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NOVEL GRINDING SYNTHESIS OF PYRANOPYRAZOLE ANALOGUES AND THEIR EVALUATION AS ANTIMICROBIAL AGENTS

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Abstract – The paper describes the results of a new four-component synthesis of pyranopyrazole heterocycles by solvent-free one-pot grinding of malononitrile, hydrazine, ethyl acetoacetate, and various aldehydes in the presence of a base. The reaction proceeded smoothly at room temperature with good yields in very short reaction time. The synthesised compounds were evaluated for their *in vitro* antibacterial activity against three different bacterial and three different fungal strains. The highlight of this work is that the synthesis was activity-driven. The brief SAR correlation found that the tested compounds showed better activity against fungal strains.

Environmental considerations and simplicity of preparative procedure have bound organic chemists to look for greener synthetic choices and hence multicomponent reactions (MCRs) are now emerging as a responsible alternative tool in organic synthesis.[1] MCRs are also crucial in the design of new and bioactive small organic molecules[2] as these procedures have distinctive green-chemistry[3] and atom-economy benefits.[4] With the emergence of drug-resistant pathogens, there has been increasing research towards the discovery of new small organic molecules that target these pathogens.[5] Moreover, there has been a more than 40 year gap between the discovery of new small molecule antibiotics: fluoroquinolones (in 1962) and the oxazolidinone linezolid (in 2000).[6] Hence, the search for better and resilient antimicrobials continues. Similarly, fungal-infections have been unremittingly increasing especially in immunity-deficient patients such as those suffering from AIDS, cancer, etc.[7] Fluconazole, itraconazole, and ketoconazole are some of the azole containing compounds that have been well established as first line of defense against fungal

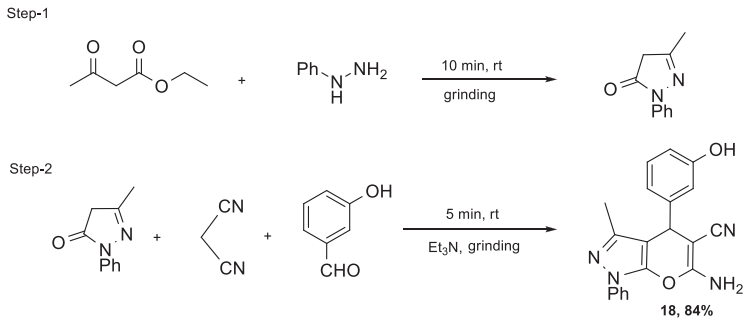
infections.

Our efforts to prepare pyrazole and pyran based heterocyclic compounds as potential bioactive molecules, led us to the synthesis of pyranopyrazoles, i.e. pyran fused pyrazoles. 4*H*-Pyrans have been reported as the basic structural pharmacophores for a range of useful compounds such as natural products,[8] other biologically and pharmacologically active compounds,[9] agrochemicals,[10] etc. Therefore, the synthesis of such compounds has generated strong scientific interest. Similarly, pyrazoles have also been reported as excellent starting materials for various bioactive small organic molecules.[11] Pyranopyrazoles have also been described as physiologically active compounds having applications such as antimicrobial,[12] antiviral,[13] sex pheromone,[14] etc. Therefore, the designing and synthesis of such heterocyclic fused-core compounds continues to draw scientific curiosity.

Regarding the chemical synthesis of the pyranopyrazole pharmacophore, several reports are available in the literature. The first reported synthesis related to the reaction between 3-methyl-1-phenylpyrazolin-5-one and tetracyanoethylene.[15] Subsequently, other groups reported related syntheses of a series of 6-amino-5-cyano-4-aryl-*4H*-pyrazolo[3,4-*b*]pyrans.[16] A useful three-component reaction between *N*-methylpiperidone, pyrazolin-5-one and malonodinitrile was reported by Shestopalov and co-workers.[17] Subsequently, the same group described a new four-component MCR assisted synthesis of pyranopyrazoles.[18] Peng and co-workers[19] outlined an interesting variation of the MCR strategy by utilising the environmentally safe technology of combined microwave and ultrasound irradiation. Vasuki *et al*. reported a fast four-component reaction in water.[20] Schlager and co-workers disclosed an altered multistep synthesis of pyranopyrazoles starting with 1-phenylpyrazole.[21] Recently, a few papers have revealed the use of catalyst,[22] nanoparticles,[23] or traditional refluxing conditions.[24] A three-component proline-catalysed grinding method was disclosed by Guo and co-workers[25] followed by a similar DBU-catalysed report by Bhavanarushi *et al*.[26] However, none of the groups reported an exclusive four component, solvent-free grinding method assisted synthesis. In the same vein, while many reports are available regarding the applications of pyranopyrazoles but the full extent of their antimicrobial potential has not been explored given the fact that the search for multidrug resistant antimicrobials is the need of the hour. Furthermore, we wanted a novel and quick green route to the synthesis of these interesting pharmacophores, which encouraged us to take up their detailed synthesis and evaluation. In this manuscript we report an efficient, eco-friendly, and solvent-free four-component reaction protocol for the synthesis of pyranopyrazole derivatives by grinding method. To the best of our knowledge, this is the first report of four-component grinding synthesis of pyranopyrazoles. The prepared compounds were subsequently tested for their *in vitro* antibacterial and antifungal activities.

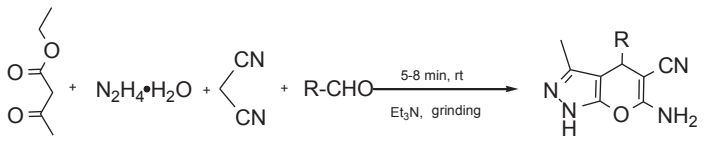
Initially, we followed the reported two-step grinding methodology where in the first step 3-methyl-1*H*-pyrazol-5(4*H*)-one was synthesised by grinding together ethyl acetoacetate and hydrazine

hydrate in a mortar with pestle. In the next step, the resulting pyrazolone was collected without further purification and subjected to additional grinding by adding the aldehyde, malononitrile, and triethylamine as base for 5 min as shown in Scheme 1. The reaction was continuously monitored by TLC.



Scheme 1. Route A, two-step strategy for the synthesis of *N*-phenyl substituted pyranopyrazole **18**

But, when we tested **18** against the *E. coli* bacterial strain and then against *F. oxyparum* fungal strain, it was found to be inactive (see Table 2). The latter SAR (Structure-Activity Relationship) result coupled with the observation that the whole synthetic procedure took two steps, prompted us to look for: i) a modified pharmacophore and ii) a simpler swifter synthetic procedure. Hence, instead of going for the tedious two-step synthetic protocol we investigated the faster one-step route. In the swifter one-step protocol, all the four components viz. ethyl acetoacetate, hydrazine hydrate, aldehyde, malononitrile, and triethylamine were collected in a mortar and ground thoroughly using the pestle. Thus, by employing the one-step grinding assisted protocol the whole reaction sequence was over in 5 to 8 minutes. To get a better comparative idea, all the subsequent pyranopyrazoles were prepared by both the routes: route A (two-step grinding) and route B (one-step grinding), an appraisal of which is shown in Table 1.

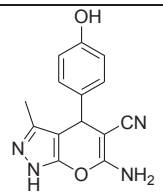
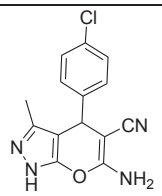
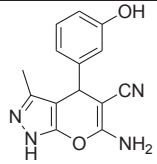
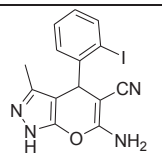
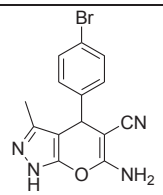
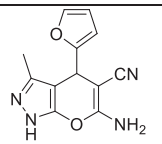
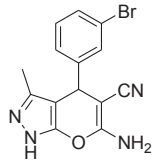
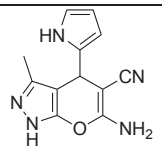
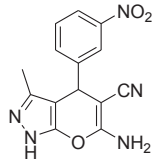
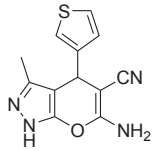
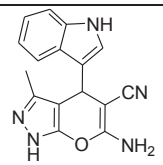
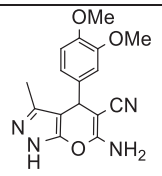


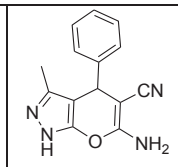
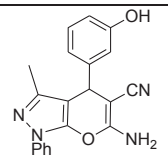
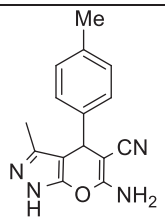
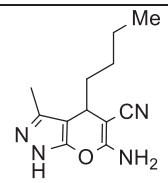
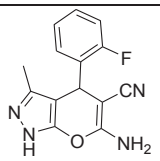
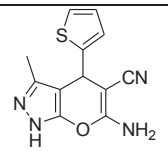
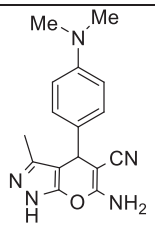
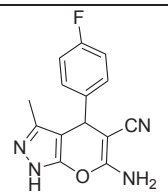
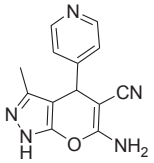
Scheme 2. Route B, one-step grinding protocols for synthesizing pyranopyrazoles

Table 1 presents all the pyranopyrazole compounds that were prepared for antimicrobial activity. The compounds were synthesised by both route A and route B, and their reaction timings and the respective isolated yields have been detailed in Table 1. A look at the table brings out the clear conclusion that the one-step scheme gave comparatively better yields to the two-step scheme with the additional benefit of

significantly reduced reaction run times. A range of variously substituted aldehydic compounds were selected for synthesis. As shown in Table 1, the strongly electron-donating OH-substituted compound **2** that mimicked **18**, was prepared followed by its 4-hydroxy analogue **1**. Similarly, mildly electron-withdrawing group (**3, 4, 9, 12, 13**), strongly electron-withdrawing (**5**), heterocyclic (**6, 11, 14, 15, 16, 20**), and some compounds having non-polar moieties (**7, 8, 19**) were also inducted into the pyranopyrazole core and tested consequently for antibacterial and antifungal activity.

Table 1. Characterization of the synthesised pyranopyrazoles by route A and B

No.	Structure	% yield[a] (time)[b]		Mp (°C)	Ref[c]	No.	Structure	% yield[a] (time)[b]		Mp (°C)	Ref[c]
		A[d]	B[e]					A[d]	B[e]		
1		84 (15)	85 (5)	223-225	20	12		79 (25)	80 (8)	175-176	14
2		85 (20)	88 (5)	230-232	-	13		75 (18)	80 (8)	250-252	-
3		80 (22)	82 (5)	177-178	14	14		90 (15)	82 (5)	240-242	15(a)
4		79 (18)	81 (6)	223-224	19	15		89 (16)	85 (5)	220-222	-
5		85 (24)	82 (7)	191-192	20	16		76 (20)	79 (6)	230-231	-
6		87 (15)	91 (5)	210-211	-	17		89 (23)	89 (7)	194-195	11

7		90 (18)	90 (8)	169-170	20	18		85 (15)	84 (5)	169-170	22
8		85 (20)	90 (6)	220-221	20	19		72 (25)	70 (10)	239-240	13(b)
9		70 (19)	74 (6)	267-268	-	20		87 (20)	85 (7)	240-241	15(b)
10		85 (18)	90 (5)	232-234	22	21		78 (18)	80 (6)	244-246	11
11		92 (15)	92 (6)	228-230	9(c)						

[a]isolated yields; [b]time in minutes; [c]end-of-text reference; [d]Route A; [e]Route B

The SAR analysis of the microbial activity results shown in Table 2 and their graphical illustration as depicted in Figure 2 and Figure 3 brings out the following facts. Considering only the antibacterial activity first, it is found that compound **4** bearing the *m*-Br substituent is the solitary compound that is mildly active against all the three strains. In fact, most of the pyranopyrazoles are only slightly active against the Gram-negative strains while only analogue **4** showed activity against Gram-positive strains. Significantly, none of the compounds is active against *S. aureus*, the Gram-positive bacterial strain. At least a couple of inferences are unequivocal. Comparing the activities of analogs **2** (bearing N-H) and **18** (bearing N-Ph), it becomes obvious that the presence of N-H bearing HBD (Hydrogen-Bond Donor) ability as opposed to the presence of N-Ph in the pyrazole moiety increases the activity. Furthermore, analogues **2** and **6** bearing HBD capacity are the most potent among all the compounds. Although it may be too early to generalise, nevertheless based on the preliminary activity data available in Table 2, the SAR reveals that the presence of HBD capable group at the 3-position (Figure 1) of the top right sector of the pharmacophore might be crucial for improved activity against Gram-negative bacterial strains. Similarly, looking at the antifungal activity data in isolation, it is found that in general, the synthesised pyranopyrazoles are relatively more

active against tested fungal strains than the tested bacterial strains. A total of eleven compounds are active against fungal strains. Also, the fact emerges that among the compounds active against fungi there seems discrimination on the basis of position or nature of the attached groups. Now, considering the overall of the activity results, it can be safely concluded that the compounds that are active against bacteria inactive against the fungal strains and vice versa. In other words, the tested compounds show complementary activity. Interestingly, comparing our antifungal results with a previous report,[12] our compounds returned better activity. Of course, this inference requires further investigation before a generalization can be made.

Table 2. Antibacterial and antifungal activities of the synthesised pyranopyrazoles

	Bacterial strains						**Fungal strains**					
No	*E. coli*		*S. aureus*		*Pseudomonas putida*		*F. oxysporum*		*F. gramillarium*		*F. monalliforme*	
	inhib zone[a]	MIC[b]	inhib zone	MIC	inhib zone	MIC	inhib zone	MIC	inhib zone	MIC	inhib zone	MIC
1	13	128	-	-	-	-	-	-	-	-	-	-
2	18	64	-	-	16	64	-	-	-	-	-	-
3	14	128	-	-	-	-	-	-	-	-	-	-
4	12	128	13	>128	14	>128	-	-	-	-	-	-
5	12	128	-	-	14	>128	-	-	-	-	-	-
6	18	64	-	-	14	128	14	64	15	64	14	64
7	14	128	-	-	-	-	14	64	14	64	15	64
8	15	128	-	-	-	-	14	64	15	64	15	64
9	15	128	-	-	-	-	15	128	15	128	-	-
10	12	128	-	-	12	>128	14	>64	-	-	16	64
11	0	-	-	-	-	-	-	-	-	-	-	-
12	0	-	-	-	-	-	16	64	14	64	14	64
13	0	-	-	-	-	-	14	64	14	>64	14	>64
14	0	-	-	-	-	-	16	>64	16	>64	16	>64
15	0	-	-	-	-	-	16	64	16	64	18	64
16	0	-	-	-	-	-	16	128	16	128	18	>64
17	0	-	-	-	-	-	14	>64	14	>64	16	>64
18	0	-	14	>128	15	>128	-	-	-	-	-	-
19	0	-	-	-	-	-	-	-	-	-	-	-
20	0	-	-	-	-	-	-	-	-	-	-	-
21	0	-	-	-	-	-	-	-	-	-	-	-
22[c]	22	16	22	16	22	16	-	-	-	-	-	-
23[d]	-	-	-	-	-	-	20	30	18	30	20	30

[a]zone of inhibition in mm; [b]Minimum Inhibitory Concentration assay in μg/mL; [c]control: Chloramphenicol (used for antibacterial assay only); [d]control: Amphotericin-B (used for antifungal assay only).

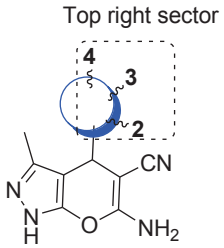


Figure 1. Depicting the top right sector (dotted) where the 3-position is found crucial for antibacterial activity

In conclusion, we have developed a novel, benign, and fast four-component, one-pot, solvent-free grinding based MCR strategy for obtaining a variety of useful pyranopyrazole compounds. The three-component and the four-component protocols were fully investigated and their relative reaction yields and reaction timings well contrasted. The analogues were designed and chosen for synthesis based on the initial activity of compound **18.** The synthesized compounds were tested against a Gram-positive and two Gram-negative bacterial strains and three different fungi and many of the molecules were found to be active against the fungal assays. Two of the synthesized compounds (**2** and **6**) turned out to be more potent against Gram-negative bacteria than the others. The results of these observation and the subsequent SAR pointed to the significant conclusion that the presence of H-bond donors at the 3-postion of the top right sector of the heterocyclic moiety of the pyranopyrazole pharmacophore may play a crucial role in enhancing the antimicrobial activity. Interestingly, compounds active against bacteria were generally inactive against fungi and vice versa. Further investigations as proof of concept of our findings are currently being established in our lab as the ultimate aim of such a study is to design compounds against multidrug resistant microbes.

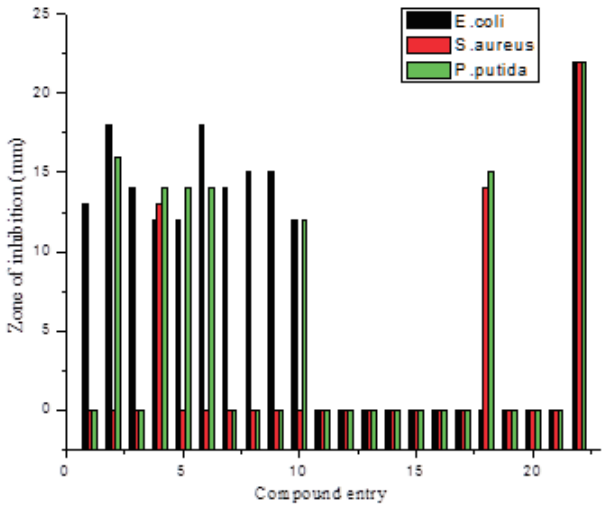


Figure 2. Shows Zone of Inhibition vs Compound entry for *E. coli, S. aureus, Pseudomonas putida*

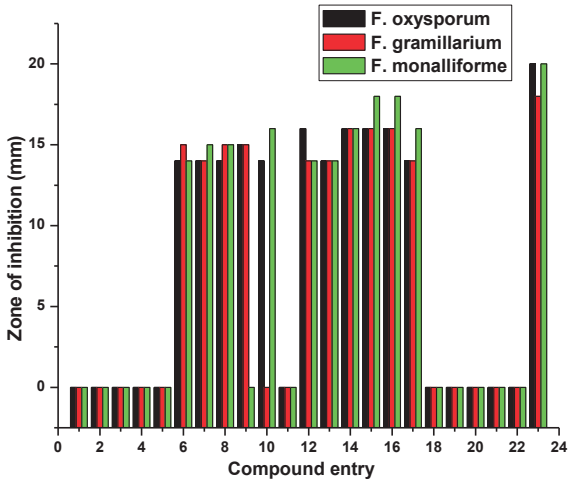


Figure 3. Zone of Inhibition vs Compound entry for *F. oxysporum, F. gramillarium, F. monalliforme*

EXPERIMENTAL

Materials & Methodology for antibacterial assay[27]

All the synthesized compounds were evaluated for their *in vitro* antibacterial activity against two Gram-negative bacterial strains: *E. coli* and *Pseudomonas putida* and one Gram-positive bacteria *Staphylococcus aureus* and their activities were compared with a commercially available broad spectrum antibiotic Chloramphenicol. For the experimental work, 20 mL of autoclaved Mueller-Hinton (Himedia, India) agar medium was poured into glass petri plates and the agar plates were swabbed with 100 µL inocula of each test organism (10^6 cfu/mL). After the adsorption, well size of 6 mm diameter was made by the sterile metallic borer and the solution of working compound (128 µg/mL) was poured into the wells. The plates were incubated at 37 °C for 18-24 h. DMSO was used as a negative control. MIC assay (Minimum Inhibitory Concentration assay) was performed to determine the lowest concentrations of compound necessary to inhibit the visible growth of microorganisms. MIC values were evaluated for all the compounds using broth microdilution method. Assay was carried out for the compounds at 16, 32, 64, 128 µg/mL concentrations. A set of tubes containing Mueller-Hinton broth medium with different concentrations of compound were prepared. The tubes were inoculated with bacterial cultures (10^6 cfu/mL) and incubated on a rotary shaker (180 rpm) at 37 °C for 18-24 h under dark conditions. MIC value was defined as lowest concentrations of compound that prevented the visible growth of the bacteria after the incubation period. Chloramphenicol was used as a positive control at a concentration of 16 µg/mL.

Materials & Methodology for antifungal assay:[28] Antifungal activities of chemically synthesized compounds were determined by agar well diffusion method at a varying concentration for all the compounds. For the experimental work, Potato dextrose broth (PDB, Himedia, India) was freshly

prepared and autoclaved at 121 °C at 15 psi for 15 min. After cooling to room temperature a loopful of fungal mycelium was inoculated into freshly prepared PDB broth for 4-5 days at 28 °C. After the proper growth, 100 µL of this inoculum was uniformly spread on the Potato dextrose agar plate. Following the adsorption of inoculum, well size of 9 mm diameter was prepared by the sterile metallic borer and compound solution was added in respective wells. Plates were incubated at 28 °C for 4 days under dark conditions. Mean diameter of inhibition zone was measured to determine the antifungal activity. The experiment was performed in triplicates. MIC assay of all the compounds was performed at 16.0, 32.0, 64.0, 128.0 µg/mL concentrations. Tubes containing 10 mL of sterilized czapeks dox broth medium was inoculated with 100 µL of freshly grown culture. Appropriate amount of compound was added to achieve the desired concentrations. The tubes were incubated at 28 °C for 4 days under dark conditions and carefully observed for the presence of turbidity.

Synthesis of 6-amino-1,4-dihydro-4-(4-hydroxyphenyl)-3-methylpyrano[2,3-*c*]pyrazole-5-carbonitrile (1): Route A (Two-step, three component grinding protocol): ethyl acetoacetate (2.53 mL, 20.0 mmol) and hydrazine hydrate (0.98 mL, 20.0 mmol) were ground together in a mortar with pestle for 5 min without adding any solvent or catalyst. 3-Methyl-1*H*-pyrazol-5(4*H*)-one so formed was collected and then grinded again by adding the 3-hydroxybenzaldehyde (2.68 g, 22.0 mmol), malononitrile (1.10 mL, 20.0 mmol) and triethylamine (5.55 mL, 40.0 mmol) as base for further 10 min. The weight of the product (**1**) obtained was 4.50 g (84% yield). **Route B** (one-step, four component grinding protocol synthesis): all the four reactants were added together followed by triethylamine in the same proportion as in route A in the mortar and ground with the pestle for 5 min. The reaction was continuously monitored by TLC. After the reaction, the contents of the flask were diluted with dichloromethane (5 mL), filtered, and subjected to column chromatography to get 4.56 g (85% yield) of the pure compound **1**.

6-Amino-1,4-dihydro-4-(3-hydroxyphenyl)-3-methylpyrano[2,3-*c*]pyrazole-5-carbonitrile (2): synthesised by the same method as described above. The compound was obtained as an off-white solid (mp 230-232 °C, 230 mg, 88% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.97 (s, 1H), 9.17 (2H), 7.06 (m, 4H), 4.45 (s, 1H), 1.84 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 160.6, 157.2, 145.3, 135.5, 128.8, 78.5, 78.4, 78.2, 77.8, 58.2, 9.7. HRMS calcd for $C_{14}H_{12}N_4O_2$ 268.096, found 268.099.

6-Amino-1,4-dihydro-4-(*1H*-indol-3-yl)-3-methylpyrano[2,3-*c*]pyrazole-5-carbonitrile (6): synthesised by the same method as described above. The compound was obtained as a white solid (mp 210-211 °C, 265 mg, 91% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.01 (s, 1H), 10.86 (1H), 6.84-7.36 (5H), 6.75 (2H), 4.85 (s, 1H), 1.77 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 160.5, 154.9, 136.8, 135.5,

125.5, 121.0, 120.7, 118.1, 116.7, 11.5, 78.7, 58.1, 28.2, 9.6. HRMS calcd for $C_{16}H_{13}N_5O$ 291.112, found 291.111.

6-Amino-4-(2-fluorophenyl)-1,4-dihydro-3-methylpyrano[2,3-*c*]pyrazole-5-carbonitrile (9): synthesised by the same method as described above. The compound was obtained as a dirty white solid (mp 267-268 °C, 200 mg, 74% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.13 (s, 1H), 6.94 (2H), 7.24 (m, 4H), 4.87 (s, 1H), 1.81 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 161.3, 154.9, 135.2, 129.7, 128.5, 124.5, 115.4, 99.5, 96.5, 78.8, 55.6, 29.8, 9.4. HRMS calcd for $C_{14}H_{11}FN_4O$ 270.092, found 270.095.

6-Amino-4-(2-iodophenyl)-3-methyl-1,4-dihydropyrano[2,3-*c*]pyrazole-5-carbonitrile (13): synthesised by the same method as described above. The compound was obtained as an off-white solid (mp 250-252 °C, 302 mg, 80% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.15 (s, 1H), 7.01-7.35 (multiplet, 4H), 6.75 (2H), 4.96 (s, 1H), 1.76 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 160.9, 154.8, 135.4, 128.6, 120.0, 78.4, 56.3, 18.3, 9.9. HRMS calcd for $C_{14}H_{11}IN_4O$ 377.998, found 377.996.

6-Amino-3-methyl-4-(1H-pyrrol-2-yl)-1,4-dihydropyrano[2,3-*c*]pyrazole-5-carbonitrile (15): synthesised by the same method as described above. The compound was obtained as a light creamy yellow solid (mp 220-222 °C, 205 mg, 85% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.05 (s, 1H), 10.51 (1H), 6.56-6.73 (3H), 5.84 (2H), 4.64 (s, 1H), 1.84 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 160.68, 154.66, 135.61, 133.22, 120.85, 116.87, 106.67, 105.10, 96.71, 78.60, 56.94, 29.56, 9.44. HRMS calcd for $C_{12}H_{11}N_5O$ 241.096, found 241.098.

6-Amino-3-methyl-4-(thiophen-3-yl)-1,4-dihydropyrano[2,3-*c*]pyrazole-5-carbonitrile (16): synthesised by the same method as described above. The compound was obtained as a pale solid (mp 230-231 °C, 205 mg, 79% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.10 (s, 1H), 7.28 (1H), 7.26 (2H), 6.83 (2H), 4.73 (s, 1H), 1.86 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 160.70, 154.59, 145, 135.50, 126.62, 125.95, 120.72, 96.89, 78.53, 57.07, 31.34, 9.62. HRMS calcd for $C_{12}H_{10}N_4OS$ 258.058, found 258.057.

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