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Dr. Shivakumara Kanchidoddi Nanjaiah
Department of Chemistry, Maharani's Science College for Women, Bangalore, Karnataka, India

Correspondence:
Dr. Shivakumara Kanchidoddi Nanjaiah
Department of Chemistry, Maharani's Science College for Women, Bangalore, Karnataka, India

Synthesis and antimicrobial evaluation of novel amino acids coupled piperidine hybrids

Dr. Shivakumara Kanchidoddi Nanjaiah

Abstract
A range of new amino acids-4-benzylpiperidine conjugates 3(a-l) were successfully synthesized with high yield, by coupling of Boc-protected amino acids with 4-benzylpiperidine using EDCI and HOBt as coupling agents. The reaction took place in basic media (NMM) at a temperature of -15^{0}C for a duration of 24 hours. The synthesized compounds were thoroughly characterized using techniques such as ^{1}HNMR, elemental analysis, R_f values, and recording melting point. Additionally, the antimicrobial activities of these compounds were assessed, revealing significant activities in some cases. The compounds were tested against antibacterial strains including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pnemoniae*, and *Pseudomonas aeruginosa*, as well as antifungal strains such as Aspergillus Niger, *Aspergillus flavus*, and *Fusarium monoliforme*. Notably, compounds 3e, 3i, and 3j exhibited good to significant activities compared to the starting material, although they were less potent than conventional drugs. Consequently, these compounds hold promise as valuable tools for further research endeavours.

Keywords: Antimicrobial drugs, 4-Benzylpiperidine, conjugated amino acids, hydrophobicity and biocompatibility

1. Introduction
Antibiotic resistance has emerged as a significant concern for public health, impacting economies and societies worldwide. This issue encompasses both community-acquired infections such as Streptococcal infections, pneumonia, and typhoid fever, as well as hospital-acquired infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), vancomycin intermediate *S. aureus* (VISA), and extended-spectrum beta-lactamase (ESBL) enzyme-producing Gram-negative bacteria. These infections result in higher hospitalization rates, prolonged stays, and increased treatment costs, placing a greater economic burden on communities. In the past, the development of new and effective antibiotics kept up with the emergence of antibiotic resistance. However, the current situation is such that the available antibiotics have become ineffective in treating diseases caused by proven bacterial sources, particularly in a hospital environment. Consequently, research efforts are now focused on identifying novel targets and drugs to combat infectious diseases [1].

The growing interest in heterocyclic compounds is due to their increased biological activity and potential for unique material development. Piperidine and its derivatives are a particularly fascinating and promising group within this category. In the field of medicinal chemistry, arylpiperidine derivatives have shown a wide range of biological activities [2]. Studies have shown that aryl group located at the 4th position of the piperidine ring exhibits a variety of activities. Notably, 4-benzylpiperidine derivatives have displayed substantial biological activities like as CCR5 antagonist [3], antimycobacterial agent [4], hyperalgesia treatment [5], NR2B selective NMDA receptor antagonist [6], NMDA receptor modulator [7], CC Chemokine receptor (CCR3) antagonist [8], NR1A/2B receptor antagonist [9], NK1 antagonist [10], CCR3 antagonist [11], antimicrobial agent [12] and biocidal activity [13].

In contrast, amino acids play a vital role in the metabolism of living organisms as essential components. They have gained attention in the field of biomedical research due to their low toxicity, biocompatibility, and similarity to small bioactive heterocyclic motifs. Numerous successful studies have demonstrated the enhanced potency, selectivity, *in vivo* stability, solubility, cell permeability, and reduced toxicity of bioactive heterocycles through the coupling reaction between amino acids and heterocyclic moieties.

Building upon these rational points, our ongoing work focuses on heterocyclic conjugated amino acids/peptides [16-18]. Surprisingly, there is a lack of literature reports on the conjugation of amino acids with 4-benzylpiperidine. Therefore, our present investigations aim to study the effects of conjugating different amino acids with 4-benzylpiperidine.

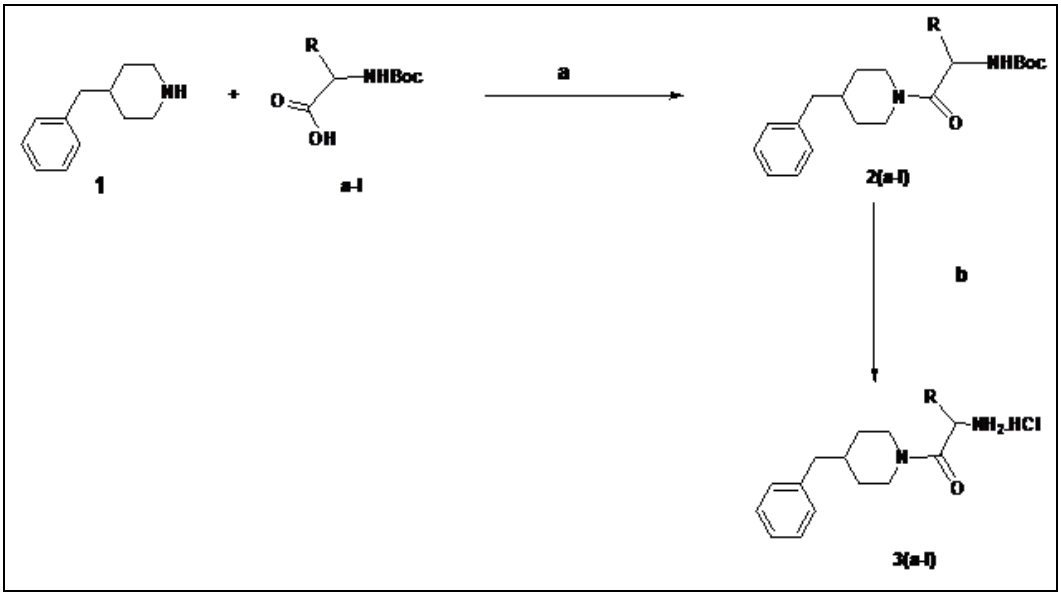
2. Materials and Methods

Unless otherwise stated, all amino acids used were in the L-configuration. All tert-butyloxycarbonyl (Boc) amino acid derivatives and 1-hydroxybenzotriazole (HOBt) were purchased from Advanced Chem. Tech., (Louisville, Kentucky, USA). Isobutylchloroformate (IBCF), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) and N-methyl morpholine (NMM) were purchased from Sigma Chemicals Co. (St. Louis, USA). All solvents and reagents used for synthesis and analysis were of analytical grade. TLC was performed on silica gel plates that were pre-coated in the laboratory using chloroform/methanol/acetic acid (95:5:3, 85:15:3) as solvent systems. Sigma-Aldrich India supplied 4-Benzylpiperidine (1). ^{1}H NMR spectra were obtained using DMSO/$CDCl_3$ as solvent and TMS as an internal standard on a 400 MHz Bruker FT-NMR Spectrometer instrument. Elemental analysis was conducted using VARIO EL III CHNS Elementar.

a) General procedure for the coupling of *N$^{\alpha}$*-Boc amino acids with 4-benzylPiperdine 2(a-l): In a cooled solution of DMF (10 mL), N$^{\alpha}$-Boc-amino acid (2 mmol) and HOBt (0.31 g, 2 mmol) were added, followed by NMM (0.22 mL, 2 mmol). The mixture was then further cooled to -15 °C ± 1 °C and EDCI (0.39 g, 2 mmol) and 4-benzylpiperidine (1, 2 mmol) were added. After 20 minutes, the pH of the solution was adjusted to 8 by adding NMM and the reaction mixture was stirred overnight while slowly warming to room temperature. The reaction mixture was quenched with water (2 mL) and the solvent was condensed. The residue was dissolved in chloroform (25 mL) and washed with 5% $NaHCO_3$ (3 X 20 mL), H_2O (1 X 20 mL), 0.1N cold HCl (3 X 20 mL) and brine solution (3 X 20 mL). The product was then dried over anhydrous Na_2SO_4. The desired products 2(a-l) were obtained after removing the chloroform under reduced pressure. The analytical data of these compounds are presented in Table 1.

b) Deprotection of Boc group

The Boc group of the synthesized compounds (1 mmol) deblocked by the treatment with 4N HCl in dioxane (10 mL/g of the compound) for a duration of 1.5 hours. Excess HCl and dioxane were eliminated through reduced pressure, followed by triturating with ether, filtering, washing with ether, and drying. This process resulted in the production of hydrochloride salts of amino acid-4-benzylpiperidine conjugates 3(a-l) with a yield of 100%. Subsequently, these compounds were utilized for antimicrobial studies.

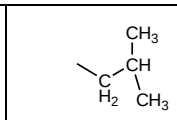
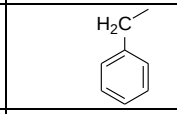
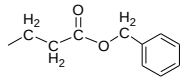
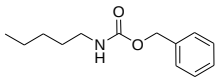
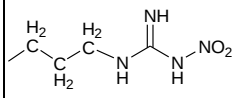
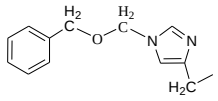
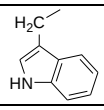


Where R = Side chain of amino acids (Gly, Ala, Val, Leu, Phe, Glu, Be, Arg, Lys, His, Trp, Pro) Reagents and Conditions (a) EDCI, HOER, NIMIM, DIMF, -15°C, 24 hrs. (b) 4NHCI Adixxxane

3. Results and Discussion

Table 1: Analytical and spectroscopic data of the synthesized compounds 2(a-l)

Entry	Side chain of amino acids (R)	R_f Value	Molecular formula	Yield (%)	Elemental analysis (%) Found (Calculated)			^{1}H-NMR ($CDCl_3$), δ ppm
					C	H	N	
3a.	H	0.49	$C_{19}H_{28}N_2O_3$	93	68.63 (68.65)	8.48 (8.49)	8.41 (8.43)	7.20-7.40 (m, 5H, Ar-H); 1.41(s, 9H, Boc); 2.4 (d, 2H, -$ArCH_2$-); 2.65(t, 4H, -CH_2-); 1.70 (m, 4H, -CH_2-); 1.9(m,1H,-CH-piperidine);7.85 (s,1H,NH-Gly); 3.85(s, 2H, -CH_2)
3b.	CH_3	0.45	$C_{20}H_{30}N_2O_3$	90	69.32 (69.33)	8.71 (8.73)	8.09 (8.09)	7.20-7.40 (m, 5H, Ar-H); 1.43(s, 9H, Boc); 7.8 (s, 1H, NH-Ala); 2.45(d, 2H,-$ArCH_2$-); 2.65 (t, 4H, -CH_2-); 1.7 (q, 4H, -CH_2-); 1.91(m, 1H,-CH-piperidine); 4.5 (q, 1H, α-CH-);1.10(d, 3H, CH_3)
3c.	CH_3 / CH_3	0.47	$C_{22}H_{34}N_2O_3$	85	70.54 (70.55)	9.13 (9.15)	7.46 (7.48)	7.10-7.40 (m, 5H, Ar-H); 1.43 (s, 9H, Boc); 8.00 (s, 1H, NH-Val); 2.45 (d, 2H, -$ArCH_2$-); 2.65(t, 4H, -CH_2-); 1.70 (m, 4H, -CH_2-); 1.90 (m, 1H, -CH-piperidine); 4.5 (q, 1H, α-CH-); 1.70 (m, 1H, βCH-); 1.2 (d, 6H, γCH_3).

3d.		0.45	$C_{23}H_{38}N_2O_3$	92	71.09 (71.10)	9.33 (9.34)	7.20 (7.21)	7.15-7.40 (m, 5H, Ar-H); 1.43 (s, 9H, Boc); 7.85 (s, 1H, NH-Leu); 2.4 (d, 2H, -ArCH$_2$-); 2.65 (t, 4H, -CH$_2$-); 1.72 (q, 4H, -CH$_2$-);1.92 (m, 1H, -CH-piperidine); 4.45 (t, 1H, -αCH-); 1.70 (t, 2H, βCH$_2$-); 1.45 (m, 1H, -γCH-); 1.2(d, 6H, δCH$_3$).
3e.		0.54	$C_{26}H_{34}N_2O_3$	90	73.90 (73.91)	8.10 (8.11)	6.62 (6.63)	7.1-7.30 (m, 10H, Ar-H); 1.43 (s, 9H, Boc); 7.90 (s, 1H, NH-Phe); 2.49 (d, 2H, -ArCH$_2$-); 2.7 (t, 4H, -CH$_2$-); 1.80 (q, 4H, -CH$_2$-); 1.90 (m, 1H, -CH-piperidine); 4.55 (t, 1H, -αCH-);3.1 (d, 2H, βCH$_2$-).
3f.		0.40	$C_{29}H_{38}N_2O_5$	95	70.41 (70.42)	7.75 (7.76)	5.65 (5.66)	7.2-7.40 (m, 10H, Ar-H); 1.43 (s, 9H, Boc); 8.0 (s, 1H, NH-Glu); 2.40 (d, 2H, -ArCH$_2$-); 2.69 (t, 4H, -CH$_2$-); 1.75(q, 4H, -CH$_2$-); 1.95 (m, 1H, -CH$_2$-); 4.35(t, 1H, -αCH-); 1.7 (m, 2H, - βCH$_2$-); 1.99 (t, 2H, -γCH$_2$-); 5.15 (s, 2H, CH$_2$-Ph).
3g.		0.51	$C_{31}H_{43}N_3O_5$	90	69.23 (69.25)	8.05 (8.06)	7.82 (7.83)	7.25-7.45(m, 10H, Ar-H); 1.45(s, 9H, Boc); 7.92(s, 1H, NH-Lys); 2.42 (d, 2H, -ArCH$_2$-); 2.60 (t, 4H, -CH$_2$-); 1.91 (m, 1H, -CH-); 1.80 (q, 4H, -CH$_2$-); 4.40 (t, 1H, -αCH-); 1.59 (m, 2H, βCH$_2$-); 1.35 (m, 2H, γCH$_2$-);1.45 (m, 2H, δCH$_2$-); 2.10 (t, 2H, εCH$_2$-); 4.85 (s, 2H, -O-CH$_2$-Ar); 8.0 (s, 1H, NH).
3h.		0.40	$C_{23}H_{36}N_6O_5$	92	57.98 (57.99)	7.60 (7.61)	17.62 (17.63)	7.30-7.45 (m, 5H, Ar-H); 1.45 (s, 9H, Boc); 7.90 (s, 1H, NH-Arg); 2.45 (d, 2H, -ArCH$_2$-); 2.60 (t, 4H, -CH$_2$-); 1.90 (m, 1H, -CH-); 1.7 (q, 4H, -CH$_2$-); 4.50 (t, 1H, -αCH-); 1.30(m, 2H, βCH$_2$-); 1.20 (m, 2H, γCH$_2$-); 2.55 (t, 2H, δCH$_2$-); 8.05 (m, 1H, guanidine).
3i.		0.43	$C_{31}H_{40}N_4O_4$	89	69.89 (69.90)	7.55 (7.57)	10.52 (10.53)	7.25-7.45(m, 10H, Ar-H); 1.43 (s, 9H, Boc); 7.90 (s, 1H, NH-His); 2.49 (d, 2H, -ArCH$_2$-); 2.75 (t, 4H, -CH$_2$-); 2.00 (m, 1H, -CH-); 1.77 (q, 4H, -CH$_2$-); 4.58 (t, 1H, -αCH-); 3.15 (d, 2H, -βCH$_2$-); 6.48 (s, 2H, imidazole); 4.53 (s, 2H, CH$_2$); 5.55 (s, 2H, CH$_2$).
3j.		0.49	$C_{28}H_{35}N_3O_3$	90	72.85 (72.86)	7.63 (7.64)	9.09 (9.10)	7.20-7.45 (m, 9H, Ar-H); 1.43 (s, 9H, Boc); 7.90 (s, 1H, NH-Trp); 2.45 (d, 2H, -ArCH$_2$-); 2.77 (t, 4H, -CH$_2$-); 1.99 (m, 1H, -CH-); 1.80 (q, 4H, -CH$_2$); 4.50 (t, 1H, -αCH-); 3.12 (d, 2H, βCH$_2$-); 10.00 (d, 1H, NH-indole);6.70(s, 1H, -CH-).
3k.		0.41	$C_{23}H_{36}N_2O_3$	90	71.09 (71.10)	9.33 (9.34)	7.19 (7.21)	7.20-7.40 (m, 5H, Ar-H); 1.43 (s, 9H, Boc); 7.90 (s, 1H, NH-Ile); 2.50(d, 2H, -CH$_2$-); 2.72 (q, 4H, -CH$_2$-); 1.95 (m, 1H, -CH-); 1.70 (q, 4H, -CH$_2$-); 4.45(d, 1H, -αCH-); 2.5(m, 1H, βCH-); 1.90 (m, 2H, γCH$_2$-); 1.0 (d, 6H, δCH$_3$).
3l.		0.45	$C_{22}H_{32}N_2O_3$	90	70.92 (70.94)	8.64 (8.66)	7.50 (7.52)	7.2-7.5 (m, 5H, Ar-H); 1.45 (s, 9H, Boc); 2.49 (d, 2H, -ArCH$_2$-); 2.70 (t, 4H, -CH$_2$-); 1.90 (m, 1H, -CH-); 1.75 (m, 4H,-CH$_2$-); 3.85 (t, 1H, -αCH-); 1.67 (m, 2H, -βCH$_2$); 1.45 (m, 2H, γCH$_2$); 3.1 (t, 2H, -δCH$_2$);

a) General method for antibacterial assay

In order to assess their effectiveness against various bacteria, antibacterial assays were carried out *in vitro* using the agar well diffusion system [14]. The bacterial strains, including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pnemoniae*, and *Pseudomonas aeruginosa*, were cultivated in Muller-Hinton broth. The inoculum concentration was adjusted using the mid-logarithmic phase system (OD 600 = 0.5). To prepare the agar media, sterile distilled water was added to Muller-Hinton agar and autoclaved for one hour. The autoclaved media was then poured into pre-sterilized 90 mm petriplates and allowed to solidify. Using an 8 mm sterile cork borer, the media was removed from the centre, creating a well for the assay. The inoculum was spread evenly over the media. Additionally, 50 µL of a stock solution of compounds (10 µg/well) was added to the wells created in the petriplates. The petriplates were then incubated at 37 °C for 3-4 days. All synthesized compounds were tested in triplicate, with streptomycin serving as the positive control and water as the negative control. The zone of inhibition, measured in millimetres, was recorded and presented in Table 2 and Fig 1.

Table 2: Antibacterial activity of synthesized compounds:

Compounds [a]	Inhibitory Zone (diameter) mm[b]			
	Staphylococcus aureus	***Escherichia coli***	***Klebsiella pneumoniae***	***Pseudomonas aeruginosa***
1.	01	02	02	02
3a.	03	04	04	03
3b.	04	04	03	03
3c.	05	04	05	04
3d.	06	05	06	05
3e.	08	07	06	07
3f.	04	06	06	04
3g.	06	06	05	06
3h.	04	06	05	05
3i.	08	06	07	08
3j.	08	08	07	07
3k.	06	05	06	05
3l.	06	05	05	06
Streptomycin	12	12	10	11

[a] The compounds and reference drug were concentrated at 10 µg per well.

[b] The mean values were obtained from three determinations, with ranges less than 5% of the mean in all cases.

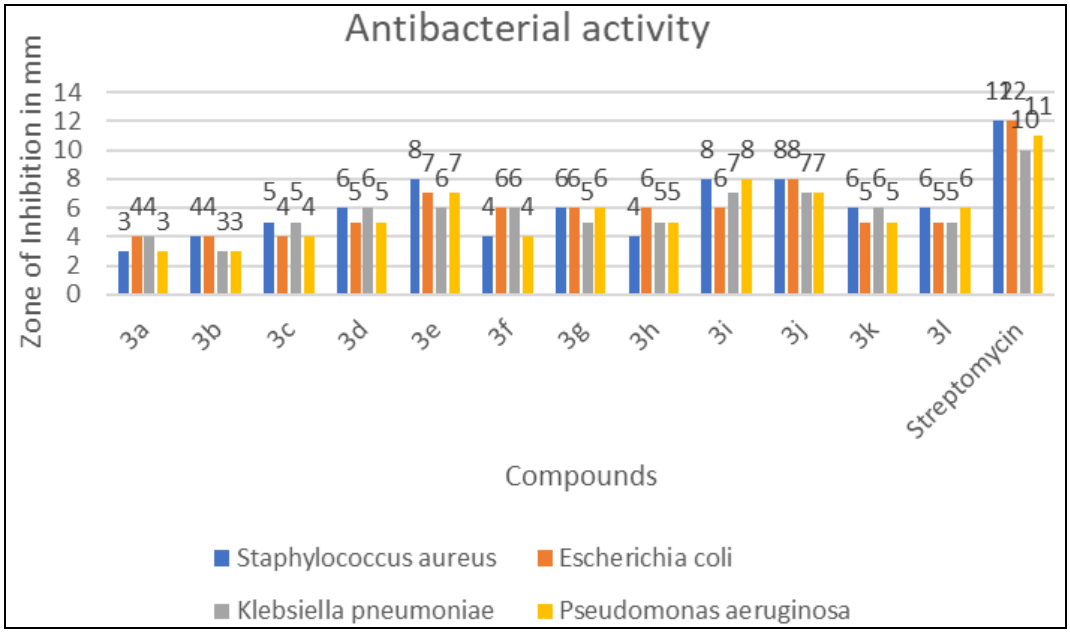


Fig 1: Antibacterial activity of synthesized compounds

b) General method of antifungal assay

Antifungal tests were carried out *in vitro* using the agar well diffusion technique to assess their efficacy against Aspergillus Niger, *Aspergillus flavus*, and *Fusarium monoliforme* [15].

The fungal cultures were cultivated on PDA media with a p^H of 7.4 for six days at a temperature of 25 °C. The spores were collected in sterilized normal saline solution and adjusted to a concentration of $1x10^6$/ml using a Haemocytometer. Autoclaved molten media (20 mL) was poured into each sterilized 90 mm petriplate and allowed to solidify. To evaluate the growth response of the fungal species, 0.4 mL of the synthesized compounds (10 µg/mL) was evenly distributed over the agar media in each plate. Subsequently, a volume of 10 µL spore suspension was added to the small depression created at the centre of each plate, and the plates were incubated for six days at 25 °C. Following the incubation period, the plates were examined and compared to their respective control plates. The control plates contained only distilled water, representing 100% fungal growth (no inhibition). The fungicidal activity of the synthesized compounds was assessed by comparing the zone of fungal growth in the treated plates to that of the control plates in millimetres. The results are presented in Table 3 and Fig 2.

Table 3: Antifungal activity of activity of synthesised compounds:

Compounds [a]	Inhibitory Zone (diameter) mm[b]		
	Aspergillus Niger	***Aspergillus flavus***	***Fusarium monoliforme***
1	02	02	02
3a	01	02	02
3b	03	04	04
3c	04	04	03
3d	05	04	05
3e	06	05	06
3f	08	07	06
3g	04	06	06
3h	06	06	05
3i	04	06	05
3j	08	06	07
3k	08	08	07
3l	06	05	06
Bavistin	09	10	09

[a] The compounds and reference drug were concentrated at 10 µg per well.

[b] The mean values were obtained from three determinations, with ranges less than 5% of the mean in all cases.

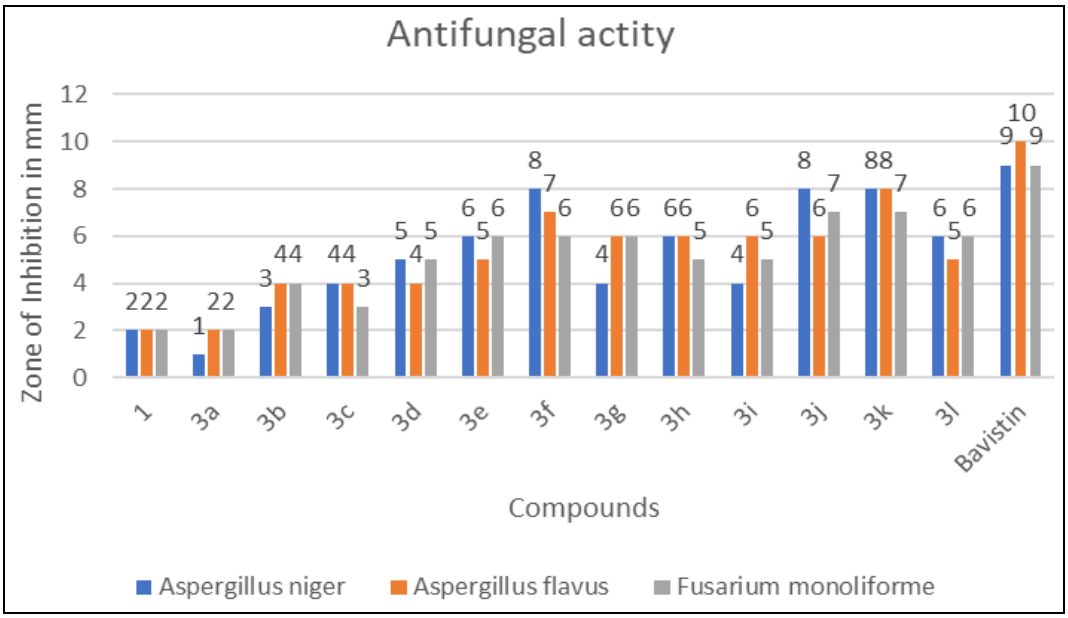


Fig 2: Antifungal activity of synthesized compounds

A series of 3(a-l) derivatives, conforming of amino acids conjugated with 4-benzylpiperidine, have been successfully synthesized. This was achieved by coupling 4-benzylpiperidine (1) with N-α Boc-amino acids (a-l) using EDCI / HOBt as the coupling agent and NMM as a base. The performing product displayed a sticky texture and was characterized through TLC, elemental analysis, and ^{1}H-NMR. The Boc-deprotected analogues of the synthesized composites were employed to estimate their antimicrobial activity.

Structural-activity relationship study

Antibacterial activity

The Boc deprotected compounds 3(a-l) were subjected to testing against gram +ve and gram -ve bacteria strains including *Staphylococcus aureus*, Klebsiella pneumoniae, *Pseudomonas aeruginosa*, and *Escherichia coli*. Streptomycin was utilized as a positive control while water served as a negative control. The concentration used for both test compounds and standard remained constant. Among the synthesized compounds, phenylalanine (Phe) 3e, histidine (His) 3i, and tryptophan (Trp) 3j conjugates exhibited the highest activity, albeit lower than conventional antibiotics, while the remaining compounds showed moderate activity. The presence of both the heterocyclic moiety and amino acid functionalities in the synthesized compounds may have contributed to the observed enhancement in antibacterial activity. The hydrophobicity of amino acid side chains, the presence of an aromatic group in phenylalanine, the presence of a heterocyclic indole ring in tryptophan, and the presence of an imidazole ring in the side chain of histidine may be responsible for the enhancement of antibacterial activity. These factors help the molecule to interact/penetrate more with the cell membrane of the microorganisms, thereby inactivating them. Previous studies have reported the significance of activity revealed by aromaticity and hydrophobicity, and hence more hydrophobic and aromatic amino acids such as Phe, Trp, and Tyr conjugates showed good activity.

Antifungal activity

The synthesized compounds were tested for their antifungal activity against fungal strains including Aspergillus Niger, *Aspergillus flavus*, and *Fusarium monoliforme*. Among all the compounds, the highest antifungal activity was observed for certain ones. Specifically, the phenylalanine analogues 3e, histidine analogues 3i, and tryptophan analogues 3j exhibited better activity compared to the other compounds, although their activity was lower than the conventional standard drug Bavistin. The rest of the compounds across all series showed mild to moderate antifungal activity. Similar to the antibacterial activity results, the compounds with Trp, Phe, and His residues displayed enhanced antifungal activities due to the presence of aromatic systems in both the amino acid residue and heterocyclic system. The aromaticity present in both moieties improved the antimicrobial properties of these synthesized compounds. However, this was not observed for other molecules where the amino acid residues lacked aromatic rings (having only aliphatic side chains) and the heterocycles alone did not confer good to moderate properties despite having aromaticity. Therefore, the molecules showing enhanced properties can be considered lead compounds in their respective series. Overall, the results highlight the importance of conjugating amino acids with heterocycles for achieving antibacterial and antifungal activities. Explained the antibacterial activity also held true here. Therefore, the compounds containing Trp, Phe, and His residues showed enhancement in both antibacterial and antifungal activities. This enhancement can be attributed to the presence of aromatic systems in both the amino acid residue and the heterocyclic system. The presence of aromaticity in both moieties enhanced the antimicrobial properties of the synthesized compounds. However, this was not the case with other molecules where the amino acid residues lacked the aromatic ring system (having only aliphatic side chains) and the presence of aromaticity in heterocycles only resulted in good to moderate properties. Therefore, the molecules exhibiting enhanced properties can be considered as lead molecules in the series. Overall, the results of antibacterial and antifungal activities highlight the importance of amino acid conjugation with heterocycles.

Conclusion

In our pursuit to discover a fresh bioactive molecule, we have come across promising antimicrobial activity in phenylalanine, tryptophan, histidine, and proline analogues. These amino acid conjugates, when compared to their parent compounds, have exhibited enhanced antibacterial and antifungal activities. This enhancement can be attributed to the synergistic effect of the

heterocyclic skeleton and amino acid residues. The results obtained suggest that further investigation into the conjugation of these heterocycles with peptides of varying chain length and composition could lead to the identification of a new class of antimicrobial agents.

Conflict of Interest

The authors have no conflicts of interest regarding this investigation.

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