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ISSN: 2278-1862

Journal of Applicable Chemistry

2019, 8 (4): 1685-1697

(International Peer Reviewed Journal)

Synthesis and Antimicrobial Evaluation of new *N*-acyl bis *n*-butyl glycine Derivatives

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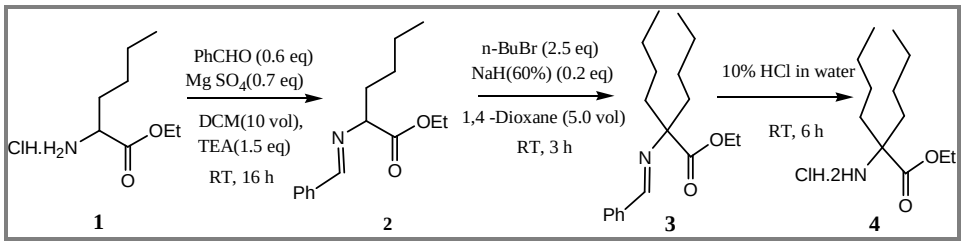
Accepted on 18th June, 2019

ABSTRACT

Antibacterial and antifungal activity of N-acylbis n-butylglycine and corresponding Ethylester derivatives was examined against 30 strains of Gram-positive and Gram-negative bacteria, and 8 species of yeasts. The level of antimicrobial activity was established using the in vitro agar assay and the standard broth dilution susceptibility test. N-acylbis n-butylglycine derivatives with free acid group have highest lipophilicity (log P), showed the best antibacterial activity, especially against Gram-positive bacteria. Minimum inhibitory concentration of these derivatives were ranging from 0.008 to mg mL^{-1} in the activity against Yersinia enterocolitica O_3, confirmed by a large inhibition zone (30 mm) by the diffusion test. Hydroxamates inhibit growth by chelation of the PDF enzyme metal in both Gram-positive and Gram-negative bacteria and LpxC enzyme in Gram-negative enzyme. These amide derivatives appear to contribute to inhibition by destabilizing m-RNA. Antifungal activity of substances **5–7** *is not very expressed.*

Graphical Abstract



Synthesis of αα-di *n*-butylglycine ethyl ester.

Keywords: Hydroxamic acid, Phthalimide, Antibacterial activity, Lipophilicity.

INTRODUCTION

Group of membrane-active peptides having a length of 5 to 20 residues, biosynthesized in soil fungi are termed as peptaibols [**1, 2**] These peptide contain symmetric α,α-dialkyl glycine α, β (α-aminoisobutyric acid) and an alcohol group in C-terminal. Hydroxyproline (Hyp) and isovaleric acid (Iva) are also very commonly found noncanonical amino acids in these peptaibol [**1, 3**]. This family of peptides exhibit antibacterial and antifungal properties and potential clinical applications. Furthermore, these peptides are very useful for investigating transmembrane ion transport through model lipid membranes, cells and organelles [**4-7**]. In fact, the role of noncanonical amino acids on conformation and design of peptidomimetics with biomedical applications is well studied [**8-11**]. It is proposed that the α,α-dialkylation induces a more constrained conformation of the φ and ψ main-chain dihedral angle pair, this will induce specific types of secondary structure in peptides with a significant increase on the bioavailability and stability in physiological conditions [**12-17**]. So for the dialkylated amino acid studies are mainly concentrated on contribution of these residues on conformational features in peptide but the contribution of these residue on biological activity is not well explored [**18-20**]. Therefore in this study, we focus on biological activity single dialkylated amino acid derivative so that the result of this will help to design more potent peptaibols in future.

MATERIALS AND METHODS

General route of synthesis for *N*-acyl bis *n*-butylglycine amide esters and amide acids:

General Procedure for the synthesis of (E)-ethyl 2-(benzylideneamino)hexanoate (2): To a solution of amino acid hydrochloride (0.026) in DCM (10 vol) was added magnesium sulphate (0.021 mol) at 25±5°C under N_2 atmosphere. Reaction mass was stirred for 10 min at 25±5 °C, then reaction mass was cooled to 20±5°C. Added triethyl amine (0.039 mol) over a period of 30 min by maintaining temperature of 20±5°C. Reaction mass was stirred at same temperature for 1 h under N_2 atmosphere. Benzaldehyde (0.016 mol) was added to the above stirred reaction mass over a period of 60 min at 20±5°C. The reaction mass allowed to 25±5°C and stirred at same temperature for 16 h for the completion of reaction. Monitored reaction by TLC and after the completion of the reaction, reaction mass was concentrated to thick syrup at < 50°C under reduced pressure. Added methyl tert butyl ether (10.0 vol) for above gummy mass, stirred for 30 min, filtered through celite bed, filtrate concentrated to dryness and used as such for next step without further purification.

General procedure for the synthesis of Intermediate (3): 1,4–Dioxane (3.0 vol), 60% Sodium hydride (0.005 mol) were charged at 25±5°C under N_2 atmosphere. Added **(2)** (0.026) in 1,4-dioxane (2.0 vol) to the above reaction mass over a period of 2h under N_2 atmosphere. Reaction mass was stirred for 3.0 h at 25±5°C, then N-butyl bromide was added to the reaction mass over a period of 1 h at 25±5°C under N_2 atmosphere. The reaction mass allowed to 25±5°C and stirred at same temperature for 3 h for the completion of reaction. Monitored reaction by TLC and after the completion of the reaction, reaction mass cooled to 5±5°C, charged ethyl acetate (10 vol) at 5±5°C, stirred for 30 min. Slowly quenched reaction mass with ammonium chloride solution (10% in water, 5 vol) at 5±5°C for 2-3 h. Stirred reaction mass for 15 min separated the layers, aqueous layer extracted back with ethyl acetate (10 vol), combined organic layer taken as such for next step.

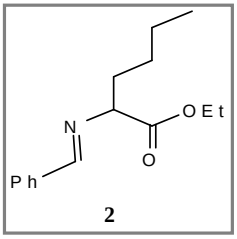
General procedure for the synthesis of αα-di n-butylglycine ethylester hydrochloride (4): Above obtained organic layer cooled to 5±5°C and added slowly 10% HCl solution in water under stirring. After addition allowed to 25±5°C, stirred the mixture for 6 h at 25±5°C. Monitored reaction by TLC and after the completion of the reaction separated the layers and aqueous layer adjusted the pH to 10-11 using sodium bicarbonate solution (10% in water). Again extracted aqueous layer with ethyl acetate, given water (5.0 vol) and brine (5.0 vol) wash to the organic layer, then organic layer dried over an Na_2SO_4, filtered off. Concentrated the organic layer to thick syrup at <50°C under reduced pressure and used as such for next step without further purification.

General procedure for the synthesis of *N*-acyl bis *n*-butylglycine amide esters (7a-g): To a solution of amino acid (0.026 mol) in DMF (10 vol) was added EDCI. HCl (0.039 mol) and HOBT (0.034 mol) at 25±5°C under N_2 atmosphere. Reaction mass was stirred for 10 min at 25±5°C, and then added anthranilic acid (0.028 mol). Reaction mass was stirred for 10 min under N_2 atmosphere, heated reaction mass to 100°C and stirred for 6h. The reaction was monitored by TLC and after the completion of the reaction, quenched with 10% sodium bicarbonate solution (5.0 vol), extracted product with DCM (10 vol). Organic layer was given water wash (5.0 vol), brine wash (5.0 vol), dried over a Na_2SO_4, filtered and concentrated under reduced pressure at <40 °C. Crude product obtained was taken as such to next step without further purification.

General procedure for the synthesis of *N*-acyl (±) Norleucine amide acids (8a-g): The above obtained crude material (0.029 mol) taken in acetonitrile (10 vol) and cooled to 0-5°C. Added aq HCl (20%) (10.0 vol) to above cooled reaction mass. Slowly heated to 50°C and the resulting reaction mixture was stirred at 50 °C for 16h. Reaction was monitored by TLC and after completion of reaction, the reaction mixture concentrated to remove solvent at <45°C, residue neutralized with aq NaOH (10% solution in water) at 0-5°C, around 6-7 pH solids thrown out. Filtered off, washed with pet ether and suck dried for 5-6h to get required product.

Characterization data

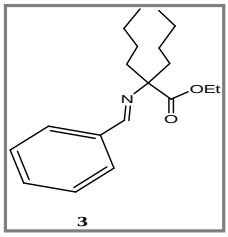
(E)-ethyl 2-(benzylideneamino)hexanoate (2): Pale yellow gummy mass; yield 75 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.81 (t, 3H), 1.07-1.14 (m, 7H), 1.74-1.92 (m, 2H), 4.01-4.2 (m, 3H), 7.43-7.49(t,3H), 7.71-7.79 (d, 2H), 8.38 (s, 1H); *m/z* (ESI-MS) [M + H]+ Calcd (found): 247.33 (248).



2

Intermediate (3): Pale brown gummy mass; yield 75 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.68-0.88 (t, 3H), 1.16-1.32 (m, 7H), 1.73-1.92 (m, 2H), 4.01-4.2 (m, 3H), 7.43-7.49 (t, 3H), 7.71-7.79 (d, 2H), 8.38 (s, 1H);

Intermediate (4): Pale brown gummy mass; yield 75 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.81 (t, 6H), 1.16-1.33 (m, 3H), 1.38-1.42 (m, 11H), 1.57-1.61 (m, 4H), 4.04-4.07 (t, 2H); *m/z* (ESI-MS) [M + H]+ Calcd (found): 215.33 (216.2).



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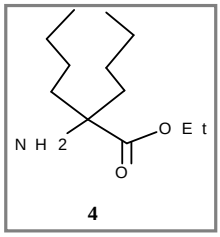

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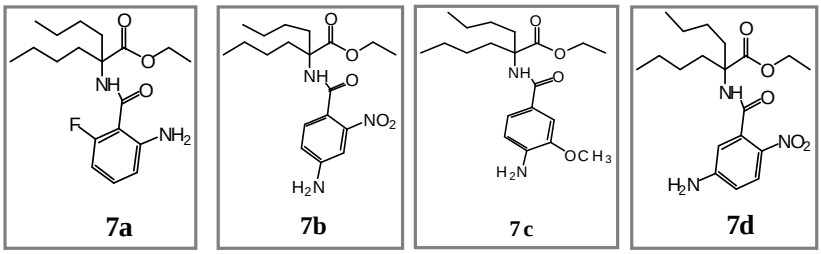
Ethyl 2-(2-amino-6-fluorobenzamido)-2-butylhexanoate (7a): Pale yellow gummy mass; yield 75 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.81 (t, 3H), 1.07-1.14 (m, 4H), 1.15-1.69 (m, 1H), 1.74-1.77 (t, 2H), 1.91-1.99 (s, 1H), 6.582-6.702 (d, 1H), 7.04-7.10 (t, 1H),7.39-7.47 (t,1H), 7.50-7.57 (t, 1H), 7.71-7.74 (d, 1H), 7.94-7.99 (t, 1H), 8.28 (s, 1H), 13.5 (s,1H); ^{13}C NMR (400 MHz, DMSO d_6): δ 14.13, 21.52, 22.56, 25.35, 35.88, 62.89, 110.113, 114.92, 117.15, 129.72, 131.75, 149.37, 172.43-172.92.; *m/z* (ESI-MS) [M + H]+ Calcd (found): 337.41 (338.5).

Ethyl 2-(4-amino-2-nitrobenzamido)-2-butylhexanoate (7b): Pale brown solid; yield 78 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.81 (t, 6H), 1.16-1.18 (m, 13H), 1.76-1.87 (m, 4H),4.01-4.08 (m, 2H), 6.075 (s, 2H), 6.77-6.79 (d, 2H), 6.97 (s,1H), 7.23-7.26 (d, 1H) 8.26 (s, 1H),; ^{13}C NMR (400 MHz, DMSO d_6): δ 14.34-14-50, 22.84, 25.45, 33.01, 60.61, 62.13, 107.99, 116.23, 118.34, 130.58, 150.02, 151.49,165.09, 173.28 *m/z* (ESI-MS) [M + H]+ Calcd (found): 379.45 (380.0).

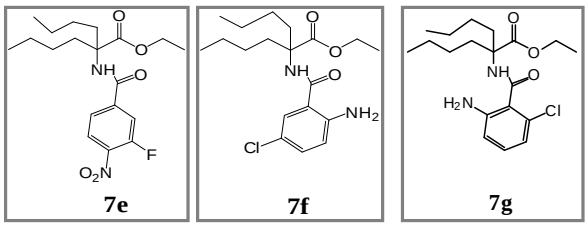
Ethyl 2-(4-amino-3-methoxybenzamido)-2-butylhexanoate (7c): Pale yellow gummy mass; yield 70 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.82-0.85 (t, 9H), 1.03-1.12 (m, 19H), 1.80-1.92 (m, 3H),1.94-1.98 (m, 4H), 3.8 (s, 3H), 4.01-4.08 (m,4H), 6.60 (d, 1H), 7.26-7.46 (m, 2H), 7.77 (s, 1H); ^{13}C NMR (400 MHz, DMSO d_6): δ 14.36-14-62, 21.21, 22.84, 25.66, 33.20, 55.85, 60.21-60.45, 62.07, 110.145, 112.659, 121.66, 141.66, 145.75, 165.75, 173.83; *m/z* (ESI-MS) [M + H]+ Calcd (found): 364.48 (366.0).

Ethyl 2-(4-amino-2-nitrobenzamido)-2-butylhexanoate (7d): Pale yellow gummy mass; yield 72 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.82-0.88 (t, 9H), 1.12-1.30(m, 19H), 1.80-1.92 (m, 4H),1.89-1.90 (m, 2H), 1.98 (s, 1H), 4.16-4.18 (m,4H), 6.46 (d, 1H), 6.80 (d, 1H), 7.85(d, 1H), 7.94 (d, 1H), 8.3 (s, 1H); ^{13}C NMR (400 MHz, DMSO d_6): δ 14.33-14-62, 21.20, 22.64-22.88, 25.35-25.50, 33.20, 60.20, 62.15, 110.77, 119.01, 123.78, 128.10, 143.37, 155.08, 162.75, 173.23; *m/z* (ESI-MS) [M + H]+ Calcd (found): 379.45 (380.5).



Ethyl 2-butyl-2-(3-fluoro-4-nitrobenzamido)hexanoate (7e): Pale brown gummy mass; yield 72 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.81-0.88 (t, 9H), 1.12-1.30 (m, 17H), 1.75-1.92 (m, 4H), 4.03-4.08 (m,3H), 6.46 (d, 1H), 7.68—7.75 (m, 2H), 7.90-7.91 (s, 1H), 8.8 (s, 1H); ^{13}C NMR (400 MHz, DMSO d_6): δ 14.27-14-63, 22.62-22.95, 25.41-26.01, 32.98, 60.46-60.82, 62.68, 124.87, 128.45, 131.35, 132.59-133.04, 172.63, 176.89; *m/z* (ESI-MS) [M + H]+ Calcd (found): 382.43 (383.6).

Ethyl 2-(2-amino-5-chlorobenzamido)-2-butylhexanoate (7f): Pale brown solid; yield 79 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.81-0.88 (t, 7H), 1.12-1.30 (m, 14H), 1.71-1.76 (m, 5H), 4.01-4.08 (m,3H), 6.35 (d, 2H), 6.68—6.71 (d, 1H), 7.15-7.18 (d, 1H), 7.55-7.56 (d, 1H), 8.16 (s, 1H); ^{13}C NMR (400 MHz, DMSO d_6): δ 14.33-14-58, 22.88, 25.51, 32.83, 60.45, 61.92, 115.93, 118.13-118.26, 128.18, 131.91, 148.87, 167.46, 173.41; *m/z* (ESI-MS) [M + H]+ Calcd (found): 368.89 (370.0).



Ethyl 2-(2-amino-6-chlorobenzamido)-2-butylhexanoate (7g): Pale yellow solid; yield 80 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.83-0.86 (t, 9H), 1.12-1.36 (m, 19H), 1.71-1.73 (m, 3H), 1.9-1.98 (m,3H), 4.03-4.11 (m,3H), 5.25 (d, 2H), 6.56—6.61(d, 1H), 7.01-7.05 (d, 1H), 7.92 (d, 1H),; ^{13}C NMR (400 MHz, DMSO d_6): δ 14.33-14-58, 22.88, 25.51, 32.83, 60.45, 61.92, 115.93, 118.13-

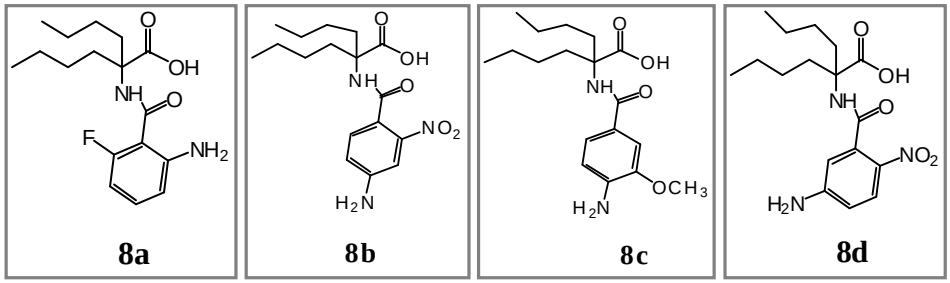
118.26, 128.18, 131.91, 148.87, 167.46, 173.41; *m/z* (ESI-MS) [M + H]+ Calcd (found): 368.89 (370.0).

2-(2-amino-6-fluorobenzamido)-2-butylhexanoic acid (8a): Off white solid; yield 80 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.83-0.84 (t, 9H), 0.98-1.02 (m, 16H), 1.71-1.89 (m, 6H), 4.10-4.12 (m,3H), 5.75 (d, 2H), 6.30—6.50 (d, 2H), 7.06-7.26 (d, 1H), 7.48 (d, 1H),7.95 (d, 1H),8.20 (s,1H); ^{13}C NMR (400 MHz, DMSO d_6): δ 14.16-14-30, 22.66-22.72, 25.53, 31.22, 33.45, 60.98, 62.71, 102.08-102.40, 110.75, 111.68, 125.51, 131.42, 150.02, 162.75, 164.04,; *m/z* (ESI-MS) [M + H]+ Calcd (found): 324.39 (325.5).

2-(4-amino-2-nitrobenzamido)-2-butylhexanoic acid (8b): Off white solid; yield 72 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.83 (t, 6H), 1.04 (m, 7H), 1.60-1.98 (m, 6H), 4.5 (m,3H), 6.08 (d, 2H), 6.76—6.78 (d, 1H), 6.91 (d, 1H), 7.18-7.20 (d, 1H),8.02 (d, 1H); ^{13}C NMR (400 MHz, DMSO d_6): δ 14.63, 23.08, 27.00, 35.90, 64.80, 108.19, 116.29, 119.91, 129.10, 150.31, 151.30, 163.32, 175.65; *m/z* (ESI-MS) [M + H]+ Calcd (found): 351.39 (353.0).

2-(4-amino-3-methoxybenzamido)-2-butylhexanoic acid (8c): Off white solid; yield 78 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.83 (t, 8H), 1.18-1.26 (m, 12H), 1.84-2.00 (m, 9H), 3.8 (m,3H), 5.5 (d, 2H), 6.62—6.64 (d, 1H), 7.43-7.45 (d, 2H), 7.87 (d, 2H), 12.2 (s, 2H); ^{13}C NMR (400 MHz, DMSO d_6): δ 14.39, 21.50, 22.82, 26.02, 33.75, 55.83, 62.56, 110.07, 112.76, 121.15, 122.32, 141.23, 146.00, 165.46, 172.44, 175.69; *m/z* (ESI-MS) [M + H]+ Calcd (found): 336.43 (337.2).

2-(4-amino-2-nitrobenzamido)-2-butylhexanoic acid (8d): Off white solid; yield 69 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.88 (t, 6H), 1.23-1.28 (m, 8H), 1.83-198 (m, 4H), , 6.5-6.59 (d, 2H), 6.77 (d, 2H), 7.85-7.88 (d, 1H), 8.00 (d, 1H), 12.6 (s, 1H); ^{13}C NMR (400 MHz, DMSO d_6): δ 14.39, 22.91, 25.71, 33.55, 62.68, 112.42-112.74, 127.80,133.42, 137.47, 155.11, 166.12, 175.05; *m/z* (ESI-MS) [M + H]+ Calcd (found): 351.39 (352.10).

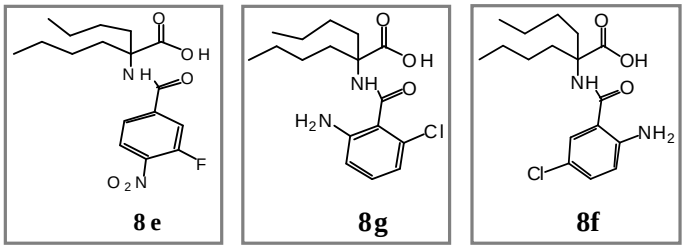


2-butyl-2-(3-fluoro-4-nitrobenzamido)hexanoic acid (8e): Off white solid; yield 74 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.86 (t, 6H), 1.06-1.28 (m, 11H), 1.83-1.88 (m, 4H), 4.06 (d, 2H), 7.67-7.73 (d, 2H), 7.89-7.91 (d, 1H), 8.85 (d, 1H); ^{13}C NMR (400 MHz, DMSO d_6): δ 14.26-14.41,22.75, 25.41, 32.99, 60.82, 62.71, 124.86, 128.45, 131.36, 132.59-133.03, 147.39, 163.00, 172.62; *m/z* (ESI-MS) [M + H]+ Calcd (found): 354.37 (356.0).

2-(2-amino-5-chlorobenzamido)-2-butylhexanoic acid (8f): Off white solid; yield 76 %; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.82-0.87 (t, 6H), 1.1-1.28 (m, 8H), 1.87-1.91 (m, 4H), 6.38 (d, 2H), 6.69-6.73 (d, 1H), 7.15- 7.18 (d, 1H), 7.46-7.49 (d, 1H), 7.97 (s,1H); ^{13}C NMR (400 MHz, DMSO d_6): δ 14.38, 22.86, 26.01, 33.58, 62.71, 116.53, 118.22-118.43, 127.73, 131.77, 148.81, 167.02, 175.23; *m/z* (ESI-MS) [M + H]+ Calcd (found): 340.85 (341.10).

2-(2-amino-6-chlorobenzamido)-2-butylhexanoic acid (8g): Off white solid; yield 72%; ^{1}H NMR (400 MHz, DMSO d_6): δ 0.83-0.87 (t, 7H), 1.24 (m, 9H), 1.68-1.78 (m, 4H), 5.32 (d, 2H), 6.55-6.59 (t, 1H), 6.99- 7.05 (t, 1H), 8.43 (s, 1H), 12.69 (s,1H); ^{13}C NMR (400 MHz, DMSO d_6): δ 14.43, 22.89,

25.62, 33.49, 62.25, 113.69, 116.08, 122.13, 130.35-130.41, 147.70, 165.26, 175.28; *m/z* (ESI-MS) [M + H]+ Calcd (found): 340.85 (341.0).



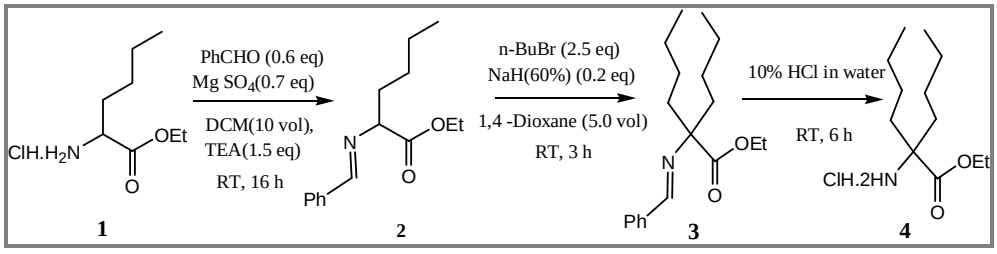
Structural Analysis: The structures of all the *N*-acyl derivatives were confirmed on the basis of MS, ^{1}H NMR, ^{13}C NMR, and elemental analysis. The ESI-MS of compounds **8a–g** and **7a-g** displayed quasimolecular ion peaks which confirmed their molecular weights. The ^{1}H-NMR spectra of compounds **8a–g** and **7a-g** were similar except for the aromatic protons.

Microbiological tests: Antimicrobial activity testing was performed with 4 strains of Gram-positive and 4 strains of Gram-negative bacteria and 5 species of yeasts, which were collected from the Department of Studies in Microbiology, University of Mysore. The bacterial growth inhibition assays were carried out by the diffusion test and the dilution susceptibility test. The agar diffusion method was carried out according to literature protocol. Testing inocula 10^{4}–10^{5} cells (0.5 mL portion) were swabbed onto solidified Mueller-Hinton agar for bacteria and Sabouraud dextrose agar for yeasts. Wells were made on LB plates using sterile borer and water solutions with 0.05 % DMSO of compounds were filled in a volume of 0.25 mL. After 2 h of diffusion at 4°C, the agar plates were incubated for 18–24 h at either 37°C for bacteria, or 48 h at 25°C for yeasts. The diameters of clear inhibition zones around the wells were measured if the tested substance inhibited bacterial or yeast growth.

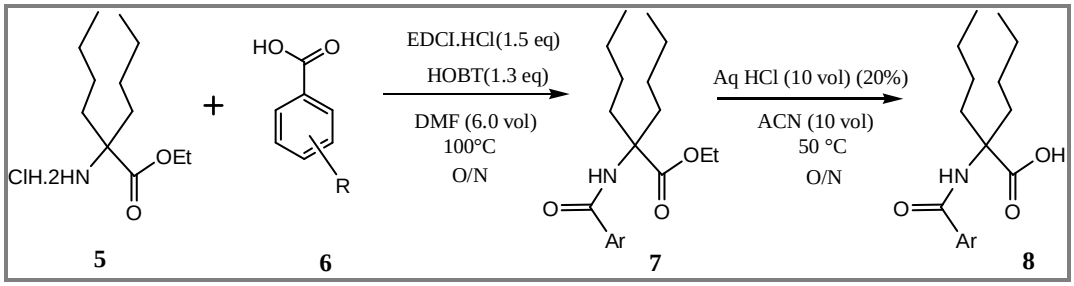
Minimal inhibition concentrations (MIC) (µg mL^{-1}): The antimicrobial activity of the sample was tested and the minimal inhibitory concentration (MIC) was determined using micro dilution method. The 96 well microtiter plates were filled with 0.1 mL of LB broth with tested substance in appropriate concentration. Dilutions of samples were made in the range from 1 to 0.004 mg mL^{-1} for all compounds. The prepared test dilutions were incubated at 37°C for bacteria or 25°C for yeasts. The last well as a positive growth control, was free of test compounds and two growth controls were made, with and without DMSO. The MIC value was determined in spectrophotometer by measuring OD at 600nm.

RESULTS AND DISCUSSION

For antimicrobic examinations, αα-di *n*-butylglycine (Dnbg) **8** and its ethyl ester **7** were converted to a series of *N*-acyl derivatives containing different benzoicacid moieties by following our previously published method with slight modification as shown in scheme 1 and 2.



Scheme 1. Synthesis of αα-di *n*-butylglycine ethyl ester.



Scheme 2. Synthesis of *N*-acyl bis *n*-butylglycine amide esters and amide acids

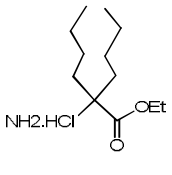
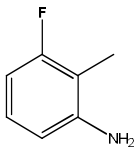
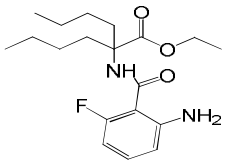
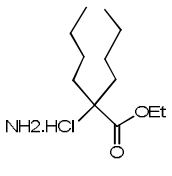
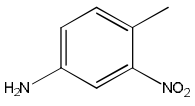
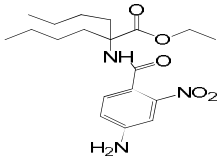
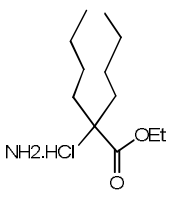
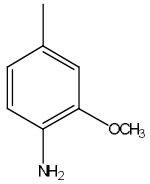
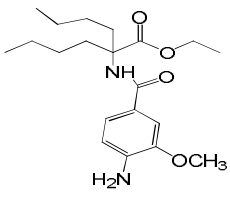
Acylation of the different substituted benzoic acids **6** with αα-di-*n*-butylglycine ethylester hydrochloride **5** using T_3P as coupling and triethylamine as base in DCM offered very low yield. This may due to steric hindrance at α-carbon atom, hence we first optimize the acylation condition using different coupling agent and solvents, the results are outlined in table 1.

Table 1. Optimization of coupling reaction conditions.

Solvent	Base	Temperature (°C)	Time (h)	Yield of 7 (%)
DCM	T_3P	40	8	40
DMF	Na_2CO_3	75	8	20
DMF	K_2CO_3	75	8	25
DMSO	Na_2CO_3	90	8	0
DMSO	K_2CO_3	90	8	0
THF	K_2CO_3	60	8	20
THF	Et_3N	60	8	15
CH_3CN	Et_3N	75	8	30
DMF	**EDCI. HCl and HOBT**	**75**	**8**	**76**

After having the optimized coupling conditions following acyl derivatives were prepared with different substitution groups on aromatic rings.

Table 2. Substrate scope for *N*-acyl bis *n*-butylglycine amide esters **7a-g.**

Substrate 5	Substrate 6	Product 7	Yield (%)
			75
			78
			70

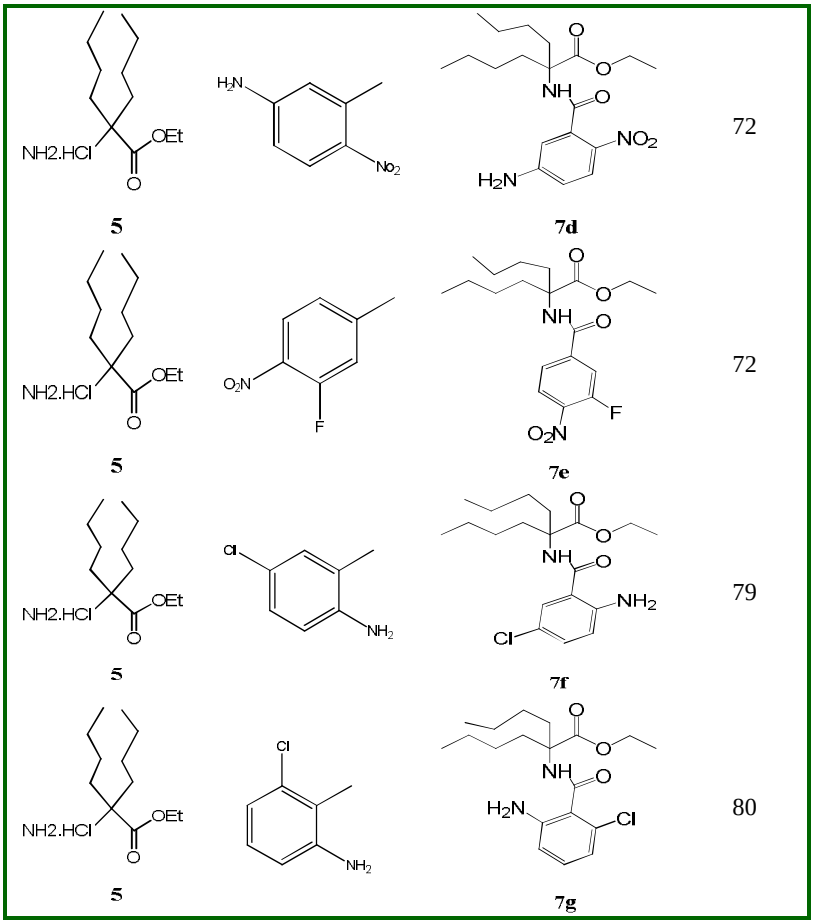
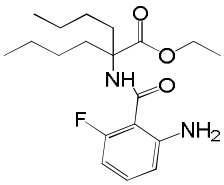
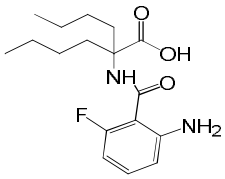
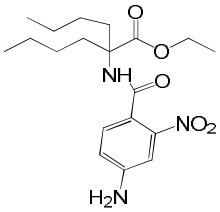
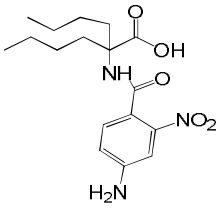
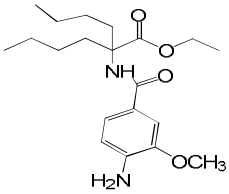
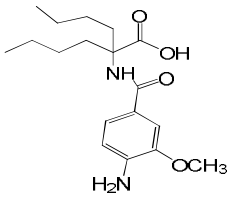
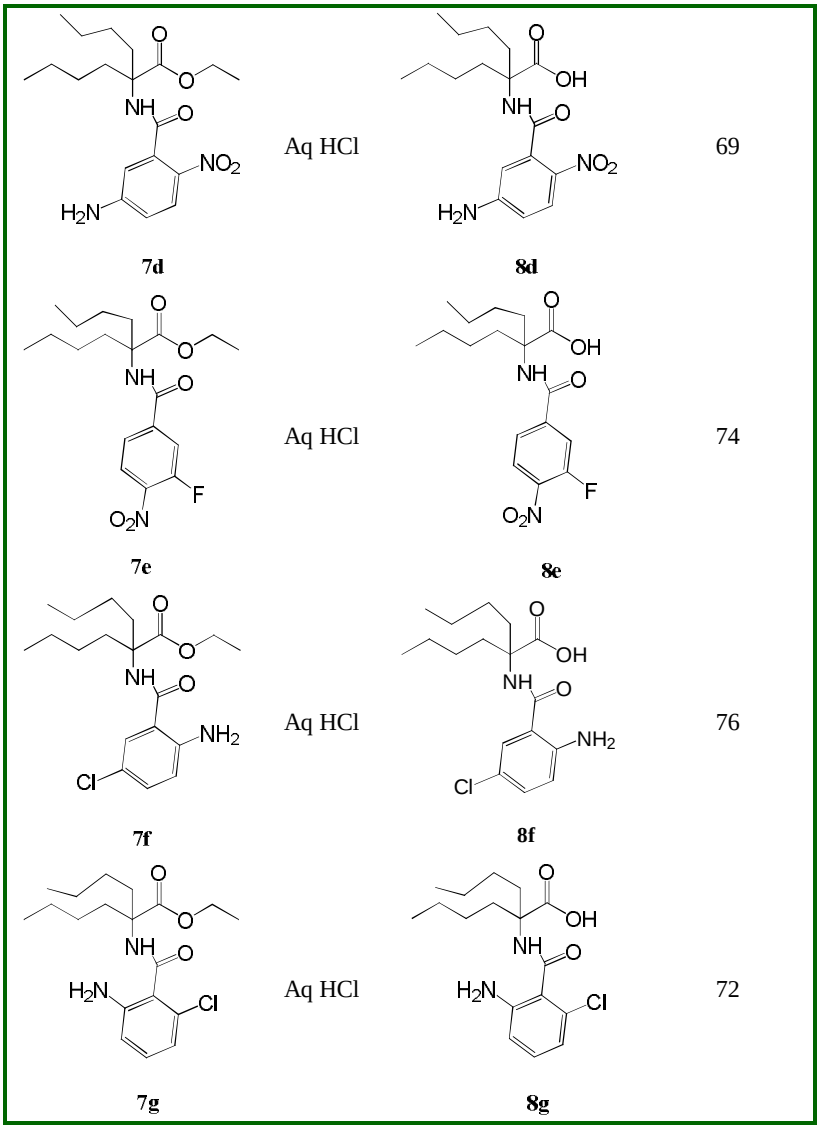


Table 3. Substrate scope for *N*-acyl bis *n*-butylglycine amide acids**8a-g**

Product 7	Reagent	Product 8	Yield (%)
 **7a**	Aq HCl	 **8a**	80
 **7b**	Aq HCl	 **8b**	72
 **7c**	Aq HCl	 **8c**	78



Antibacterial activities of amide ester compounds 7a-g: It has been established by the diffusion antibacterial test that compounds **7a-g** (Table 4) inhibits 7/8 strains of bacteria. The zones of inhibition were also bigger in compounds **7a,7f and 7g** with strong electron withdrawing substituent's (average 31.12 mm) compared to those of compounds **7b, 7c, 7d and 7e** (12.53 mm) having less electron withdrawing and electron releasing substituent's comparatively. The minimal inhibitory concentration (MIC) values of these compounds against the test microorganisms were listed in table 5.

Table 4. Antibacterial activity of amide ester compounds **7a-g** (diffusion test)

Compounds	Inhibition zone (mm)							
	Gram-positive				Gram-Negative			
	Bacillus Cereus ATCC 11778	*Bacillus Subtilis* NCTC 8236	*Staphylococcus Aureus* SR2	*Staphylococcus Aureus* SR5	*E.coli* R 16	*E.coli* R19	*Pseudomonas Aeruginosa*	*Yersinia Enterocolitica* O_3
7a	-	30	31	29	32	37	39	37
7b	-	11	13	12	15	13	11	14
7c	-	12	14	16	13	16	14	11
7d	-	13	12	15	17	14	21	22
7e	8	12	15	13	11	14	13	16
7f	7	35	34	27	35	38	38	37
7g	9	39	32	30	38	36	39	38

The result revealed that the MICs of the tested compounds differed slightly and it was found that ester derivative **7e**, **7f**, and **7g** which show bigger inhibition zones have exhibited three fold more significant activities (0.04–0.016 μg mL^{-1}) against all tested bacterial stains except *Bacillus cereus* ATCC 11778. Among them, compounds **7g** showed the strongest activities (0.004 μg mL^{-1}) against *Escherichia coli* R 16, *Pseudomonas aeruginosa, Bacillus subtilis* NCTC 8236 and *Bacillus Subtilis* NCTC 8236 bacterial stains.

Table-5. Antibacterial activities of amide ester compounds **7a–g**.

Compounds	Minimum inhibitory concentration (μg mL^{-1})							
	Gram-positive				Gram-Negative			
	Bacillus Cereus ATCC 11778	*Bacillus Subtilis* NCTC 8236	*Staphylococcus Aureus* SR2	*StaphylococcusAureus* SR5	*E.coli* R 16	*E.coli* R19	*Pseudomonas Aeruginosa*	*Yersinia Enterocolitica* O_3
7a	>2.0	2.0	1.0	2.0	2.0	2.0	1.0	0.063
7b	>2.0	2.0	1.0	2.0	1.0	1.0	1.0	0.063
7c	>2.0	2.0	2.0	1.0	1.0	0.063	0.050	0.031
7d	>2.0	2.0	2.0	1.0	0.050	1.0	0.002	0.031
7e	1.0	0.016	0.016	0.031	0.016	0.008	0.004	0.004
7f	1.0	0.008	0.008	0.016	0.008	0.008	0.004	0.004
7g	0.250	0.004	0.016	0.016	0.004	0.004	0.004	0.004

The influence of compounds **7a-g** on the growth of yeasts, especially *Candida*, is shown in table 6. Growth inhibitions were not prominent. The lowest inhibitory concentration was 0.063 mg mL^{-1} determined for compound 3g against *Candida parapsilosis*. The growth control of the water solution with 0.05 vol. % DMSO (used for dissolution of compound 3) was positive for bacteria and yeasts.

Table-6. Antifungal activities of amide ester compounds **7a–g**.

Compounds	Minimum inhibitory concentration (μg mL^{-1})							
	Candida tropicalis	*Candida albicans*	*Candida glabrata*	*Candida kefyr*	*Candida krusei*	*Geotrichum* sp	*Candida parapsilosis*	*Criptococus neoformans*
7a	>2.0	2.0	1.0	2.0	2.0	2.0	1.0	0.125
7b	>2.0	2.0	1.0	2.0	1.0	1.0	1.0	0.125
7c	>2.0	2.0	0.50	1.0	1.0	1.0	0.050	0.125
7d	>2.0	2.0	0.50	1.0	0.050	0.500	0.063	0.125
7e	1.0	1.0	0.025	0.031	0.016	0.063	0.016	0.125
7f	1.0	1.0	0.025	0.016	0.008	0.063	0.016	0.125
7g	1.0	1.0	0.025	0.125	0.125	0.063	0.016	0.125

Antibacterial activities of amide acid compounds 8a-g: In order to find the influence of charge on the antimicrobial resistance the ester group of the compound **7a-g** was converted to free carboxylic group by subjected to safonification. The newly obtained acid amide derivatives **8a-g** were then studied for their antibacterial and antifungal activities with same bacterial and fungal stains mentioned

Table 7. Antibacterial activities of amide acid compounds **8a–g**

Compounds	Minimum inhibitory concentration (μg mL^{-1})							
	Gram-positive				Gram-Negative			
	Bacillus cereus ATCC 11778	*Bacillus subtilis* NCTC 8236	*Staphylococcus aureus* SR2	*Staphylococcus aureus* SR5	*Escherichia coli* R16	*Escherichia coli* R19	*Pseudomonas aeruginosa*	*Yersinia enterocolitica* O3
8a	2.0	0.250	0.250	0.250	0.250	0.250	0.250	0.008
8b	2.0	0.250	0.250	0.250	0.250	0.250	0.250	0.008
8c	2.0	0.031	0.016	0.031	0.008	0.016	0.050	0.031
8d	2.0	0.031	0.016	0.031	0.008	0.016	0.063	0.031
8e	0.250	0.004	0.004	0.004	0.004	0.008	0.004	0.004
8f	0.250	0.004	0.002	0.002	0.002	0.002	0.002	0.002
8g	0.125	0.002	0.002	0.002	0.002	0.002	0.002	0.002

in tables 5 and 6 respectively by following similar procedure mentioned above. The results obtained are tabulated in tables 7 and 8. From the table it was found that, amide acid derivatives exhibited two to three folds more significant activities against both Gram-negative and Gram-positive bacteria compared to corresponding amide ester derivatives among them, compounds **8f** and **8g** showed the strongest activities (0.002 µg mL^{-1}) against all bacterial strains except *Bacillus cereus* ATCC 11778. This trend was also observed in antifungal activities.

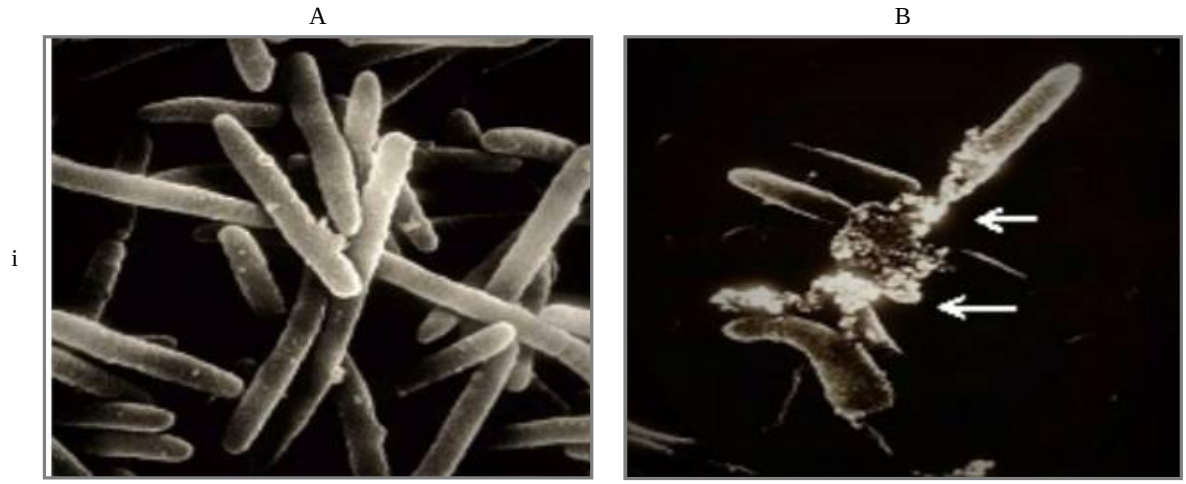
Table 8. Antifungal activities of amide acid compounds **8a–g**

Compounds	Minimum inhibitory concentration (µg mL^{-1})							
	Candida tropicalis	*Candida albicans*	*Candida glabrata*	*Candida kefyr*	*Candida krusei*	*Geotrichum* sp	*Candida parapsilosis*	*Criptococus neoformans*
8a	2.0	1.0	0.50	1.0	1.0	1.0	1.0	0.063
8b	2.0	1.0	0.50	1.0	0.050	0.050	1.0	0.063
8c	2.0	1.0	0.025	1.0	0.050	0.050	0.050	0.063
8d	2.0	1.0	0.025	1.0	0.025	0.025	0.063	0.063
8e	0.50	0.50	0.063	0.025	0.025	0.025	0.016	0.063
8f	0.50	0.50	0.063	0.025	0.025	0.025	0.016	0.063
8g	0.50	0.50	0.063	0.025	0.025	0.025	0.016	0.063

The differences in inhibition of investigated amide ester (**7a-g**) and amide acid (**8a-g**) derivatives can be explained by the level of lipophilicity. In general, the free carboxylic acid group is most lipophilic compare its corresponding ester hence showed the 2 fold more inhibition.

Scanning electron microscopy (SEM): The effects of compound **8g** on the surface morphology of Gram-positive and Gram-negative bacteria during its logarithmic phase of growth were studied using Scanning electron microscopy. The spectrum of antimicrobial activity on the surface morphology and bacterial populations visualized by SEM was showed in figure 1. The SEM analysis showed that morphological changes by cellular lysis in *S. Aureus* ATCC 43300 (MRSA), *P. Aeruginosa* ATCC 27853 (Amp C β-lactamase producing strain) and *K. pneumoniae* ATCC BAA1705 (*Carbapenemase*; KPC-producing strain).

The effects of compound **8g** on the surface morphology of *Pseudomonas aeruginosa* and *Escherichia coli* R16 were generally similar. Untreated organisms (Figure1: Ai and Aiii) appeared rod-shaped. Exposure to compound **8g** resulted in morphologic defects characterized by tubular out pouchings from the cell wall (Figure 1: Bi and Biii).



ii

iii

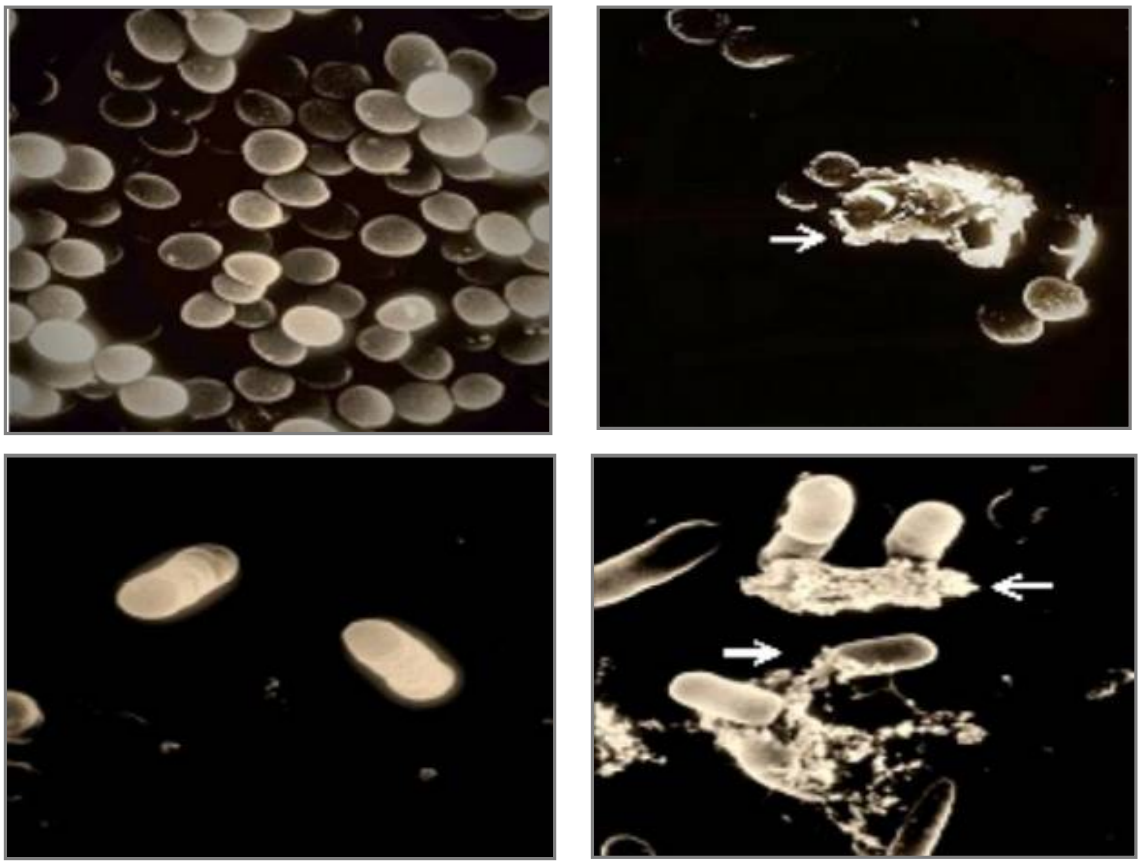


Figure 1. Scanning electron microscopy images of the antibacterial effect of compound **8g** on the bacterial strains i) *Pseudomonas Aeruginosa*, ii) *Bacillus Subtilis NCTC 8236*and iii) *Escherichia Coli* R 16. A series are bacterial strains without treatment and B series are with treatment with compound **8g**. Note morphological alterations in B series with clearly broken cells and leakage from cells

The effects of compound **8g** on the surface morphology of *Bacillus subtilis NCTC 8236* during its logarithmic phase of growth study reveals that untreated *Bacillus subtilis NCTC 8236* appeared to be smooth and spherical in grape like clusters. Exposure to the compound **8g** resulted in the appearance of ruff road-like structures. Irregular spherical structures lying free or appearing to extrude from cells were also observed (Figure 1: A ii and B ii)

APPLICATION

These compounds as antimicrobial agents in new drugs for therapy and can be subjected to identification of the therapeutic antimicrobials and undergo further pharmacological screening.

CONCLUSION

The results of the present study showed that the bis alkylated glycine amides were more effective against the bacterial species tested. This can be used to treat various diseases like pimples, food borne infections, typhoid, oral and throat sores and nosocomial infections. This investigation has opened up the possibility of the use of amide derivatives of α α-disubstituted amino acids as drug for human consumption possibly for the treatment of bacterial infections. These findings are a preliminary scientific validation for the use of these classes of compounds for antibacterial activity. The results of the present study also support the medicinal usage of these compounds as antimicrobial agents in new drugs for therapy and can be subjected to identification of the therapeutic antimicrobials and undergo further pharmacological screening that can be used as sources for new drugs.

ACKNOWLEDGEMENTS

The authors are thankful to Department of studies in Microbiology, University of Mysore, Mysore, Karnataka, for providing test pathogens to carrying this work.

Conflict of interest: The authors declare that there is no conflict of interest, financial or otherwise regarding the publication of this paper.

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