Chemistry & Biology Interface, 2018, 8, 1, 11-21

RESEARCH PAPER ISSN: 2249 –4820

CHEMISTRY & BIOLOGY INTERFACE

An official Journal of ISCB, Journal homepage; www.cbijournal.com

Synthesis, designing and biological evaluation of 4-(1'-(6''-chloro/substitutedpyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ols as antimicrobial agents

Ranjana Aggarwal[a]*, Swati[a], Neeraj Kumar[b], Anita[b]

[a]*Department of Chemistry, Kurukshetra University, Kurukshetra, 136119, India*
[b]*Department of Microbiology, Kurukshetra University, Kurukshetra, 136119, India*
Phone: +91 (1744) 238734 ; Fax no: +91 (1744) 238277
Email: ranjanaaggarwal67@gmail.com, shswati2@gmail.com
Received 21 November 2017; Accepted 25 January 2018

Abstract: 2-phenyl-1-[1-(4-hydroxy-6-methylpyran-2-one-3-yl)ethylidene]hydrazine, obtained by reaction of DHA and phenyl hydrazine, underwent smooth skeletal rearrangement to 1(5-hydroxy-3-methyl-1-phenylpyrazol-4-yl)-1,3-butanedione in glacial acetic acid, which inturn yielded 4-(1'-(6''-chloropyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol as an exclusive product on reaction with 3-chloro-6-hydrazinopyridazine. The structure of the synthesized compounds were established by nuclear magnetic resonance (^{1}H and ^{13}C), infrared, mass spectral and elemental analysis. In *in vitro* analysis, candidates 7f and 7i showed good activity against both Gram-positive bacteria, Gram-negative bacteria, and the Fungi, could be probed further as an antimicrobial agent with application in the pharmaceutical industry, after testing its toxicity in humans.

Keywords: Pyrazole, bipyrazole, pyridazine, antimicrobial agents.

Introduction:

In designing of novel molecules, the remarkable position of pyrazole motif in biological system has been highlighted as it shows diverse therapeutic properties, such as antimicrobial[1], anti-inflammatory[2], anticancer[3], antitubercular[4], xanthine oxidoreductase inhibitors[5], Chk-2 inhibitors[6], CDK1/CDc2 inhibitors[7] etc. Celebrex (selective COX-2 inhibitor)[8], a currently marketed drug endowed with pyrazole nucleus. Several other drugs are known, including antipyrine, aminopyrine, mepirizole, phenylbutazone, fluazolate, and, remifenazone which entitles the medicinal efficacy of pyrazole nucleus.

Bipyrazole skeleton act as an active component

in medicines which helps in medicating adult diseases such as cerebral ischemia, cardiovascular disease, carcinoma and inflammation caused by active oxygen and free radicals.[9] More particularly, the present invention relates to a bipyrazole derivative capable of capturing active oxygen and *in vivo* free radicals, and useful as an agent for preventing or treating various diseases induced by active oxygen or *in vivo* free radicals. Moreover, bipyazole nucleus is also associated with several pharmacological activities such as anti-inflammatory[10], antibacterial[11], antifungal[12], cytotoxic activities[13] etc.

As pyridazine moiety does not show its existence in any natural product but have received considerable attention in biological system because of its bioavailability to cell membrane, notably to CNS due to water soluble nature, hence known for its therapeutic value[14]. In the recent studies, the growing medicinal application of pyridazine motif has been proven by its existence in number of pharmaceutical drugs such as emorfazone (**anti-inflammatory**)[15], pipofezine (**antidepressant**)[16], monopolar spindle 1,MPs 1 and ponalrestat (**anticancer**)[17], CRTH2 antagonist (**anti-asthamatic**)[18], PF-04254644 (***c*-Met inhibitor**)[19], Irdabisant (**H_3R antagonist**)[20], indolidan, bemoradan and imazodan (**cardiotonic**)[21], pimobendan (**vasodilator**)[22], zardaverine (**antiasthmatic**)[23], gabazine (**antagonist**)[24] etc.

Gelin *et al*[25] reported the reaction of DHA with phenylhydrazine to generate (5-hydroxy-3-methyl-1-phenylpyrazol-4-yl)-1,3-butanedione and its synthetic application to bipyrazoles. Moreover, considering our research work on the synthesis of bipyrazoles,[26–29] 1-(4,6-dimethylpyrimidin-2-yl)-1′-aryl/heteroaryl-3,3′-dimethyl-(4,5′-bipyrazol)-5-ols (reference molecule) reported as antimicrobial agents and above-cited applications, the pyrazole, bipyrazole and pyridazine moieties are considered privileged structures to fulfill an additional urgency in biological system and consequently provoked us to design 4-(1'-(6''-chloro/substitutedpyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol as antimicrobial agents (Fig. 1).

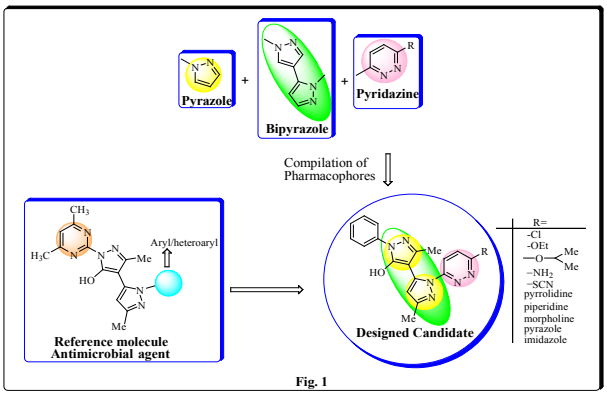


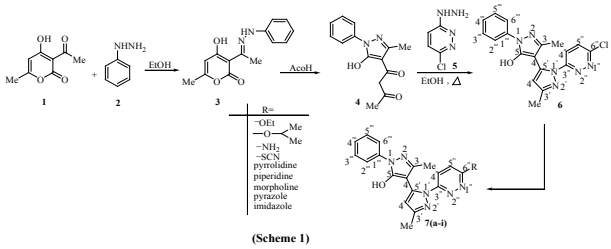
Fig. 1

Designing of 4-(1'-(6''-chloro/substitutedpyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol

Results and Discussion

The synthesis of title compounds **6**, **7**(**a-i**) is outlined in **Scheme 1** and candidate **6** was achieved by the condensation of 3-chloro-6-hydrazinopyridazine with 1(5-hydroxy-3-methyl-1-phenylpyrazol-4-yl)-1,3-butanedione[25] **4**, obtained by the rearrangement 2-phenyl-1-[1-(4-hydroxy-6-methylpyran-2-one-3-yl)ethylidene]hydrazine[30] **3** which inturn was obtained by the reaction of dehyroacetic acid (DHA) **1** and phenyl hydrazine **2**. The key precursor, 3-chloro-6-hydrazinopyridazine **5**, synthesized by treating 3,6-dichloropyridazine with hydrazine hydrate following the literature procedure[31]. The structures of the compounds were established by NMR (^{1}H and C^{13}) and (IR) spectroscopy. The ^{1}H NMR spectra of pyridazinylbipyrazole (**6**) having a 4,5'-junction displayed a sharp singlet around δ 6.3 ppm (C_4'-H), pyridazinyl protons appeared

around δ 8.2 ppm (C_4''-H, J = 9.2), δ 7.7 ppm (C_5''-H, J = 9.2) and enolic OH showed a broad signal around δ 11.5 ppm (C_3-OH) whereas in IR spectra, enolic OH appeared at 3240 cm^{-1}. Nucleophilic substitution of chloro group of bipyrazole **6** by ethoxy, amino, thiocyanato, morpholine, pyrazole, imidazole (**7a,c,d,g-i**) resulted into downfield shift of 4'-H δ value whereas isopropoxy, pyrrolidine, piperidine (**7b,e,f**) resulted into upfield shift of 4'-H δ value and same effects were observed on pyridazine protons. C^{13} spectra showed a characteristic peak of C-4' at δ 95 ppm of bipyrazoles.



(Scheme 1)

Synthesis of title compound 4-(1'-(6''-chloro/substitutedpyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ols

The plausible mechanism for these reaction steps is outlined in (Fig. 2). Initial nucleophilic attack of the amino group of hydrazine 2 on the carbonyl of the acetyl side chain at the 3 position of dehydroacetic acid 1 followed by loss of one molecule of water yielded hydrazone 3. This hydrazone 3 then underwent smooth skeletal rearrangement, involving a nitrogen nucleophilic attack at the C_2 lactone carbonyl with ring opening, thus generating 4. In the last step, this bielectrophilic pyrazolyl diketone 4 reacts with binucleophilic 3-chloro-6-hydrazinopyridazine to give 4,5′-bipyrazol-5-ol, **6** (Fig. 2).

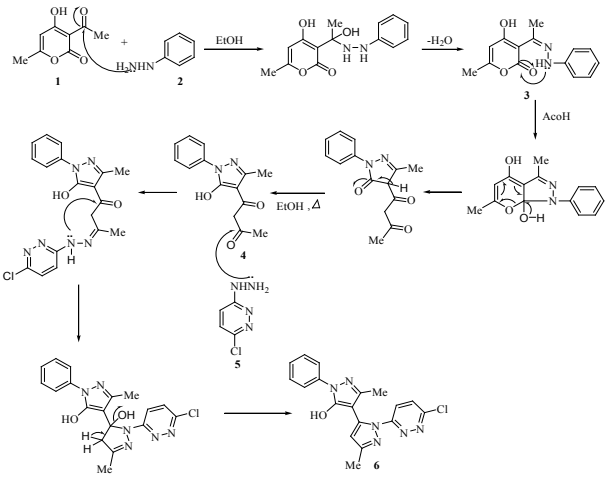


Fig. 2 Plausible mechanism of formation of 4-(1'-(6''-chloro/substitutedpyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ols **(6)**

Antimicrobial activity

All the synthesized compounds (6, 7a–i) were assayed *in vitro* for their antibacterial and antifungal activity against two Gram-positive bacteria (*Staphylococcus aureus* MTCC 3160 and *Bacillus subtilis* MTCC 441), two Gram-negative bacteria (*Escherichia coli* MTCC 1302 and *Pseudomonas aeruginosa* MTCC 424), and two Fungi, *Candida albicans* (MTCC 227) and *Saccharomyces cerevisiae* (MTCC 170).

Standard drugs used for evaluating antibacterial and antifungal activity, were Ciprofloxacin and Amphotericin B respectively. The zones of inhibition of antimicrobial activity are summarized in Table 1. The results obtained in the primary screening for minimum inhibitory concentrations (MIC) presented in Table 2. All the tested compounds (6,7a–i) have shown moderate to good activity against the Gram-positive bacteria, *B. subtilis*, Gram-negative bacteria, *E. coli*, and Fungi, *C. albicans* whereas only few candidates were found to be active against Gram-positive bacteria, *S.*

Table 1: *In vitro* antimicrobial activity of compounds 6, 7a–i using the agar diffusion assay technique

Compound No.	Diameter of growth of inhibition zone (mm)[a]					
	Gram positive bacteria		Gram negative bacteria		Fungi	
	Bacillus subtilis	*Staphylococcu s aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albicans*	*Saccharomyces cerevisiae*
6	11	9	14	-	12	-
7a	14	-	10	-	10	8
7b	13	-	14	13	11	9
7c	-	10	14	-	14	-
7d	-	-	10	-	12	-
7e	12	10	15	10	10	-
7f	10	-	19	11	16	-
7g	15	11	10	13	10	-
7h	15	10	15	-	10	9
7i	-	-	-	16	13	9
Ciprofloxacin	24.0	26.6	25.0	22	-	-
Amphotericin-B	-	-	-	-	16.6	19.3

Notes:- No activity; [a] Values, including diameter of the well (8mm), are means of three replicates

Abbreviations: *S. aureus*, *Staphylococcus aureus*; *E. coli*, *Escherichia coli*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *C. albicans*, *Candida albicans*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *B. subtilis*, *Bacillus subtilis*.

aureus (6, 7c, 7e, 7g, 7h), Gram-negative bacteria, *P. aeruginosa* (7b-e, 7i) and Fungi, *S. cerevisiae* (7a, 7b, 7h, 7i). Candidates 7e was found to be effective against *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *C. albicans* with diameter of growth of inhibition zone 12, 10, 15, 10, 10 mm, respectively, 6f exhibited zone of inhibition against *B. subtilis*, *E. coli*, *P. aeruginosa*, *C. albicans* 10, 19, 11, 16 mm, respectively whereas 7g shown zone of inhibition against *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *C. albicans* with 15, 11, 10, 13, 10 mm, respectively and 7h exhibited inhibition zone against *B. subtilis*, *S. aureus*, *E. coli*, *C. albicans*, *S. cerevisiae* with 15, 10, 15, 10, 9 mm diameter, respectively (Table 1 and Fig. 3), with commercial drugs, ciprofloxacin and amphotericin B. Compounds 7e, 7f, 7g and 7h, found to be most active, containing pyrrolidine, piperidine, morpholine and pyrazole moieties at 6''-position of bipyrazole, respectively. Moreover, 7a, 7b, 7h, 7i were the candidates active against both the fungi, *C. albicans* and *S. cerevisiae*, and only candidates active against *S. cerevisiae* containing ethoxy, isopropoxy, pyrazole, imidazole moieties at 6''-position of bipyrazole.

Furthermore, as depicted from Table 1 replacement of chloro group at 6''-position of bipyrazole 6 with ethoxy, isopropoxy, pyrrolidine, morpholine, pyrazole entities in (7a, 7b, 7e, 7g, 7h) caused enhancement in antibacterial activity against *B. subtilis* (Gram-positive bacteria) whereas amino, thiocyanato, piperidine, imidazole substitutes at 6''-position in (7c, 7d, 7f, 7i) caused a decline. On the other hand, replacement of chloro group at 6''-position of bipyrazole 6 with amino, pyrrolidine, morpholine, pyrazole in (7c, 7e, 7g, 7h) caused

increment in antibacterial activity against *S. aureus* (Gram-positive bacteria) and no activity was found on substitution with ethoxy, isopropoxy, thiocyanato, piperidine, imidazole in (7a, 7b, 7d, 7f, 7i). In case of Gram-negative bacteria, *E. coli*, replacement of chloro group at 6''-position of bipyrazole 6 with pyrrolidine, piperidine, pyrazole (7e, 7f, 7h) laid to increase in activity whereas isopropoxy, amino groups in (7b, 7c) has the same biological effect and a decline effect was observed with ethoxy, thiocyanato, morpholine, imidazole in (7a, 7d, 7g, 7i) whereas replacement of chloro group at 6''-position of bipyrazole 6 with isopropoxy, pyrrolidine, piperidine, morpholine, imidazole in (7b, 7e, 7f, 7g, 7i) shown antibacterial activity and no activity was seen with compounds (6, 7a, 7c, 7d, 7h) against *P. aeruginosa*.

Antifungal activity against *C. albicans* was good in compounds containing amino, piperidine, imidazole at 6''-position of bipyrazoles (7c, 7f, 7i) and a decline was seen in (7a, 7b, 7e, 7g, 7h) with ethoxy, isopropoxy, pyrrolidine, morpholine, pyrazole substitutes at 6''-position of bipyrazole whereas candidate 7d containing thiocyanato at 6''-position of bipyrazole shown same biological effect as bipyrazole 6. The only candidates which were active against *S. cerevisiae* (7a, 7b, 7h, 7i) containing ethoxy, isopropoxy, pyrazole, imidazole substitutes at 6''-position of bipyrazoles whereas candidates having chloro, amino, thiocyanato pyrrolidine, piperidine, morpholine, substitutes at 6''-position in (6, 7c, 7d, 7e, 7f, 7g) shown nil effects in biological results.

The MIC of the various chemical compounds tested was in the range of 25–50 μg/mL against bacteria and 25–50 μg/mL against fungi.

Table 2: Minimum inhibitory concentration (μg/mL) values for compounds 6,7a–i and reference drugs against the study micro-organisms

Compound No.	Gram positive bacteria		Gram negative bacteria		Fungi	
	Bacillus subtilis	***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Candida albicans***	***Saccharomyces cerevisiae***
6	50	nt	50	nt	50	nt
7a	50	nt	nt	nt	nt	nt
7b	50	nt	50	50	50	nt
7c	nt	nt	50	nt	nt	nt
7d	nt	nt	nt	nt	50	nt
7e	50	nt	50	nt	nt	nt
7f	50	nt	25	50	25	nt
7g	50	50	nt	50	nt	nt
7h	50	nt	50	nt	50	nt
7i	50	nt	nt	25	50	nt
Ciprofloxacin	6.25	6.25	6.25	12.5	-	-
Amphotericin-B	-	-	-	-	12.5	12.5

Notes:- nt –not tested

Abbreviations: *S. aureus*, *Staphylococcus aureus*; *E. coli*, *Escherichia coli*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *C. albicans*, *Candida albicans*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *B. subtilis*, *Bacillus subtilis*.

Compound 7f exhibited the most promising results, showing the lowest MIC of 25 µg/mL against *E. coli*, and 25 µg/mL against *C. albicans*. Also, good results were shown by candidate 7i with lowest MIC of 25 µg/mL against *P. aeruginosa* (Table 2). In conclusion, compounds having a piperidine (7f) and an imidazole (7i) substitutes at 6''-position of bipyrazole 6 showed activity against both Gram-positive bacteria, Gram-negative bacteria, and the Fungi, could be probed further as an antimicrobial agent with application in the pharmaceutical industry, after testing its toxicity in humans.

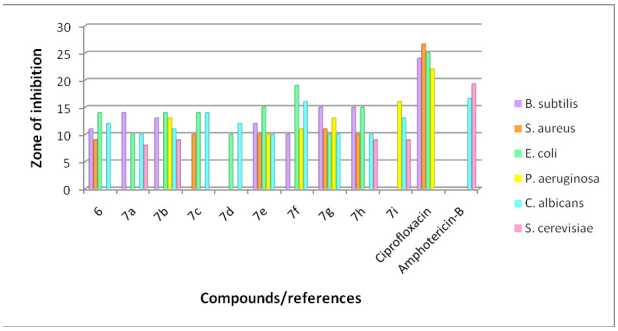


Fig. 3 *In vitro* antimicrobial assay of test compounds/reference at concentration 4 mg/mL.

Abbreviations: *S. aureus*, *Staphylococcus aureus*; *E. coli*, *Escherichia coli*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *C. albicans*, *Candida albicans*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *B. subtilis*, *Bacillus subtilis*.

Materials and methods

Melting points were determined in open capillaries on digital instrument Make. Infrared spectra were recorded on a spectrophotometer (IR M-500, Buck Scientific Inc, Norwalk, CT) in KBr pellets (ν_{max} in cm^{-1}). ^1H and ^{13}C NMR spectra for analytical purpose were recorded in $CDCl_3$ on a Bruker instrument (Billerica, MA) at 400 MHz. Tetramethylsilane was taken as an integral standard. Chemical shifts (δ) were measured in ppm. Coupling constants (J) are given in Hertz (Hz). Mass and elemental analyses were performed at Sophisticated Analytical Instrumentation Facility, Panjab University, Chandigarh.

Synthetic procedure: 4-(1'-(6''-chloropyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol (*6*)

Equimolar amount of 1-(5-hydroxy-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)butane-1,3-dione **(4)** (0.258g, 1mmol) and 3-chloro-6-hydrazinopyridazine **(5)** (0.144g, 1mmol) were refluxed in 10 ml ethanol. The reaction was monitored on TLC at regular intervals and indicated that reaction is complete in one hour and solid separated out during refluxing was filtered, washed with ethanol.

Yield 83%; M.Pt. 258 °C; IR (KBr, cm^{-1}): 3240 (OH); ^1H NMR (400 MHz, $CDCl_3$) δ: 2.24 (s, 3H, C_3-CH_3); 2.41 (s, 3H, $C_{3'}$-CH_3); 6.30 (s, 1H, 4'-H); 7.27 (t, 1H, *J*= 7.5 Hz, 4'''-H); 7.44 (t, 2H, 3''', 5'''-H); 7.75 (d, 1H, *J*= 9.2 Hz, 5''-H); 7.84 (d, 2H, *J*= 7.6 Hz, 2''', 6'''-H); 8.20 (d, 2H, *J*= 9.2 Hz, 4''-H); 11.54 (bs, 1H,C_5-OH); C^{13} NMR (400 MHz, TFA/$CDCl_3$) δ: 10.6, 12.7, 95.6, 105.7, 117.8, 123.9, 126.1, 128.4, 130.1, 131.1, 135.1, 138.9, 144.6, 148. 0, 153.8, 155.5; Ms: *m/z* $[M+1]^+$ 367/369 (3:1). Anal. Cal. for $C_{18}H_{15}ClN_6O$: C, 58.94; H, 4.12; N, 22.91. Found: C, 58.81; H, 4.02; N, 22.85.

4-(1'-(6''-ethoxypyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol (*7a*)

4-(1'-(6''-chloropyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol **(6)** (0.366g, 1mmol) with sodium ethoxide (0.068g, 1mmol) were refluxed on water bath and completion of reaction was observed on TLC within 4-5 hrs. Once the

reaction was completed, reaction mixture was concentrated, neutralised by concentrated HCl (dropwise addition) till solid separated out, filtered through suction and washed first with water than ethanol.

Yield 73.2 %; M.Pt. 241 °C; IR (KBr, cm^{-1}): 3063 (OH); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.60 (t, 3H, *J*= 7.0 Hz, $C_{6''}$-O--CH_3); 2.45 (s, 3H, C_3-CH_3); 2.51 (s, 3H, $C_{3'}$-CH_3); 4.70 (q, 2H, *J*= 7.0 Hz, $C_{6''}$-OCH_2); 6.66 (s, 1H, 4'-H); 7.44 (m, 2H, 3''', 5'''-H); 7.54 (m, 3H, 2''', 4''', 6'''-H); 7.92 (d, 1H, *J*= 9.8 Hz, 5''-H); 8.66 (d, 1H, *J*= 9.8 Hz, 4''-H); C^{13} NMR (400 MHz, TFA/$CDCl_3$) δ: 10.6, 12.4, 13.3, 70.2, 95.1, 110.0, 115.0, 124.1, 125.7, 130.0, 131.0, 131.4, 133.5, 133.9, 148.0, 148.7, 155.4, 155.5; Ms: *m/z* $[M+1]^+$ 377. Anal. Cal. for $C_{20}H_{20}N_6O_2$: C, 63.82; H, 5.36; N, 22.33. Found: C, 63.70; H, 5.25; N, 22.27.

4-(1'-(6''-isopropoxypyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol (*7b*)

4-(1'-(6''-chloropyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol **(6)** (0.366g, 1mmol) with sodium isopropoxide (0.082g, 1mmol) were refluxed on water bath and completion of reaction was observed on TLC within 4-5 hrs. Once the reaction was completed, reaction mixture was concentrated, neutralised by concentrated HCl (dropwise addition) till solid separated out, filtered through suction and washed first with water than ethanol.

Yield 42.3 %; M.Pt. 311 °C; IR (KBr, cm^{-1}): 3290 (OH); ^{1}H NMR (400 MHz, DMSO) δ: 1.35 (d, 6H, *J*= 6.1 Hz, $C_{6''}$-O-- $(CH_3)_2$); 2.30 (s, 3H, C_3-CH_3); 2.33 (s, 3H, $C_{3'}$-CH_3); 5.39 (bs, 1H, $C_{6''}$-OCH); 6.23 (s, 1H, 4'-H); 7.26 (bs, 2H, 4''', 5''-H); 7.38 (bs, 2H, 3''', 5'''-H); 7.66 (m, 2H, 2'', 6''-H); 7.91 (bs, 1H, 4''-H), 11.85 (bs,1H, C_5-OH); C^{13} NMR (400 MHz, TFA/$CDCl_3$) δ: Not evaluated because of poor solubility. Ms: *m/z* $[M+1]^+$ 391. Anal. Cal. for $C_{21}H_{22}N_6O_2$: C, 64.60; H, 5.68; N, 21.52. Found: C, 64.55; H, 5.57; N, 22.49.

4-(1'-(6''-aminoypyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol (*7c*)

4-(1'-(6''-chloropyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol **(6)** (0.366g, 1mmol) was stirred in 10 ml ammonia. Reaction was monitored on TLC and took 8 hrs of completion. Excess of solvent was distilled off to about 1/3 of the volume, neutralised by concentrated HCl (dropwise addition) till solid separated out, filtered through suction and washed first with water than ethanol.

Yield 63.5 %; M.Pt. 264.5 °C; IR (KBr, cm^{-1}): 3101 (symm.) and 3063 (asymm.) NH strech; 3441 (OH); ^{1}H NMR (400 MHz, TFA/$CDCl_3$) δ: 2.52 (s, 3H, C_3-CH_3); 2.63 (s, 3H, $C_{3'}$-CH_3 6.80 (s, 1H, 4'-H); 7.55 (m, 2H, 3''', 5'''-H); 7.63 (m, 3H, 2''', 6''', 4'''-H); 8.13 (d, 1H, *J*= 8.9 Hz, 5''-H); 8.40 (d, 1H, *J*= 9.0 Hz, 4''-H); C^{13} NMR (400 MHz, TFA/$CDCl_3$) δ: 10.7, 12.0, 95.7, 110.8, 115.1, 124.8, 128.1, 130.8, 131.7, 132.0, 135.6, 148.5, 153.0, 155.5, 155.7, 157.3; Ms: *m/z* $[M+1]^+$ 348. Anal. Cal. for $C_{18}H_{17}N_7O$: C, 62.24; H, 4.93; N, 28.23. Found: C, 62.19; H, 4.86; N, 28.17.

3-methyl-4-(3'-methyl-1'-(6''-thiocyanatopyridazin-3''-yl)-1*H*-pyrazol-5'-yl)-1-phenyl-1*H*-pyrazol-5-ol (*7d*)

Basic alcoholic (0. 040g, 1mmol NaOH + 10 ml EtOH) solution of 4-(1'-(6''-chloropyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol **(6)** (0.366g, 1mmol) refluxed with KSCN (0. 097g, 1mmol) and the reaction was monitored on TLC, took 6-8 hrs of completion, concentrated, neutralised by concentrated HCl (dropwise addition) till

solid separated out, filtered through suction and washed first with water than ethanol.

Yield 70.8 %; M.Pt. 262.0 °C; IR (KBr, cm^{-1}): 3047, 3742 (OH); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.46 (s, 3H, C_3-CH_3); 2.54 (s, 3H, $C_{3'}$-CH_3); 6.67 (s, 1H, 4'-H); 7.51 (m, 2H, 3''', 5'''-H); 7.58 (t, 3H, 2''', 6''', 4'''-H); 8.07 (d, 1H, *J*= 9.3 Hz, 5''-H); 8.36 (d, 1H, *J*= 9.3 Hz, 4''-H); C^{13} NMR (400 MHz, TFA/$CDCl_3$) δ: 10.6, 12.3, 95.7,109.9, 110.0, 114.8, 123.9, 128.1, 130.1, 131.2, 133.2, 134.9, 148.0, 152.7, 154.5, 155.4, 156.4; Ms: *m/z* $[M+1]^+$ 390. Anal. Cal. for $C_{18}H_{16}N_6O_2$: C, 62.06; H, 4.63; N, 24.12. Found: C, 61.09; H, 4.57; N, 23.08.

3-methyl-4-(3'-methyl-1'-(6''-(pyrrolidin-1-yl)pyridazin-3''-yl)-1*H*-pyrazol-5'-yl)-1-phenyl-1*H*-pyrazol-5-ol (*7e*)

4-(1'-(6''-chloropyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol **(6)** (0.366g, 1mmol) treated with pyrrolidine (0.142g, 2mmol) in 10 ml ethanol and reaction mixture was refluxed on water bath. Completion of reaction was observed on TLC within 4-5 hrs. Once the reaction was completed, reaction mixture was concentrated, neutralised by concentrated HCl (dropwise addition) till solid separated out, filtered through suction and washed first with water than ethanol.

Yield 77.6 %; M.Pt. 268 °C; IR (KBr, cm^{-1}): 3063 (OH); ^{1}H NMR (400 MHz,$CDCl_3$) δ: 2.04 (quintet, pyrrolidine, 4H, *J*= 4.0 Hz, N-$(CH_2)_2$); 2.25 (s, 3H, C_3-CH_3); 2.40 (s, 3H, $C_{3'}$-CH_3); 3.52 (t, pyrrolidine, 4H, N--$(CH_2)_2$); 6.20 (s, 1H, 4'-H); 6.90 (d, 1H, *J*= 9.6 Hz, 5''-H); 7.24 (t, 1H, *J*= 9.0 Hz, 4''' -H); 7.43 (t, 2H, *J*= 7.7 Hz, 3''', 5'''-H); 7.79 (d, 1H, *J*= 9.6 Hz, 4''-H); 7.88 (d, 2H, *J*= 7.7 Hz, 2''', 6'''-H), 13.76 (bs, 1H, C_5-OH); C^{13} NMR (400 MHz, $CDCl_3$) δ: 13.5, 13.7, 25.4, 47.1, 93.5, 109.9, 116.9, 121.8, 125.7, 126.0, 128.7, 136.1, 139.0, 147.1, 148.1, 151.4, 152.0, 156.9; Ms: *m/z* $[M+1]^+$ 402. Anal. Cal. for $C_{22}H_{23}N_7O$: C, 65.82; H, 5.77; N, 24.42. Found: C, 65.79; H, 5.68; N, 24.38.

3-methyl-4-(3'-methyl-1'-(6''-(piperidin-1-yl)pyridazin-3''-yl)-1*H*-pyrazol-5'-yl)-1-phenyl-1*H*-pyrazol-5-ol (*7f*)

4-(1'-(6''-chloropyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol **(6)** (0.366g, 1mmol) treated with piperidine (0.170g, 2mmol) in 10 ml ethanol and reaction mixture was refluxed on water bath. Completion of reaction was observed on TLC within 4-5 hrs. Once the reaction was completed, reaction mixture was concentrated, neutralised by concentrated HCl (dropwise addition) till solid separated out, filtered through suction and washed first with water than ethanol.

Yield 70.5 %; M.Pt. 254.5 °C; IR (KBr, cm^{-1}): 3060 (OH); ^{1}H NMR (400 MHz, TFA/$CDCl_3$) δ: 1.89 (bs, piperidine, 6H, N--$(CH_2)_3$); 2.52 (bs, 3H, C_3-CH_3); 2.62 (s, 3H, $C_{3'}$-CH_3); 3.80 (s, piperidine, 4H, N-$(CH_2)_2$); 6.78 (s, 1H, 4'-H); 7.87 (d, 1H, *J*= 10.1 Hz, 5''-H); 7.45 (t, 2H, *J*= 7.1 Hz, 3''', 5'''-H); 8. 08 (d, 1H, *J*= 10.0 Hz, 4''-H); 7.58 (m, 3H, 2''', 6''', 4'''-H); C^{13} NMR (400 MHz, TFA/$CDCl_3$) δ: 13.6, 13.7, 24.4, 25.3, 46.2, 93.5, 110.1, 117.2, 121.9, 125.7, 126.0, 128.8, 136.1, 139.0, 147.4, 148.1, 151.5, 152.0, 158.9; Ms: *m/z* $[M+1]^+$ 416. Anal. Cal. for $C_{23}H_{25}N_7O$: C, 66.49; H, 6.06; N, 23.60. Found: C, 66.45; H, 6.58; N, 23.46.

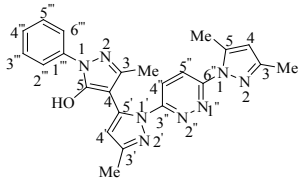
3-methyl-4-(3'-methyl-1'-(6''-morpholinopyridazin-3''-yl)-1*H*-pyrazol-5'-yl)-1-phenyl-1*H*-pyrazol-5-ol (*7g*)

4-(1'-(6''-chloropyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol **(6)** (0.366g, 1mmol) treated with morpholine (0.174g, 2mmol) in 10 ml ethanol and reaction mixture was refluxed on water bath. Completion of reaction was observed on TLC within 4-5 hrs. Once the reaction was completed,

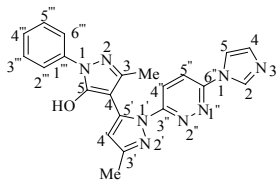
reaction mixture was concentrated, neutralised by concentrated HCl (dropwise addition) till solid separated out, filtered through suction and washed first with water than ethanol.

Yield 74.6 %; M.Pt. 303 °C; IR (KBr, cm^{-1}): 3070 (OH); ^{1}H NMR (400 MHz, TFA/$CDCl_3$) δ: 2.42 (s, 3H, C_3-CH_3); 2.52 (s, 3H, $C_{3'}$-CH_3); 3.84 (t, morpholine, 4H, *J*= 5.0 Hz, N-$(CH_2)_2$); 4.05 (t, morpholine, 4H, *J*= 4.4 Hz, O-$(CH_2)_2$); 6.63 (s, 1H, 4'-H); 7.41 (m, 2H, 3''', 5'''-H); 7.52 (m, 3H, 2''', 6'', 4''''-H); 7.82 (d, 1H, *J*= 10.2 Hz, 5''-H); 8.22 (d, 1H, *J*= 10.2 Hz, 4''-H); C^{13} NMR (400 MHz, TFA/$CDCl_3$) δ: 10.6, 11.9, 46.0, 65.0, 94.3, 113.6, 124.1, 124.6, 130.0, 130.6, 131.0, 131.5, 134.6, 144.6, 148.0, 151.4, 153.7, 156.0; Ms: *m/z* $[M+1]^+$ 418. Anal. Cal. for $C_{22}H_{23}N_7O_2$: C, 63.30; H, 5.55; N, 23.49. Found: C, 63.22; H, 5.51; N, 23.42.

4-(1'-(6''-(3,5-dimethyl-1*H*-pyrazol-1-yl) pyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol (*7h*)



7h



7i

4-(1'-(6''-chloropyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol **(6)** (0.366g, 1mmol) was dissolved in 7 ml DMF and Na metal (0.012g, 0.5mmol) was added to the solution. Reaction mixture was refluxed till a clear solution was observed, then 3,5-dimethylpyrazole (0.192g, 2mmol) was added and reaction was monitored on TLC, completed in about 4-5 hrs, excess of solvent was distilled off, cooled, poured into water, neutralised by concentrated HCl (dropwise addition) till solid separated out, filtered through suction and washed first with water than ethanol. Yield 30.0 %; M.Pt. 209 °C; IR (KBr, cm^{-1}): 3070 (OH); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.28 (s, diMepyrazole, 3H, C_3-CH_3); 2.31 (s, 3H, C_3-CH_3); 2.43 (s, diMepyrazole, 3H, C_5-CH_3); 2.72 (s, 3H, $C_{3'}$-CH_3); 6.08 (s, diMepyrazole, 1H, 4-H); 6.31 (s, 1H, 4'-H); 7.43 (m, 3H, 3''', 5''', 4'''-H); 7.87 (d, 1H, *J*= 7.5 Hz, 2''', 6'''-H); 8.25 (d, 1H, *J*= 9.6 Hz, 5''-H); 8.47 (d, 1H, *J*= 9.3 Hz, 4''-H); 12.58 (bs, 1H, C_5-OH); C^{13} NMR (400 MHz, $CDCl_3$) δ: 13.5, 13.6, 13.7, 15.1, 93.5, 110.9, 111.7, 122.0, 124.6, 126.1, 126.8, 128.8, 131.0, 138.8, 148.3, 151.5, 152.8, 155.5, 158.0, 158.9; Ms: *m/z* $[M+1]^+$ 427. Anal. Cal. for $C_{23}H_{22}N_8O$: C, 64.77; H, 5.20; N, 26.27. Found: C, 64.72; H, 5.17; N, 26.19.

4-(1'-(6''-(1*H*-imidazol-1-yl)pyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol (*7i*)

4-(1'-(6''-chloropyridazin-3''-yl)-3'-methyl-1*H*-pyrazol-5'-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-ol **(6)** (0.366g, 1mmol) was dissolved in 7 ml DMF and Na metal (0.012g, 0.5mmol) was added to the solution. Reaction mixture was refluxed till a clear solution was observed, then imidazole (0.136g, 2mmol) was added and reaction was monitored on TLC, completed in about 4-5 hrs, excess of solvent was distilled off, cooled, poured into water, neutralised by concentrated HCl (dropwise addition) till solid separated out, filtered through suction and washed first with water than ethanol.

Yield 32.3 %; M.Pt. >315 °C; IR (KBr, cm^{-1}): 3117 (OH); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.51 (s, 3H, C_3-CH_3); 2.58 (s, 3H, $C_{3'}$-CH_3); 6.70 (s, 1H, 4'-H); 7.45 (s, 2H, 3''', 5'''-H); 7.58 (s, 3H, 2''', 6'', 4''''-H); 7.73 (s, imidazole, 1H, 4-H); 8.25 (s, 1H, 5''-H); 8.51 (bs, 2H, 4''-H, 5-H imidazole); 9.67 (bs, imidazole, 1H, 2-H); C^{13} NMR (400 MHz, TFA/$CDCl_3$) δ: 10.8, 12.0, 95.7, 110.8, 114.7, 120.0, 122.3, 122.3, 124.7, 128.2, 130.7, 131.7, 132.0, 134.5, 135.3, 148.6, 150.9, 154.5, 155.7, 155.8; Ms: *m/z* $[M+1]^+$ 399. Anal. Cal. for $C_{21}H_{18}N_8O$: C, 64.77; H, 5.20; N, 26.27. Anal. Cal. for $C_{21}H_{18}N_8O$: C,

63.31; H, 4.55; N, 28.12. Found: C, 63.27; H, 4.49; N, 28.15.

Antimicrobial assay

All the microbial cultures were sourced from the Microbial Type Culture Collection (MTCC, Imtech, Chandigarh). The microbial isolates representing Gram-positive and Gram-negative bacteria were sub cultured on nutrient agar, whereas the fungi was sub cultured on yeast peptone dextrose agar. All the synthesized ten compounds (6, 7a–i) were screened *in vitro* using the agar well diffusion method[32]. Preparation of stock solutions (4 mg/mL) of the test compounds was undertaken by dissolving 4 mg of test compound in 1 mL of dimethyl sulfoxide. Sterilization of all samples was done through a 0.2 mm membrane filter and stored at 4°C until further use. 24-hour-old cultures were used for the preparation of microbial inoculums and turbidity was adjusted equivalent to the 0.5 McFarland turbidity standard, which is visually comparable with a microbial suspension of approximately 1.5 × 108 cfu/mL. Seed layers were prepared (separate flasks were used for each bacterial culture), by inoculating 100 µL of each test bacterial culture in 20 mL of warm, melted, autoclaved Mueller Hinton agar and poured into sterilized labeled Petri plates (150 mm × 20 mm) after mixing . With the help of a sterile cork borer, 8 mm wells were punched in the solidified Petri plates. 100 mL of each test compound (stock 4 mg/mL) was added aseptically to the individual wells using a micropipette. At 37°C ± 1°C, the loaded plates were incubated in an upright position for 24 hours. After incubation, the diameter of the zone of growth inhibition around each well was measured in millimeters using a zone reader (Hi Antibiotic zone scale, Table 2). Ciprofloxacin 4 mg/mL was used as the standard antibiotic for bacteria and amphotericin B for fungi, with dimethyl sulfoxide as a negative control under similar conditions for comparison. This procedure was performed in three replicate plates for each organism.

Determination of minimum inhibitory concentration

After overnight incubation, the MIC is the lowest concentration of an antimicrobial compound that will inhibit visible growth of a microorganism. The MIC of the various compounds against bacterial strains was tested using a modified agar well diffusion method. According to National Committee for Clinical Laboratory Standards recommendations, MICs were determined using the broth micro dilution method[33]. At pH 7.4 ± 0.1, testing was performed. The inoculums were prepared using a 16-hour broth culture of each bacterial strain adjusted to a turbidity equivalent to a 0.5 McFarland standard, and diluted in Mueller Hinton agar broth medium to give a concentration of 1 × 106 cfu/mL for bacteria. To prepare 4 mg/mL stock solutions the test compounds were dissolved in dimethyl sulfoxide and to ensure that the solvent had no effect on microbial growth, a control test was performed using test medium supplemented with dimethyl sulfoxide at the same dilution as used in the experiments. Preparation of positive control (containing inoculum but no compound) and a negative control (medium only, without inoculum) was also undertaken. Using this method, a two-fold serial dilution of each chemically synthesized compound was prepared by first reconstituting the compound in dimethyl sulfoxide followed by dilution in sterile distilled water to achieve a decreasing concentration range of 512 to 1 µg/mL. A 100 µL volume of each dilution was introduced into wells (in triplicate) in the agar plates already seeded with 100 µL of standardized inoculum (106 cfu/mL) of the test microbial strain. All test plates were incubated aerobically at 37°C for 24 hours and observed for the inhibition zones. MIC, taken as the lowest concentration of the chemical compound that completely inhibited

the growth of bacteria, shown by a clear zone of inhibition, was recorded for each test organism. Ciprofloxacin was used as a positive control for bacteria and amphotericin B for Fungi, with dimethyl sulfoxide as a negative control.

Conclusion

These nitrogen compounds generally forms complexes with metalloenzymes, particularly those which are responsible in basic physiology such as cytochrome oxidase. It may found that the compounds having nitrogen content generally reacted with peptidoglycan layer of bacterial cell wall and damage it by penetrating it followed by death of bacterial cell. Sometimes, the nitrogen containing compounds at lower concentration may cause the bacteriostatic condition by slow down the growth of bacterial cell.

Acknowledgments

We are thankful to University Grants Commission, New Delhi, India, for providing financial assistance to Swati, in the form of Senior Research Fellowship. Thanks are also extended to the Sophisticated Analytical Instrument Facility, Panjab University, for use of NMR, mass spectrometer facilities and elemental analyses.

References

1. M. F. El Shehry, M. M. Ghorab, S. Y. Abbas, E. A. Fayed, Y. A. Ammar, Eur. J. Med. Chem. **2018**, 143, 1463–1473.
2. B. M. Chougala, S. Samundeeswari, M. Holiyachi, L. A. Shastri, V. A. Sunagar, Eur. J. Med. Chem. **2017**, 125, 101-116.
3. M. M.A. Hawash, D. C. Kahraman, F. Eren, R. C. Atalay, S. N. Baytas, Eur. J. Med. Chem. **2017**, 129, 12-26.
4. 4. Z. Xu, C. Gao,Q. Ren, X. Song, Z. Lv, Eur. J. Med. Chem. **2017**, 139, 429-440.
5. J. Li, F. Wu, X. Liu, Y. Zou, L. Zhang, Eur. J. Med. Chem. **2017**, 140, 20-30.
6. S. A. Galal, A. S. Abdelsamie, S. A. Shouman, Y. M. Attia, H. I. El-Diwani, Eur. J. Med. Chem. **2017**, 134, 392-405.
7. R. T. Srinivasa, K. V. Hitesh, R. Ganga, B. Vipul, K. Ahmed, S. Ravi, Eur. J. Med. Chem. **2016**, 122, 164-177.
8. L. Weimin, C. Yuhchyau, W. Wei, K. Peter, F. Jacob, H. Dongping, L. Li, G, Min, F. Bruce, O. Paul, D. Ivan, American J. Cli. Oncology **2003**, 26,103-109.
9. I. Takashi, S. Kazuhisa, O. Tetsuo, O. Hietaro, O. Hiroaki, K. Hitoshi, Free Radical Bio. Med. **1999**, 26, 1339–1345.
10. R. Smaail, T. Yahya, D. Nihed, F. Olivier, R. Olivier, Lett. Drug Des. Discov. **2012**, 9, 305–309.
11. A. Adnan, A. Tarek, Bioorg. Med. Chem. **2004**, 12, 1935–1945.
12. R. Smaail, T. Yahya, H. Imad, et al. Res. J. Chem. Sci. **2012**, 2, 40–44.
13. I. Bouabdallahy, L. Barekz, A. Zyadz, A. Ramdaniy, I. Zidaney, A. Melhaoui, Nat. Prod. Res. **2007**, 21, 298–302.
14. J. P. Chambon, P. Feltz, M. Heaulme, S. Restlet, R. Schlichtert, K. Biziere, C. G.Wermuth, Proc. Nad. Acad. Sci. **1985**, 82, 1832-1836.
15. A. Mohammad, Annals Med. Chem. Res. **2015**, 00, 00.
16. V.V. Glushchenko, Rat. Pharma. Cardio. **2012**, 8, 447-452.
17. B. Erden, S. Murat, C.E. Burcu, N.B. Sultan, A. Eda, A. R. K. Mustafa, Turk. J. Chem. **2007**, 31, 677-687.
18. K. Kusakabe, N. Ide, Y. Daigo, T. Itoh, T. Yamamoto, H. Hashizume, J. Med. Chem. **2015**, 58, 1780-1775.
19. N. Kaila, A. Huang, A. Moretto, B. Follows, K. Janz, J. Med. Chem. **2012**, 55, 5088-5109.
20. J. Jean, H. Shen, M. T. Dube, M. Nambu, M. McTigue, N. Grodsky, J. Med. Chem. **2013**, 56, 6651-6665.
21. K. Abouzid, M. A. Hakeem, O. Khalil, Y. Maklad, Bioorg. Med. Chem. **2008**, 16, 382.
22. J. B. Moore, D. W. Combs, A. J. Tobia, Biochem. Pharmacology **1991**, 42, 679-683.
23. T. Brunee, R. Engelslatter, V. W. Steinjans, G. Kunkel, Eur Respire J. **1992**, 5, 982-5.
24. A. R. J. Graham, Br. J. Pharmacol. **2013**, 169, 328–336.
25. S. Gelin, A. Chantegral, A. Nadi, A. Fruchier, J. Org. Chem. **1983**, 48, 4078–4082.
26. D. Kumar, S.P. Singh, J. Indian Chem. Soc. **2006**, 83, 419–426.
27. S.P. Singh, O. Prakash, R. K. Vaid, Indian J. Chem. **1984**, 23B, 191–192.
28. D. Kumar, S.P. Singh, A. Martinez, et al. Tetrahedron **1995**, 51, 4891–4906.
29. R. Aggarwal, C. Rani, C. Sharma, K. Aneja, Reports Org. Chem. **2013**, 3, 13.
30. W. H. Perkin, C. Benhart, Ber. **1884**, 17, 1522.
31. J. A. Elvidge, J. A. Pickett, J. Chem. Soc. Perkin 1, **1972**, 1483-1488.
32. K. Aman, J. Gul, K. Afsar, F. G. Jan, A. Bahadur, M. Danish, Hindwai, **2017**, DOI. Org/10.1155/2017/4040254.
33. G. Mikayel, P. Margarit, T. Armen, Research Article, **2017**, DOI. 10.1186/s12906-017-1573-y.