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Synthesis, characterization and evaluation of antitubercular and analgesic activity of some novel pyrazolopyrimidine and pyrazolopyridine derivatives

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ABSTRACT

Desired fused ring system4{1.phenyl-3(Substituted phenyl)-1H-pyrazole-4-yl}-6-phenyl-1,2,3,4 tetrahydro pyrimidine-2thione & 5-phenyl –s-triazolo[1,5,a]pyrimidine (5,a,b) have been synthesized respectively with reaction of (1-phenyl-3-(substituted phenyl)-1Hpyrazol-4yl)-3-phenyl-1-propen-3-one.using a mixture Thiourea & ethanolic sodium ethaoxide & 3.amino .s.triazole. these derivatives 4(a-c) & 5(a-b) screened for analgesic & Antitubercular activity.

Keywords: Pyrazolopyrimidine, Pyrazolopyridine, analgesic, Antitubercular.

INTRODUCTION

Tuberculosis[TB] is an infectious disease, caused by several species of mycobacteria, collectively termed the tubercle bacilli. The important human pathogens of this class are M- tuberculosis and M. bovis. Tuberculosis is a systemic disease, the commonest form in man being the chronic pulmonary variety, acute fulminating forms such as tuberculous pneumonia. Tuberculosis can also involve other organs [1].

Worldwide, TB causes in 4 preventable adult death, 95% of the 8 million new cases and 98% of the million deaths due to TB, each year occur in the developing countries. India accounts for nearly 1/3rd of global tuberculosis burden and almost 1000 die of it every day HIV and multi drug resistant TB threaten to make the situation even worse. Infection with M. bovis is now less common than in the past but infection with atypical mycobacteria is now known to account for 1-4 % of cases considered to have tuberculosis. The atypical mycobacteria are in general, of low virulence but the disease caused by them is chronic, and usually resistant to routine therapy [1-2].

Heterocyclic compounds are acquiring more importance in recent years because of their immense biological and pharmacological potency. Various biologically active synthetic compounds have five membered nitrogen containing heterocyclic ring in their structures. Many compounds bearing pyrazoles and their reduced forms Pyrazolines constitute an interesting class of heterocycles due to their synthetic versatility and effective biological activities such as antimicrobial [3,4], antiviral [5], anti-inflammatory [6,7], antidepressant [8], antitubercular [9], antiamoebic

[10],analgesic [11] activities. Literature survey reveals several synthetic protocols for the synthesis of these compounds and the presence of this core in any molecule plays a key role in enhancing the activity.

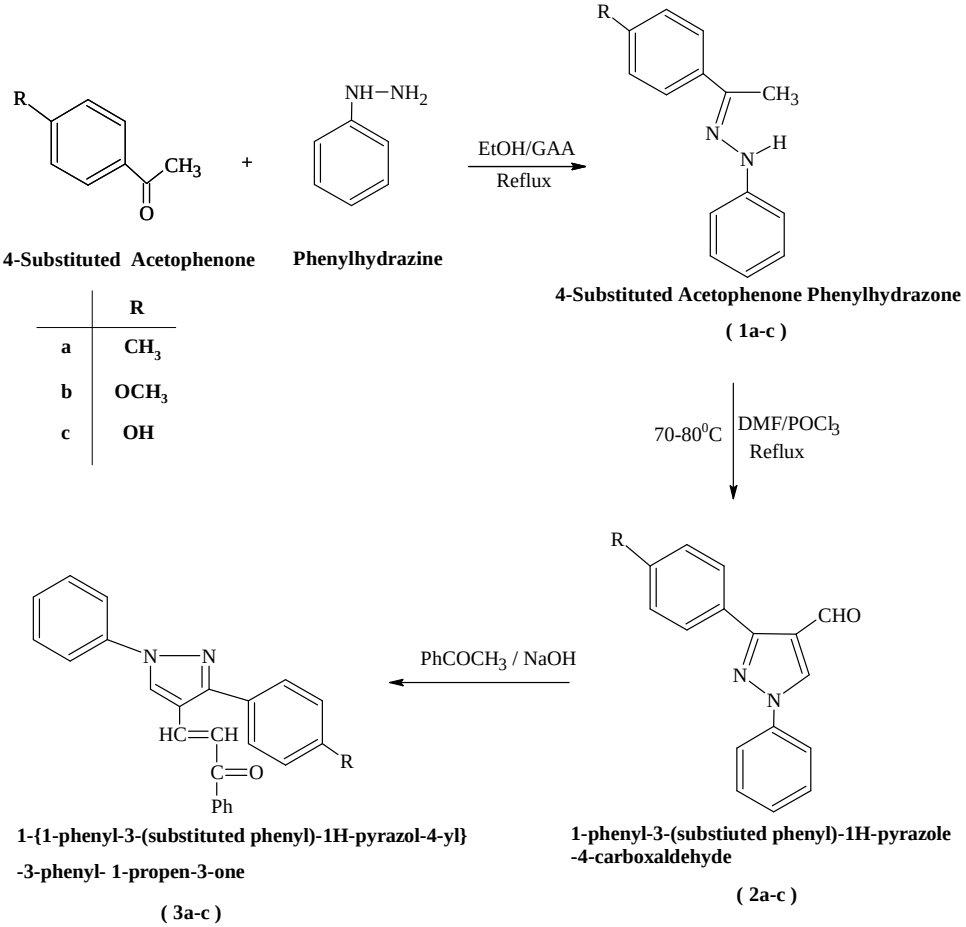
MATERIALS AND METHODS

All chemicals and solvents were procured from commercial sources and purified and dried using standard procedures from literature whenever required.

Melting points were taken on Electro thermal digital melting point apparatus and are uncorrected. Thin layer chromatography was used to assess the course of reaction and the purity of the intermediates and the final compounds, giving a single spot on TLC plate (Silica gel G), using various solvent systems. Visualization of the compounds on chromatographic plates was done by exposure to iodine vapors.

Infrared (IR), Proton Nuclear Magnetic Resonance (H1-NMR), confirmed structures of all the synthesized compounds, IR spectra were recorded using KBR disc on a Jasco FTIR-410, H1-NMR spectra were recorded in CDCl3 solution on FTNMR, Varian mercury 300 MHz and proton chemical shift are relative to tetramethylsilane as internal standard.

Scheme I



4-Substituted Acetophenone **Phenylhydrazine**

	R
a	**CH_3**
b	**OCH_3**
c	**OH**

4-Substituted Acetophenone Phenylhydrazone

(1a-c)

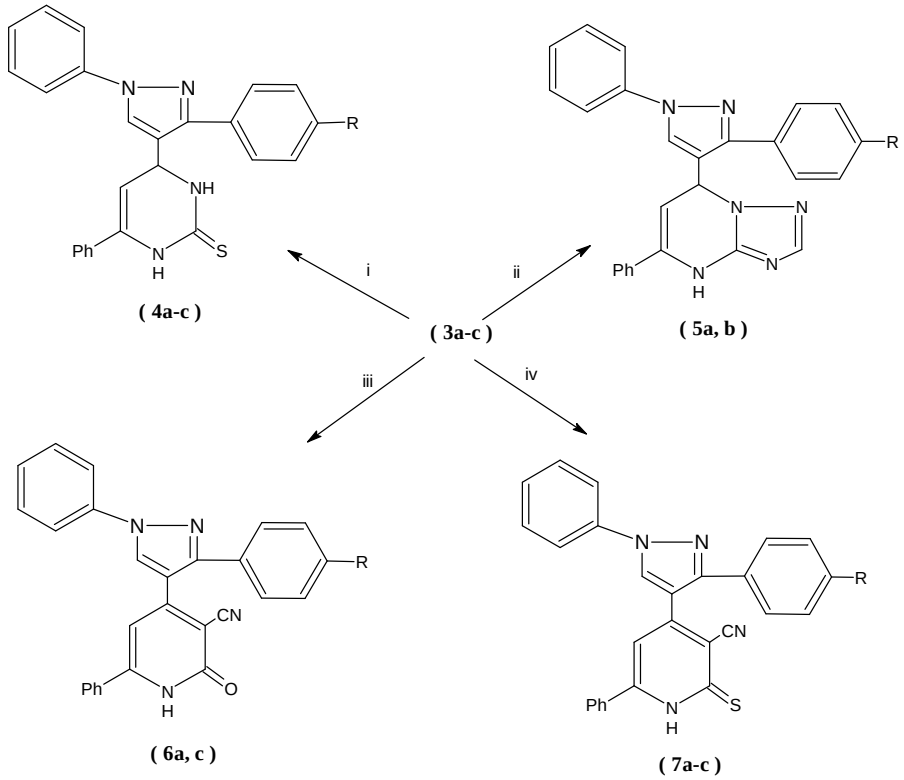
1-phenyl-3-(substiuted phenyl)-1H-pyrazole -4-carboxaldehyde

(2a-c)

1-{1-phenyl-3-(substituted phenyl)-1H-pyrazol-4-yl} -3-phenyl- 1-propen-3-one

(3a-c)

Scheme II



i. NH_2CSNH_2
ii. 3-amino-s-triazole
iii. $CNCH_2CO_2Et$
iv. $CNCH_2CSNH_2$

General procedure for Synthesis of 4-substituted Acetophenonephenylhydrazone (1a-c).[12-17]
Concentrated acetic acid (1ml) and phenylhydrazine hydrochloride (3g, 20 mmol) were added to solution of substituted acetophenone (24 mmol) in 90 ml of ethanol. Then, the reaction mixture was refluxed for 1 hour. The precipitate was filtered and washed with ethanol. After drying in vacuum, product (1) was obtained, **SchemeI**

General procedure for Synthesis of 1-phenyl-3-(substituted phenyl)-1H-pyrazoles-4 carboxaldehyde (Vilsmeier-Haack Reaction) (2a-c).[12-14,17]
Vilsmeier-Haack reagent was prepared from previously from separately cooled Dimethylformamide (2.58g, 35.3 mmol) and $POCl_3$ (5.4g, 35.5 mmol) at 0^0 C and stirred at 0^0 C. A solution of **1** (3g, 11.76 mmol)in DMF (3ml) was added dropwise to the Vilsmeier- Haack reagent, which was then warmed at room temperature and refluxed at 70-80^0 C for 4-5 hours. After cooling at room temperature the mixture was basified with a cool saturated K_2CO_3 solution. The precipitate was filtered, strongly washed with water and recrystallized from ethanol. **Scheme I**

General procedure for Synthesis of 1-{1-phenyl-3-(substituted phenyl)-1H-pyrazol-4-yl}-3-phenyl-1-propen-3-one (Chalcone) (3a-c).[18]
0.01 mole of ethanolic solution of acetophenone and 0.01 mole of 1,3– diphenylpyrazoles-4-carboxaldehyde were mixed together and stirred. 10ml of 40% sodium hydroxide solution was added to it. The mixture mixture was kept overnight at room temperature. The content was then poured over crushed ice and acidified with dilute hydrochloric acid. The solid obtained was filtered, dried and recrystallised with ethanol, **Scheme I**

General procedure for Synthesis of 4-{1-phenyl-3-(substituted phenyl)-1H-pyrazol-4-yl}-6-phenyl-1,2,3,4-tetrahydropyrimidine-2-thione (4a-c).[12]
A mixture of chalcone 3 (3.5gm, 0.01mol) and thiourea (0.76g, 0.01 mol) in ethanolic C_2H_5ONa solution (0.25g, Na in 30 ml abs C_2H_5OH) was heated under reflux for 2-4 hours, cooled and neutralised with diluted CH_3COOH. The precipated solid was collected and recrystallised from benzene as fine white needles, **Scheme II**

General procedure for Synthesis of 4,7-Dihydro-7-{1-phenyl-3-(substituted phenyl)-1H-pyrazol-4-yl}-5-phenyl-s-triazolo[1,5-a]pyrimidine (5a,b).[12]
A mixture of chalcone 3 (3.5g ,0.01 mol) and 3-amino-s-triazole (0.84g, 0.01 mol) in CH_3COOH (40ml) was heated under reflux for 2-4 hours and allowed to cool. The product thus separated was collected and recrystallised from C_2H_5OH to give white crystals, **Scheme II**

General procedure for Synthesis of 3-Cyano-4-{1-phenyl-3-(substituted phenyl)-1H-pyrazol-4-yl}-6-phenylpyridine-2(1H)-one (6a,c).[12]
A mixture of chalcone 3 (3.5g ,0.01mol), ethyl cyanoacetate (2.26,0.02mol) and CH_3COONH_4(7.7g, 0.1 mol) was heated at 150^0C in an oil bath for 5-7hour . The solid which precipated after cooling and dilution with water was collected and recrystallized from CH_3COOH as white crystals, **Scheme II.**

General procedure for Synthesis of 3-Cyano-4-(1,3-Diphenyl-1H-pyrazol-4-yl)-6-phenylpyridine-2(1H)-thione(7a-c) .[12]
To a suspension of chalcone 3 (7.0g,0.02mol) and cynothioacetamide (2.0g,0.02 mol) in absolute C_2H_5OH (150ml), triethylamine (1.0 ml) was added . The reaction mixture was heated under reflux for 6-8 hour , concentrated and left to cool . The product thus formed was collected and recrystallised form CH_3COOH as yellow fine needles**Scheme II.**

Biological Activity:
Preparation of Media
Lowenstein–Jensen Medium[19]
Lowenstein–Jensen is the most widely used solid medium for TB culture. The modification introduced by the IUATLD is recommended and is described in detail. LJ medium containing glycerol favors the growth of M. tuberculosis. while replacement of glycerol by sodium pyruvate enhances the growth of *M. bovis*and *M. africanum*.

1. Mineral salt base solution
7.24gm of Lowenstein–JensenMediumBase w/o starch was dissolved in 600ml distilled water containing 12ml of glycerol (heat if necessary to dissolve the medium completely). Sterilized it in autoclave at 15 lbs pressure (121^oC) for 15 minutes.Cooled to room temperature.

2. Malachite green solution
Malachite green dye 2.0 g
Sterile distilled water 100 ml
Using aseptic techniques the dye was dissolved in sterile distilled water by placing it in the incubator for 1–2 hours. (Note: If precipitation occurs or the solution becomes less deeply coloured, discard and prepare a fresh solution.)

3. Homogenized whole eggs
Fresh hens' eggs (not more than 7 days old), from hens that have not been fed antibiotic containing feed, are cleaned by scrubbing thoroughly with a brush in warm water and a plain alkaline soap. The eggs were soaked for 30 minutes in the soap solution, then rinsed them thoroughly in running water and soaked them in 70% ethanol for 15 minutes. Before handling the clean dry eggs, hands were scrubbed and washed. The eggs were cracked with a sterile knife into a sterile flask and beated them in a sterile blender.

Dilution of the compounds
1) All the synthesized compounds were dissolved in dimethylsulfoxide (DMSO) so as to get concentration of 100µg/ml of DMSO.

2) Standard Anti TB Drug, Isoniazid was dissolved in water so as to get concentration of 0.2 µg/ml of water, Rifampicin was dissolved in DMSO so as to get concentration of 40 µg/ml and used as standard drug for antitubercular activity

Preparation of medium
The following ingredients are aseptically pooled in a large, sterile flask and mixed well:
• Mineral salt solution 600 ml
• Malachite green solution 20 ml
• Homogenized eggs (20–25 eggs, depending on size) 1000 ml

The complete egg medium was distributed in 8ml volumes in sterile 14 ml McCartney bottles and the tops are securely fastened. Inspissited the medium within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

Coagulation of medium
Before loading, preheated the inspissator to 85^0 C. The bottles were placed in a slanted position in the inspissator and coagulated the medium for 45 minutes at 85^0 C .

Sterility check
After inspissation, a representative sample of culture bottle was incubated at 35–37 °C for 24 hours as a sterility check.

Proportion Method [20]
Only one concentration per drug is used. The concentrations are as follows:
Isoniazid 0.2 µg/ml
Rifampicin 40 µg/ml

Inoculum
With a spatula, a representative sample of 5–10 mg was taken from the primary culture and placed in a spherical, flat-bottomed flask containing 30 glass beads of diameter 3 mm. The flask was shaked for 20–30 seconds; 5 ml of distilled water was added slowly under continuous shaking. The opacity of the bacterial suspension was then adjusted by the addition of distilled water and compared with Mactarland standard solution.

Loop
The loop of platinum wire (diameter 0.7 mm) having an internal diameter of 3 mm, which delivers 0.01 ml (Delivery volume must be verified by weighing10 loopfuls of distilled water deposited on a filter paper.) was used. The bacterial dilutions required for inoculations with the loop were 10^{-2} mg/ml, the inoculum respectively of bacilli for each slope. The dilution 10^{-2} mg/ml was produced by discharging two loopful of the bacterial suspension, standardized at 1 mg/ml, into a small tube containing 2 ml of distilled water, and shaking. Similarly, the dilution 10^{-4} mg/ml was produced by discharging two loopful of the dilution 10^{-2} mg/ml into a small tube containing 2 ml of distilled water, and shaking Three slopes of medium without drug and three slopes of medium with each drug were inoculated with a loopful of each dilution.

The inoculated slopes were plugged with cotton-wool; no screw cap was used. The slopes were put in a stand at a very slight angle from the horizontal and placed in the incubator at 37°C. The liquid should cover as much surface of the medium as possible, without touching the cotton-wool plug. When the liquid part of the inoculum was evaporated (24–48 hours) the slopes were covered with screw caps and left in the incubator at 37°C.

Reading of Tests:
The results were read for the first time on the 28^{th} day. The colonies were counted only on the slopes seeded with the inoculum that has produced exact readable counts or actual counts (up to 100 colonies on the slope). The high inoculum (10^{-4} mg of bacilli) was used for the control slopes and the drug-containing slopes. The average number of colonies obtained for the drug-containing slopes indicates the number of resistant bacilli contained in the inoculum. Dividing the second figure by the first gives the proportion of resistant bacilli existing in the strain. Below a certain value (1%) – the critical proportion – the strain was classified as sensitive; above that value, it was classified as resistant. The proportions were reported as percentages. If, according to the criteria indicated below, the result of the

reading made on the 28th day is “resistant”, no further reading of the test for that drug was required: the strain was classified as resistant. If the result at the 28th day is “sensitive”, a second reading is made on the 42nd day: this provides the definitive result.

Criteria of Resistance

Any strain with 1% (the critical proportion) of bacilli resistant to any of the two drugs rifampicin and isoniazid is classified as resistant to that drug.

For calculating the proportion of resistant bacilli, the highest count obtained on the drug free and on the drug containing medium should be taken, regardless of whether both counts are obtained on the 28th day, both on the 42nd day, or one on the 28th day and the other on the 42nd day.

Experimental

Animals and drugs

Species	**:**	Swiss mice
Age/weight/ size	**:**	8-10 weeks (20-25g)
Gender	**:**	male/female
Number of days each will be housed	**:**	30 days
Number of animals in group	**:**	6
Standard Drug	**:**	Indomethacin

METHODS

Principle of writhing test[13]

Painful reactions in animals may be produced by chemicals also. Intraperitoneally injections of phenylquinone, bradykinin or acetic acid produces pain reaction which is characterized as a writhing response. Constriction of abdomen, turning of trunk (twist) and extension of hind legs are taken as reaction to chemically induced pain. Analgesics both narcotic and non-narcotic type, inhibit writhing response.

Writhing test

All the compounds were screened using the method of ghosh.[14] Albino mice (20-25g) of either sex were used for the study. Percentage protection exhibited by the test compounds administered at a dose of 20 mg/kg in DMSO solution intraperitoneally against the acetic acid (0.6% w/w) induced writhing or stretching syndrome was recorded. Indomethacin (10 mg/kg body weight) was employed as reference standard under similar conditions.

Observations

Analgesic activity

Sr. No.	Compound	Dose (mg/kg)	Number of Writhing (± S.E.M.)	Percent Decrease in Writhing
1	Control (Saline)	1	54±0.7071	-
2	4a	10	18.2±0.3742	66.29
3	4b	10	15±0.8367	72.22
4	4c	10	12.2±1.393	77.4
5	5a	10	18.8±1.158	65.18
6	5b	10	16±0.7071	70
7	6a	10	20.2±0.5831	62.59
8	6c	10	14.6±1.166	72.96
9	7a	10	20.8±1.497	61.48
10	7b	10	16.8±0.663	68.88
11	7c	10	14.2±1.158	73.7
12	Standard (Indomethcin)	10	3.2±0.3742	94.07

RESULTS AND DISCUSSION

Table 1: Physicochemical Data for the Synthesized Compound

Compound	Mol. Formula	Mol. Wt.	Melting point[^{0}C]	Yield (%)	Colour	*R_f Value
1a	$C_{15}H_{16}N_2$	224	85	89.74	Yellow	0.84
1b	$C_{15}H_{16}N_2O$	240	141	78.125	Faint Off white	0.87
1c	$C_{15}H_{14}N_2O$	226	128-130	54.71	Light Orange	0.50
2a	$C_{17}H_{14}N_2O$	262	105	91.42	Yellow	0.60
2b	$C_{17}H_{14}N_2O_2$	278	150-155	84.62	Off White	0.73
2c	$C_{16}H_{12}N_2O_2$	264	256-260	85	Orange	0.63
3a	$C_{25}H_{20}N_2O$	364	180	81.96	Yellow	0.96
3b	$C_{25}H_{20}N_2O_2$	378	168-172	91	Dark Yellow	0.53
3c	$C_{24}H_{18}N_2O_2$	366	120-122	67.21	Brownish Yellow	0.41
4a	$C_{26}H_{22}N_4S$	422	210	77.80	Lemon Yellow Crstals	0.90
4b	$C_{25}H_{22}N_4SO$	438	165	82.25	Dark Yellow Crystals	0.82
4c	$C_{25}H_{20}N_4SO$	424	135	43.82	Faint Bronish Yellow	0.62
5a	$C_{27}H_{22}N_6$	430	195	86.79	Dark Yellow Crystals	0.76
5b	$C_{27}H_{22}N_6O$	446	120	71.02	Yellow Crystals	0.92
6a	$C_{28}H_{20}N_4O$	428	265	62.50	Brownish Yellow	0.86
6c	$C_{27}H_{18}N_4O_2$	430	165	57.78	Dark Brown	0.95
7a	$C_{28}H_{20}N_4S$	444	188	73.89	Golden Yellow	0.63
7b	$C_{28}H_{20}N_4SO$	460	190	81	Dark Reddish Brown	0.80
7c	$C_{27}H_{18}N_4SO$	446	155	75	Brown	0.7

**Mobile phase - Chloroform: Benzene (1:1) for 1a-1c, 5a-7c*
Mobile phase – Ethyl acetate: Carbontetrachloride: Acetic acid(few drops) (3.5:1.5) for 4a-c

Table :-2 Antitubercular activity of Synthesized compounds

Sr. No.	Compound	Growth of observed		
		I	II	III
1	Control	+++	++ +	+ + +
2	Standard 1 (Rifampicin 40µg/ml)	-	-	-
	Standard 2 (Isoniazid 0.2µg/ml)	-	-	-
3	4a	+	+ +	+ + +
4	4b	-	+	+
5	4c	-	-	-
6	5a	+	+ +	-
7	5b	-	-	-
8	6a	+ +	+	-
9	6c	-	+	+ +
10	7a	-	-	-
11	7b	+	+ +	-
12	7c	-	-	-

Key to symbols:
No growth = - (below 1%)
Mild growth = + (1-50%)
Moderate growth = + + (50-100 %)
Severe growth = + + + (100% above)

From above observation it has been observed that the Compound 4c, 5b, 7a and 7c show the activity similar to that of standard used for assessment of antitubercular activity. Compound 4b, 6a, 6cand 7b have moderate activity against the *Mycobacterium tuberculosis.* Compound 5a have slight antitubercular activity while the compound 4a is inactive.

The Indomethacin was used as standard for the measurement of analgesic activity of the synthesized derivatives. The formula for computing percent inhibition is average writhes in the control group minus writhes in the drug group divided by writhes in the control group times into 100%. The time period with the greatest percent of inhibition is considered the peak time. A dose range is reserved for interesting compounds or those which inhibit writhing more than 70%. Compounds with less than 70% inhibition are considered to have minimal activity. We can

say that compounds 4b, 4c, 6c, 7c and 5b showed maximum analgesic activity, while compounds 4a, 6a, 7a, 7b and 5a showed minimum activity.

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