Synthesis, Bacterial Biofilm Inhibition and Hemolytic Activity of 4-Chloro-*N*-(dimethylphenyl)butamide regio-isomers

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Summary: The goal of this work was to synthesize some 4-chloro-*N*-(dimethylphenyl)butamide regio-isomers starting from dimethylated phenylamines and to examine their biofilm inhibition against *Staphylococcus aureus* and *Escherichia coli* strains along with hemolytic activity. The synthetic approach for the synthesis of regio-isomers involved the reaction of 4-chlorobutanoyl chloride (**1**) with three different dimethylphenylamines (**2a-c**) in alkaline medium under vigorous shaking for 1-2 h under dynamic control of pH ranging from 9-10 to obtain the final products, 4-chloro-*N*-(dimethylphenyl)butamides **(3a-c)**. The structural confirmation of these products was accomplished using IR, ^{1}H-NMR, ^{13}C-NMR and CHN analysis data.

Keywords: Dimethylphenylamines, 4-chlorobutanoyl chloride, Structure characterization, Antibacterial, Hemolytic activity.

Introduction

Amides are basically the derivatives of carboxylic acids having general formula of R-CO-NH_2, where R may be hydrogen, alkyl or an aryl group. It is the most essential bond which sustains life by making the peptide bonds present in proteins and is necessary for almost all biological processes. Amides are also present in many polymers, agrochemicals, natural products and many pharmacological important molecules. The data present in Comprehensive Medicinal Chemistry tells us that in most of the 25% known drugs; carboxamide group is present [1]. In last two decades, the commonness of infections caused by pathogens has increased considerably in immune compromised patients. Due to resistance of these pathogen strains against different drugs, it is the need of the hour to new structural classes of compounds along with novel mechanism of action. It is revealed from the literature survey that many of the nitrogen compounds [2,3] are potentially active against viral, fungal and cancerous diseases [4,5]. Many of the amines derivatives are biologically active [6]. In the presence of different groups on amine moiety, they also act as antimicrobial and antifungal agents [7,8]. Many of the compounds are available containing amino acids are potentially active against various fungal strains and they got acceptance in clinical trials [9,10]. On the other hand, the compounds containing amide moiety attracts the attention due to their unique role in various processes occurring on industrial scale and in biological things [11]. Moreover, they also have fundamental interests in understanding the role of metallo proteins which is responsible to control the cell metabolism. In the light of important behavior of amides, our present research work was focused to synthesize some butamides regio-isomers which could be useful to overcome the resistance of pathogenic microbes.

General

All the chemicals, along with analytical grade solvents used for synthetic purposes were purchased from Merck or Sigma Aldrich, Alfa Aesar (Germany) through local suppliers. TLC was done by using Pre-coated silica gel Al-plates by using ethyl acetate and *n*-hexane as solvent system. Detection of the Spots was done by UV_{254}. Melting points in capillary tubes were detected by using Gallonkamp apparatus. Similarly, IR spectra (ν, cm^{-1}) were recorded by using Jasco-320-A spectrometer through KBr pellet method. ^{1}H-NMR spectra (δ, ppm) of synthesized compounds were recorded at 600 MHz (^{13}C-NMR spectra, at 150 MHz) in $CDCl_3$ using the Bruker Advance III 600 As- cend spectrometer using BBO probe. The coupling constant (J) is given in hertz (Hz) and chemical shifts δ in ppm.

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The abbreviations used in spectral interpretation of ^{1}H-NMR were; s = singlet, d = doublet, dd = doublet of doublet, t = triplet, br.t = broad triplet, q = quartet, quint = quintet, sex = sextet, sep = septet and m = multiplet.

Synthesis of 4-chloro-N-(dimethylphenyl)butamide regio-isomers **(5a-c)**

The respective dimethylphenylamine (1.38 m.mol; **2a-c**, one in each reaction) was suspended in distilled water (5 mL) contained in a round bottom flask of 25 mL and pH of the solution was adjusted at 9-10 by adding the aqueous solution of Na_2CO_3. The reaction mixture was stirred for 15 min at 25℃ . Then, 4-chlorobutanoyl chloride (1.38 m.mol; **1**) was added into the reaction mixture and vigorously shaken manually for 1-2 h to obtain final product, 4-chloro-*N*-(dimethylphenyl)butamide regio-isomers (**3a-c**). The advancement of the reaction was monitored by Thin Layer Chromatography till single spot. After the completion of the reaction the product was quenched with ice cubes. The precipitate of respective product was filtered out, washed, dried and utilized for further study.

Spectral Characterization

4-Chloro-N-(2,4-dimethylphenyl)butamide (**3a**)

Off white solid; Yield: 83%; m.p.: 104-105 °C, Molecular formula: $C_{12}H_{16}ClNO$; Molecular mass: 225 g/mol; IR (KBr, υ, cm^{-1}): 3413 (N-H stretching), 3095 (C-H stretching of aromatic ring), 2887 (C-H stretching of aliphatic), 1665 (C=O stretching), 1586 (C=C stretching of aromatic ring), 1112 (C-N-C bond stretching). ^{1}H-NMR ($CDCl_3$, 600 MHz, δ, ppm): 9.25 (s, 1H, H-7, CO-NH), 7.21 (br.d, J = 7.9 Hz, 1H, H-6'), 7.01 (br.s, J = 7.01 Hz, 1H, H-3'), 6.95 (br.d, J = 7.9 Hz, 1H, H-5'), 3.70 (t, J = 6.5 Hz, 2H, CH_2-4), 2.48 (t, J = 7.3 Hz, 2H, CH_2-2), 2.24 (s, 3H, CH_3-7'), 2.14 (s, 3H, CH_3-8'), 2.09 (quintet, J = 7.0 Hz, 2H, CH_2-3); ^{13}C-NMR ($CDCl_3$, 150 MHz, δ, ppm): 170.47 (C=O), 134.63 (C-1'), 134.20 (C-2'), 132.24 (C-4'), 126.81 (C-3'), 125.73 C-6'), 45.48 (C-4), 33.22 (C-2), 28.68 (C-3), 20.92 (C-8'), 18.20 (C-7'). Anal. Calc. for $C_{12}H_{16}ClNO$ (225.09): C, 63.85; H, 7.14; N, 6.21. Found: C, 63.90; H, 7.21; N, 6.25.

4-Chloro-N-(3,4-dimethylphenyl)butamide (**3b**)

Light yellow solid; Yield: 70%; m.p.: 109-110 °C; Molecular formula: $C_{12}H_{16}ClNO$; Molecular mass: 225 g/mol; IR (KBr, υ, cm^{-1}): 3417 (N-H stretching), 3091 (C-H stretching of aromatic ring), 2881 (C-H stretching of aliphatic), 1668 (C=O stretching), 1583 (C=C stretching of aromatic ring), 1117 (C-N-C bond stretching). ^{1}H-NMR ($CDCl_3$, 600 MHz, δ, ppm): 7.41 (dist.s, 1H, H-2'), 7.36 (dd, J = 2.04,8.16 Hz, 1H, H-6'), 7.11 (d, J = 8.16 Hz, 1H, H-5'), 3.79 (br.t, J = 7.02 Hz, 2H, CH_2-4), 2.46 (br.t, J = 7.92 Hz, 2H, CH_2-1), 2.24 (s, 3H, CH_3-8'), 2.19 (s, 3H, CH_3-7'), 2.04 (quintet, J = 7.56 Hz, 2H, CH_2-3). ^{13}C-NMR ($CDCl_3$, 150 MHz, δ, ppm): 170.87 (C=O), 137.90 (C-1'), 136.68 (C-3'), 132.14 (C-4'), 129.89 (C-5'), 121.19 (C-2'), 48.65 (C-4), 32.71 (C-2), 20.08 (C-7'), 19.19 (C-8'), 17.90 (C-3). Anal. Calc. for $C_{12}H_{16}ClNO$ (225.09): C, 63.85; H, 7.14; N, 6.21. Found: C, 63.94; H, 7.19; N, 6.17.

4-Chloro-N-(3,5-dimethylphenyl)butamide (**3c**)

Light yellow solid; Yield: 82%; m.p.: 102-103 °C; Molecular formula: $C_{12}H_{16}ClNO$; Molecular mass: 225 g; IR (KBr, υ, cm^{-1}): 3415 (N-H stretching), 3097 (C-H stretching of aromatic ring), 2883 (C-H stretching of aliphatic), 1660 (C=O stretching), 1583 (C=C stretching of aromatic ring), 1117 (C-N-C bond stretching). ^{1}H-NMR ($CDCl_3$, 600 MHz, δ, ppm): 7.26 (br.s, 2H, H-2' & H-6'), 6.77 (br.s, 1H, H-4'), 3.79 (br.t, J = 6.96 Hz, 2H, CH_2-4), 2.47 (br.t, J = 8.04 Hz, 2H, CH_2-2), 2.26 (s, 6H, CH_3-7' & CH_3-8'), 2.04 (quintet, J = 7.44 Hz, 2H, CH_2-3). ^{13}C-NMR ($CDCl_3$, 150 MHz, δ, ppm): 174.05 (C=O), 140.01 (C-1'), 138.01 (C-3' & C-5'), 125.82 (C-4'), 117.78 (C-2' & C-6'), 48.70 (C-4), 32.83 (C-2), 21.59 (C-7' & 8'), 17.94 (C-3). Anal. Calc. for $C_{12}H_{16}ClNO$ (225.09): C, 63.85; H, 7.14; N, 6.21. Found: C, 63.89; H, 7.23; N, 6.29.

Assessment of biofilm inhibition

The microliter-plate method as described by the Stepanovic *et. al.* [12] was used for the inhibition of bacterial (*Escherichia coli/S. aureus)* biofilm formation. Flat bottomed plastic tissue culture plate wells of a sterile 24-well were filled with 100 μ L of nutrient broth (Oxoid, UK). Two testing samples (dissolved in 1 mL of DMSO), having concentrations 2.5 and 5.0 μ g were added in different wells. At last, a bacterial suspension of 20 μ L containing 1×10^9 CFU/mL was inoculated. A well of Positive control contained nutrient broth (Oxoid, UK) and Ampicillin. On the other hand, well of negative control contained microbial strain and nutrient broth. After that, the plates were sheltered and then aerobically incubated at 37 ºC for next 24 h. Subsequently, each well having contents were beheld thrice with sterile phosphate buffer (pH: 7.2) of 200 μ L. The plates were vigorously shaken to remove all non-adherent bacteria. Then, leftover bacteria attached were fixed with 220 mL having 99% methanol per well and after 15 minutes, the plates were emptied and left to dry. Then, for 5 minutes the plates were stained with 220 mL of 50% crystal violet per well. Distilled water

was used to rinse the surplus stains. Then plates were dried by air re-solubilized with 220 μ L of 33% (v/v) glacial acetic acid each well. The micro-plate reader (Biotek, USA) operating at 630 nm was used to measure the optical density (OD) of each well. All the tests were repeated thrice against desired bacterial strains and the results were averaged. Following formula was used to calculate the bacterial growth inhibition (Inhibition %).

$$\text{Inhibition \%} = 100 - \frac{[(OD]_{630\ sample} \times 100)}{OD_{630\ control}}$$

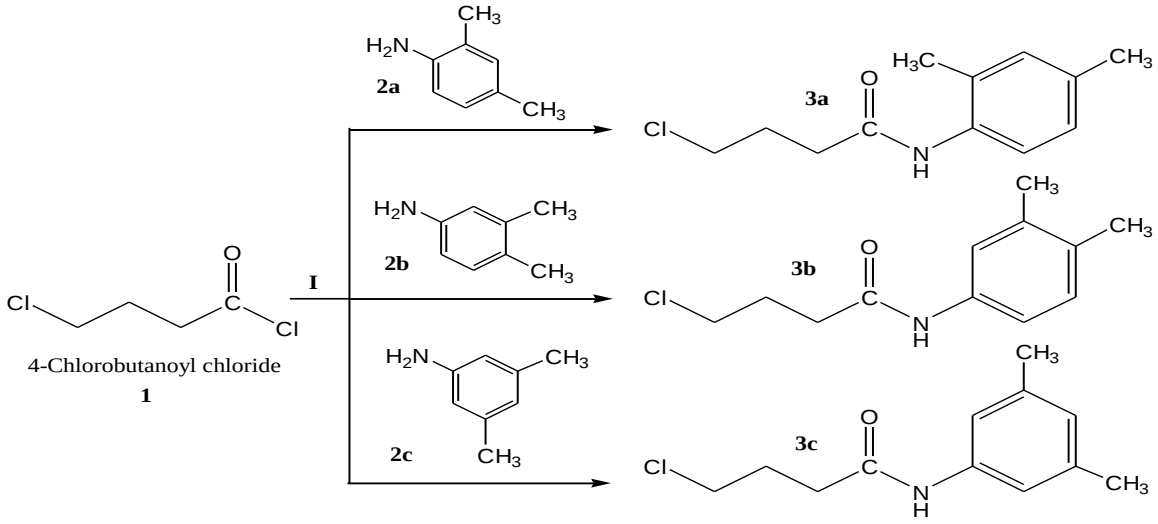
Hemolytic activity

Different samples of bovine blood were collected in EDTA and diluted with saline (0.9% NaCl) solution. After that, it was centrifuged at 1000xg for 10 minutes. The separated erythrocytes were diluted in phosphate buffer saline having pH 7.4 and then a suspension was made. Then added 20 µL of synthetic compounds solution (10 mg/mL) in 180 µL of RBCs suspension and incubate for 30 minutes at room temperature. Triton-X was used as positive control and PBS was taken as negative control [13-15]. The experiment was performed in triplicate. The hemolysis percentage was taken as by using the formula:

$$(\%)\ of\ Hemolysis = \frac{Absorbance\ of\ Sample - Absorbance\ of\ Negative\ Control}{Absorbance\ of\ Positive\ Control} \times 100$$

Results and Discussion:

In our present investigated work; we synthesized 4-chloro-*N*-(dimethylphenyl)butamide regio-isomers according to the outline illustrated in Scheme-1. The structures of the derivatives obtained were recognized by ^{1}H-NMR, ^{13}C-NMR, IR and elemental analysis. The spectral characterization of these derivatives is specified in the experimental section. Their antibacterial data was acquired through biofilm inhibition study against two bacterial strains i.e. *Escherichia coli* and *Staphylococcus aureus* and is tabulated in Table-1. The cytotoxicity of the synthesized compounds was also profiled through hemolytic study and these results are also shown in Table-1.



Scheme-1: Outline for the synthesis of 4-chloro-*N*-(dimethylphenyl)butamide regio-isomers. Reagents & Conditions: (I) Respective dimethylphenylamine/aq. Na_2CO_3 soln./pH 9-10/vigorous shaking at room temperature for 1-2 h, after addition of **1**.

Table-1: Bacterial biofilm inhibition of 4-chloro-*N*-(dimethylphenyl)butamide regio-isomers (**3a-c**).

Compound	Percentage Biofilm Inhibition		Hemolysis (%)
	E. coli	*S. aureus*	
3a	23.348 ± 0.034	38.090 ± 0.024	3.71 ± 0.02
3b	16.153 ± 0.021	9.749 ± 0.019	1.64 ± 0.01
3c	16.593 ± 0.022	53.367 ± 0.027	12.68 ± 0.04
Ampicillin	78.869 ± 0.003	80.271± 0.029	-
Negative control	1.28 ± 0.001	1.28 ± 0.001	-
Triton X	-	-	87.67 ± 0.01

Note: Ampicillin was used as a positive control for biofilm inhibition. For hemolysis; Phosphate-buffered saline (PBS) = 1.03 ± 0.01%.

Chemistry

The synthesis of 4-chloro-*N*-(dimethylphenyl)butamide regio-isomers **(3a-c)** was consummated through a facile strategy and the isomers were obtained in very good yields. The structures of the synthesized molecules were characterized by the spectral data of ^{1}H-NMR, ^{13}C-NMR and IR analysis. Among the synthesized derivatives, the compound **3b,** which was obtained as light yellow solid is discussed hereby in elaborated manner for the pragmatism of the readers. The molecular formula of this compound $C_{12}H_{16}ClNO$ was recognized by counting the total number of protons in its ^{1}H-NMR spectrum (Figure 1a), number of carbons from the resonances obtained from ^{13}C-NMR spectrum (Figure 2) and elemental analysis. The different functional groups present in the molecule were identified through IR spectrum. The distinguishing peaks appeared at υ 3417 (N-H stretching), 3091 (C-H stretching of aromatic ring), 2881 (C-H stretching of aliphatic), 1668 (C=O stretching), 1583 (C=C stretching of aromatic ring), 1117 (C-N-C bond stretching) cm^{-1}. A distorted singlet and a doublet was appeared at δ 7.41 (dist.s, 1H, H-2'), 7.11 (d, J = 8.16 Hz, 1H, H-5') along with a doublet of doublet at 7.36 (dd, J = 2.04, 8.16 Hz, 1H, H-6') with respect to *meta* and *ortho*-positions of respective protons. These values confirmed the presence of tri-substituted aromatic ring in the compound (Figure-1c). The aliphatic part of the compound was characterized by two broad triplets and a quintet obtained at δ 3.79 (br.t, J = 7.02 Hz, 2H, CH_2-4), 2.46 (br.t, J = 7.92 Hz, 2H, CH_2-1) and 2.04 (quintet, J = 7.56 Hz, 2H, CH_2-3) respectively (Figure-1c). Similarly, two singlets obtained at δ 2.24 (s, 3H, CH_3-8'), 2.19 (s, 3H, CH_3-7'), respectively, confirmed the presence of two methyl groups present on aromatic ring. The ^{13}C-NMR spectrum confirmed overall twelve carbon resonances because various sets of duplet symmetrical carbons were present in the compound and certainly each duplet resonated at same position, thus reducing the total signals of carbon showing in the spectrum relative to molecular formula ($C_{12}H_{16}ClNO$) of the compound. The six carbon atoms of the dimethylated phenyl group attached with the nitrogen of the amino group were characterized by the peaks at δ 137.90 (C-1'), 136.68 (C-3'), 132.14 (C-4'), 129.89 (C-5'), 121.19 (C-2'), respectively. The four carbons of the butanamide unit were ensured by the four peaks obtained at δ 170.87 (C=O), 48.65 (C-4), 32.71 (C-2) and 17.90 (C-3). Similarly, two peaks obtained at δ 20.08 (C-7') and 19.19 (C-8') were symbolic for two methyl groups attached to aromatic ring. So, based on the description of aforementioned evidences, the structure of **3b** was confirmed and it was named as 4-chloro-*N*-(3,4-dimethylphenyl)butamide. A similar approach was followed for the structural characterization of other region-isomer

s.

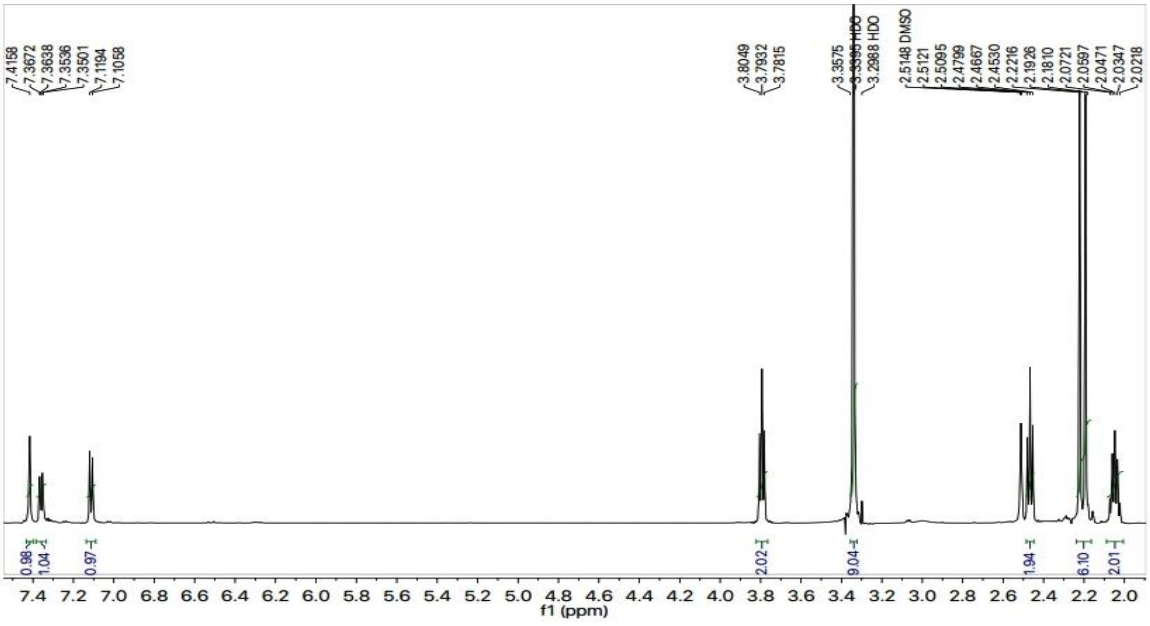


Fig. 1a: ^{1}H-NMR spectrum of **3b**.

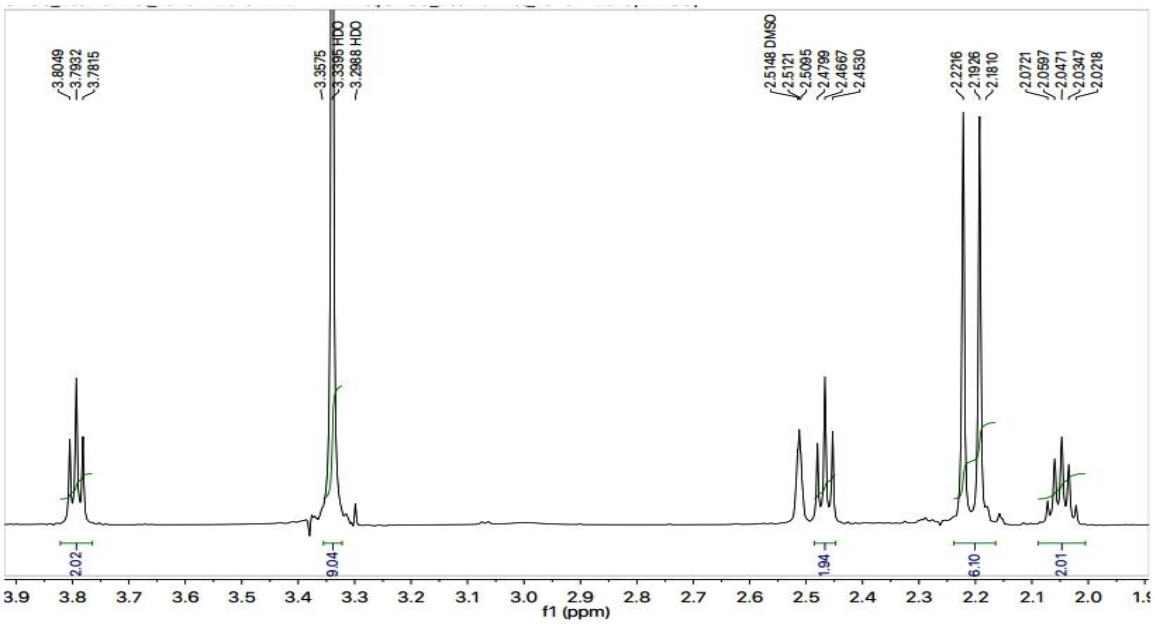


Fig. 1b: Expanded aliphatic region of ^{1}H-NMR spectrum of **3b**.

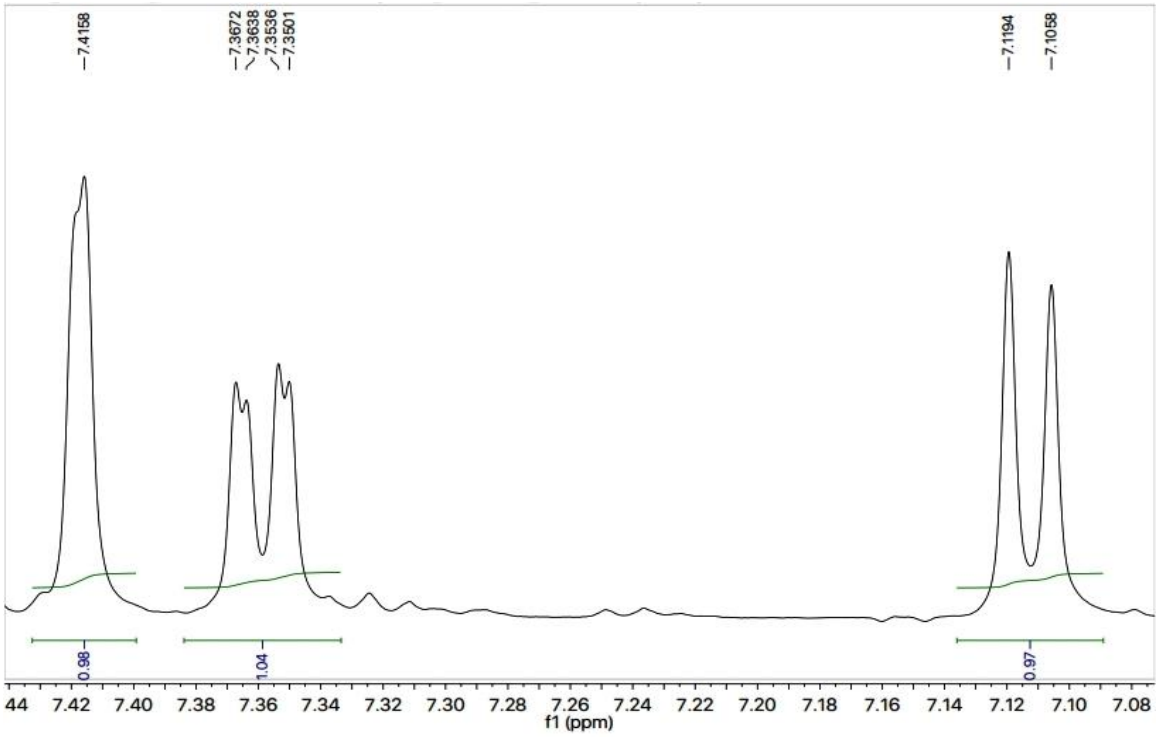


Fig. 1c: Expanded aromatic region of ^{1}H-NMR spectrum of **3b**.

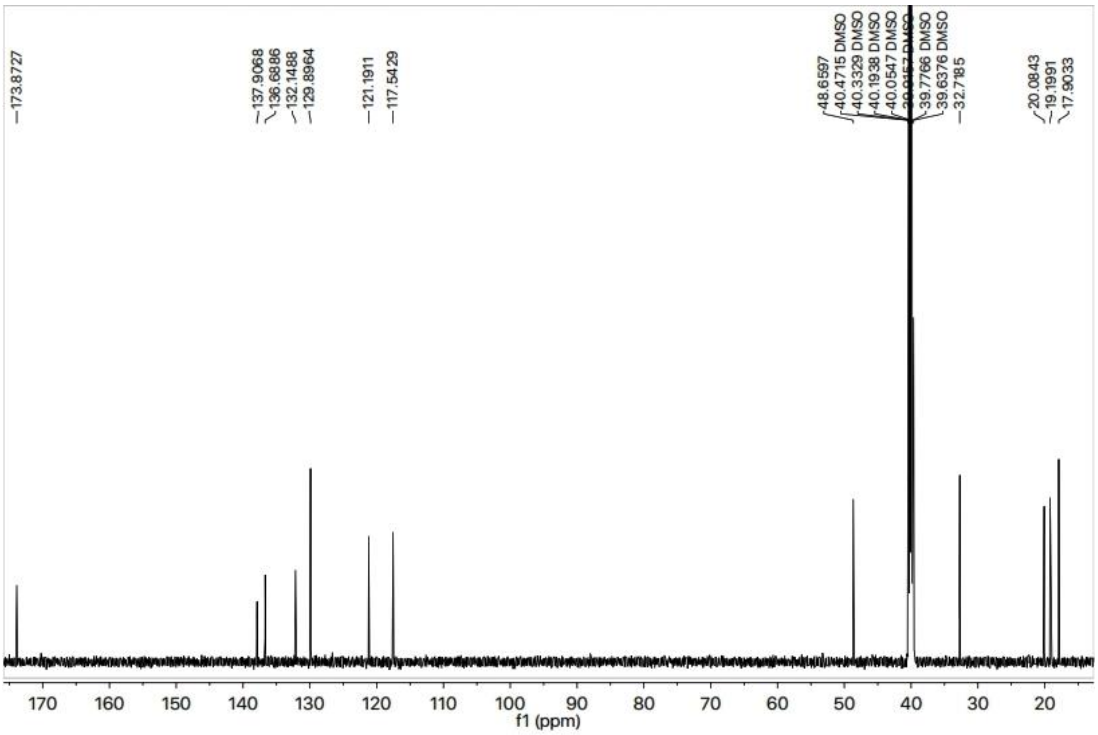


Fig. 2: ^{13}C-NMR spectrum of **3b**.

Antibacterial Activity

The antibacterial activity of synthesized derivatives of butamides, **3a-c**, was checked against two pathogenic bacterial strains. The bacterial strains included a gram-positive (*S. aureus*), and other as a gram negative (*E. coli*) strain. *Ampicillin* was used as standard drug against *S. aureus* and *E. coli* to compare the antimicrobial behavior of these synthesized compounds. It was manifest from our results (Table-1) that the presence of methyl groups at different positions on benzene ring in **3a-c** molecules, resulted in a decrease or increase in the antibacterial activity of the particular compounds. Among these synthesized compounds, the weak antibacterial activity was showed by the compound **3a** against the *Escherichia coli* and moderate activity was showed by the compound **3c** relative to the standard (Ampicillin). So, it was conclusion that variation of the position of methyl groups was resulting in varied antibacterial potential of these molecules.

Hemolytic activity

The synthesized regio-isomers, **3a-c**, were also subjected to hemolytic assay so as to profile their cytotoxicity. Results of the percentage hemolysis are shown in Table-1. The maximum toxicity towards red blood cell membrane was exposed by **3c** (12.68 ± 0.04%) and minimum by **3b** (1.64 ± 0.01%). Overall, these molecules exhibited weak cytotoxicity relative to the Triton-X having % hemolysis of 87.67 ± 0.01%.

Conclusion

It was concluded that some variations on substitution patterns of the aromatic system showed a varied antibacterial activity. Among these regio-isomers, **3a** exhibited better antibacterial potential against *Escherichia coli* while **3c** possessed better activity against *Staphylococcus aureus* strain. All the molecules showed mild cytotoxicity. So, these molecules might be considered as possible therapeutic agents in antibacterial drug designing program.

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