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Synthesis and antimicrobial studies of novel derivatives of 4-(4-formyl-3-phenyl-1H-pyrazol-1-yl)benzoic acid as potent anti-*Acinetobacter baumannii* agents

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ABSTRACT

Microbial resistance to antibiotics is a global concern. The World Health Organization (WHO) has identified antimicrobial resistance as one the three greatest threats for human beings in the 21st century. Without urgent and coordinated action, the world is moving toward a post-antibiotic era, in which normal infections or minor injuries may become fatal. In an effort to find new agents, we report the synthesis and antimicrobial activities of 40 novel 1,3-diphenyl pyrazole derivatives. These compounds have shown zones of growth inhibition up to 85 mm against *Acinetobacter baumannii*. We tested the active compounds against this Gram-negative bacterium in minimum inhibitory concentration (MIC) tests and found activity with concentration as low as 4 μg/mL.

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Antibiotic resistance to infection has become a worldwide problem in recent years. According to the latest Center for Disease Control (CDC) report more than two million people are infected every year with antibiotic-resistant infections and at least 23,000 are dying as a result of these diseases in the US alone.[1] In healthcare settings, Gram-negative bacterial infection causes pneumonia, bloodstream infections, wound or surgical site infections, and meningitis. Gram-negative bacteria are increasingly resistant to most of the available antibiotics. Most of the Gram-negative bacterial infections are caused by *Klebsiella*, *Acinetobacter*, *Pseudomonas aeruginosa*, and *Escherichia coli*.[2]

There are many species of *Acinetobacter* commonly found in soil and water. All the species of *Acinetobacter* can cause human disease, but *A. baumannii* alone accounts for about 80% of *Acinetobacter* infections. The main victims of *Acinetobacter* infections are people with weakened immune system, chronic lung disease, and diabetes. Outbreaks of *Acinetobacter* infections normally happen in intensive care units and healthcare settings. *Acinetobacter* can colonize tracheostomy sites or open wounds without causing infection. These bacteria can survive on the skin or surfaces for several days and can be spread to susceptible persons by person-to-person contact or contact with contaminated surfaces.[3] *A. baumannii* infection to US service members has become a major problem since the OPERATION Iraqi Freedom began in 2003.[4,5] In particular, multidrug resistant (MDR) *A. baumannii* is a rising class of extremely pathogenic bacteria. The medical community is in a desperate need of finding new antibiotics to treat MDR *A. baumannii* infection as some of the clinical strains are resistant to all known antibiotics approved to treat infections.[6]

The outer membrane of certain non-fermenting Gram-negative bacteria such as *A. baumannii* and *P. aeruginosa* can be highly impermeable to the vast majority of molecules. These non-fermenters are opportunistic and nosocomial pathogens, and many of these pathogens are multi-drug resistant.[7] There are several components associated with the *A. baumannii* cell wall that prevent the penetration of antibiotics.[8] Therefore, finding novel compounds with growth inhibition properties against non-fermenting bacteria is extremely important and may offer exciting new opportunities to treat these infections.

Phenylpyrazole, a privileged scaffold, is found in a great number of drugs and drug candidates including best selling drugs.[9] Several

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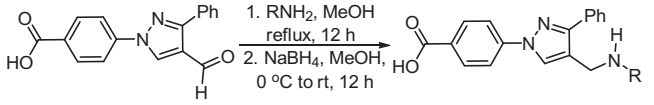
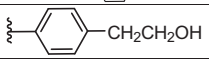
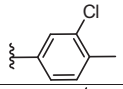
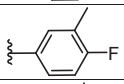
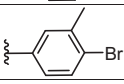
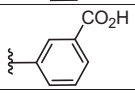
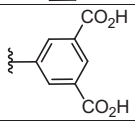
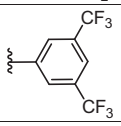
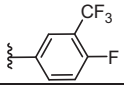
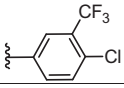
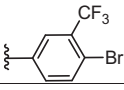
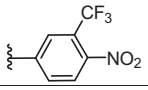
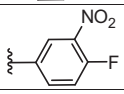
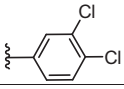
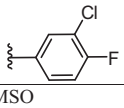
pyrazole derivatives have been found as analgesic, anti-inflammatory, antimicrobial, anticonvulsant, antidepressant, antimycobacterial, antiviral, and antitumor agents among others.[10–14] Pyrazole derivatives have been reported as antimicrobial agents in a number of publications,[15–17] but as anti-*Acinetobacter*, they are unknown. In our efforts to get potent antimicrobial agents,[18] we have synthesized several pyrazole-derived terphenyl like novel molecules. We have found pyrazole-derived N-aryl amines as anti-methicillin resistant *Staphylococcus aureus* (anti-MRSA) agents. Herein, we report the synthesis and antibacterial studies of forty pyrazole derivatives.

We have synthesized the starting material by using our recently published manuscript.[18] Aromatic amine derivatives are an integral part of many potent antimicrobial agents.[19] All these novel molecules are characterized by ^{1}H and ^{13}C NMR spectroscopy. We reported several N-aryl derivatives of the pyrazole as anti-MRSA agents up to 16 μM concentration.[18] To get the N-aryl pyrazole derivatives as potent antimicrobial agents, we synthesized several novel molecules (Scheme 1) to test against Gram-positive and Gram-negative bacteria. Nearly all of the test compounds showed moderate activity against Gram-positive bacteria in zone of inhibition studies. Compounds having electron donating groups such alkyl along with halogen atoms in the N-aryl moiety (**1**, **2**, **3**, **4**, & **5**) have shown moderate activity against Gram-positive bacteria, *S. aureus* and *B. subtilis*. These compounds showed slightly better growth inhibition against *B. subtilis* than *S. aureus*. N-Aryl moiety with a carboxylic acid functional group has eliminated the activity of the resultant molecule (**6**). Addition of one more carboxylic acid group (**7**) showed some activity against Gram-positive bacteria. Replacing carboxylic groups with trifluoromethyl group increased the activity significantly and the resultant molecule (**8**) showed activity up to 22 mm and 25 mm against *S. aureus* and *B. subtilis* respectively. One trifluoro group with a fluoro substituent (**9**) also showed good activity against Gram-positive bacteria. Trifluoro substituent with other halogen atoms (Cl & Br) also showed moderate activity in zone of inhibition studies (**10** & **11**). Very strong electron withdrawing group (NO_2) substitution also showed moderate activity against Gram-positive bacteria (**12** & **13**). Dihalogen substituted compounds (**14** & **15**) also showed some activity against Gram-positive bacteria in zone of inhibition studies (Scheme 1). The positive control, chloramphenicol, showed 25 mm and 32 mm zone of inhibition against *S. aureus* and *B. subtilis* respectively.

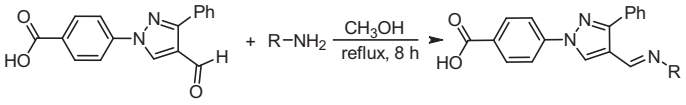
Hydrazone derivatives show a wide range of biological properties such as antimicrobial, antitubercular, anti-inflammatory, and anticancer activities.[20] Based on the excellent pharmacological profile in the literature, we synthesized several hydrazones of the aldehyde derivative, 4-(4-formyl-3-phenyl-1H-pyrazol-1-yl)benzoic acid. We synthesized the target molecules by the reaction of aldehyde with the corresponding hydrazine in methanol and acetic acid as a catalyst (Scheme 2). Refluxing the reaction mixture for 8 h followed by filtration of the solid precipitate afforded the hydrazones in excellent yield. All hydrazone derivatives are characterized by NMR (^{1}H & ^{13}C) spectroscopy and mass spectrometry.

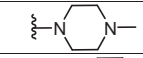
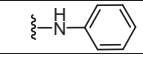
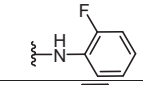
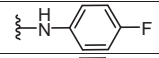
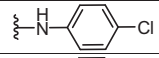
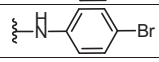
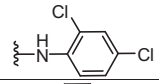
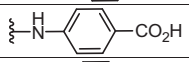
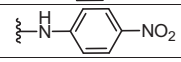
Synthesized hydrazones were evaluated against Gram-positive and Gram-negative bacteria. The simplest hydrazone derivative (**16**) showed moderate activity against both the Gram-positive bacteria (*S. aureus* & *B. subtilis*) in zone of inhibition studies. N-Methyl piperazine derived hydrazone (**17**) did not show any activity against the tested microorganisms. N-Phenyl derived hydrazone (**18**) showed moderate activity against tested Gram-positive bacteria. To our surprise, this hydrazone (**18**) also exhibited potent activity, 28 mm size zone of inhibition, against a Gram-negative bacterium, *A. baumannii*. Addition of a fluoro substituent (**19**) at the ortho position increased the activity of the resultant molecule. Positioning the fluoro group at the para carbon (**20**) increased the activity exponentially. At 0.1 M concentration, 10 μL of the solution of the compound completely cleared the 85 mm Petri dish plate. After tenfold dilution, 0.01 M concentration, it showed 32 mm zone of inhibition. Based on our literature search, this molecule (**20**) is the most potent pyrazole derivative as anti-*A. baumannii* agent. This hydrazone derivative (**20**) also showed moderate activity against *E. aerogenes* in addition to showing good activity against Gram-positive bacteria. Corresponding chloro



Compound No	R	Gram-positive bacteria (Zone of inhibition)		Product yield (%)
		S a (mm)	*B s* (mm)	
1		14	16	85
2		9	10	88
3		13	16	84
4		15	16	90
5		15	17	92
6		NA	NA	82
7		10	9	80
8		22	25	92
9		21	22	90
10		17	17	90
11		15	12	90
12				
13		14	12	84
14		11	11	90
15		11	11	90
DMSO		NA	NA	
Chloramphenicol		36	36	

Scheme 1. Synthesis and antimicrobial studies of pyrazole derived N-arylamines, Gram-positive bacteria: *S. aureus* (*S a*) and *B. subtilis* (*B s*), NA = no activity.



Compound No	R	Microorganism (zone of inhibition in mm)				Product yield (%)
		S a	*B s*	*A b*	*E a*	
16		7	7	NA	NA	95
17		NA	NA	NA	NA	79
18		10	11	28	NA	88
19		13	17	34	NA	90
20		16	18	>85 (0.1M) 32 (0.01M)	8	94
21		16	13	54	8	92
22		10	10	42	NA	93
23		10	11	NA	NA	92
24		NA	NA	NA	NA	78
25		NA	NA	22	NA	89
DMSO		NA	NA	NA	NA	
Ciprofloxacin		36	44	32	39	
Chloramphenicol		36	36	18	39	

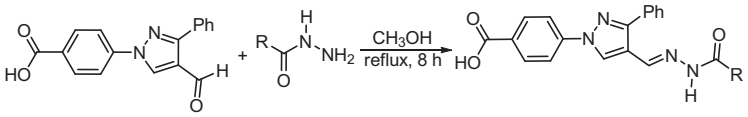
Scheme 2. Synthesis and antimicrobial studies of hydrazone derivatives, Gram-positive bacteria: *S. aureus* (*S a*) and *B. subtilis* (*B s*), Gram-negative bacteria: *P. aeruginosa* (*P a*) & *E. coli* (*E c*), and NA = no activity.

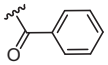
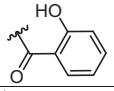
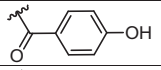
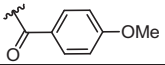
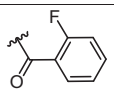
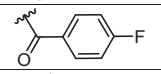
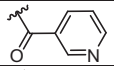
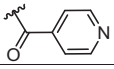
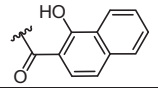
substituted compound (**21**) showed up to 54 mm zone of inhibition against *A. baumannii* and almost the same activity against other bacteria. Bromo substituted hydrazone derivative (**22**) exhibited good activity against *A. baumannii* and moderate activity against Gram-positive bacteria. Dichloro substitution (**23**) completely eliminated the activity against *A. baumanni* but showed moderate activity against Gram-positive bacteria. Strong electron withdrawing groups such as CO_2H and NO_2 completely ceased the activity of the resultant hydrazone derivatives (**24** & **25**). We found a clear structure activity relationship (SAR) of the synthesized compounds for the growth inhibition against the *A. baumanni*. Moderate electron withdrawing groups (halogens) showed good activity compared to other functional groups. Among the halogens, greater the electronegativity, better the activity of the molecule. Thus the fluoro substituted hydrazone showed the highest activity against the microorganisms, particularly against the *A. baumannii*. The positive controls, chloramphenicol and ciprofloxacin have shown 22 mm and 37 mm zone of inhibition respectively. Thus, these synthesized novel hydrazone derivatives have shown better activity than that of positive controls.

After finding lead molecules in the hydrazone series, we were encouraged to synthesize N-acylhydrazones to test against different bacteria (Scheme 3). We synthesized semicarbazone (**26**), and methyl hydrazinocarboxylate (**27**) derivatives by reacting with the corresponding hydrazide in refluxing methanol. Similarly N-acyl and N-benzoyl hydrazones (**28** & **29**) were synthesized and the products were isolated by filtration followed by recrystallization in acetonitrile to get the pure products. None of these compounds showed any activity except the simple benzoyl substituted hydrazone in zone of inhibition studies. Hydroxy substituted N-benzoyl hydrazones (**30** & **31**) were also synthesized but these products did not show any appreciable growth inhibition of bacteria. 4-Methoxy (**32**) and 2-fluoro (**33**) substituted N-benzoyl derivatives showed moderate activity against Gram-positive bacteria. Surprisingly, 4-fluoro substituted N-benzoyl hydrazone (**34**) did not show any activity in contrast to the corresponding hydrazone derivative (**20**). Synthesis and antimicrobial studies of isoniazid derivatives (**35** & **36**) also did not give any positive result. 2-Hydroxy naphthoic acid derived hydrazone (**37**) also did not show any activity. Although most of the molecules in this series did not show any activity, hydrophobic N-benzoyl hydrazones exhibited moderate activity against Gram-positive bacteria (Scheme 3) (see Scheme 4).

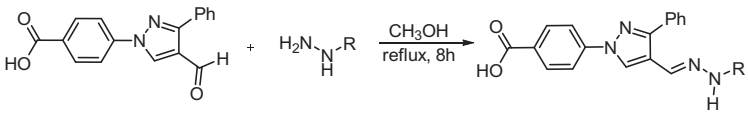
Sulfonylhydrazones are well known to show a wide range pharmacological properties such as antiviral,[21] antifungal,[22] antitumor,[23] anti-inflamatory,[24] among other activities. We synthesized a thiosemicarbazide derivative (**38**) and two sulfonylhydrazones (**39** & **40**). The sulfonylhydrazones showed moderate activity against Gram-positive bacteria. These two molecules provided leads to synthesize more sulfonamide derivatives as potent anti-Gram-positive bacterial agents.

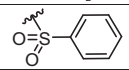
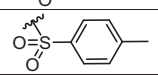
Compounds showing activity against Gram-positive bacteria were tested against methicillin resistant *S. aureus* (MRSA) for MIC studies. Seven of these compounds have shown promising activity against this drug resistant bacteria. Methyl substitution along with the chloro and bromo substituted N-arylamine derivatives (**3** & **5**) have shown activity up to 16 μg/mL concentration in MIC studies. Trifluoromethyl substituted N-arylamines (**8**, **9**, **10**, & **11**) have shown better activity in zone of inhibition studies, but these molecules are less potent in MIC studies than the methyl substituted compounds. Dichloro substituted N-arylamine-derived pyrazole showed activity up to 16 μg/mL concentration against MRSA. All four molecules have shown activity at 32 μg/mL concentration. Hydrazone derivatives (**20**, **21**, & **22**) showing activity against *A.*



Compound No	R	Gram-positive bacteria (Zone of inhibition)		Product yield (%)
		S a (mm)	*B s* (mm)	
26		NA	NA	72
27		NA	NA	75
28		NA	NA	78
29		8	8	93
30		NA	NA	91
31		NA	NA	92
32		8	11	90
33		10	11	88
34		NA	NA	89
35		NA	NA	72
36		NA	NA	70
37		NA	NA	93
DMSO		NA	NA	
Chloramphenicol		36	36	

Scheme 3. Synthesis and antimicrobial data of semicarbazones derivatives.



Compound No	R	Gram-positive bacteria (Zone of inhibition)		Product yield (%)
		S a (mm)	*B s* (mm)	
38		NA	NA	95
39		9	10	89
40		12	11	91
DMSO		NA	NA	
Chloramphenicol		36	36	

Scheme 4. Synthesis and antimicrobial data of thiosemicarbazone sulfonyl hydrazone.

baumannii in disk diffusion assay were also subjected to MIC studies. Fluoro and bromo substituted compounds (**20** & **22**) have shown activity against *A. baumannii* in 8 μg/mL concentration. Surprisingly, the chloro derivative (**21**) which was less active in zone of inhibition studies showed better activity (4 μg/mL) in MIC studies against *A. baumannii* (Table 1). The positive control, chloramphenicol, inhibited the growth of *A. baumannii* at 16 μg/mL in MIC studies. Thus, our potent compound (**21**) is four times more potent than the approved drug, chloramphenicol (positive control) (see Fig. 1).

Table 1
MIC values for active compounds: *S. aureus* ATCC 43300 (MRSA), *A. baumannii* ATCC 19606 (type strain).

Compounds	MRSA MIC (μg/mL)
3	16
5	16
8	32
9	32
10	32
11	32
14	16
A. baumannii	
20	8
21	4
22	8
Chloramphenicol	16
Ciprofloxacin	1.56

Table 2
CC_{50} values of potent compounds against HEK293 cells.

Compounds	HEK293, ATCC CRL-1573 CC_{50} (μg/mL)
3	20
5	17.9
8	>32
9	>32
10	>32
11	>32
14	18
20	>32
21	>32
22	>32

To predict the mode of action of potent molecules, we carried out the docking studies of compounds (**20**, **21**, & **22**) with several druggable proteins of Gram-negative bacteria. We found the best affinity (~−9.8 kcal/mol) of compound (**21**) against LpxC enzyme. These three potent molecules show similar interactions with enzyme except the chlorine atom of **21**, which forms a halogen bond-like interaction with phenyl ring phenyl alanine (PHE 182 & PHE 155) of LpxC enzyme (Fig. 2). This additional interaction of **21** with the LpxC protein could be the reason of its potent activity against *A. baumannii* in MIC studies.

To study the druglike properties of the active compounds, all ten compounds were evaluated for cytotoxic properties for 50% cytotoxic concentration (CC_{50}) values against healthy human (HEK293) cells. N-Aryl derivatives (**3**, **5**, & **14**) without trifluoromethyl showed significant cytotoxicity against HEK293 cells, and N-aryl amines (**8**, **9**, **10**, & **11**) with trifluoromethyl as a substituent did not show significant cytotoxicity against this healthy cell line up to 32 μg/mL concentration. This finding is very significant to design N-arylamines as noncytotoxic antibacterial agents. Hydrazone derivatives (**20**, **21**, & **22**) did not show any noticeable cytotoxicity against HEK293 cell lines (Table 2). Therefore, these three hydrazone derivatives have given an excellent starting point to design noncytotoxic anti-Gram-negative bacterial agents.

We reported an efficient synthesis of new 1,3-dipenyl pyrazole derived N-aryl amines, hydrazones, semicarbazones, and sulfahydrazones from readily available reagents and starting materials. From these compounds, we found seven molecules possessing promising anti-MRSA activity and three molecules that demonstrated potent anti-*Acinetobacter baumannii* activities in MIC studies. Hydrazone derivatives have shown potent activity without any toxic effect on healthy mammalian cells (HEK293). Many variables in the lead molecules and ease of synthesis will help us to optimize the activity and antimicrobial potential of these novel agents.

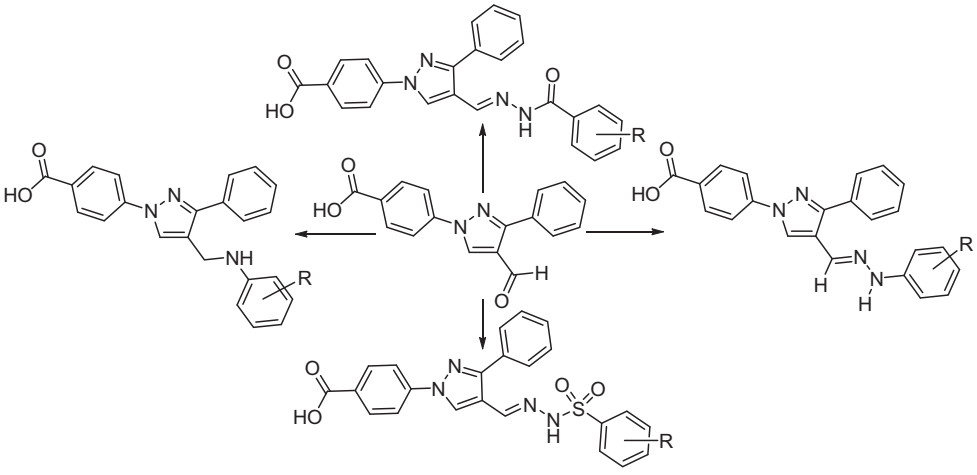


Fig. 1. Synthesized pyrazole derivatives as antibacterial agents.

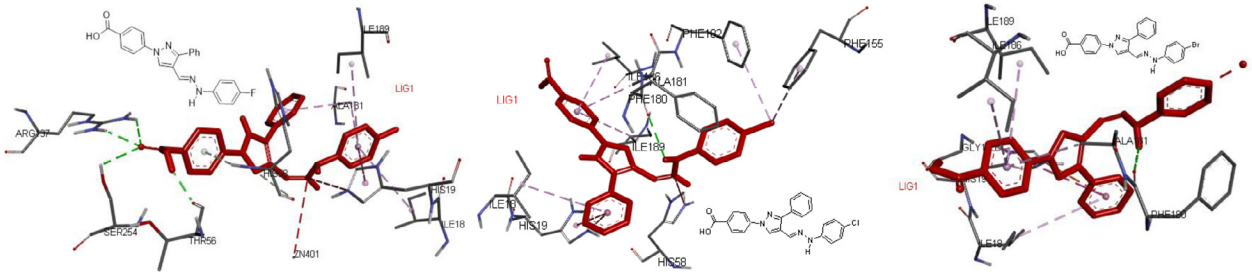


Fig. 2. Docking of compounds **20**, **21**, & **22** in the active site of LpxC enzyme (PDB ID: 5DRO).

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by College of Science and Mathematics Arkansas State University, Jonesboro, AR. USA. Arkansas Statewide MS facility, Grant Number P30 GM103450 from the National Institute of General Medical Sciences of the National Institutes of Health (NIH) for recording mass spectrometry. This publication was made possible by the Arkansas INBRE program, supported by grant funding from the National Institutes of Health (NIH), USA, National Institute of General Medical Sciences (NIGMS) (P20 GM103429) (formerly P20RR016460). Arkansas Biosciences Institute – Arkansas State University (ABI-A-state) start-up fund helped to complete this project. Anti-MRSA screening was performed by CO-ADD (The Community for Antimicrobial Drug Discovery), funded by the Welcome Trust (UK) and the University of Queensland (Australia).

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