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Drug design, development and biological screening of pyridazine derivatives

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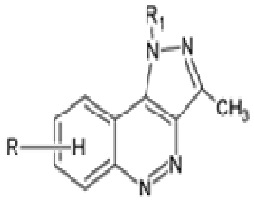
ABSTRACT

A series of Pyridazine derivatives were synthesised by diazotization of substituted anilines followed by Friedel–Crafts acylation and coupling to form corresponding hydrazones which on Intramolecular cyclisation forms 3-acetyl- substituted benz pyridazine-4(1H)-one. Further, condensation reaction by treatment with hydrazine hydrate yields the expected 3'-methyl-substituted-pyrazolo [4, 3-C] Cinnoline derivatives.The synthesised compounds were characterized by analytical techniques like TLC, UV, IR, NMR Spectral studies. All the synthesised compounds were checked for drug likeliness using Molinspiration software and toxicity prediction studies were conducted using Protox and Gusar softwares and found to be efficacious and Screening for anti-microbial activity studies. All the synthesised analogues showed good anti-fungal activity against various pathogenic bacteria and fungi.The compound PZ-5 was found to be safe and moderate drug in comparison to standard drug.

Keywords: Antimicrobial, Cinnoline, *Gusar*, *Protox*, Pyrazolo pyridazine , Ciprofloxacin, Fluconazole

INTRODUCTION

The heterocyclic nitrogen compounds like Pyrazolo benzpyridazine derivatives has a crucial role in synthetic drugs and biological processes. A wide number of pyridazines and pyridazinones has been reported to possess broad spectrum of biological activities such as antimicrobial, analgesic, anticancer, antifeedant, antitubercular, antidiabetic, antifungal, antihypertensive, antiplatelet, anticonvulsant, anti-HIV, antiasthma, anti-inflammatory, phosphodiesterase (PDE) inhibitors, cyclooxygenase(COX) inhibitors, antipyretic, insecticidal, neurological activity like anti anxiety &depressant, and intermediates for drugs synthesis, agrochemicals and other anticipated biological properties[1]. Cinoxacin is a cinnoline [Benzpyridazine] isosteric analogue of the Quinoline antibacterials used for urinary tract infection. Most of azoles are used as effective anti-fungal agents. This incited us in the synthesis of new congeners as analogs of 4-methyl Benzo pyridazine and fusing pyrazole hoping to get more potent anti bacterial and anti fungal activity[2]



Pyrazolc benzpyridazine derivatives

In the present investigation it has been designed and synthesized some novel compounds which take account of both the advantage of pyrazole and Benzo Pyridazine[Cinnoline] nucleus in the single molecule. All the designed

compounds (PZ-1 to PZ-6) were subjected to ADMET drug likeness studies in order to filter the drugs for synthesis and biological screening and to reduce enormous wastage of expensive chemicals and precious time.

EXPERIMENTAL SECTION

Drug Likeliness Studies

All the title compounds (**PZ-1 to PZ-6**) were subjected to molecular properties prediction to check drug likeliness by Molinspiration software**[3]**, ADMESAR software and Environmental toxicity (eco-toxicity) were screened using Protox and GUSAR in order to filter the drugs for synthesis and biological screening.

Table1: Molecular properties Predictions (Molinspiration) table

Compound code	Mol. Wt	Log P	No of H Donors	No of H acceptors	No of violations	Mol. Polar Surface Area
Pz-1	218.65	2.594	1	3	0	54.46
Pz-2	294.75	3.94	0	3	0	43.6
Pz-3	278.29	3.42	0	4	0	43.6
Pz-4	305.30	3.22	1	5	0	100.28
Pz-5	263.28	0.61	1	6	0	123
Pz-6	339.38	1.95	0	6	0	112.14

ADME Properties predictions

Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties of drug candidates or environmental chemicals play a key role in drug discovery and environmental hazard assessment**.** All the synthesised benzpyridazine derivatives were subjected to ADME Properties predictions using ADMESAR software **[4]** , and the predicted Absorption and Distribution properties are given in table: 2 and the predicted metabolic properties are given in table: 3.

Table 2: Absorption and Distribution Properties Prediction using ADMESAR

Compound code	Blood-Brain Barrier	Human Intestinal Absorption	Caco-2 Permeability	LogPapp, cm/s	P-glycoprotein Substrate
PZ-1	BBB+	HIA+	Caco2-	1.0776	Non-substrate
PZ-2	BBB+	HIA+	Caco2+	1.4446	Non-substrate
PZ-3	BBB+	HIA+	Caco2+	1.3634	Non-substrate
PZ-4	BBB+	HIA+	Caco2+	1.0872	Non-substrate
PZ-5	BBB+	HIA+	Caco2+	0.9354	Non-substrate
PZ-6	BBB+	HIA+	Caco2-	0.5929	Non-substrate

Table 3: Metabolic properties predictions of synthesised benzpyridazine derivatives

Comp code	CYP450 2D6 Substrate	CYP450 2D6 Inhibitor	CYP450 2C9 Substrate	CYP450 2C9 Inhibitor
PZ-1	Non-substrate	Non-inhibitor	Non-substrate	Non-inhibitor
PZ-2	Non-substrate	Non-inhibitor	Non-substrate	Inhibitor
PZ-3	Non-substrate	Non-inhibitor	Non-substrate	Inhibitor
PZ-4	Non-substrate	Non-inhibitor	Non-substrate	Non-inhibitor
PZ-5	Non-substrate	Non-inhibitor	Non-substrate	Non-inhibitor
PZ-6	Non-substrate	Non-inhibitor	Non-substrate	Non-inhibitor

Toxicity Prediction [5, 6]

The common toxicity studies were conducted in animal experiments is time-consuming and take animal lives. In silico toxicity predictions are a quick and inexpensive choice to animal experiments. They rely on known toxicity data which is used to build up a model capable of predicting toxicities of new compounds. PROTOX is a webserver for the prediction of oral toxicities of small molecules in rodents based on chemical similarities between compounds with known toxic effects and the presence of toxic fragments.

Table 4 : Oral toxicity properties predictions of synthesised benzpyridazine derivatives

Comp code	Predicted Oral toxicity LD50 value mg/kg	Predicted Toxicity Class	Tox related fragments	Toxicity targets binding[AA2AR, ADRB2, ANDR, AOFA, CRFR1DRD3, ESR1, ESR2, GCR, HRH1, NR1I2, OPRK, OPRM, PDE4D, PGH1, PRGR]	Rat IP LD50 (mg/kg)	Rat IV LD50 (mg/kg)	Rat Oral LD50 mg/kg	Rat SC LD50 (mg/kg)
PZ-1	1000	4	No toxicity Fragments	No Binding	265,200	110,500	778,000	488,300
PZ-2	1680	4	No toxicity Fragments	No Binding	382,100	124,700	1520,000	1158,000
PZ-3	1680	4	No toxicity Fragments	No Binding	353,300	180,600	979,200	564,700
PZ-4	2200	5	No toxicity Fragments	No Binding	591,400	124,000	547,700	265,400
PZ-5	295	3	No toxicity Fragments	No Binding	916,500	881,000	4298,000	1327,000
PZ-6	1680	4	No toxicity Fragments	No Binding	979,100	617,900	4484,000	1578,000

GUSAR software was used for Quantitative prediction of ecotoxicity for chemical compounds and it utilises in silico prediction of LD50 values for rats with four types of administration (oral, intravenous, intraperitoneal, subcutaneous, inhalation) .The training sets were created on the basis of data from SYMYX MDL Toxicity Database.

Table 5: Environmental Toxicity predictions

Comp. code	Predicted activity						Biodegradation
	Bioaccumulation factor Log10(BCF)	Daphnia magna LC50 - Log10(mol/L)	Fathead Minnow LC50 Log10 mmol/L	Tetrahymena pyriformis IGC50 Log10 (mol/L)	AMES Toxicity	Carcinogens	
PZ-1	1,152	4,986	-1,218	1,392	AMES toxic	Non-carcinogen	Not readily biodegradable
PZ-2	1,716	5,146	-2,082	1,755	AMES toxic	Non-carcinogen	Not readily biodegradable
PZ-3	1,445	5,925	-1,966	1,520	AMES toxic	Non-carcinogen	Not readily biodegradable
PZ-4	0,812	5,119	-1,159	1,223	AMES toxic	Non-carcinogen	Not readily biodegradable
PZ-5	0,467	4,418	-0,244	0,711	Non-AMES toxic	Non-carcinogen	Not readily biodegradable
PZ-6	0,596	5,337	-1,210	0,848	Non-AMES toxic	Non-carcinogen	Not readily biodegradable

Synthesis of pyrazolo pyridazine derivatives (PZ-1 to PZ-6) [7]

STEP 1: Synthesis of Ethyl 3 –oxo-2-(2-substituted phenyl hydrazinylidene) butanoate.

Various substituted anilines (0.39mol) were dissolved in a mixture of concentrated Hydrochloric acid (15ml) and water (15ml) and cooled to 0-5°C in ice bath, it was then added to a cold saturated solution of sodium nitrite (0.58mol) with constant stirring. The diazonium salt thus formed was filtered into a cooled solution of ethyl acetoacetate (0.39mol) in ethanol and sodium acetate in water (to make it alkaline).The solid was collected and recrystallized from methanol.

STEP 2: Synthesis of 3-Acetyl Cinnoline-4(1*H*)-one derivative:

To Ethyl 3–oxo-2-(2-phenylhydrazinylidene) butanoate(0.01mol) was added anhydrous Aluminium chloride (0.02 mol). Chlorobenzene (30ml) was added in order to dissolve the solids and the mixture was then refluxed for 1hr.the complex formed was decomposed with concentrated hydrochloric acid(30 mL) and diluted with cold water.The product was filtered, washed with water, dried and recrystallized from methanol.

STEP 3: Synthesis of Pyrazolo Cinnoline derivatives:

A mixture of Ethyl 3 –oxo-2-(2- phenylhydrazinylidene)butanoate(0.005mol) and hydrazine hydrate/phenyl hydrazine (0.02mol) in ethanol was refluxed for 3 hrs. The product formed was collected and recrystallized from ethanol.

The physico chemical parameters and spectral data of all synthesised were analysed. Melting points were recorded on SMP1 Stuart apparatus and are uncorrected. Melting point and percentage yield of the compounds (PZ-1 to PZ-6) are determined and given in table:6. The ^{1}H and ^{13} CNMR spectra were recorded on a Bruker DPX-300 spectrometer in CDCl3 with TMS as an internal standard. Mass spectra were acquired using Bruker APEX-4 instrument.

SCHEME

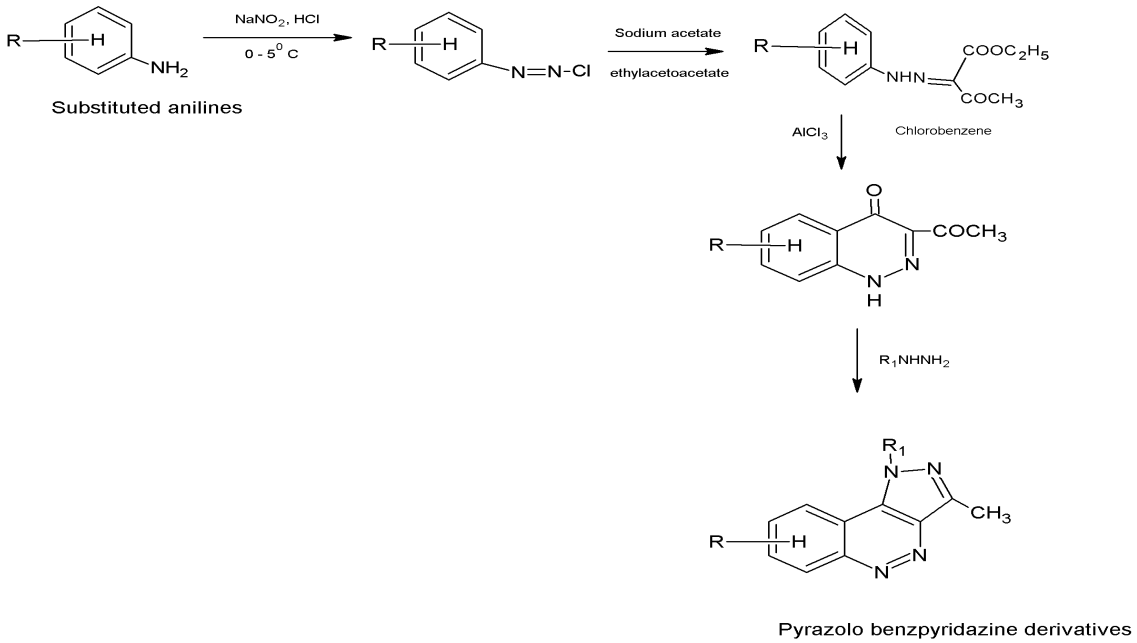


Table 6: Physicochemical parameters of synthesised compounds

Comp Code	**R**	**R_1**	Molecular Formula	Mol. Weight	% Yield	Colour	Solubility	M.P °C	R^f value*	λmax (nm)
PZ-1	Cl	H	$C_{10}H_7ClN_4$	218.65	74.5	Orange	DMSO	232	0.76	432
PZ-2	Cl	C_6H_5	$C_{16}H_{11}ClN_4$	294.75	95.6	Orange	DMSO	180	0.42	207.5
PZ-3	F	C_6H_5	$C_{16}H_{11}FN_4$	278.29	92.3	Orange	DMSO	200	0.96	272
PZ-4	NO_2	H	$C_{10}H_7N_5O_2$	305.30	78.68	Brown	DMSO	100	0.61	207.5
PZ-5	SO_2NH_2	H	$C_{10}H_9N_5SO_2$	263.28	88.3	Yellow	DMSO	210	0.21	401.5
PZ-6	SO_2NH_2	C_6H_5	$C_{16}H_{13}N_5SO_2$	339.38	94.32	Yellow	DMSO	200	0.73	272.5

ɤ Mobile phase - Chloroform: methanol - 0.2: 9.8.

7-Chloro-3-methyl-1H-pyrazolo[4,3-c]Cinnoline

IR(KBR,CM^{-1}) : 2911(CH stretching), 3162.69(NH stretching), 1588.59(N=N stretching), 1667(C=N), 1045.71(C-N), ^{1}H NMR δppm: 7-8.2(Ar H) 2.1 (Hin CH3 group), ^{13}C NMR δppm: 109 -116c[benzene] 12.6 -CH3-methyl, 145 –C-pyrazole.MS (m/z, m^{+}) 219.

7-Chloro-3-methyl-1phenyl-1H-pyrazolo[4,3-c]Cinnoline

IR(KBR,CM^{-1}) : 2924.04(Aromatic CH stretching), 3484.26(NH stretching), 1618.95(N=N stretching), 1468.05(C=N stretching), 1249.16(C-N stretching), 900-675 (Aromatic group), 3162.69(Aromatic C-H Stretching), 600-800 (C-Cl stretching). ^{1}H NMR δppm: 7-7.48 (Aromatic H), 2.132(H in CH_3 group)

7-Fluoro-3-methyl-1phenyl-1H-pyrazolo[4,3-c]Cinnoline

IR(KBR,CM^{-1}) : 2926.93(Aromatic CH stretching),1614.13(N=N stretching),1561.09(C=N str), 900-675 (Aromatic group),1400-1000 (C-F stretching), ^{1}H NMR δppm: 7-7.5 (Aromatic H), 2.23(H in CH_3 group)

3-Methyl-7-nitro-1H-pyrazolo[4,3-c]Cinnoline

IR(KBR,CM-1) : 2921.63(Aromatic CH stretching), 3411.46(NH stretching), 1629.55(N=N stretching), 1405.37(C=N stretching), 613.735(Aromatic group). 1H NMR δppm: 10.69-11.29(H in NH group), 7.247-7.97(Aromatic H group), 2.022(H in CH_3 group).

3-Methyl-7sulphonamido-1H-pyrazolo[4,3-c]Cinnoline

IR(KBR,CM^{-1}) : 2921.63(CH stretching),3411.94(NH stretching),1617.5(N=N stretching), 1410.67(C=N stretching), 1160.45(C-N),900-675(Aromatic group),3302.02(C-SO_2NH_2 group), 1334.5(SO_2 stretching), ^{1}H NMR δppm: 11.618(H in NH group),7.46-7.99 (Aromatic H group), 2.17(H in CH_3 group). ^{13}C NMR δppm: 11.612(C of CH_3),112-145.5(C of Aromatic ring), 129.306(C-N Cinnoline),146.902(C =N of pyrazole ring),159.993(C-C Cinnoline**)** .MS (m/z, m+)
263.75

3-Methyl-7-sulphonamido-1phenyl -1H-pyrazolo[4,3-c]Cinnoline

IR(KBR,CM^{-1}) : 2926.93(Aromatic CH stretching),3450-3390(NH stretching),1617.98(N=N stretching,C=N stretching),1160.45(C-N),900-675(Aromatic group),3228.74(Aromatic C-SO_2 NH_2 group),1332.57(SO_2 stretching). ^{1} H NMR δppm: 7.23-7.93 (Aromatic H group),2.33(H in CH_3 group). ^{13}C NMR δppm: 11.648(C of CH_3), 114.47-145.513(C of Aromatic ring),129.0346(C-N Cinnoline),145.513(C =N of pyrazole ring),156.327(C-C Cinnoline**).**

Anti microbial activity of pyrazolo pyridazine derivatives

Antibacterial Screening [15]

Nutrient broth medium was prepared and transferred into sterile Petri plates aseptically (thickness of 5-6mm). The standardized inoculums was inoculated in the sterilized plates prepared earlier (aseptically) by dipping a sterile in the inoculums removing the excess of inoculums by passing and rotating the swab firmly against the side of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3 times rotating the plate through an angle of 60º after each application. Finally pass the swab round the edge of the agar surface. Leave the inoculums to dry at room temperature with the lid closed. The sterile discs were soaked overnight in sample solutions, PZ-1 to PZ-6. Each Petri dish is divided into 4 parts. First, second and third compartment were loaded with sample disc (10µg/disc) and Standard Ciprofloxacin disc (10µg/disc), is placed on the fourth compartment of the plate with the help of sterile forceps. After that petri dishes are placed in the refrigerator at 4º C or at room temperature for 1 hour for diffusion. Incubate at 37 º C for 24 hours. Observe the zone of inhibition produced by different samples. Measure it using a scale and record the average of two diameters of each zone of inhibition.

Table 7: Zone of inhibition for Gram +ve and Gram –ve organisms

Organism	Zone of inhibition (in mm) Compound (10 µg/disc)						
	Pz-1	Pz-2	Pz-3	Pz-4	Pz-5	Pz-6	STD
Bacillus subtilis	12	30	15	13	25	30	33
Staphylococcus aureus	5	8	13	6	28	12	30
Saccharomyces cervaceae	13	29	27	10	23	29	29
E.coli	12	16	31	16	24	26	32
Klebsella pneumonia	30	7	32	10	29	23	30
Salmonella paratyphi	20	10	20	10	25	18	31

Table 8: Anti-bacterial activity [Minimum inhibitory concentration] of the synthesized compounds by Serial Dilution method

Microorganisms	MIC Values(µg/ml)					
	PZ-1	PZ-2	PZ-3	PZ-4	PZ-5	PZ-6
Bacillus subtilis	1.2	2.5	3.0	2.8	1.5	1.3
Staphylococcus aureus	2.8	3.5	4.8	5.2	2.5	2.2
Saccharomyces cervaceae	2.7	1.5	2.4	1.5	1.5	1.5
E.coli	2.3	3.5	2.0	1.6	1.6	2.0
Klebsella	2.8	2.0	2.5	1.2	1.2	1.2
Salmonella paratyphi	3.5	4.2	1.2	2.8	2.7	2.4

Anti fungal screening [16]

Disc diffusion method

Sabourands dextrose broth medium was prepared and transferred into sterile Petri plates aseptically (thickness of 5-6mm). The plates were allowed to dry at room temp. The plates were inverted to prevent condensate falling on the agar surface. The layers of the medium are uniform in thickness, is done by placing the plates on a levelled surface. Standardized fungal inoculums of *Aspergillus niger, Aspergillus fumigatus, Candida albicans, Monascus purpureus* were applied to the plates and spreaded uniformly over the surfaceof medium by using a sterile Non-absorbent cotton swab and finally the swab was passed around the edge of the medium. The inoculated plates were closed with the lid and allowed to dry at room temperature. The sample impregnated discs (10µg /disc) in dimethyl sulphoxide and standard clotrimazole 10µg/disc were placed on the inoculated agar medium. All petri plates were incubated at 27°C -28°C for 48 hrs. After the incubation diameter of zone of inhibition produced by the sample were measured.

Table:9 Anti-fungal activity of the synthesized compounds by Disc Diffusion method

Organism	Zone of inhibition (in mm) Compound (10 µg/disc)						
	Pz-1	**Pz-2**	**Pz-3**	**Pz-4**	**Pz-5**	**Pz-6**	**STD**
Candida albicans	11	20	24	9	20	18	30
Aspergillus fumigatus	13	28	15	10	28	24	30
Monascus purpureus	7	15	16	10	25	25	31
Aspergillus niger	7	16	18	8	7	17	22

Table 10: Anti-fungal activity [Minimum inhibitory concentration] of the synthesized compounds by Serial Dilution method

Microorganisms	MIC Values(µg/ml)					
	PZ-1	PZ-2	PZ-3	PZ-4	PZ-5	PZ-6
Candida albicans	2.5	2.5	3.0	2.8	1.5	1.3
Aspergillus fumigatus	2.5	2.0	2.5	1.2	1.2	1.2
Monascus purpureus	2.6	1.5	2.4	1.5	1.5	1.5
Aspergillus niger	2.3	3.5	2.0	1.6	1.6	2.0

RESULTS AND DISCUSSION

The Pyrazolo pyridazine derivatives have been prepared by the Intramolecular cyclisation of the phenyl hydrazones and the physico chemical parameters and spectral data are given in Table 2&3.All the title compounds (Pz-1 to Pz-6) were subjected to molecular properties prediction to check drug likeliness by Molinspiration software and toxicity predictions using GUSAR and PROTOX webserver in order to filter the drugs for biological screening and the prediction scores are given in Table (1-5).The antimicrobial activity of compounds (Pz-1 to Pz-6) by disc diffusion assay and is shown in table (7-10)

The Pyrazolo pyridazine derivatives have been synthesised by the Intramolecular cyclisation of the corresponding phenyl hydrazones acquired from diazotization of substituted Anilines followed by Friedel crafts acylation with ethyl aceto acetate in aqueous ethanolic solution containing sodium acetate and by reaction with hydrazine according to the mentioned procedure[10].

Some novel Pyrazolo benzpyridazine and its substituted derivatives were synthesized. The melting points were found and were uncorrected. The purity of the synthesized compounds, determineded by thin layer chromatography was found to be pure. The structures of the compounds were elucidated by UV, IR and NMR spectral studies and was found to be in specified ranges. From the Molinspiration values, none of the compounds violated drug likeliness parameters, making them potentially promising agents for anti-microbial therapy. Evaluation of the results from anti-bacterial and anti-fungal studies showed that synthesised Pyridazine derivatives exhibits moderate to good antibacterial and anti-fungal activity with zone of inhibition was found to be in the range of (5-30mm) as compared to standard. The MIC of the synthesized compounds against *Candida albicans , Aspergillus fumigates, Monascus purpureus* was determined by serial diluton method, was found to be in the range of 1.2 to3µg/ml and antibacterial in the range of 1.2 to 5.2 µg/ml.

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