylglycine was testedsimilarly.

Test culture: To determine antimicrobial properties of protected non-protein amino acid, the conditionally pathogenic Gram-negative *Salmonella typhimurium* and Gram-positive *Bacillus subtilis G17-89* from the Microbial Culture Collection of the Microbial Depository Center (MDC) at the SPC "Armbiotechnology" NAS RA, were used. Test cultures were grown on solid Nutrient agar (Himedia, India) at pH 7.2 for 16–18 h and at 37 °C. Then cells were harvested and suspended in the Nutrient broth at a concentration of approximately $2.2x10^6$ CFU/ml.

Detection of antimicrobial activity: The spot-on-lawn method on the test culture pre-plated in the solid medium was applied. For the spot-on-lawn method, suspension of the overnight test culture in the Nutrient broth (containing 10^6 CFU/ml) speeded across the surface and aliquots of investigated samples of 20, 40, and 60 µg were applied above the test culture using a micropipette. Plates remained at 4 °C for 1–2 h to promote diffusion of samples. Then plates were incubated in temperature-controlled conditions at 30 °C during 24–48 h. Antimicrobial activity was assessed by measuring the size of the inhibition zone (diameter) of test culture growth (Ø, mm) after 24 h incubation in a thermostat at 30 °C [8].

Determination of resistance to antibiotics: To determine the resistance of test cultures to antibiotics, the method with antibiotic disks was applied. Each strain was inoculated into appropriate broth, and incubated at 37 °C for 16 h. By spread plate technique the cultures were inoculated in the plates using sterile swabs. The antibiotic disks were placed on the plates. Agar plates with antibiotic disks were then incubated at 37 °C for 24 h. The diameters of the inhibition zones were measured. The results were expressed as sensitive (+) and resistant (-) [8–10].

3. Results and discussion

Developing a method of synthesis of potentially biologically active new non-protein protected 9-fluorenylmethoxycarbonyl-*(S)*-β-(N-imidazolyl)-α-alanine amino acids, as well as synthesizing other well-known protected amino acids in the specified way. The schematic diagram of the reaction is given in Fig. 1.

The addition reaction was optimized by variation of the solvent and base: methanol/NaOH, methanol/KOH, methanol/Na_2CO_3, methanol/K_2CO_3, acetone/NaOH, acetone/KOH, acetone/Na_2CO_3, 1,4-dioxane/K_2CO_3, 1,4-dioxane/NaOH, 1,4-dioxane /KOH, 1,4-dioxane/Na_2CO_3, 1,4-dioxane/K_2CO_3. The best result both in terms of conversion was obtained when the reaction was carried out in 1,4-dioxane using Na_2CO_3 as the base, at room temperature.

A non-protein amino acid was selected considering several important properties of heterocyclic amino acids.

Amino acids and peptides combined with heterocycles represent an important class of therapeutic agents. Biologically active heterocycles are combined with amino acids or peptides to increase drug stability. Besides, drugs based on amino acids and peptides generally have low toxicity, high bioavailability, and permeability, as well as good metabolic and pharmacokinetic properties. Synthetic heterocyclic substituted amino acids and peptides synthesized on their bases are a promising choice shortly for the development of new, less toxic, and safe drugs, which exhibit multiple properties [3, 11–12].

Nitrogen-containing heterocyclic compounds have become the subject of great interest due to their wide application. Studies have shown their positive activity as anti-inflammatory, antioxidant, anti-tumor, anti-ulcer, antidepressant, anti-malarial, anti-tuberculosis, antiviral, anti-hypertensive, anti-diabetic and cholinesterase inhibitory agents [13–14].

By combining two potential antimicrobial compounds, we synthesized new, not described in literature 9-fluorenylmethoxycarbonyl-*(S)*-β-(N-imidazolyl)-α-alanine protected (3) compounds with potential antimicrobial activity.

9-Fluorenyl-methoxycarbonyl-*(S)*-α-methyl-phenylalanine (4), 9-fluorenylmethoxy-carbonyl-*(S)*-α-allylglycine (5), 9-fluorenylmethoxycarbonyl-*(S)*-α-propargylglycine (6), 9-fluorenylmethoxycarbonyl-*(S)*-leucine (7) were also selected and synthesized by the same method (Fig. 2, Table 2).

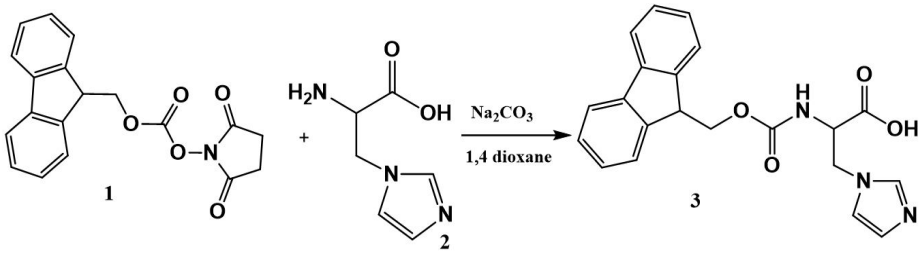

Fig. 1. The protecting reaction for obtaining 9-fluorenylmethoxycarbonyl-*(S)*-β-(N-imidazolyl)-α-alanine (3) amino acids.

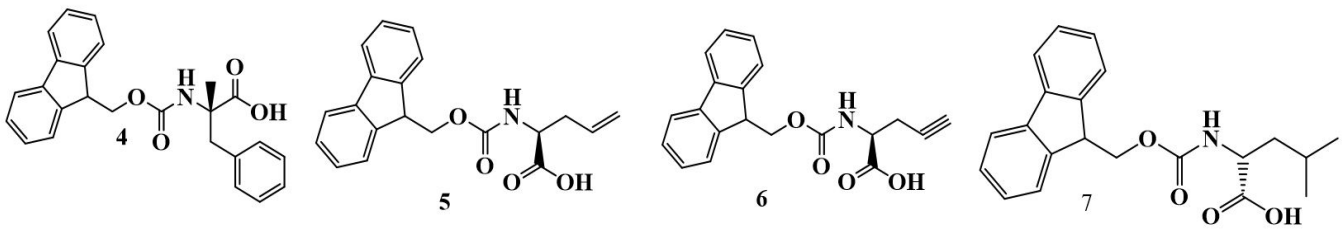

Fig. 2. Structure of N-F-moc protected amino acids.

Table 2. Physicochemical properties of synthesized protected amino acids

Names	$[\alpha]D^{20}$ (C=1 in DMF)^0		Melting temperature		Chemical purity %
	Obtained	Literature	Obtained	Literature	
9-fluorenylmethoxycarbonyl-(*S*)-β-(N-imidazolyl)-α-alanine (3)	-3.3	-	175±2	-	96.3
9-fluorenylmethoxycarbonyl-(*S*)-α-methylphenyl-alanine (4)	-29±2	-28±2	-	-	99.5
9-fluorenylmethoxycarbonyl-(*S*)-α-allylglycine (5)	-9±2	-8±2	137±2	136±2	96.5
9-fluorenylmethoxycarbonyl-(*S*)-α-propargylglycine (6)	-18±2	-19±2	176±2	177±2	99.5
9-fluorenylmethoxycarbonyl-(*S*)-leucine (7)	-25 ± 2	-24± 2	154±2	155±2	99.8

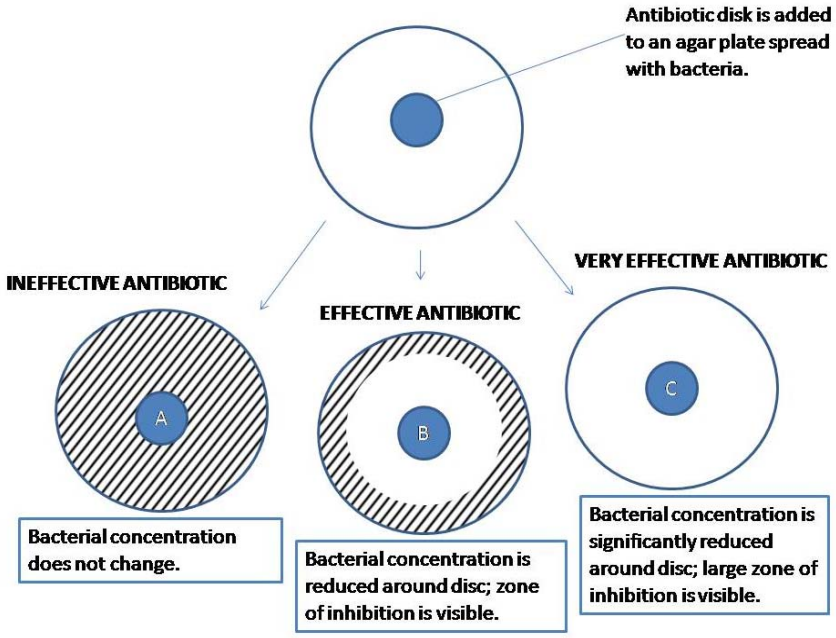

Fig. 3. Schematic diagram of the research on the example of antibiotics.

In the next stage of the research, the antimicrobial activity of the synthesized compounds was carried out along with the evaluation of the resistance of the given test cultures' to a number of antibiotics. For the investigation of test cultures sensitivity to antibiotics, the disk diffusion method was used. It is one of the oldest approaches to antimicrobial susceptibility testing and remains one of the most widely used antimicrobial susceptibility testing standardized methods. A schematic diagram of the study of antibiotic susceptibility testing is shown in Fig. 3.

As seen from the diagram, antibiotics can be classified as bactericidal, then after using of antibiotics total number of viable bacteria significantly reduced around the disk, and zone of inhibition is visible and more than 20 mm (C), bacteriostatic, then after using of antibiotics total number or viable bacteria also reduced around the disk, but visible zone of inhibition about 15–20 mm (B), more than 20 mm, the replication of bacteria are arrests, thus limited the spread of bacterial growth (c). Then antibiotics are ineffective, the bacterial growth is observed and bacterial concentration does not change (A).

The sensitivity of test cultures to the most commonly prescribed antibiotics was performed. The data are given in Table 3.

As can be seen from the presented data, antibiotics have different effects on test culture growth. *Bacillus subtilis 17-89* was more sensitive to examined antibiotics. Growth inhibition zones occur

Table 3. Determination of resistance of *Salmonella typhimurium G-38* and *Bacillus subtilis G17-89* test cultures to antibiotics

Antibiotics (Antibiotics Sensitivity Discs)	Discs Dosage	Test cultures			
		Salmonella typhimurium G-38		*Bacillus subtilis 17-89*	
		Growth inhibition zone, Ø, mm	Sensitivity	Growth inhibition zone, Ø, mm	Sensitivity
Clarithromycin (CLR)	15 μg	18±2	+	28±2	+
Doxycyclin (DXT)	30 μg	14±1	-	18±2	+
Azithromycin (AZM)	15 μg	25±2	+	22±2	+
Tetracycline (TE)	30 μg	12±1	-	16±1	+
Ampicillin (AMP)	10 μg	18±2	+	18±2	+
Amicacin (AN)	30 μg	30±2	+	28±2	+
Ofloxacin (OFX)	5 μg	0	-	24±2	+
Erythromycin (E)	15 μg	0	-	27±2	+
Vancomycin (VA)	5 μg	0	-	16±1	+
Piperacillin (tazobactam, TZP)	110 μg	20±2	+	26±2	+
Cefazolin (KZ)	30 μg	17±2	+	30±2	+
Penicillin G (P)	10 μg	10±1	-	22±2	+
Amoxicillin (AML)	10 μg	28±2	+	26±2	+
Rifampicin (RD)	5 μg	15±1	-	16±1	+
Note: sensitive (+) and stable (-)					

Table 4. Antimicrobial effect of the protected amino acids on the growth of *Salmonella typhimurium G 38* and *Bacillus subtilis G17-89*

Protected non-protein amino acids	Test cultures					
	Salmonella typhimurium G-38			*Bacillus subtilis 17-89*		
	20 μg	40 μg	60 μg	20 μg	40 μg	60 μg
9-fluorenylmethoxycarbonyl-*(S)*-β-(N-imidazolyl)-α-alanine (3)	20±2	22±2	25±2	neg.	neg.	neg.
9-fluorenyl-methoxycarbonyl-*(S)*-α-methyl-phenylalanine (4)	10±1	12±1	12±1	20±2	20±2	22±2
9-fluorenylmethoxycarbonyl-*(S)*-α-allyl-glycine (5)	15±1	15±1	15±1	14±1	17±2	17±2
9-fluorenylmethoxycarbonyl-*(S)*-α-propargyl-glycine (6)	12±1	16±2	16±2	10±1	18±2	18±2
9-fluorenylmethoxycarbonyl-*(S)*-leucine (7)	12±1	14±1	14±1	12±1	18±2	20±2
Note: growth – growth pressure; negative – lack of growth pressure; μg – injected doses						

about 20–30 mm. While *Salmonella typhimurium G-38* showed high resistance to almost all investigated antibiotics.

The investigation of the antimicrobial activity of the synthesized compounds was carried out. The results are presented in Table 4.

As can be seen from the results of the research, amino acids suppress the growth of the mentioned bacteria with different efficiency. The amount of injected non-protein amino acids does not affect antimicrobial activity, the test culture growth inhibition zones are practically the same. Sample (3) shows the highest activity against *Salmonella typhimurium G-38* test culture but does not inhibit the growth of *Bacillus subtilis 17-89* strain. Sample (4) more efficiently inhibited the growth of *Bacillus subtilis*

17-89 test culture. Other samples (5-7) showed the same activity against investigated test cultures. Growth inhibition zones occur about 12–18 mm.

The obtained results were compared with antibiotic disk susceptibility testing data. As was shown during in vitro investigation, the samples (3) and (4) own bactericidal activity. The amino acids 9-fluorenylmethoxycarbonyl-*(S)*-β-(N-imidazolyl)-α-alanine (3) inhibited the growth of Gram-negative *Salmonella typhimurium G-38* and 9-fluorenyl-methoxycarbonyl-*(S)*-αmethyl-phenylalanine (4) inhibited the growth Gram-positive *Bacillus subtilis 17-89* bacteria better than some antibiotics (see Table 4).

4. Conclusion

Thus, using the 9-fluorenylmethoxycarbonyl protecting group, 9-fluorenylmethoxycarbonyl-*(S)*-β-(N-imidazolyl)-α-alanine protected non-protein amino acid, not described in the literature, were developed. Then 9-fluorenylmethoxycarbonyl-*(S)*-α-methylphenylalanine, 9-fluorenylmethoxycarbonyl-*(S)*-α-allylglycine, 9-fluorenylmethoxycarbonyl-*(S)*-α-propargylglycine were synthesized by the same method. The 9-fluorenylmethoxycarbonyl-*(S)*-β-(N-imidazolyl)-α-alanine (3) suppressed the growth of Gram-negative *Salmonella typhimurium G-38* and 9-fluorenyl-methoxycarbonyl-*(S)*-α-methyl-phenylalanine (4) suppressed the growth Gram-positive *Bacillus subtilis 17-89* bacteria. The prospects for the use of non-protein amino acids in the fight against pathogenic microbes are obvious. They can discuss alternatives to antibiotics in the prevention or treatment of certain infectious diseases caused by antibiotic-resistant bacteria of various origins.

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