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Synthesis of pyrazolo[4′,3′:5,6]pyrano[2,3-*d*]pyrimidine derivatives and their antimicrobial, antimalarial and antituberculosis evaluation

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This article deals with the synthesis of a new pyrazolo[4′,3′:5,6]pyrano[2,3-*d*]pyrimidine derivatives by the reaction of 1,4-dihydropyrano[2,3-*c*]-pyrazole-5-carbonitriles with ethyl cyanoacetate catalyzed by triethylamine (TEA) in ethanol under reflux conditions. All the synthesized compounds have been checked for their purity by melting point and TLC, and have been characterized through various spectroscopic techniques such as ^{1}H and ^{13}C NMR, IR, ESI-MS and elemental analysis. All synthesized compounds have been screened for antimicrobial, antimalarial and antituberculosis activity.

Keywords: Pyrazolo[4,3-*d*]isoxazol, isoxazol-5(4*H*)-ones, green synthesis, antimicrobial, antimalarial, antituberculosis

The green synthesis of heterocyclic compounds in the field of medicinal chemistry can be considered as an attractive research area, because of waste reduction, energy savings, atom economy, easy work-up processes and avoiding the use of hazardous chemicals are advanced features associated with these syntheses[1,2]. The expansion of a green, clean and eco-friendly methodologies for the synthesis of highly potent bio-active molecules is an exciting and to be discovered area of medicinal chemistry[3,4].

The development of effective antimicrobial compoundsbecomes one of the most important areas of antibacterial research today, because of their resistance to the existing antibacterial and antifungal drugs[5-7]. Thus new antimicrobial agents are urgently essential to survive this situation. This is the goal why it seems crucial in order to examine for novel antimicrobial molecules with new mechanisms of action, to overcome antimicrobial resistance[8].

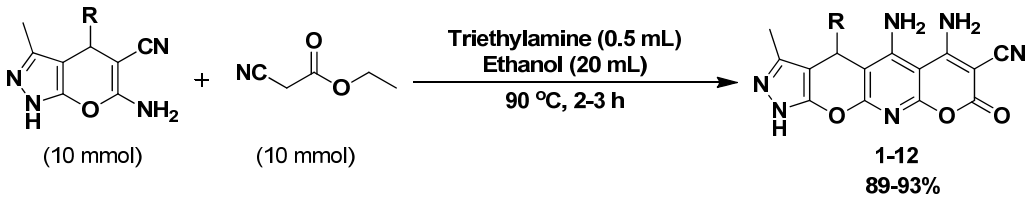
Pyrano[2,3-*d*]pyrimidine derivatives are one of asignificant class of heterocyclic compounds in medicinal chemistry that attracted much consideration due to their wide range of biological activities, such as antitumor[9], hepatoprotective[10], antibronchitic[11] and anti-HIV[12]. Recently, Saundaneand co-workers have synthesised 4,5-diamino-2-oxo-8-phenyl-6-(2-phenyl-1*H*-indol-3-yl)-2,6-dihydrodipyrano[2,3-*b*:3′,2′-*e*]pyridine-3-carbonitrile derivatives from the reaction of 2-amino-6-phenyl-4-(2-phenyl-1*H*-indol-3-yl)-4*H*-pyran-3-carbonitrilesand ethyl cyanoacetatein ethanol in the presence of catalytic amount of triethyl-amineunder reflux condition[13]. This result showed that pyrano[2,3-*d*]pyrimidine exhibit excellent biological activity, which encouraged us to investigate other pyrano[2,3-*d*]pyrimidine derivatives.

Herein, we report pyrazolo[4′,3′:5,6]pyrano[2,3-*d*]pyrimidine derivatives by the reaction of 1,4-dihydropyrano[2,3-*c*]-pyrazole-5-carbonitriles with ethyl cyanoacetate catalyzed by triethylamine (TEA) in ethanolunder reflux at the 90°C (Scheme I).

Experimental Section

All chemicals of the highest purity available were purchased from commercial sources and used as received. The progress of the reaction was monitored by thin layer chromatography (TLC) analysis on Merck pre-coated silica gel 60 F 254 aluminum sheets, visualized by UV light. Various 1,4-dihydropyrano[2,3-*c*]-pyrazole-5-carbonitriles were synthesized as per green protocols[14].

The reactions were performed under CEM Discover microwave system as well as Samsung modified microwave oven. Melting points were measured on an Optimelt MPA 100 melting point apparatus and are uncorrected. Fourier transform infrared spectroscopy (FT-IR) spectra were recorded



Scheme I — General reaction scheme for the preparation of pyrazolo[4′,3′:5,6]pyrano[2,3-d]pyrimidine (1-12)

on a Perkin-Elmer FT-IR 377 spectrometer using KBr. ^{1}H NMR spectra were recorded on Bruker AV 400 MHz spectrometer using DMSO-d_6 as solvent and TMS as the internal reference. ^{13}C NMR was recorded on a Bruker AV 100 MHz spectrometer using DMSO-d_6 as solvent. Mass spectra were recorded at Advion Expression CMS, USA. Acetone was used as the mobile phase, and electron spray ionization (ESI) was used as the ion source. Elemental analysis was performed on a CHNS elemental analyzer.

General procedure for the synthesis of pyrazolo[4′,3′:5,6]pyrano[2,3-*d*]pyrimidine, 1-12

To the mixture of 1,4-dihydropyrano[2,3-*c*]-pyrazole-5-carbonitriles (10 mmol) and ethyl cyanoacetate (20 mmol) in absolute ethanol (20 mL), triethylamine (0.5 mL) was added. The reaction mixture was refluxed for 2-3 h, excess of ethanol was removed under vacuum to about one third of its original volume and the remaining mixture left overnight at room temperature. To the clear solution thus obtained, a few drops of concentrated hydrochloric acid were added, whereby the precipitate formed was filtered off and washed thoroughly with diethyl ether. The crude product was purified by recrystallization from ethanol to afford pure compounds.

Microbiology

Antibacterial and antifungal activity

Mueller-Hinton broth and Sabouraud's broth were used as nutrient medium to grow bacteria and fungus, respectively. Inoculum size for the test strain was adjusted to 10^6 colony-forming unit (CFU) per milliliter by comparing the turbidity. Serial dilutions were prepared in primary and secondary screening. The control tube containing no antibiotic was immediately subcultured (before inoculation) by spreading a loopful evenly over a quarter of plate of medium suitable for the growth of test organism and incubated at 37°C for bacteria and 22°C for fungi overnight. The MIC of the control organism was read to check the accuracy of the drug concentrations. The lowest concentration inhibiting growth of the organism was recorded as the MIC. Each test compound was diluted, obtaining 2000 µg/mL concentration, as a stock solution. In primary screening 1000, 500, 250 and 125 µg/mL concentrations of the test compounds were taken. The active synthesized compounds found in this primary screening were further tested in a second set of dilutions against all organisms. The drugs found active in primary screening were similarly diluted to obtain 100, 50, 62.5, 25, 12.5 and 6.25 µg/mL concentrations. The highest dilution showing at least 99% inhibition is taken as MIC.

All newly synthesized pyrazolo[4′,3′:5,6] pyrano[2,3-*d*]pyrimidine derivatives (**1-12**) were examined for antimicrobial activity against two gram-positive bacterial strains (*Staphylococcus aureus* MTCC 96, *Streptococcus pyogenes* MTCC 442), two gram-negative bacterial strains (*Escherichia coli* MTCC 443, *Pseudomonas aeruginosa* MTCC 1688) as well as three fungal strains (*Aspergillus clavatus* MTCC 1323, *Candida albicans* MTCC 227 and *Aspergillus niger* MTCC 282) using the agar dilution method[15]. Ampicillin, Ciprofloxacin and Chloramphenicol were used as standard control drugs for antibacterial activity, whereas Nystatin and Greseofulvin were used as standard control drugs for antifungal activity.

Anti-malarial activity

A stock solution of 5 mg/mL of each of the test samples as well as standards was prepared in DMSO and subsequent dilutions were prepared with the culture medium. The diluted samples in 20 µL volume were added to the test wells so as to obtain final concentrations (at five-fold dilutions) ranging between 0.4 and 100 µg/mL in duplicate well containing parasitized cell preparation. The *in vitro* antimalarial assay was carried out in 96 well plates according to the microassay protocol of Reickmann and co-workers with minor modifications[16]. The cultures of *P. falciparum* strain were maintained in medium RPMI 1640 supplemented with 25 mM

HEPES, 1% D-glucose, 0.23% sodium bicarbonate and 10% heat inactivated human serum. The asynchronous parasites of *P. falciparum* were synchronized after 5% D-sorbitol treatment to obtain only the ring stage parasitized cells. For carrying out the assay, an initial ring stage parasitaemia of 0.8-1.5% at 3% haematocrit in a total volume of 200 µL of medium RPMI-1640 was determined by Jaswant Singh Bhattacharya (JSB) staining[17] to assess the percent parasitaemia (rings) and uniformly maintained with 50% RBCs (O^{+ve}). The culture plates were incubated at 37°C in a candle jar. After 36-40 h incubation, thin blood smears from each well were prepared and stained with JSB stain. The slides were microscopically observed to record maturation of the ring stage parasites into trophozoites and schizonts in the presence of different concentrations of the test agents. The test concentration which inhibited the complete maturation into schizonts was recorded as the minimum inhibitory concentration (MIC). Chloroquine and Quinine were used as the reference drugs.

Anti-tuberculosis activity

MIC of the test compounds against *M. tuberculosis* $H_{37}Rv$ was determined by L. J. agar (MIC) method[18,19] where primary 1,000, 500 and 250 µg/mL and secondary 200, 100, 50, 25, 12.5, 6.250 and 3.125 µg/mL dilutions of each test compound were added to liquid L. J. medium and then media were sterilized by inspissation method. A culture of *M. tuberculosis* $H_{37}Rv$ growing on L. J. medium was harvested in 0.85% saline in Bijou bottles. For all test compounds, first a stock solution of 2000 µg/mL concentration was prepared in DMSO. These tubes were then incubated at 37°C for 24 h followed by streaking of *M. tuberculosis* $H_{37}Rv$ (5×10^4 bacilli per mL). These tubes were then incubated at 37±1°C. Growth of bacilli was seen after 12, 22 and finally 28 days of incubation. Tubes having the compounds were compared with control tubes where medium alone was incubated with *M. tuberculosis* H37Rv. The concentration at which no development of colonies occurred or less than 20 colonies was taken as MIC of test compound. The standard strain *M. tuberculosis* $H_{37}Rv$ was tested with known drug Rifampicin.

Results and Discussion

After successful synthesis of various 1,4-dihydropyrano[2,3-*c*]-pyrazole-5-carbonitriles *via* green methodology, we have planned to develop an efficient protocol for the preparation of pyrazolo[4′,3′:5,6]-pyrano[2,3-*d*]pyrimidine derivatives (**1-12**).

To recognize the optimization of the reaction conditions, the reaction was studied by employing several solvents with the hope to maximize the product yield in short reaction times (Table I). In a model reaction, we used 6-amino-3-methyl-4-phenyl-1,4-dihydropyrano[2,3-*c*]pyrazole-5-carbonitrile (10 mmol) with ethyl cyanoacetate (20 mmol) as reactants in the presence of triethylamine as a catalyst in ethanol at 90°C and found that pyrazolo[4′,3′:5,6]-pyrano[2,3-*d*]pyrimidine derivatives (**1**) could be produced in 92% yield in 2 h (Table I, Entry 1). The same reaction was carried out in the presence of solvents like methanol, toluene, ethyl acetate, dioxane, chloroform and acetonitrile at reflux temperature for 2 h. Although they afforded product in moderate yields (Table I, entries 2-7), variation of the amount of triethylamine (TEA) like 0.2 mL, 0.3 mL,0.4 mL and 0.6 mL led to product **1** in 51%, 65%, 80% and 92% yields, respectively (Table I, entries 8-11). These results indicated that, 20 mol% of triethylamine gives high yield of the product within shorter period of time.

The optimal conditions found for the compound **1** was successfully applied to the reactions of various 1,4-dihydropyrano[2,3-*c*]-pyrazole-5-carbonitriles and ethyl cyanoacetate in the presence of catalytic amount of triethylamine in ethanol under reflux at 90°C, which afforded corresponding pyrazolo[4′,3′:5,6]-pyrano[2,3-*d*]pyrimidine (**1-12**) in excellent yields. The reaction proceeded smoothly and provided excellent yields in all cases (Table II). Both electron-withdrawing and electron-donating substituents bearing 1,4-dihydropyrano[2,3-*c*]-pyrazole-5-carbonitriles reacted smoothly with this protocol to afford excellent yields of the products. Furthermore, all synthesized products were easily purified by recrystallized from

Table I — Optimization of the reaction conditions for the synthesis of compound **1**

Entry	Solvent (10 mL)	Catalysts (mL)	Conditions	Time (h)	Yield[a] (%)
1	Ethanol	TEA (0.5)	90°C	2	92
2	Methanol	TEA (0.5)	Reflux	2	62
3	Toluene	TEA (0.5)	Reflux	2	Trace
4	Ethyl acetate	TEA (0.5)	Reflux	2	Trace
5	Dioxane	TEA (0.5)	Reflux	2	45
6	Chloroform	TEA (0.5)	Reflux	2	Trace
7	Acetonitrile	TEA (0.5)	Reflux	2	Trace
8	Ethanol	TEA (0.2)	90°C	2	51
9	Ethanol	TEA (0.3)	90°C	2	65
10	Ethanol	TEA (0.4)	90°C	2	80
11	Ethanol	TEA (0.6)	90°C	2	92

[a] = Isolated yield.

ethanol, thus avoiding extraction steps and chromatographic separations. The purity of the synthesized compounds was confirmed by TLC and elemental analysis. The structure of the final products were well characterized by using spectral (IR, mass spectroscopy, ^{1}H and ^{13}C NMR).

Proposed reaction mechanism

We have also described possible reaction mechanism of formation of pyrazolo[4′,3′:5,6]-pyrano[2,3-*d*]pyrimidine derivatives (**1-12**) (Scheme II).

Table II — Preparation of pyrazolo[4′,3′:5,6]pyrano[2,3-*d*]pyrimidine (**1-12**)

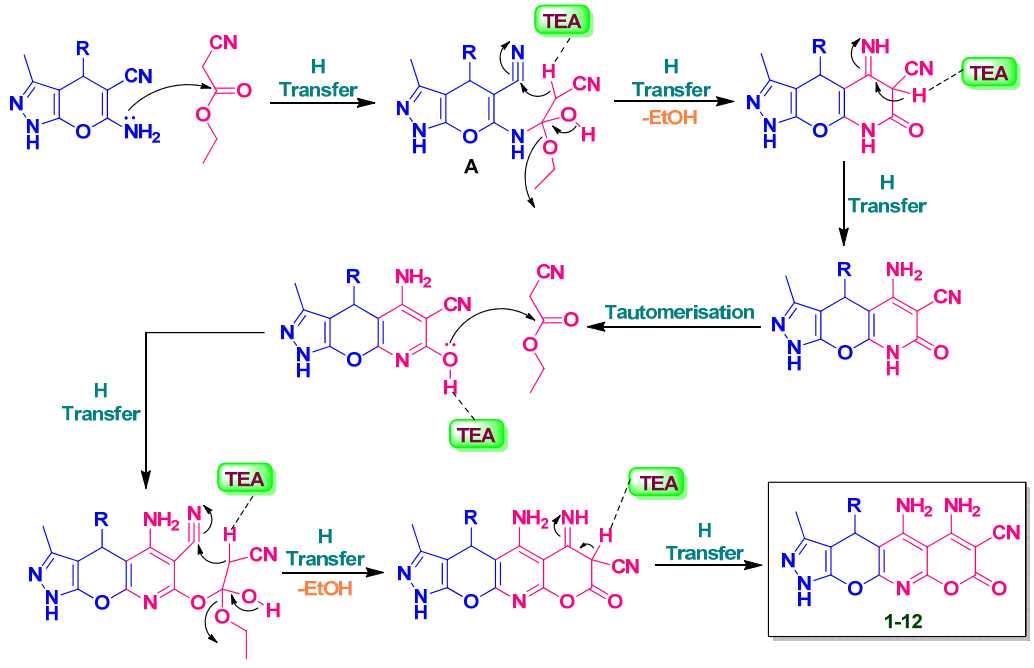
Compd	R	Thermal Heating (90°C)		m.p. (°C)
		Time (h)	Yield[a] (%)	
1	C_6H_4	2	90	230-32
2	4-N(Me)$_2$-C_6H_4	3	93	250-52
3	4-OMe-C_6H_4	2	91	208-10
4	4-Cl-C_6H_4	2	92	223-24
5	4-OH-C_6H_4	2	90	228-30
6	4-F-C_6H_4	3	90	201-203
7	4-Br-C_6H_4	2	92	238-40
8	4-Me-C_6H_4	3	90	199-201
9	4-NO$_2$-C_6H_4	2	90	216-18
10	3,5-diOMe-4-OH-C_6H_2	2	92	224-26
11	3,4-diOMe-C_6H_3	3	91	220-21
12	3,4,5-triOMe C_6H_2	3	93	235-37

[a] = Isolated yields

According to this, initially, lone pair electron of–NH_2 group of pyrano[2,3-*c*]-pyrazole derivatives attacks on carbonyl group of ethyl cyanoacetate followed by hydrogen transfer to form adduct A. This is followed by hydrogen from active methylene to form hydrogen bond with TEA leading to adduct B *via* intramolecular cyclization and hydrogen transfer followed by removal of ethanol molecule. Similarly, second molecule of ethyl cyanoacetate reacted to form desired products (**1-12**) *via* intramolecular cyclization and hydrogen transfer followed by removal of ethanol molecule.

Spectroscopic characterization of pyrazolo [4′,3′:5,6]pyrano[2,3-*d*]pyrimidines, 1-12

IR spectra showed characteristic NH stretching peaks between 3100 and 3200 cm^{-1} corresponding to NH group and two NH starching peaks between 3200-3400 cm^{-1} corresponding to NH_2 group. In addition, C-N starching peaks near 2170 cm^{-1} is corresponding to CN group. The ^{1}H NMR spectrum exhibited a singlet at δ 4.6, which indicated a proton of the pyrane nucleus, while singlet at δ 12.2, indicated a proton of the NH group of pyrazole nucleus. In addition, two singlets at δ 7.0 and 8.5 indicated protons of the two NH_2 groups, In addition, peaks between δ 7.0 and 8.5 were observed for respective aromatic protons. Furthermore, singlet at δ 3.14 indicated protons of CH_3 group of the pyrazole. The ^{13}C NMR spectrum exhibited peak at δ 180, which indicated Ar-NH_2



Scheme II — Proposed reaction mechanism

group. In addition, peaks at δ 9.7 indicated CH_3 group of thepyrazole. The ESI-MS spectra of compounds (1-12), show corresponding $(M+1)^+$ peak.

Spectral data of synthesized compounds 5,6-Diamino-3-methyl-8-oxo-4-phenyl-4,8-dihydro-1*H*-pyrano[2,3-b]pyrazolo[4′,3′:5,6]pyrano[3,2-e]-pyridine-7-carbonitrile, 1: Off-white solid. IR (KBr): 3387.4, 3312.4, 3174.6, 2967.8, 2825.4, 2172.5, 1657.8, 1607.9, 1482.9, 1393.4, 1145.1, 1042.8, 933.4, 775.8 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.14 (s, 3H, CH_3), 4.64 (s, 1H-pyrane), 6.97 (s, 2H, NH_2), 7.21-7.32 (m, 5H, ArH), 8.46 (s, 2H, NH_2), 12.18 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.74, 35.51, 42.32, 56.62, 90.23, 97.29, 108.54, 120.69, 126.19, 129.27, 131.24, 135.54, 143.42, 149.57, 154.69, 158.64, 160.81, 163.68, 180.75; ESI-MS: *m/z* for (386.36): 387.2 $(M+1)^+$. Anal. Calcd for $C_{20}H_{14}N_6O_3$ (386.11): C, 62.17; H, 3.65; N, 21.75. Found: C, 62.15; H, 3.62; N, 21.77%.

5,6-Diamino-4-(4-(dimethylamino)phenyl)-3-methyl-8-oxo-4,8-dihydro-1*H*-pyrano[2,3-b]pyrazolo[4′,3′:5,6]pyrano[3,2-e]pyridine-7-carbonitrile, 2: Orange solid. IR (KBr): 3370.4, 3322.6, 3162.6, 2952.9, 2822.7, 2124.9, 1657.6, 1614.9, 1480.6, 1388.8, 1149.4, 1046.8, 930.4, 767.8 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.12 (s, 3H, CH_3), 3.36 (s, 6H, $N(CH_3)_2$), 4.64 (s, 1H-pyrane), 6.94 (s, 2H, NH_2), 7.23-7.25 (d, 2H, ArH, *J* = 8.2), 7.32-7.34 (d, ArH, 2H, *J* = 8.4), 8.45 (s, 2H, NH_2), 12.16 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.72, 35.54, 42.35, 56.67, 90.26, 97.20, 108.55, 120.68, 129.09, 129.80, 131.24, 135.79, 143.22, 149.47, 154.68, 158.69, 160.82, 163.65, 180.72; ESI-MS: *m/z* for (386.36): 387.2 $(M+1)^+$. Anal. Calcd for $C_{20}H_{14}N_6O_3$ (386.11): C, 62.17; H, 3.65; N, 21.75. Found: C, 62.15; H, 3.62; N, 21.77%.

5,6-Diamino-4-(4-methoxyphenyl)-3-methyl-8-oxo-4,8-dihydro-1*H*-pyrano[2,3-b]pyrazolo[4′,3′:5,6]-pyrano[3,2-e]pyridine-7-carbonitrile, 3: Light yellow solid. IR (KBr): 3380.1, 3302.7, 3176.2, 2962.7, 2820.6, 2179.8, 1652.5, 1605.8, 1486.4, 1396.7, 1144.2, 1049.4, 936.2, 760.4 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.12 (s, 3H, CH_3), 3.84 (s, 3H, OCH_3), 4.64 (s, 1H-pyrane), 6.92 (s, 2H, NH_2), 7.23-7.25 (d, 2H, ArH, *J* = 8.2), 7.45-7.47 (d, 2H, ArH, *J* = 8.4), 8.40 (s, 2H, NH_2), 12.19 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.71, 35.53, 46.28, 56.71, 90.44, 97.25, 108.72, 120.62, 129.17, 129.83, 131.27, 135.70, 143.26, 149.49, 154.61, 158.67, 160.82, 163.64, 180.80; ESI-MS: *m/z* for (416.39): 417.2 $(M+1)^+$. Anal. Calcd for $C_{21}H_{16}N_6O_4$ (416.12): C, 60.57; H, 3.87; N, 20.18. Found: C, 60.55; H, 3.84; N, 20.17%.

5,6-Diamino-4-(4-chlorophenyl)-3-methyl-8-oxo-4,8-dihydro-1*H*-pyrano[2,3-b]pyrazolo[4′,3′:5,6] pyrano[3,2-e]pyridine-7-carbonitrile, 4: White solid. IR (KBr): 3384.9, 3304.6, 3171.5, 2954.6, 2818.3, 2187.1, 1644.2, 1603.7, 1484.2, 1394.8, 1141.9, 1046.2, 935.12, 756.2 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.11 (s, 3H, CH_3), 4.64 (s, 1H-pyrane), 6.96 (s, 2H, NH_2), 7.19-7.21 (d, 2H, ArH, *J* = 8.4), 7.37-7.39 (d, 2H, ArH, *J* = 8.4), 8.44 (s, 2H, NH_2), 12.17 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.71, 35.51, 56.68, 90.21, 97.15, 108.60, 120.65, 129.07, 129.70, 131.20, 135.76, 143.20, 149.44, 154.65, 158.68, 160.87, 163.63, 180.67. MS (ESI) *m/z* for (420.81): 421.3 $(M+1)^+$, 422.3 $(M+2)^+$. Anal. Calcd for $C_{20}H_{13}ClN_6O_3$ (420.07): C, 57.08; H, 3.11; N, 19.97%. Found: C, 57.09; H, 3.62; N, 19.98%.

5,6-Diamino-4-(4-hydroxyphenyl)-3-methyl-8-oxo-4,8-dihydro-1*H*-pyrano[2,3-b]pyrazolo[4′,3′:5,6]pyrano[3,2-e]pyridine-7-carbonitrile, 5: Yellow solid. IR (KBr): 3540.4, 3382.1, 3306.7, 3160.6, 2956.8, 2819.4, 2189.4, 1648.4, 1599.9, 1480.1, 1397.4, 1148.2, 1055.4, 942.4, 751.4 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.14 (s, 3H, CH_3), 4.63 (s, 1H-pyrane), 6.94 (s, 2H, NH_2), 7.25-7.27 (d, 2H, ArH, *J* = 8.2), 7.43-7.45 (d, 2H, ArH, *J* = 8.4), 8.45 (s, 2H, NH_2), 9.82 (s, 1H, OH), 12.16 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.74, 35.55, 56.68, 90.27, 97.26, 108.48, 120.60, 129.17, 129.75, 131.22, 135.66, 143.08, 149.33, 154.61, 158.64, 160.84, 163.64, 180.82; ESI-MS: *m/z* for (402.36): 403.4 $(M+1)^+$. Anal. Calcd for $C_{20}H_{14}N_6O_4$ (402.11): C, 59.70; H, 3.51; N, 20.89. Found: C, 59.72; H, 3.53; N, 20.90%.

5,6-Diamino-4-(4-fluorophenyl)-3-methyl-8-oxo-4,8-dihydro-1*H*-pyrano[2,3-b]pyrazolo[4′,3′:5,6] pyrano[3,2-e]pyridine-7-carbonitrile, 6: Off-white solid. IR (KBr): 3390.7, 3322.4, 3191.6, 2953.2, 2827.8, 2168.4, 1654.3, 1609.1, 1499.1, 1392.7, 1260.5, 1140.9, 1049.0, 946.2, 778.8 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.13 (s, 3H, CH_3), 4.67 (s, 1H-pyrane), 6.94 (s, 2H, NH_2), 7.19-7.21 (d, 2H, ArH, *J* = 8.4), 7.29-7.31 (d, 2H, ArH, *J* = 8.4), 8.46 (s, 2H, NH_2), 12.18 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.74, 35.51, 56.57, 90.27, 97.18, 108.64, 120.45, 129.27, 129.82, 131.24, 135.78,

143.29, 149.43, 154.61, 158.67, 160.89, 163.66, 180.45; ESI-MS: *m/z* for (404.35): 404.5 $(M+1)^+$. Anal. Calcd for $C_{20}H_{13}FN_6O_3$ (404.10): C, 59.41; H, 3.24; N, 20.78. Found: C, 59.43; H, 3.26; N, 20.79%.

5,6-Diamino-4-(4-bromophenyl)-3-methyl-8-oxo-4,8-dihydro-1*H*-pyrano[2,3-b]pyrazolo[4′,3′:5,6]pyrano[3,2-e]pyridine-7-carbonitrile, 7: Pale yellow solid. IR (KBr): 3386.7, 3308.2, 3166.8, 2968.9, 2827.1, 2182.0, 1649.9, 1609.2, 1489.3, 1392.4, 1147.1, 1040.3, 929.4, 755.9 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.12 (s, 3H, CH_3), 4.67 (s, 1H-pyrane), 6.95 (s, 2H, NH_2), 7.21-7.22 (d, 2H, ArH, *J* = 8.2), 7.30-7.32 (d, 2H, ArH, *J* = 8.4), 8.45 (s, 2H, NH_2), 12.19 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.72, 35.58, 56.73, 90.24, 97.15, 108.65, 120.71, 129.06, 129.75, 131.32, 135.71, 143.26, 149.49, 154.62, 158.67, 160.90, 163.62, 180.64; ESI-MS: *m/z* for (465.26): 466.1 $(M+1)^+$, 468.2 $(M+2)^+$. Anal. Calcd for $C_{20}H_{13}N_6O_3$ (464.02): C, 51.63; H, 2.82; N, 18.06. Found: C, 51.62; H, 2.84; N, 18.08%.

5,6-Diamino-3-methyl-8-oxo-4-(p-tolyl)-4,8-dihydro-1*H*-pyrano[2,3-b]pyrazolo[4′,3′:5,6]pyrano-[3,2-e]pyridine-7-carbonitrile, 8: White solid. IR (KBr): 3360.3, 3298.4, 3178.4, 2963.2, 2825.7, 2352.4, 2172.1, 1658.4, 1608.2, 1467.2, 1380.7, 1142.8, 1040.6, 930.1, 764.1 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 2.48(s, 3H, Ar-CH_3), 3.14 (s, 3H, CH_3), 4.64 (s, 1H-pyrane), 6.96 (s, 2H, NH_2), 7.21-7.23 (d, 2H, ArH, *J* = 8.4), 7.31-7.32 (d, 2H, ArH, *J* = 8.4), 8.45 (s, 2H, NH_2), 12.18 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.72, 22.12, 35.56, 56.67, 90.18, 97.24, 108.42, 120.77, 129.37, 129.75, 131.26, 135.77, 143.29, 149.54, 154.61, 158.72, 160.85, 163.61, 180.69; ESI-MS: *m/z* for (400.39): 401.2 $(M+1)^+$. Anal. Calcd for $C_{21}H_{16}N_6O_3$ (400.13): C, 62.99; H, 4.03; N, 20.99. Found: C, 62.98; H, 4.02; N, 21.01%.

5,6-Diamino-3-methyl-4-(4-nitrophenyl)-8-oxo-4,8-dihydro-1*H*-pyrano[2,3-b]pyrazolo[4′,3′:5,6]pyrano[3,2-e]pyridine-7-carbonitrile, 9: Dark yellow solid. IR (KBr): 3382.4, 3390.5, 3172.7, 2974.8, 2828.2, 2171.1, 1659.5, 1612.8, 1490.1, 1399.0, 1124.6, 1048.9, 930.1, 767.3 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.12 (s, 3H, CH_3), 4.54 (s, 1H-pyrane), 6.94 (s, 2H, NH_2), 7.17-7.19 (d, 2H, ArH, *J* = 8.2), 7.32-7.34 (d, 2H, ArH, *J* = 8.4), 8.45 (s, 2H, NH_2), 12.15 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO): ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.74, 35.56, 56.72, 90.48, 97.22, 108.77, 120.65, 129.15, 129.82, 131.29, 135.72, 143.28, 149.39, 154.59, 158.63, 160.84, 163.62, 180.82; ESI-MS: *m/z* for (431.36): 231.8 $(M+1)^+$. Anal. Calcd for $C_{20}H_{13}N_7O_5$ (431.10): C, 55.69; H, 3.04; N, 22.73. Found: C, 55.68; H, 3.03; N, 22.75%.

5,6-Diamino-4-(4-hydroxy-3,5-dimethoxyphenyl)-3-methyl-8-oxo-4,8-dihydro-1*H*-pyrano[2,3-b]pyrazolo[4′,3′:5,6]pyrano[3,2-e]pyridine-7-carbonitrile, 10: Yellow solid. IR (KBr): 3520.5, 3383.4, 3298.1, 3166.8, 2964.0, 2826.8, 2171.4, 1658.4, 1609.1, 1482.4, 1398.7, 1142.2, 1047.7, 930.1, 764.3 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.11 (s, 3H, CH_3), 3.85 (s, 6H, 2-OCH_3), 4.63 (s, 1H-pyrane), 6.94 (s, 2H, NH_2), 7.24 (s, 1H, ArH), 7.32 (s, 1H, ArH), 8.48 (s, 2H, NH_2), 9.91 (s, 1H, OH), 12.19 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.74, 35.42, 46.25, 56.76, 90.46, 97.28, 108.77, 126.64, 128.28, 130.74, 135.79, 140.21, 143.22, 149.46, 154.64, 158.68, 160.81, 163.62, 180.78; ESI-MS: *m/z* for (462.41): 463.8 $(M+1)^+$. Anal. Calcd for $C_{22}H_{18}N_6O_6$ (462.13): C, 57.14; H, 3.92; N, 18.17. Found: C, 57.18; H, 3.90; N, 18.19%.

5,6-Diamino-4-(3,4-dimethoxyphenyl)-3-methyl-8-oxo-4,8-dihydro-1*H*-pyrano[2,3-b]pyrazolo-[4′,3′:5,6]pyrano[3,2-e]pyridine-7-carbonitrile, 11: Light orange solid. IR (KBr): 3382.2, 3299.6, 3160.4, 2969.1, 2820.1, 2360.3, 2178.2, 1651.8, 1623.5, 1488.9, 1290.9, 1143.8, 1040.1, 937.4, 760.1 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.12 (s, 3H, CH_3), 3.84 (s, 6H, 2-OCH_3), 4.66 (s, 1H-pyrane), 6.96 (s, 1H, NH_2), 7.15-7.17 (d, 1H, ArH, *J* = 7.6), 7.24-7.26 (d, 1H, ArH, *J* = 7.6), 7.36 (s, 1H, ArH), 8.46 (s, 2H, NH_2), 12.15 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.73, 35.44, 46.21, 56.77, 90.56, 97.32, 108.78, 126.66, 128.32, 130.72, 135.82, 137.26, 138.98, 140.26, 143.27, 149.43, 154.66, 158.63, 160.83, 163.67, 180.74; ESI-MS: *m/z* for (446.42): 447.2 $(M+1)^+$. Anal. Calcd for $C_{22}H_{18}N_6O_5$ (446.13): C, 59.19; H, 4.06; N, 18.83. Found: C, 59.18; H, 4.04; N, 18.80%.

5,6-Diamino-3-methyl-8-oxo-4-(3,4,5-trimethoxyphenyl)-4,8-dihydro-1*H*-pyrano[2,3-b]pyrazolo-[4′,3′:5,6]pyrano[3,2-e]pyridine-7-carbonitrile, 12: Dark Yellow solid. IR (KBr): 3394.6, 3298.4, 3162.5, 2968.6, 2826.4, 2364.7, 2170.8, 1659.2, 1629.4, 1489.1, 1295.4, 1146.4, 1045.7, 938.2, 762.4 cm^{-1};

^{1}H NMR (400 MHz, DMSO-d_6): δ 3.14 (s, 3H, CH_3), 3.84 (s, 9H, 3-OCH_3), 4.65 (s, 1H-pyrane), 6.96 (s, 2H, NH_2), 7.22 (s, 1H, ArH), 7.34 (s, 1H, ArH), 8.46 (s, 2H, NH_2), 12.21 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.72, 35.41, 46.27, 50.44, 56.78, 90.42, 97.18, 108.72, 126.69, 130.71, 135.69, 138.30, 140.28, 143.24, 149.49, 154.68, 158.72, 160.84, 163.66, 180.75; ESI-MS: *m/z* for (476.44): 477.6 $(M+1)^+$. Anal. Calcd for $C_{23}H_{20}N_6O_6$ (476.14): C, 57.98; H, 4.23; N, 17.64. Found: C, 57.95; H, 4.22; N, 17.66%.

Pharmacology

Antimicrobial activity

In vitro antibacterial activity

All novel synthesized scaffolds were investigated for their *in vitro* antibacterial activity (Table III). The bioassay results demonstrated that pyrazolo [4′,3′:5,6]pyrano[2,3-*d*]pyrimidine derivatives (**1-12**) have succeeded in showing excellent activity against the mentioned microorganisms as compared to standard drugs. In general, most of the tested compounds exhibited better activity against the Gram-positive as well as the Gram-negative bacteria.

Among the Gram-positive bacterial strain, *Staphylococcus aureus* showed relatively higher sensitivity towards the tested compounds. In this view, compound **4** bearing an electron donating -Cl group at 4th positions of the phenyl ring was found to be the most active compound that inhibits the gram positive *S. aureus* bacterial growth at the lowest minimum inhibitory concentration (MIC) value of 50 μg/mL, which is most active as compared to Ampicillin (MIC 250 μg/mL), but equally active to Chloramphenicol (MIC 50 μg/mL) and Ciprofloxacin (MIC 50 μg/mL). Furthermore, compounds **7** and **10** displayed excellent effectiveness with MIC value 62.5 μg/mL against *S. aureus* as compared to standard drug Ampicillin.

Table III — *In vitro* antibacterial activity of compounds (**1-12**) MIC [μg/mL]

Compd	*E. coli* MTCC 443	*P. aeruginosa* MTCC 1688	*S. aureus* MTCC 96	*S. pyogenus* MTCC 442
1	100	200	100	200
2	62.5	100	200	250
3	200	250	100	200
4	125	62.5	50	100
5	200	500	200	200
6	125	200	100	100
7	100	62.5	62.5	125
8	200	100	200	250
9	125	125	250	200
10	62.5	50	62.5	125
11	100	100	100	125
12	100	200	200	250
Ampicillin	100	100	250	100
Ciprofloxacin	25	25	50	50
Chloramphenicol	50	50	50	50

Moreover, compounds **1**, **3**, **6** and **11** displayed excellent activity with MIC value 100 μg/mL against *S. aureus* as compared to standard drug Ampicillin. While analogues **2**, **5**, **8** and **12** (MIC 200 μg/mL) displayed good activity against *S. aureus* as compared to standard drug Ampicillin (MIC 2500 μg/mL). Only compound **9** (MIC 2500 μg/mL) exhibited equipotent activity to standard drugAmpicillin (MIC 2500 μg/mL) against *S. aureus*. With regard to the activity against *S. pyogenus*, the best activity was exhibited by compounds **4** and **6** (MIC 100 μg/mL) having electron donating –Cl and −F groups on phenyl ring respectively, which is equally potent to the Ampicillin (MIC 100 μg/mL) but 50% less active than Chloramphenicol (MIC 50 μg/mL) and Ciprofloxacin (MIC 50 μg/mL).

On the other hand, investigation of antibacterial activity of all newly synthesized analogs against the two tested Gram-negative strains revealed that analogs **2** and **10** (MIC 62.5 μg/mL) displayed excellent activity against *E. coli* as compared to standard drug Ampicillin (MIC 100 μg/mL). Furthermore, analogs **1**, **7**, **11** and **12** (MIC 100 μg/mL) were equipotent to Ampicillin (MIC 100 μg/mL) against *E. coli*, but 50% less active than Chloramphenicol (MIC 50 μg/mL). With regard to the activity against *P. aeruginosa*, the best activity was exhibited by compound **10** (MIC 50 μg/mL) having electron donating −OMe and −OH groups on phenyl ring, which was found to be the most active compound that inhibits the *P. Aeruginosa* bacterial growth at the lowest minimum inhibitory concentration (MIC) value of 50 μg/mL, which is most active as compared to Ampicillin (MIC 100 μg/mL), but equally active to Chloramphenicol (MIC 50 μg/mL) and 50% less active to Ciprofloxacin (MIC 25 μg/mL). Moreover, compounds **4**, **7** and **10** inhibited the gram negative *P. Aeruginosa* bacterial growth at the lowest minimum inhibitory concentration (MIC) value of 62.5 μg/mL as compared to Ampicillin (MIC 100 μg/mL). Moreover, compounds **2**, **8** and **11** displayed equipotent activity to standard drug Ampicillin with MIC value 100 μg/mL against *P. Aeruginosa*.

In vitro antifungal activity

Concerning the antifungal activity of tested compounds, only one strain, *i.e. C. albicans* showed

Table IV — *In vitro* antifungal activity of compounds (**1-12**) MIC [μg/mL]

Compd	*C. albicans* MTCC 227	A. *niger* MTCC 282	A. *clavatus* MTCC 1323
1	250	500	1000
2	500	1000	1000
3	500	200	200
4	250	250	500
5	200	250	250
6	1000	500	250
7	250	1000	1000
8	500	500	1000
9	100	200	500
10	200	250	500
11	250	500	250
12	500	1000	500
Nystatin	100	100	100
Greseofulvin	500	100	100

Table V — *In vitro* antimalarial activity of compounds (**1-12**).

Compd	Mean IC50 values (μg/mL)
1	1.35
2	1.62
3	1.22
4	0.84
5	1.32
6	1.48
7	1.85
8	1.09
9	1.18
10	1.04
11	0.96
12	1.10
Quinine	0.268
Chloroquine	0.02

certain sensitivity against some of the tested compounds, whereas rest of other two fungal strains were insensitive to the same compounds (Table IV).

Antifungal activity data indicates that among the (**1-12**) analogues, compound **9** having electron withdrowing $–NO_2$ group, which displayed excellent activity with MIC 100 μg/mL against *C. albicans* is equipotent to Nystatin (MIC 100 μg/mL), but greater than Greseofulvin (MIC 500 μg/mL). In addition, compounds **5** and **10** (MIC 200 μg/mL), which are more potent to Greseofulvin (MIC 500 μg/mL), but 50% less active than Nystatin (MIC 100 μg/mL). Furthermore, compounds **1**, **4**, **7** and **11** displayed excellent activity with MIC 250 μg/mL against *C. albicans* as compared to Greseofulvin (MIC 500 μg/mL). Furthermore, compounds **2**, **3**, **8** and **12** displayed good activity with MIC 500 μg/mL against *C. albicans* equipotent to Greseofulvin (MIC 500 μg/mL). Moreover, compounds (**1-12**) exerted moderate inhibitory efficiency against *A. niger* and *A. clavatus*.

Table VI — *In vitro* antimalarial activity of compounds (**1-12**).

Compd	MIC (μg/mL)
1	100
2	62.5
3	500
4	100
5	50
6	62.5
7	100
8	50
9	250
10	500
11	100
12	62.5
Isoniazid	0.20
Rifampicin	0.25

In vitro antimalarial activity

The synthesized compounds were also screened for *in vitro* antimalarial activity against *Plasmodium falciparum* 3D7-chloroquine-sensitive strain (Microcare laboratory and TRC, Surat, Gujarat, India). All experiments were performed in duplicate and a mean IC50 value is mentioned in Table V. All compounds exhibited only moderate antimalarial activity (IC50 = 0.84-1.84 μg/mL).

In vitro antituberculosis activity

The preliminary screening of the title compounds (**1-12**) for their *in vitro* antituberculosis activity against *Mycobacterium tuberculosis* $H_{37}Rv$ strain was determined. The observed MIC values of these compounds are presented in Table VI. Among the screened analogs **5** and **8** showed the good activity (50 mg/mL), followed by compounds **2**, **6** and **12** (62.5 mg/mL). Isoniazid and rifampicin were used as the standard drugs.

Conclusions

In summary, we have developed pyrazolo [4′,3′:5,6]pyrano[2,3-*d*]pyrimidine derivatives by the reaction of 1,4-dihydropyrano[2,3-*c*]-pyrazole-5-carbonitriles with ethyl cyanoacetate catalyzed by triethylamine (TEA) in ethanol under reflux at 90°C. The major advantages of the present protocol are excellent product yield, short reaction time and simple

work-up procedures. Moreover, by this methodology highly pure products are obtained and there is no need for column purification. From the bioassays it is clear that the pyrazolo [4′,3′:5,6]pyrano[2,3-*d*]pyrimidine derivatives lead to more active antimicrobial activity. In the present study, compounds **2**, **4**, **7**, **10** and **11** exhibited highly potent activity against most of the tested bacteria.

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