

SYNTHESIS, ANTIMICROBIAL, AND ANTITUBERCULAR ACTIVITIES OF NOVEL N-PYRAZOLYL BENZAMIDE DERIVATIVES

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ABSTRACT

The current study investigated the antimicrobial and antitubercular activities of novel N-Pyrazolyl Benzamide derivatives. The study includes the synthesis, characterization, and ligand-based molecular docking of the designed molecules. Synthesis of N-Pyrazolyl Benzamide derivatives involves a two-step process in which 5-amino pyrazole (3) intermediate is produced by the condensation reaction of aryl hydrazine (1) and β -keto nitrile (2) in acidic conditions. The final N-Pyrazolyl Benzamide derivatives (5a-n) were synthesized by the reaction involving amide coupling between 5-amino pyrazole (3) and various substituted benzoyl halides (4a-n). The compounds (5a-n) were tested for antimicrobial activity against two gram-negative strains (*E. coli* and *S. Typhi*), two gram-positive strains (*S. aureus* and *B. subtilis*) and *M. tuberculosis* MTB H37Rv (ATCC 27294) strains were used for evaluating antitubercular activity. Molecular docking studies of designed derivatives were performed with the Schrodinger glide package against mycobacterial pantothenate synthetase (PDB ID: 4MQ6). All the tested compounds displayed moderate to good antimicrobial and antitubercular activity against the selected strains of bacteria. All the docked molecules were found occupying the active site and established a minimum of two hydrogen bond interactions in molecular docking studies.

Keywords: N-Pyrazolyl Benzamide, Antimicrobial, Antitubercular, Molecular Docking.

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INTRODUCTION

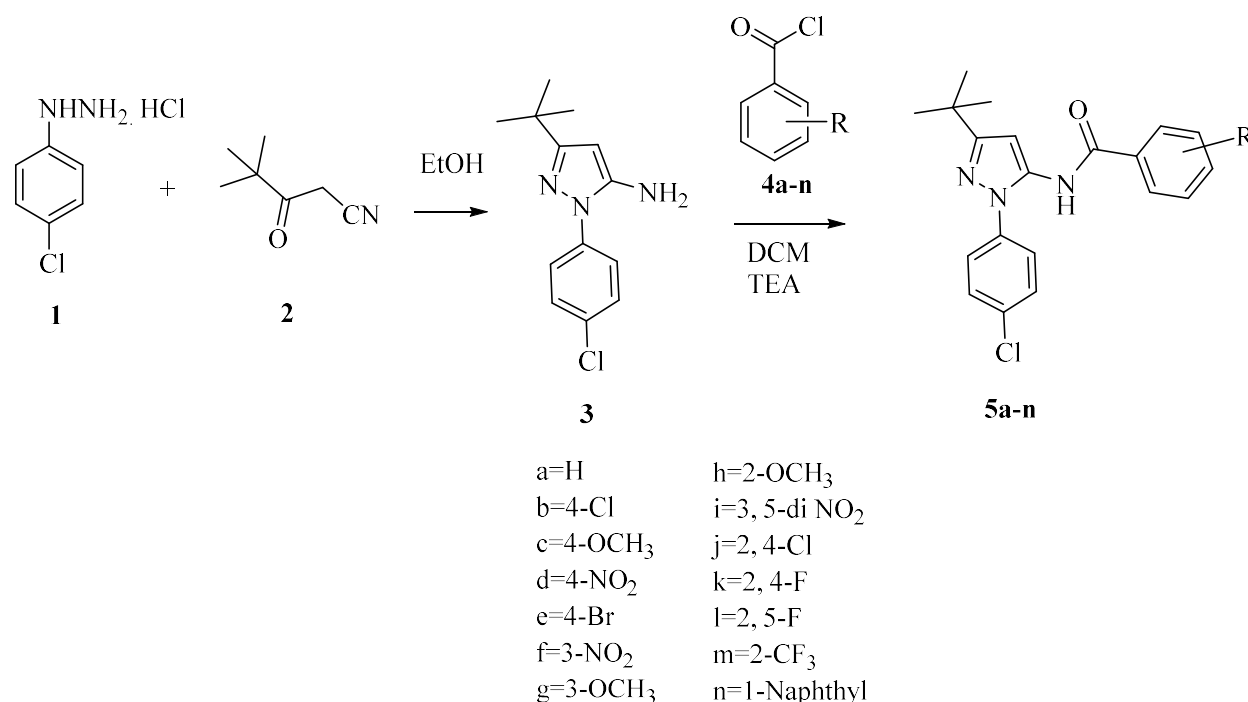
A major challenge that is faced in the healthcare field is the death of patients due to resistance to clinically used antimicrobial drugs.¹ Bacteria are now resistant to a variety of drugs making it necessary to change the standards of drug production.^{2,3} The most challenged resistant strains to public health are Methicillin Resistant *Staphylococcus Aureus* (MRSA), *Staphylococcus epidermidis* (MRSE); Vancomycin Resistant *Staphylococcus Aureus* (VRSA), *Enterococci* (VRE) and new bacteria which express genes encoded for Carbapenemase.^{4,5} Resistant gram-negative organisms categorized in “*Enterobacteriaceae*, carbapenem-resistant and 3rd generation cephalosporin-resistant” *Proteus spp.*, *Serratia spp.*, *Enterobacter spp.*, *Escherichia coli*, *Klebsiella pneumonia* and noted gram-negative bacteria are worried some for the human health. For more than three decades, not a new antibiotic was discovered however the newer clinical candidates are modifications of the existing class of drug candidates. The emergence of dreadful drug resistance bacteria coupled with the lackluster approach of big pharmaceutical industries in antimicrobial drug research has intensified the need for new antibacterial agents.⁶ Mycobacterial tuberculosis is one more frightful infectious disease caused by *Mycobacterium tuberculosis* organism which is with a thick lipoidal layer. WHO 2019 reports disclosed that worldwide over 10 million people suffered mycobacterial infection and approximately 1.5 million people lost their life. In the past two decades, the situation has still aggrandized because of the inception of resistance strains such as multidrug resistance, extremely drug-resistant strains, and coinfection with HIV. Drug discovery attempts in the search for new antitubercular drugs failed in the last 50 years, however, in the last decade two new drugs emerged namely Bedaquiline, a diarylquinoline derivative, a bacterial ATP synthase inhibitor, and Delamanid, a nitroimidazole which is a cell wall synthesis inhibitor. Despite the availability of new antitubercular drugs, still there persists the need for more such drugs which control the spread of this infection.

Compounds containing Pyrazole scaffolds have been substantiated for showing a diverse variety of pharmacological activities like kinase inhibitory activity, anti-tubercular, anti-inflammatory, and anti-angiogenic activities.^{7,8} Anti-inflammatory activity of urea derivatives and potent p38 kinase inhibitor activities of compounds bearing pyrazolo scaffold were developed in recent days.⁹⁻¹⁴ In this paper, the synthesis of new Pyrazolyl benzamides derivatives was reported and they were proved to possess antimicrobial and antitubercular activities.

EXPERIMENTAL

Materials and Methods

Chemicals, solvents, and glassware required for carrying out all the reactions were either purchased from SD Fine, Loba or purified whenever required. Polmon companie's melting point apparatus was used to measure the melting points and is uncorrected. Bruker Alfa Germany spectrophotometer in KBr pellet or ATR was used to record FT IR spectra of compounds. BrukerAvance400 MHz NMR spectrometer in CDCl₃ (δ 7.26) or DMSO- d₆ (δ 2.49) was employed to measure proton NMR spectra. Agilent mass spectrometer was employed for evaluating the M/z mass data of compounds. Pre-coated silica gel F254 (Merck) was used to check the purity of compounds and purified by using silica gel 60-120 with the solvent system as hexane and ethyl acetate.



Scheme-1: Synthesis of Pyrazole Substituted Benzamides 5a-N

Synthesis of 5-amino-1-(4'-chloro) phenyl-3-t-butyl pyrazole (3)

The synthesis of compound 3 was accomplished by following reported method in which 4-chlorophenyl hydrazine hydrochlorides 1 (0.05 mM) and β-keto nitrile 2 (0.06 mol) were refluxed in 50 mL of absolute ethanol in the presence of acetic acid (few drops) for 10-12 hours. The cooled reaction mixture was vacuum evaporated for the removal of volatilities and the crude solid was washed with ether. The crude hydrogen chloride salt was dispensed over ethyl acetate and treated with saturated NaHCO₃. Brine solution followed by distilled water was used to wash pooled ethyl acetate solution and dried over sodium sulphate. After taking away ethyl acetate, the leftover residue was additionally washed with hexane and ether (80:20) to acquire 5-amino-1-(4'-chloro) phenyl-3-*t*-butyl pyrazole 3.^{15,16}

Preparation of N-[3'-*t*-butyl-1'-(4'-chloro) phenylpyrazol-5'-yl] benzamide (5a-n)

The key intermediate 5-amino-1-(4'-chloro) phenyl-3-*t*-butyl pyrazole 3 (0.001 mol) was stirred in dichloromethane for 0.5 hours for a clear solution along with base triethyl amine 0.003 mol. To the above

solution of 3 and base, selected substituted C_6H_5COCl 4a-n (0.0025 mol) in 5 ml dichloromethane, dropped in portion & stirred well at room temperature overnight. After the reaction gets completed, the mixture was diluted with dichloromethane and brine followed by distilled water. The extract was dried with anhydrous sodium sulphate and then evaporated to remove dichloromethane. The crude products were purified over silica to acquire the pure desired products 5a-n.

Antibacterial Activity

“Agar disc diffusion method was used for evaluating antibacterial activity for synthesized compounds as per the guidelines of Clinical and laboratory standard institute.¹⁷ *B. subtilis* (ATCC 6051), *S. aureus* (ATCC 25323), *S. Typhi* (MTCC 3216) & *E. coli* (ATCC 35218) were utilized in the present study. Collected bacterial cultures were again sub-cultured to segregate pure colonies. These isolated pure colonies were shifted into a normal sterile saline solution and then vortexed to obtain a homogenous bacterial suspension. By employing the broth dilution method, minimum inhibitory concentration (MIC) of synthesized compounds was obtained. In Mueller-Hinton broth (MHB), the pure bacterial culture of individual microbes was adjusted to 0.5 McFarland standards. As per the guidelines of the clinical and laboratory standard institute, a two-fold serial dilution method was followed.¹⁸ The stock solutions of tested compounds were prepared in DMSO and were diluted with sterile water. The tested compound's concentration ranged from 0.8 to 100 $\mu\text{g/mL}$. Ciprofloxacin & sterile distilled water were used as positive and negative controls respectively”.

In vitro Antimycobacterial activity

Test organisms and preparation of inoculum

“The compounds were also evaluated for antitubercular activity by using *M. tuberculosis* MTB H37Rv (ATCC 27294) strains which are susceptible to Isoniazid. Sub cultured bacterial strains were used for the study, which was supplemented with Muller Hinton broth at 37°C for 2 weeks. Diluted bacterial suspensions were prepared with 0.5 McFarland standard turbidity, equivalent to 10^8 CFU. For dilutions, normal saline solution was used and finally diluted bacterial suspensions were vortexed for 30 seconds.¹⁹ For the inoculation, 100 μL of the microbial suspension was used.

Preparation of Test Samples and determination of MIC

100 $\mu\text{g/mL}$ concentrations of stock solutions were prepared for synthesized compounds by using DMSO. From the respective stock solutions, serial dilutions were made with varying strengths (0.8, 1.6, 3.12, 6.25, 12.5, 25, 50, and 100 $\mu\text{g/mL}$) to determine their MIC. The mycobacterium was grown using a middle brook 7H11 agar medium which was supplemented with OADC (Oleic Albumin Dextrose Catalase), then it was sterilized by moist heat method using an autoclave (121°C for 15 minutes). In appropriate volumes, the medium was diluted with varying strengths (0.8, 1.6, 3.12, 6.25, 12.5, 25, 50, and 100 $\mu\text{g/mL}$) of synthesized compounds. 5 ml of middle brook 7H11 agar medium was suspended in each labeled quadrants of sterile quad-plates under sterile conditions”. The lids are kept slightly opened to solidify under laminar airflow. After this, the culture broth of bacterial suspension was taken in a loop for inoculation and incubated for around 21 days at a constant temperature of 37 °C. The colonies produced on the medium were counted to determine the MIC in comparison to the controls. Isoniazid and DMSO were used as positive and negative controls respectively.^{20, 21}

Molecular Docking Studies

Synthesized compounds were undertaken for molecular docking simulation studies in order to identify and understand putative binding interactions of 5a-n within the active site of the pantothenate synthetase enzyme. Schrodinger package Glide module was selected for exploring molecular docking studies.²² The crystal structure of the mycobacterial pantothenate synthetase enzyme (PDB ID: 4MQ6, Resolution: 1.7 Å) was taken from the RCSB protein data bank. Structure of synthesized molecules, 5a-n were drawn in Marcush sketch and imported as mol2 in Maestro for minimization. Ligands were prepared as the part of docking protocol for energy minimization LigPrep, proteins were prepared by using the protein preparation wizard of glide, and docking studies were done by using Glide XP as per the standard procedure.²⁴ The log file generated after the completion of the docking simulation was analyzed critically

for the interaction efficiency of compounds with the macromolecule expressed as kcal/mol. Visualizations of the docking results were done in PyMOL 2.1.1 and Discovery Studio Visualizer 2020.²³

Characterization

5a N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl) benzamide

FTIR (KBr, in cm^{-1}): 3210 (amide NH stretching), 3010 (ArCH stretching), 2940 (aliphatic CHstr.), 1673 (C=O) and 1613 (NH band)(NH band); ^1H NMR (CDCl_3): (ppm): 1.3 (s, 9H, t-butyl), 6.6 (s, 1H, C4, pyrazole), 6.8-7.6 (m, 7H, aromatic), 7.8 (d $J=8.2$ Hz, 2H, C2, C6, aromatic), 8.3 (bs, 1H, NH amide). m/z $[\text{M}+1]^+$: 354.0

5b N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl)-4-chlorobenzamide

FTIR (KBr, in cm^{-1}): 3235 (amide NH), 3100 (ArCH), 2935 (alkyl CH), 1660 (C=O) and 1610 (NH bend). ^1H NMR (CDCl_3 , 400 MH): 8.8 (bs, 1H, Amide NH), 1.26 (s, 9H, CH), 7.2 (s, 1H, pyrazolo 4CH), 7.18-7.24 (d, 2H, ArCH), 7.7-7.78 (d, 2H, Ar CH), 7.9-8.0 (d, 2H, ArCH), 8.04-8.1 (d, 2H, Ar CH), m/z $[\text{M}+1]^+$: 432.00

5c N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl)-4-methoxybenzamide

FTIR (KBr, in cm^{-1}): 3223 (amide NH), 3106 (ArCH), 2918 (alkyl CH), 1648 (C=O) and 1620 (NH bend). ^1H NMR (CDCl_3 , 400 MH): 8.4 (bs, 1H, Amide NH), 1.24 (s, 9H, CH), 2.8 (s, 3H, OCH_3), 6.3 (s, 1H, pyrazolo 4CH), 6.88-6.99 (d, 2H, arCH), 7.2-7.28 (d, 2H, ArCH), 7.46-7.52 (d, 2H, ArCH), 7.9-8.0 (d, 2H, ArCH), m/z $[\text{M}+1]^+$: 384.2

5d N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl)-4-nitrobenzamide

FTIR (KBr, in cm^{-1}): 3244 (amide NH), 3095 (ArCH), 2920 (alkyl CH), 1640 (C=O) and 1625 (NH bend). ^1H NMR (CDCl_3 , 400 MH): 9.0 (bs, 1H, Amide NH), 1.2 (s, 9H, CH), 6.8 (s, 1H, pyrazolo 4CH), 7.26-7.33 (d, 2H, ArCH), 7.66-7.75 (d, 2H, ArCH), 8.28-8.35 (d, 2H, ArCH), 8.42-8.5 (d, 2H, ArCH); m/z $[\text{M}+1]^+$: 399.1

5e N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl)- 4-bromobenzamide

FTIR (KBr, in cm^{-1}): 3235 (amide NH), 3100 (ArCH), 2935 (alkyl CH), 1660 (C=O) and 1610 (NH bend). ^1H NMR (CDCl_3 , 400 MH): 8.8 (bs, 1H, Amide NH), 1.26 (s, 9H, CH), 6.4 (s, 1H, pyrazolo 4CH), 7.18-7.24 (d, 2H, ArCH), 7.7-7.78 (d, 2H, Ar CH), 7.9-8.0 (d, 2H, ArCH), 8.04-8.1 (d, 2H, Ar CH), m/z $[\text{M}+1]^+$: 432.00

5f N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl)-3-nitrobenzamide

FTIR (KBr, in cm^{-1}): 3255 (amide NH), 3110 (ArCH), 2908 (alkyl CH), 1675 (C=O) and 1618 (NH bend). ^1H NMR (CDCl_3 , 400 MH): 9.1 (bs, 1H, Amide NH), 1.22 (s, 9H, CH), 6.7 (s, 1H, pyrazolo 4CH), 7.22-7.3 (d, 2H, ArCH), 7.6-7.7 (d, 2H, ArCH), 7.7-7.88 (t, 1H, ArCH), 8.2-4 (m, 2H, Ar CH), 8.7 (s, 1H, Ar CH); m/z $[\text{M}+1]^+$: 399.1

5g N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl)-3-methoxybenzamide

FTIR (KBr, in cm^{-1}): 3240 (amide NH), 3118 (ArCH), 2923 (alkyl CH), 1656 (C=O) and 1623 (NH bend). ^1H NMR (CDCl_3 , 400 MH): 8.4 (bs, 1H, Amide NH), 1.20 (s, 9H, CH), 3.4 (s, 3H, methoxy), 6.38 (s, 1H, pyrazolo 4CH), 6.8 (s, 1H, Ar CH), 7.1-7.3 (m, 3H, Ar CH), 7.4-7.8 (m, 4H, ArCH); m/z $[\text{M}+1]^+$: 384.2

5h N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl)-2-methoxybenzamide

FTIR (KBr, in cm^{-1}): 3246 (amide NH), 3120 (ArCH), 2932 (alkyl CH), 1660 (C=O) and 1615 (NH bend). ^1H NMR (CDCl_3 , 400 MH): 8.45 (bs, 1H, Amide NH), 6.3 (s, 1H, pyrazolo 4CH), 7.0-7.7 (m, 8H, Ar CH), 3.7 (s, 3H, methoxy), 1.22 (s, 9H, CH); m/z $[\text{M}+1]^+$: 384.2

5i N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl)-3,5-dinitrobenzamide

FTIR (KBr, in cm^{-1}): 3230 (amide NH), 3106 (ArCH), 2938 (alkyl CH), 1656 (C=O) and 1610 (NH bend). ^1H NMR (CDCl_3 , 400 MH): 9.8 (bs, 1H, Amide NH), 1.4 (s, 9H, CH), 6.8 (s, 1H, pyrazolo 4CH), 7.28-7.38 (d, 2H, Ar CH), 7.54-7.6 (d, 2H, Ar CH), 8.8-9.3 (m, 3H, Ar CH), m/z $[\text{M}+1]^+$: 444.10

5j N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl)-2,4-dichlorobenzamide

FTIR (KBr, in cm^{-1}): 3240 (amide NH), 3115 (ArCH), 2922 (alkyl CH), 1640 (C=O) and 1618 (NH bend). ^1H NMR (CDCl_3 , 400 MH): 8.8 (bs, 1H, Amide NH), 7.2-7.95 (m, 7H, ArCH), 1.32 (s, 9H, CH); 6.65 (s, 1H, pyrazolo 4CH), m/z $[\text{M}+1]^+$:422.01

5k N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl)-2,4-difluorobenzamide

FTIR (KBr, in cm^{-1}): 3233 (amide NH), 3107 (ArCH), 2915 (alkyl CH), 1630 (C=O) and 1605 (NH bend). ^1H NMR (CDCl_3 , 400 MH): 8.9 (bs, 1H, Amide NH), 6.6 (s, 1H, pyrazolo 4CH), 7.3-7.9 (m, 7H, ArCH), 1.28 (s, 9H, CH); m/z $[\text{M}+1]^+$:390.1

5l N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl)-2,5-difluorobenzamide

FTIR (KBr, in cm^{-1}): 3235 (amide NH), 3110 (ArCH), 2918 (alkyl CH), 1638 (C=O) and 1610 (NH bend). ^1H NMR (CDCl_3 , 400 MH): 1.24 (s, 9H, CH), 6.66 (s, 1H, pyrazolo 4CH), 7.2-7.8 (m, 7H, ArCH), 8.8 (bs, 1H, Amide NH), m/z $[\text{M}+1]^+$:390.1

5m N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl)-2-(trifluoromethyl)benzamide

FTIR (KBr, in cm^{-1}): 3262 (amide NH), 3122 (ArCH), 2943 (alkyl CH), 1658 (C=O) and 1621 (NH bend). ^1H NMR (CDCl_3 , 400 MH): 8.68 (bs, 1H, Amide NH), 1.29 (s, 9H, CH), 6.58 (s, 1H, pyrazolo 4CH), 7.1-7.9 (m, 8H, ArCH), m/z $[\text{M}+1]^+$:422.0

5n N-(3-(tert-butyl) – 1-(4-chlorophenyl)-1H-pyrazol-5-yl)-1-naphthamide

FTIR (KBr, in cm^{-1}): 3226 (amide NH), 3092 (ArCH), 2910 (alkyl CH), 1633 (C=O) and 1605 (NH bend). ^1H NMR (CDCl_3 , 400 MH): 8.8 (bs, 1H, Amide NH), 1.22 (s, 9H, CH), 6.5 (s, 1H, pyrazolo 4CH), 7.22-7.85 (m, 8H, ArCH), 8.1-8.5 (m, 3H, ArCH), m/z $[\text{M}+1]^+$:404.1

RESULTS AND DISCUSSION

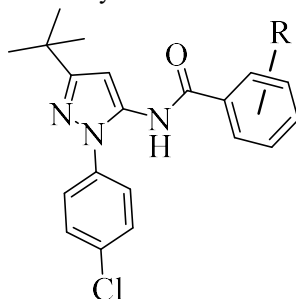
The contemplated molecule synthesis was depicted in Scheme-1. The key intermediate 3 was synthesized from pivaloyl acetonitrile and 4-chlorophenylhydrazine hydrochloride in ethanol by refluxing overnight. Upon workup which involved neutralizing with 5% NaHCO_3 and extracted with ethyl acetate. Brine followed by water is used to wash the organic layer to remove organic volatilities. Ethyl acetate & hexane (3:7) were used as eluent to purify the crude product over silica gel. The yields of the compounds with physical properties are shown in Table-1. FT IR spectra of 3 showed IR frequencies at ~ 3400 and 3300 cm^{-1} which are because of NH symmetric & asymmetric stretching vibrations. The CH_3 asymmetric & symmetric stretching frequencies were found in the range $2900\text{--}2800 \text{ cm}^{-1}$ and aromatic CH stretching vibrations were located at $\sim 3100 \text{ cm}^{-1}$. The ^1H NMR of 3 was recorded in CDCl_3 indicating a sharp singlet absorption band at a delta value of 1.6 integrate for nine protons which are assigned for protons of the tertiary butyl group and a broad peak at 4.3 which is attributed to the primary amine protons. Five protons including a vinyl CH and aromatic protons resonated between 6.8-7.5 δ . The ESI mass data of the same compound indicated an m/z molecular ion peak at 250, above FTIR, ^1H NMR, and Mass spectral data confirmed the structure of intermediate 3. Intermediate 3 was benzoylated with substituted benzoyl chlorides (5a-n) in base-mediated nucleophilic substitution reaction in dichloromethane at room temperature for overnight. Crude compounds 5a-n were purified using silica on a column with ethyl acetate & hexane as solvent system.

The FTIR spectra of compound 5a indicated a peak at 3285 cm^{-1} which attributes to NH vibration, aromatic CH stretching was located at $\sim 3100 \text{ cm}^{-1}$ and methyl group's aliphatic CH was seen in between $2950\text{--}2850 \text{ cm}^{-1}$. ^1H NMR of 5a showed two broad singlet peaks in delta range of 8.5-9.2 which are due to NH protons of urea and nine aliphatic protons of tertiary butyl group were located at delta value of 1.4. Ten protons which included nine aromatic and a vinylic resonated as a multiplet between 6.8-7.5. The molecular ion peak of 5a was found at 369 which corresponds to the molecular formula $\text{C}_{20}\text{H}_{21}\text{ClN}_4\text{O}$ thus confirming the structure of 5a.

All the molecules 5a-n were synthesized (5a&5b reported) in the ongoing search for new antitubercular agents and also screened for the antibacterial property involving *S. aureus*, *B. subtilis*, *E. coli* and *S. typhi*. The antibacterial activities of 5a-n were compared with standard ciprofloxacin which is given in Table-2. All tested molecules showed antibacterial potential in the range of 3.12 – 100 $\mu\text{g/ml}$ MIC. The

unsubstituted compound 5a showed MIC of 25µg/ml against gram +ve& showed MIC of 50µg/ml against both the gram –ve organisms.

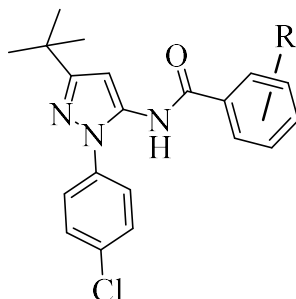
Table-1: Physical Properties of Pyrazole Substituted Benzamide Derivatives



No.	R	Molecular Formula	Molecular Weight	Melting Point	Yield in %	Rf*
5a	H	C ₂₀ H ₂₀ ClN ₃ O	353.13	202-204	78	0.66
5b	4-Cl	C ₂₀ H ₁₉ Cl ₂ N ₃ O	387.09	207-209	71	0.74
5c	4-OCH ₃	C ₂₁ H ₂₂ ClN ₃ O ₂	383.14	182-184	73	0.78
5d	4-NO ₂	C ₂₀ H ₁₉ ClN ₃ O ₃	399.1	191-193	52	0.54
5e	4-Bromo	C ₂₀ H ₁₉ BrClN ₃ O	431.04	228-230	66	0.78
5f	3-NO ₂	C ₂₀ H ₁₉ ClN ₃ O ₃	399.1	199-201	48	0.6
5g	3-OCH ₃	C ₂₁ H ₂₂ ClN ₃ O ₂	383.14	235-237	62	0.7
5h	2-OCH ₃	C ₂₁ H ₂₂ ClN ₃ O ₂	383.14	241-43	63	0.72
5i	3,5-Dinitro	C ₂₀ H ₁₈ ClN ₃ O ₅	443.10	168-70	40	0.58
5j	2,4-Dichloro	C ₂₀ H ₁₈ Cl ₃ N ₃ O	421.05	222-24	56	0.82
5k	2,4-Difluoro	C ₂₀ H ₁₈ ClF ₂ N ₃ O	389.11	244-46	58	0.64
5l	2,5-Difluoro	C ₂₀ H ₁₈ ClF ₂ N ₃ O	389.11	236-38	53	0.6
5m	2-CF ₃	C ₂₁ H ₁₉ ClF ₃ N ₃ O	421.12	231-33	49	0.73
5n	1-Naphthoyl	C ₂₄ H ₂₂ ClN ₃ O	403.15	190-92	62	0.8

*Hexane: ethyl acetate, 7:3, # in °C

Table-2: Antimicrobial Activity



No.	R	Staphylococcus aureus (ATCC25323)*	Bacillus subtilis (ATCC 6051)*	Escherichia coli (ATCC35218)*	Salmonella typhi (MTCC3216)*
5a	H	50	25	50	50
5b	4-Cl	6.25	3.12	50	50
5c	4-OCH ₃	3.12	6.25	25	12.5
5d	4-NO ₂	12.5	12.5	25	25
5e	4-Bromo	3.12	3.12	25	50
5f	3-NO ₂	12.5	6.25	25	25
5g	3-OCH ₃	25	25	12.5	12.5
5h	2-Methoxy	12.5	12.5	12.5	25
5i	3,5-Dinitro	25	25	12.5	6.25
5j	2,4-Dichloro	12.5	12.5	6.25	6.25
5k	2,4-Difluoro	100	50	50	50
5l	2,5-Difluoro	100	100	25	25

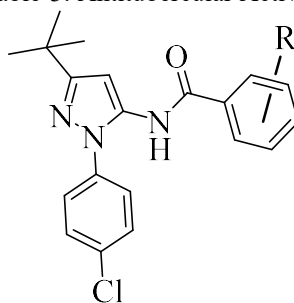
5m	2-CF ₃	12.5	25	25	25
5n	1-Napthoyl	12.5	25	50	50
Std#		3.12	3.12	3.12	3.12

* (MIC µg/ml)

#Ciprofloxacin

Four molecules 5b-e substituted with groups on the 4th position showed improved antibacterial activity against gram-positive organisms. Compounds 5c and 5e with 4-OCH₃ and 4-Br inhibited the growth of gram-positive organisms with a minimum inhibitory concentration of 3.12 µg/ml while 4-Cl & 4-NO₂ molecules 5b&5d demonstrated a minimum inhibitory concentration of 6.25 and 12.5 µg/ mL respectively. The remaining molecules with different substitutions have shown 12.5 to 100 µg/ml of minimum inhibitory conc. surprisingly; the same molecules were either moderate or weak against gram-negative organisms with minimum inhibitory conc. of 25-50 µg/ml, however, only 5c has 12.5 µg/ml of MIC against *Salmonella typhi*. Comparatively, compounds with substitutions on the 3rd position of the phenyl ring (5f-g) were with encouraging antibacterial activity against all the investigated microbes with MIC ranging from 6.25 to 25 µg/ml. Numerous compounds with substitution on the 2nd position were synthesized, 5i and 5j displayed minimum inhibitory conc. of 6.25 µg/ml against *Escherichia coli* and *Salmonella typhi*, whereas the same compounds 5i and 5j showed MIC between 12.5-25 µg/ml against gram-positive organisms. Two compounds with substitution at 2nd position 5k and 5l were weak in inhibiting the growth of both gram-negative and gram-positive organisms. Lone naphthyl substituted compound 5n was potent against *Staphylococcus aureus* while moderate against the remaining three tested organisms.

Table-3: Antitubercular Activity



No.	R	Anti Tb Activity H37Rv (ATCC 27294)*	Dock score
5a	H	100	-4.528
5b	4-Cl	12.5	-1.511
5c	4-OCH ₃	25	-2.427
5d	4-NO ₂	6.25	-4.96
5e	4-Bromo	50	-4.302
5f	3-NO ₂	12.5	-5.179
5g	3-OCH ₃	3.12	-1.274
5h	2-Methoxy	50	-5.105
5i	3,5-Dinitro	12.5	-2.67
5j	2,4-Dichloro	25	-4.199
5k	2,4-Difluoro	50	-1.493
5l	2,5-Difluoro	12.5	-1.332
5m	2-CF ₃	25	-4.357
5n	1-Napthoyl	25	-5.938
INH		0.3	

*(MIC µg/ml)

As the compounds were synthesized for exploring the antitubercular activity, compounds 5a-n were screened against H37Rv (ATCC 27294) strain. All the compounds were active in the antitubercular inhibitory test with minimum inhibitory conc. between ranges of 3.12-50 µg/ML as enumerated in above Table-3. Unsubstituted compound 5a displayed 100 µg/ml of MIC and substitution on the phenyl ring

either retained the activity or bettered with exception of 5e, 5h, and 5k. Two para-substituted compounds 5b and 5d have shown superior activity over 5a and among the mentioned compounds, 5d was found to be effective with 6.25 µg/ml of minimum inhibitory concentration. Among the two molecules, 3-substituted molecules 5f, and 5g with the 3-methoxy group were found to be the most potent for anti-TB activity having 3.12 µg/ml minimum inhibitory concentrations. Three compounds have been tested with substitution on second position 5k, 5l and 5m, among them, 5l was superior in antitubercular activity over the rest two. Lone naphthyl compound 5n demonstrated MIC of 25 µg/ml.

Molecular Docking Simulation Studies

As there is no information of inhibitors bearing pyrazole scaffold and with structural similarity to our synthesized molecules about molecular targets for antitubercular activity, the planned work pondered exploring molecular docking studies of compounds targeting pantothenate synthetase (PS). PS is an important enzyme required for the survival of mycobacteria, catalyzing the amide bond formation between pantoate and β -alanine in ATP-dependent biochemical reactions in *BiUniUni Bi Ping Pong* mechanism. The active site of PS showed a lipophilic pantoate binding pocket P1 formed by amino acids Pro 38, Met40, Val 143, Leu 146, and Phe 157. More hydrophilic pocket P2 is ATP binding domain where ATP makes two hydrogen bonds with Met40 and His47.

All the compounds have been docked in the pantothenate synthetase active site by using Schrodinger's Glide XP with standard parameters. All the docked molecules were found occupying the active site and established a minimum of two hydrogen bond interactions which are listed in Table 4. Five compounds 5a, 5c, 5h, 5j, and 5m have been involved with three hydrogen bonds whereas 5d and 5n interacted with four hydrogen bonds. All the molecules were found hydrogen bonded at least one either with His44 or His 47 or both and additional hydrogen bonds with Lys160, Val187, Ser196, and Ser197. Apart from hydrogen bond interactions, docked molecules also displayed close hydrophobic interactions at a close distance with amino acid residues Met40, Ala42, Leu50, Lys160, Met195, Arg198, and Pro261. All the compounds except 5c have demonstrated distant dependent π - π interactions with His44 or His 47 and compounds 5a, 5k and 5l have additionally displayed halogen bond interactions. Interactions of compound 5g have been included in Fig.-1.

Table-4: Interactions of Compounds with Amino Acids in the Active Site

No.	Hydrogen Bond	Hydrophobic	Pi-Pi and others if otherwise mentioned
5a	His44: 2.9, His47: 2.84, Ser197: 2.47	Met40: 3.91, Leu50: 3.96, Arg198: 3.86, Pro261: 1.92	His44: 5.1, His47: 5.35 Pro185: 3.8 (Halogen)
5b	His44: 2.99, His47: 2.88	Met40: 3.45, Leu50: 3.78, Pro261: 1.92	His47: 4.35
5c	His47: 2.94, Lys160: 2.92, Val187: 2.1	Leu50: 3.88, Pro261: 1.92,	
5d	His44: 2.77, His47: 2.56, Lys160: 2.65, Val187: 2.2	Met40: 3.17, Ala42: 3.45, Lys160: 3.96, Pro261: 1.92	His44: 4.94
5e	His44: 2.04, His47: 3.22	Leu50: 3.94, Pro261: 1.92,	His47: 5.41
5f	His47: 2.72, Ser197: 2.16	Met40: 3.92, Pro261: 1.9	His44: 4.74
5g	His47: 2.92, Lys160: 2.74	Met40: 3.43, Lys160: 3.89, Pro261: 1.92	His44: 5.39
5h	His44: 2.92, His47: 3.17, Ser197: 2.77	Lys160: 3.52, Met195: 3.4, Pro261: 1.92	His44: 4.77
5i	His44: 2.97, His47: 3.17	Lys160: 3.5, Met195: 3.24, Pro261: 1.92	His44: 4.75
5j	His44: 1.85, Lys160: 2.96, Ser197: 2.44	Lys160: 3.44, Met195: 3.52, Arg198: 3.99, Pro261: 1.92	His44: 4.9
5k	His47: 2.77	Met40: 3.38, Lys160: 3.85, Pro261: 1.92	His44: 5.32, His47: 4.38, Lys160: 3.59 (Halogen), Val187: 3.47 (Halogen)
5l	His47: 3.01, Lys160: 2.79	Met40: 3.65, Pro261: 1.92	His44: 5.37, Val187: 3.38 (Halogen)

5m	His44: 1.9, Lys160: 3.19, Ser197: 2.3	Lys160: 3.52, Met195: 3.38, Pro261: 1.92	His44: 4.84
5n	His44: 1.81, Lys160: 3.23, Ser196: 3.44, Ser197: 3.13	Lys160: 3.44, Met195: 3.37, Arg198: 3.84, Pro261: 1.92	His44: 4.86

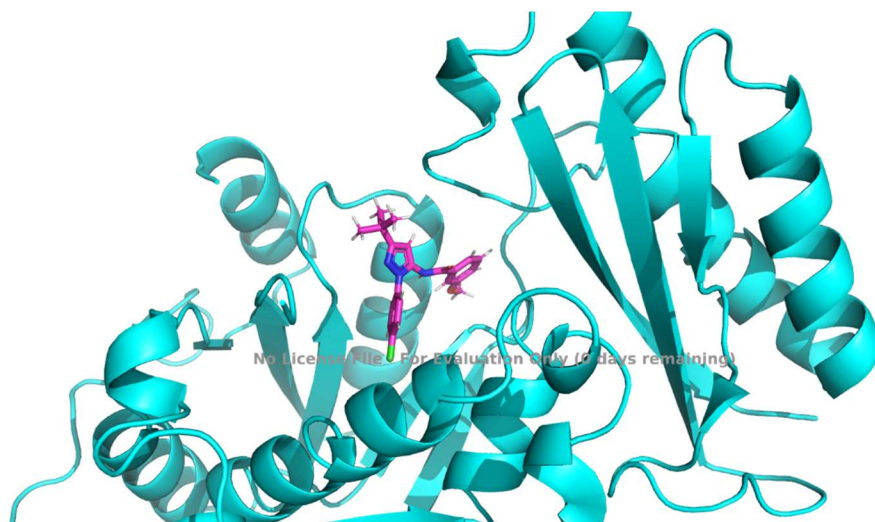


Fig.-1: Docked Pose of 5g in the Active Site

CONCLUSION

Compounds 5c and 5e with 4-OCH₃ and 4-Br inhibited the growth of gram-positive organisms with a minimum inhibitory concentration of 3.12 µg/ml while 4-Cl & 4-NO₂ molecules 5b & 5d demonstrated minimum inhibitory concentrations of 6.25, 12.5 µg/ mL respectively. However only 5c has 12.5 µg/ml of MIC against *Salmonella typhi*. Comparatively, compounds with substitutions on the 3rd position of phenyl ring (5f-g) were with encouraging antibacterial activity against all the investigated microbes with MIC ranging from 6.25 to 25 µg/ml. Numerous compounds with substitution on 2nd position were synthesized, 5i and 5j displayed minimum inhibitory conc. of 6.25 µg/ml against *Escherichia coli* and *Salmonella typhi* whereas the same compounds 5i and 5j showed MIC between 12.5-25 µg/ml against gram-positive organisms. Lone naphthyl substituted compound 5n was potent against *Staphylococcus aureus* while moderate against the remaining three tested organisms. 4. 5g with 3-methoxy group were found to be the most potent for anti-TB activity having 3.12 µg/ml minimum inhibitory concentrations. All the compounds except 5c have demonstrated distant dependent π - π interactions with His44 or His 47 and compounds 5a, 5k and 5l have additionally displayed halogen bond interactions.

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