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ORIGINAL PAPER

Microwave-assisted, solvent-free, one-pot, three-component synthesis of fused pyran derivatives containing benzothiazole nucleus catalyzed by pyrrolidine-acetic acid and their biological evaluation

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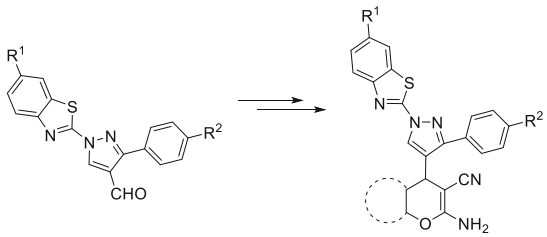
Abstract An efficient method for the synthesis of fused pyran derivatives has been developed by a one-pot, three-component, solvent-free reaction of 1*H*-pyrazole-4-carbaldehyde and various active methylenes and malononitriles under microwave irradiation in the presence of pyrrolidine-acetic acid as bifunctional catalyst. The salient features of this protocol are the solvent-free reaction, shorter reaction time, greater selectivity, and straightforward workup procedure. All the synthesized compounds were confirmed by analytical and spectral data. The synthesized compounds were investigated against a representative panel of pathogenic strains using the broth microdilution MIC method for their in vitro antimicrobial activity.

Graphical abstract



Keywords Microwave · Solvent free · Fused pyran · Benzothiazole · Pyrrolidine-acetic acid

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Introduction

Fused pyran derivatives represent an important class of compounds, which possess a broad spectrum of biological activities such as anticancer, antiviral, antimicrobial, antimalarial, anti-HIV, antituberculosis, antiinflammatory, and antifungal [1–10]. Other biologically active moiety chromenes were found in plants [11], and the synthesis of new pharmacophores for medicinal chemistry has been used as a valuable lead for the design and development of drugs. Benzothiazole is also a bioactive nucleus that covers a large domain of pharmacological activities serving as anti-microbial, antitumor, anti-inflammatory, and analgesic agents [12–14].

Multicomponent reactions (MCRs) have been developed as an efficient, environmentally friendly, and economic protocol in the current decade for a range of pharmacologically important compounds. These multicomponent reactions have become an important tool for atomic economy, simple purification of products, and rapid generation of molecular complexity, diversity with predefined functionality in chemical biology, and drug discovery [15–17]. Small organic molecules such as amino acids have shown promising and highly efficient catalytic activities for multicomponent reactions. However, among all the various organocatalysts, the pyrrolidine-acetic acid catalyst is one of the most versatile for many important organic reactions and asymmetric transformations such as Michael, Mannich, Aldol, and Diels-Alder reactions [18–21].

Previously, fused pyran derivatives were prepared by using different synthetic approaches [22–29]. However, some limitations were observed in these methods such as longer reaction times, difficulties in the workup, and poor yield with various solvents. In the present study, we have used some acid as well as base catalysts such as

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CH_3COOH, *p*-TsOH, piperidine, K_2CO_3, and NaOH; they did not lead to formation of the desired product. Also, L-proline was a weak catalyst for this reaction; however, these catalysts have some drawbacks such as side products, low yields, and lesser selectivity. We thus developed an alternative solvent-free, microwave-assisted organic synthesis (MAOS) approach using pyrrolidine-acetic acid as an efficient catalyst, which facilitates designing the title derivatives and avoids the use of volatile organic solvents.

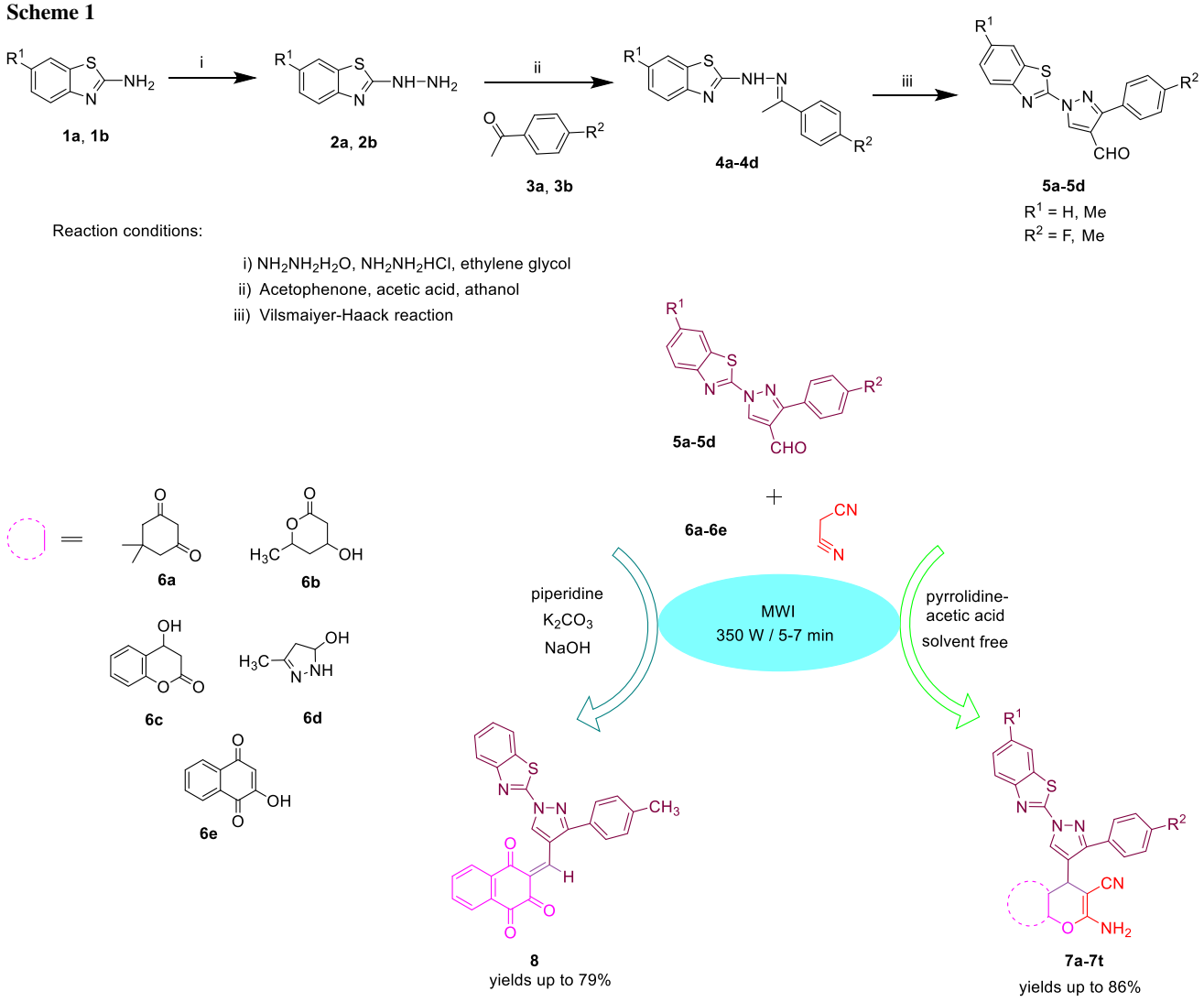
In continuation of our previous investigations on biopotent heterocycles [30–34], our efforts are focused to design and synthesize biologically potent heterocyclic systems via a one-pot, three-component, solvent-free reaction triggered by 10 mol% pyrrolidine-acetic acid (Scheme 1) under microwave assistance assuming the assimilation of more than one biopotent nucleus in a single scaffold.

Results and discussion

Analytical results

Compounds 1-(substituted benzo[*d*]thiazol-2-yl)-3-(substituted phenyl)-1*H*-pyrazole-4-carbaldehyde **5a–5d** were prepared by Vilsmeier-Haack reaction, according to the literature procedure [35, 36]. Reaction of 1-(benzo[*d*]thiazol-2-yl)-3-(4-methylphenyl)-1*H*-pyrazole-4-carbaldehyde (**5b**) (10 mmol), compound **6e** (10 mmol), malononitrile (10 mmol), and a catalytic amount of pyrrolidine-acetic acid (10 mol%) afforded compound **7j** (Table 1). Initially, the reaction was investigated using different catalysts, such as piperidine, K_2CO_3, and NaOH, which were tried as base catalysts for the model reaction. They did not lead to formation of the desired product **7a–7t** but formed compound **8**. Only pyrrolidine-acetic acid catalyst surprisingly led to

Scheme 1



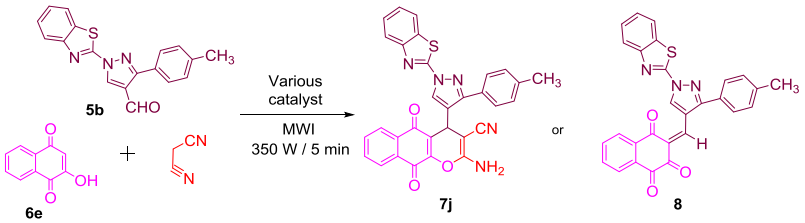
the reaction pathway being directed toward the formation of compounds **7a**–**7t**.

Different catalysts were attempted, and the result is shown in Table 1. CH_3COOH and *p*-TsOH were also used to try to obtain the desired product **7j**. The reaction did not occur even after a long reaction time of up to 10 min (entries 1 and 2). It was also investigated by employing a variety of bases including piperidine (entry 3), K_2CO_3 (entry 4), and NaOH (entry 5), which resulted the Knoevenagel adduct in all cases. In case of the catalyst-free (entry 6) condition, the reaction failed to occur. In addition, we performed the reaction with a bi-functional catalyst such as L-proline and pyrrolidine-acetic acid catalyst; L-proline was found to push the reaction efficiently in the proper direction, but it was a poor catalyst of this reaction, and we obtained a lower yield (up to 37%) (entry 7). Using pyrrolidine-acetic acid, we obtained the best result (up to 86%) under solvent-free conditions (entry 8).

Furthermore, we checked the effects of varying amounts of catalyst loading without and with different solvents (Table 2). It was very surprising to observe that the reaction pathway was directed toward the formation of compounds **7a**–**7t**. The reaction was performed using 5, 10, 15 and 20 mol% of the catalyst and was monitored for 5–7 min. It was observed that 10 mol% (entry 2) of the catalyst loading provided the maximum yield (86%) in 5 min, while 5 and 15 mol% (entries 1, 3) of the catalyst afforded 74 and 80% of the product even after irradiation for 7 min. An additional increase of the catalyst loading to 20% (entry 4) did not improve the yield (68%). On the contrary, the reaction slowed down on adding more than 20 mol% of the catalyst. These pyrrolidine-acetic acid catalysts were also screened in the same way but using a series of different solvents. When the reaction was performed in acetonitrile (entry 5), *n*-butanol (entry 7), DMF (entry 9) and THF (entry 10) were obtained in lower yields of products **7a**–**7t**, whereas in water (entry 8) we failed to obtain products **7a**–**7t**. The best results were obtained in methanol (entry 6) and ethanol (entry 11). Surprisingly, however, when the reaction was carried out under solvent-free conditions, both the yield and reaction time were significantly improved. In this study pyrrolidine-acetic acid emerged as a model catalyst under solvent-free conditions and was employed for the synthesis of all other targeted compounds **7a**–**7t**.

The structures of all the newly synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, FT-IR, elemental analysis, and mass spectrometry. The IR spectrum of compounds exhibited characteristic absorption bands 3450 and 3290 (asym. and sym. str. of NH_2), 2200 (C≡N str.), and 1682 (C=O str.) cm^{-1}. The ^{1}H NMR spectrum of compound **7j** indicated the presence of one singlet peak at the $\delta = 4.66$ ppm of –CH (H5) proton and the disappearance of a singlet from 10.12 ppm of –CHO, which clearly

Table 1 Effect of different catalysts on the model reaction under microwave irradiation



Entry	Catalyst (10 mol%)	Solvent	Time/min	Yield/%
1	CH_3COOH	Ethanol	10	–[a]
2	*p*-TsOH	Ethanol	10	–[a]
3	Piperidine	Ethanol	6	76[b]
4	K_2CO_3	Ethanol	7	65[b]
5	NaOH	Ethanol	5	79[b]
6	–	Ethanol	7	–[a]
7	L-Proline	Ethanol	7	37[c]
8	Pyrrolidine-acetic acid (1:1)	–	5	86[c]

Reaction conditions: 1*H*-pyrazole-4-carbaldehyde **5b** (10 mmol), compound **6e** (10 mmol), malononitrile (10 mmol), and different catalysts (10 mol%) under microwave irradiation, time 5–10 min

[a] Reaction failed to occur

[b] Isolated yield of compound **8**

[c] Isolated yield of compound **7j**

Table 2 Screening of catalysts and solvents in the model reaction under microwave irradiation

Entry	Catalyst (mol%)	Solvent	Yield[a]/%
1	Pyrrolidine-acetic acid (5)	–	74
2	Pyrrolidine-acetic acid (10)	–	86
3	Pyrrolidine-acetic acid (15)	–	80
4	Pyrrolidine-acetic acid (20)	–	68
5	Pyrrolidine-acetic acid (10)	Acetonitrile	49
6	Pyrrolidine-acetic acid (10)	Methanol	72
7	Pyrrolidine-acetic acid (10)	*n*-Butanol	52
8	Pyrrolidine-acetic acid (10)	Water	–
9	Pyrrolidine-acetic acid (10)	DMF	52
10	Pyrrolidine-acetic acid (10)	THF	56
11	Pyrrolidine-acetic acid (10)	Ethanol	78

Reaction conditions: 1*H*-pyrazole-4-carbaldehyde **5b** (10 mmol), compound **6e** (10 mmol), malononitrile (10 mmol), and pyrrolidine-acetic acid as catalysts in various solvents under microwave irradiation, time 5–7 min

[a] Isolated yield of compound **7j**

Table 3 Substituent pattern and reaction condition for the synthesized compounds **7a–7t**

Entry[a]	Prod.	R^1	R^2	Het.	Time/min	Yield[b]/%
1	**7a**	H	F	Dimedone	7	71
2	**7b**	H	F	4-Hydroxy-6-methylpyran-2-one	5	78
3	**7c**	H	F	4-Hydroxycoumarin	5	73
4	**7d**	H	F	3-Methyl-5-hydroxypyrazole	5	68
5	**7e**	H	F	2-Hydroxy-1,4-naphthoquinone	7	75
6	**7f**	H	Me	Dimedone	6	83
7	**7g**	H	Me	4-Hydroxy-6-methylpyran-2-one	5	72
8	**7h**	H	Me	4-Hydroxycoumarin	7	83
9	**7i**	H	Me	3-Methyl-5-hydroxypyrazole	7	73
10	**7j**	H	Me	2-Hydroxy-1,4-naphthoquinone	5	86
11	**7k**	Me	F	Dimedone	6	78
12	**7l**	Me	F	4-Hydroxy-6-methylpyran-2-one	5	75
13	**7m**	Me	F	4-Hydroxycoumarin	5	81
14	**7n**	Me	F	3-Methyl-5-hydroxypyrazole	7	75
15	**7o**	Me	F	2-Hydroxy-1,4-naphthoquinone	6	70
16	**7p**	Me	Me	Dimedone	6	79
17	**7q**	Me	Me	4-Hydroxy-6-methylpyran-2-one	6	70
18	**7r**	Me	Me	4-Hydroxycoumarin	7	84
19	**7s**	Me	Me	3-Methyl-5-hydroxypyrazole	7	76
20	**7t**	Me	Me	2-Hydroxy-1,4-naphthoquinone	5	82

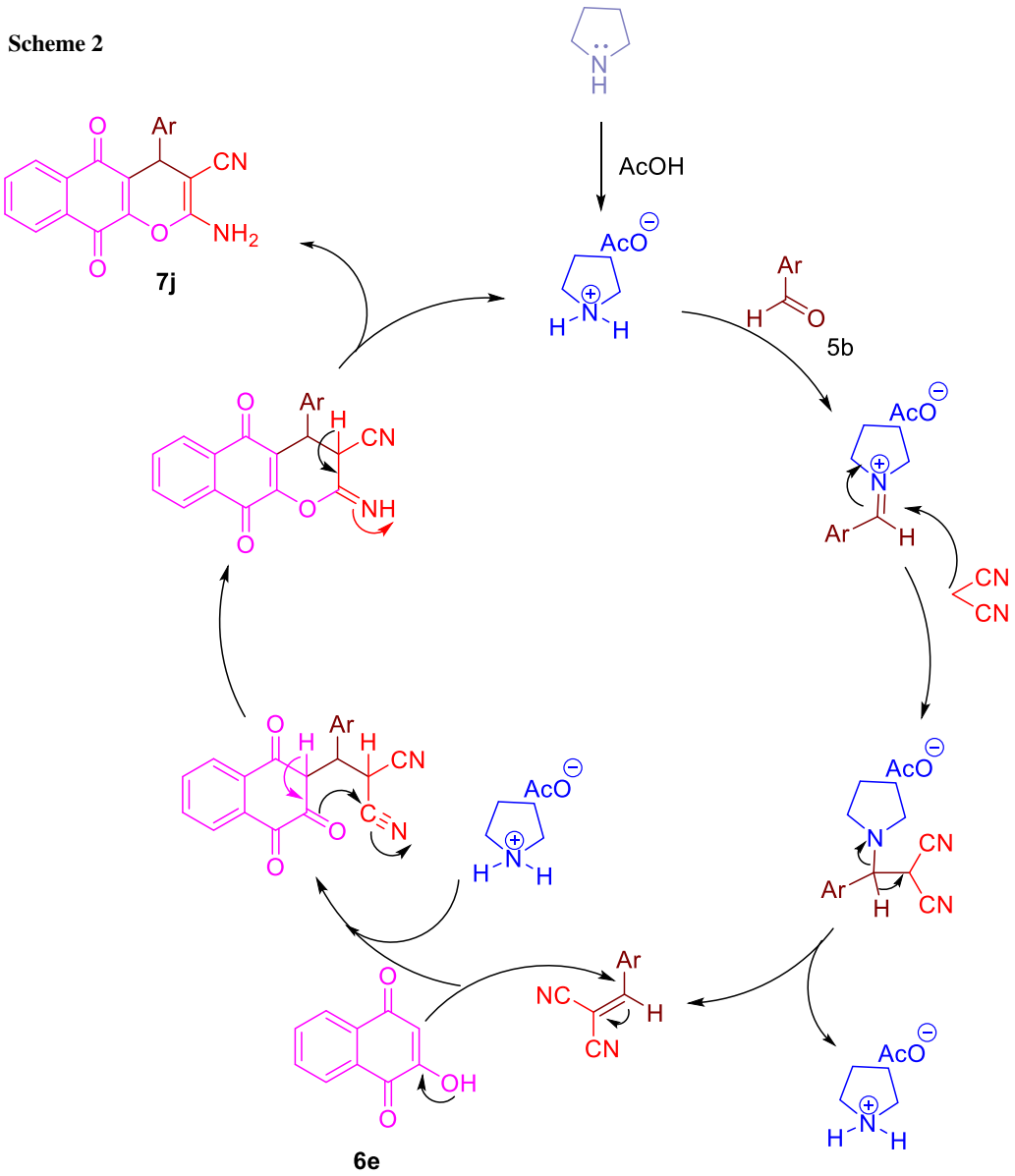
[a] Reaction conditions: 1*H*-pyrazole-4-carbaldehyde **5a–5d** (10 mmol), compound **6a–6e** (10 mmol), malononitrile (10 mmol), and pyrrolidine-acetic acid as catalyst (10 mol%) under microwave irradiation, time 5–7 min

[b] Isolated yield of compound **7a–7t**

confirmed the cyclization of the Knoevenagel intermediate. The singlet at 7.31–7.12 ppm exhibits (Ar–H+NH_2) protons of the pyran ring (Table 3).

The mechanistic pathway for the development of fused pyran derivative **7j** catalyzed by pyrrolidine-acetic acid is outlined in Scheme 2. The reaction proceeded via in situ formation of the heterylidenenitrile, containing the electron-poor C=C double bond from the Knoevenagel condensation between pyrazolyl aldehydes **5b** and malononitrile by loss of water molecules. Finally, Michael addition of compound **6e** to the formed unsaturated nitrile yielded an intermediate. The enolate O-atom of the formed

Scheme 2



intermediate attacked the nitrile group and a subsequent H-atom shift led to the final compound **7j**.

Antimicrobial activity

Investigation of the antimicrobial activity data (Table 4) revealed that some compounds showed good to excellent antibacterial and antifungal activity against the representative species when compared with the standard drugs such as ampicillin, ciprofloxacin, norfloxacin, nystatin, and griseofulvin.

Against the gram-positive bacteria *B. subtilis*, compounds **7b**, **7m**, and **7t** (MIC = 100 μg/cm^3) were found to be equipotent as compared to ampicillin (MIC = 100 μg/cm^3). Compounds **7c**, **7e**, **7f**, **7k**, **7m**, and **7t** (MIC = 100 μg/cm^3) elicited excellent activity as compared to ampicillin (MIC = 250 μg/cm^3) and had similar potency as compared to ciprofloxacin (MIC = 100 μg/cm^3) against *C. tetani*. Compound **7g** (MIC = 100 μg/cm^3) was found equipotent, whereas compound **7m** (MIC = 62.5 μg/cm^3) showed excellent activity compared to ampicillin (MIC = 100 μg/cm^3) against *S. aureus*.

Against the gram-negative bacteria *E. coli*, compound **7n** (MIC = 62.5 μg/cm^3) showed the highest activity, whereas compounds **7c** and **7d** (MIC = 100 μg/cm^3) showed comparable activity to ampicillin (MIC = 100 μg/cm^3). Against *S. typhi*, compounds **7h**, **7n**, **7o**, and **7q** (MIC = 100 μg/cm^3) were found

Table 4 In vitro antimicrobial activity of **7a–7t** MICs (μg/cm^3)

Sr. no.	Comp.	Gram-positive bacteria			Gram-negative bacteria			Fungi	
		BS MTCC 441	CT MTCC 449	SA MTCC 96	EC MTCC 443	ST MTCC 98	VC MTCC 3906	CA MTCC 227	TR MTCC 296
1	**7a**	250	250	500	200	200	250	>1000	>1000
2	**7b**	**100**	250	500	250	500	250	1000	500
3	**7c**	250	**100**	500	**100**	200	**100**	**500**	1000
4	**7d**	250	500	250	**100**	125	250	1000	250
5	**7e**	200	**100**	500	200	250	**200**	**500**	1000
6	**7f**	250	**100**	250	500	500	250	1000	>1000
7	**7g**	200	500	**100**	250	250	**200**	1000	1000
8	**7h**	200	250	500	125	**100**	250	**250**	1000
9	**7i**	500	250	250	250	250	500	1000	1000
10	**7j**	500	500	200	200	200	500	**500**	500
11	**7k**	250	**100**	125	200	250	**200**	1000	500
12	**7l**	250	250	250	200	250	250	1000	>1000
13	**7m**	**100**	**100**	**62.5**	200	125	**125**	**250**	**100**
14	**7n**	250	250	250	**62.5**	**100**	250	**250**	500
15	**7o**	125	500	500	125	**100**	**100**	**250**	500
16	**7p**	500	500	500	200	250	500	1000	1000
17	**7q**	250	250	250	125	**100**	**125**	**500**	1000
18	**7r**	500	**200**	200	**100**	200	**100**	1000	>1000
19	**7s**	250	250	500	250	500	250	1000	>1000
20	**7t**	**100**	**100**	500	200	250	**100**	**250**	500
Ampicillin		100	250	100	100	100	250	–	–
Chloramphenicol		50	50	50	50	50	50	–	–
Ciprofloxacin		50	100	25	25	25	50	–	–
Norfloxacin		10	50	10	10	10	100	–	–
Nystatin		–	–	–	–	–	–	100	100
Griseofulvin		–	–	–	–	–	–	500	100

Bold numbers indicate more or equivalent potent compounds compared to standard drugs

BS, *Bacillus subtilis*; CT, *Clostridium tetani*; SA, *Staphylococcus aureus*; EC, *Escherichia coli*; ST, *Salmonella typhi*; VC, *Vibrio cholerae*; CA, *Candida albicans*; TR, *Trichophyton rubrum*. MTCC, microbial type culture collection

equipotent to ampicillin (MIC = 100 μg/cm^3). Compounds **7c**, **7o**, **7r**, **7t** (MIC = 100 μg/cm^3), **7m**, **7q** (MIC = 125 μg/cm^3), and **7e**, **7g**, **7k** (MIC = 200 μg/cm^3) were more potent compared to ampicillin (MIC = 250 μg/cm^3), and compounds **7c**, **7o**, **7r**, and **7t** demonstrated equipotent activity to norfloxacin (MIC = 100 μg/cm^3) against *V. Cholerae*.

For exploration of anti-fungal screening (Table 4), against *C. albicans*, compounds **7h**, **7m**, **7n**, **7o**, and **7t** (MIC = 250 μg/cm^3) showed excellent activity, and compounds **7c**, **7e**, **7j**, and **7q** (MIC = 500 μg/cm^3) were equally active compared to griseofulvin (MIC = 500 μg/cm^3). Against *T. rubrum*, compound **7m** (MIC = 100 μg/cm^3) was found equipotent compared to nystatin and griseofulvin (MIC = 100 μg/cm^3); the remaining compounds showed poor antifungal activity compared to nystatin and griseofulvin.

Conclusion

In summary, we have introduced a highly efficient microwave-assisted, one-pot, three-component protocol for the synthesis of fused pyran derivatives in the presence of pyrrolidine-acetic acid under solvent-free conditions. This protocol makes use of simple and readily available precursors such as 1-(substituted benzo[*d*]thiazol-2-yl)-3-(substituted phenyl)-1*H*-pyrazole-4-carbaldehyde **5a–5d**, compounds **6a–6e**, and malononitrile. The present study expands the scope of the area dealing with fused pyran derivatives bearing benzothiazole nuclei, considering their potent antimicrobial activities. Some magnificent results have been obtained with the fused pyran scaffold. Compounds **7c**, **7e**, **7h**, **7m**, **7n**, **7o**, **7q**, **7r**, and **7t** were found to be the most efficient antimicrobials in the series. It is worth

mentioning that fused pyran derivatives bearing a benzothiazole nucleus have become a vital area of antimicrobial medicine research.

Experimental

All reactions were performed with commercially available reagents and were used without further purification. All reactions were monitored by thin-layer chromatography (TLC, on aluminum plates coated with silica gel $60F_{254}$, 0.25 mm thickness, Merck) carried out on fluorescent-coated plates, and detection of the components was made by exposure to iodine vapors or UV light. Melting points of all the title compounds were determined by the open tube capillary method (using silicon oil 350 cst). The microwave-assisted reactions were conducted in a "RAGA's Modified Electromagnetic Microwave System" whereby microwaves are generated by a magnetron at a frequency of 2450 MHz, having adjustable output power levels, i.e., ten levels from 140 to 700 W, and with an individual sensor for temperature control (fiber optic is used as an individual sensor for temperature control) with attachment of the reflux condenser with constant stirring (thus avoiding the risk of high pressure development). The IR spectra were recorded on a PerkinElmer Spectrum GX FT-IR Spectrophotometer (PerkinElmer, USA) using potassium bromide pellets in the range 4000–400 cm^{-1}, and frequencies of only characteristic peaks are expressed in cm^{-1}. Elemental analysis (% C, H, N) was carried out using a PerkinElmer 2400 series-II elemental analyzer (PerkinElmer, USA), and all compounds were within ±0.4% of the theoretical compositions. ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Bruker Avance 400F (MHz) spectrometer (Bruker Scientific Corp., Ltd., Switzerland) using TMS as an internal standard at 400 and 100 MHz, respectively. Chemical shifts are reported in parts per million (ppm). Splitting patterns are designated as s for singlet, d for doublet, and m for multiplet. The ESI mass spectra were scanned on a Shimadzu LCMS 2010 spectrometer (Shimadzu, Tokyo, Japan).

General procedure for the synthesis of fused pyran derivatives ***7a–7t***

In the typical experimental procedure, equimolar amounts of 1-(substituted benzo[*d*]thiazol-2-yl)-3-(substituted phenyl)-1*H*-pyrazole-4-carbaldehyde **5a**–**5d**, compounds **6a**–**6e**, malononitrile, and a catalytic amount of pyrrolidine-acetic acid were placed in the vessel of a microwave reactor and irradiated under microwave for 5–7 min at 350 W output power. Completion of the reaction was confirmed by TLC. After easy isolation of the obtained product, it was purified by recrystallization with methanol and dried. Products **7a**–**7t** were achieved quantitatively (up to 86% yield) with excellent purity.

2-Amino-4-[1-(benzo[d]thiazol-2-yl)-3-(4-fluorophenyl)-1H-pyrazol-4-yl]-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile (**7a**, $C_{28}H_{22}FN_5O_2S$)

Yellow powder; yield 71%; m.p.: 226 °C; IR (KBr): $\bar{\nu}$ = 3445, 3312 (asym., sym. str. of NH_2), 2197 (C≡N str.), 1694 (C=O str.), 1228 (Ar–C–F str.) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.06 (s, 3H, CH_3), 1.13 (s, 3H, CH_3), 2.20–2.48 (4H, m, 2 × CH_2), 4.47 (s, 1H, H4), 7.02 (s, 2H, NH_2), 7.32–8.10 (m, 8H, Ar–H), 8.51 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 25.9, 28.0, 28.7, 32.1, 50.5, 58.3, 112.3, 121.9, 122.4, 128.1, 128.5, 128.8, 129.0, 129.3, 129.4, 129.5, 133.1, 135.1, 138.2, 148.8, 154.0, 158.5, 159.4, 162.7, 196.5 ppm; MS (ESI): *m/z* = 512.12 ($[M+1]^+$).

2-Amino-4-[1-(benzo[d]thiazol-2-yl)-3-(4-fluorophenyl)-1H-pyrazol-4-yl]-7-methyl-5-oxo-4,5-dihydropyrano[4,3-b]pyran-3-carbonitrile (**7b**, $C_{26}H_{16}FN_5O_3S$)

Yellow powder; yield 78%; m.p.: 215 °C; IR (KBr): $\bar{\nu}$ = 3435, 3350 (asym., sym. str. of NH_2), 2190 (C≡N str.), 1690 (C=O str.), 1230 (Ar–C–F str.) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.40 (s, 3H, CH_3) 4.45 (s, 1H, H4), 6.97 (s, 2H, NH_2), 7.10–8.66 (m, 8H, Ar–H), 8.76 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 21.4, 27.1, 57.8, 105.2, 112.7, 115.2, 116.9, 122.0, 122.7, 123.1, 124.9, 127.8, 128.8, 129.6, 131.4, 133.3, 133.4, 136.1, 148.9, 152.6, 153.9, 159.7, 160.7, 161.3 ppm; MS (ESI): *m/z* = 498.10 ($[M+1]^+$).

2-Amino-4-[3-(4-chlorophenyl)-1-phenyl-1H-pyrazol-4-yl]-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile (**7c**, $C_{29}H_{16}FN_5O_3S$)

Light yellow powder; yield 73%; m.p.: 221 °C; IR (KBr): $\bar{\nu}$ = 3455, 3289 (asym., sym. str. of NH_2), 2194 (C≡N str.), 1683 (C=O str.), 1228 (Ar–C–F str.) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 4.75 (s, 1H, H4), 7.21–7.89 (m, 14H, Ar–H+NH_2), 8.80 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 27.3, 57.9, 103.3, 113.7, 115.8, 116.1, 117.1, 122.0, 122.7, 123.1, 125.1, 127.6, 128.6, 129.5, 131.2, 131.3, 133.2, 133.4, 135.3, 148.9, 152.5, 153.5, 154.0, 158.0, 159.2, 160.4, 161.6 ppm; MS (ESI): *m/z* = 534.05 ($[M+1]^+$).

6-Amino-4-[1-(benzo[d]thiazol-2-yl)-3-(4-fluorophenyl)-1H-pyrazol-4-yl]-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**7d**, $C_{24}H_{16}FN_7OS$)

White powder; yield 68%; m.p.: 214 °C; IR (KBr): $\bar{\nu}$ = 3450, 3292 (asym., sym. str. of NH_2), 2204 (C≡N str.), 1232 (Ar–C–F str.) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.81 (s, 3H, pyran), 4.93 (s, 1H, H4), 6.87 (s, 2H, NH_2), 7.32 (m, 8H, Ar–H), 8.55 (s, 1H, pyrazole H5), 12.03 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-

d_6): δ = 10.2, 27.6, 56.5, 96.9, 121.2, 121.9, 122.6, 126.7, 128.4, 128.6, 129.1, 129.2, 133.0, 135.0, 136.0, 138.2, 149.0, 154.2, 154.9, 159.3, 161.3 ppm; MS (ESI): *m/z* = 470.15 ($[M+1]^+$).

2-Amino-4-[1-(benzo[d]thiazol-2-yl)-3-(4-fluorophenyl)-1H-pyrazol-4-yl]-5,10-dioxo-5,10-dihydro-4H-benzo[g]-chromene-3-carbonitrile (**7e**, $C_{30}H_{16}FN_5O_3S$)
Light yellow powder; yield 75%; m.p.: 219 °C; IR (KBr): $\bar{\nu}$ = 3452, 3290 (asym., sym. str. of NH_2), 2201 (C≡N str.), 1679 (C=O str.), 1230 (Ar–C–F str.) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 4.68 (s, 1H, H4), 7.29–8.06 (m, 14H, ArH+NH_2), 8.73 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 27.8, 58.1, 114.2, 114.9, 116.3, 116.8, 120.3, 122.3, 123.4, 125.3, 127.6, 128.6, 129.5, 131.2, 131.3, 133.2, 133.4, 135.3, 148.9, 152.5, 159.2, 160.4, 161.6, 163.1, 176.3, 181.4 ppm; MS (ESI): *m/z* = 546.20 ($[M+1]^+$).

2-Amino-4-[1-(benzo[d]thiazol-2-yl)-3-(p-tolyl)-1H-pyrazol-4-yl]-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile (**7f**, $C_{29}H_{25}N_5O_2S$)
White powder; yield 83%; m.p.: 190 °C; IR (KBr): $\bar{\nu}$ = 3455, 3292 (asym., sym. str. of NH_2), 2203 (C≡N str.), 1682 (C=O str.) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.08 (s, 3H, CH_3), 1.12 (s, 3H, CH_3), 2.18 (s, 3H, CH_3), 2.21–2.54 (m, 4H, 2 × CH_2), 4.48 (s, 1H, H4), 7.03 (s, 2H, NH_2), 7.32–7.98 (m, 8H, Ar–H), 8.51 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 21.4, 25.9, 28.0, 28.7, 32.1, 50.5, 58.3, 112.3, 121.9, 122.4, 128.1, 128.5, 128.8, 129.0, 129.2, 129.4, 129.6, 133.1, 135.1, 138.2, 148.8, 154.0, 158.4, 159.4, 162.7, 196.5 ppm; MS (ESI): *m/z* = 508.15 ($[M+1]^+$).

2-Amino-4-[1-(benzo[d]thiazol-2-yl)-3-(p-tolyl)-1H-pyrazol-4-yl]-7-methyl-5-oxo-4,5-dihydropyrano[4,3-b]pyran-3-carbonitrile (**7g**, $C_{27}H_{19}N_5O_3S$)
Yellow powder; yield 72%; m.p.: 221 °C; IR (KBr): $\bar{\nu}$ = 3450, 3294 (asym., sym. str. of NH_2), 2210 (C≡N str.), 1684 (C=O str.) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.34 (s, 3H, CH_3), 2.41 (s, 3H), 4.47 (s, 1H, H5), 7.01 (s, 2H, NH_2), 7.13-8.56 (m, 8H, Ar–H), 8.71 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 21.4, 21.4, 27.1, 57.8, 105.2, 112.7, 115.2, 116.9, 121.9, 122.7, 123.1, 125.0, 127.8, 128.8, 129.6, 131.4, 133.3, 133.4, 136.1, 149.0, 152.6, 154.0, 159.7, 160.7, 161.3 ppm; MS (ESI): *m/z* = 494.10 ($[M+1]^+$).

2-Amino-4-[1-(benzo[d]thiazol-2-yl)-3-(p-tolyl)-1H-pyrazol-4-yl]-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile (**7h**, $C_{30}H_{19}N_5O_3S$)
White powder; yield 83%; m.p.: 231 °C; IR (KBr): $\bar{\nu}$ = 3451, 3289 (asym., sym. str. of NH_2), 2190 (C≡N str.), 1682 (C=O str.) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.35 (s, 3H, CH_3), 4.73 (s, 1H, H4), 7.23–8.10 (m, 14H, Ar–H+NH_2), 8.81(s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 21.4, 27.2, 57.8, 103.3, 113.7, 115.8, 115.9, 117.0, 121.9, 122.5, 122.9, 125.0, 127.5, 128.6, 129.2, 130.9, 131.2, 132.9, 133.2, 135.1, 148.8, 152.6, 153.5, 154.0, 158.0, 159.2, 160.4, 161.6 ppm; MS (ESI): *m/z* = 530.05 ($[M+1]^+$).

6-Amino-4-[1-(benzo[d]thiazol-2-yl)-3-(p-tolyl)-1H-pyrazol-4-yl]-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**7i**, $C_{25}H_{19}N_7OS$)
Light yellow powder; yield 73%; m.p.: 214 °C; IR (KBr): $\bar{\nu}$ = 3453, 3295 (asym., sym. str. of NH_2), 2193 (C≡N str.) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.80 (s, 3H, CH_3-pyran), 2.35 (s, 3H, CH_3), 4.96 (s, 1H, H4), 6.86 (s, 2H, NH_2), 7.21–8.11 (m, 8H, Ar–H), 8.63 (s, 1H, pyrazole H5), 12.03 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 10.2, 21.4, 27.6, 56.5, 97.0, 121.2, 121.9, 122.5, 126.6, 128.2, 128.4, 129.1, 129.4, 133.0, 135.1, 136.0, 138.4, 149.0, 154.2, 154.9, 159.3, 161.3 ppm; MS (ESI): *m/z* = 466.12 ($[M+1]^+$).

2-Amino-4-[1-(benzo[d]thiazol-2-yl)-3-(p-tolyl)-1H-pyrazol-4-yl]-5,10-dioxo-5,10-dihydro-4H-benzo[g]chromene-3-carbonitrile (**7j**, $C_{31}H_{19}N_5O_3S$)
White powder; yield 86%; m.p.: 224 °C; IR (KBr): $\bar{\nu}$ = 3450, 3290 (asym., sym. str. of NH_2), 2200 (C≡N str.), 1682 (C=O str.) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.36 (s, 3H, CH_3), 4.66 (s, 1H, H4), 7.31–7.12 (m, 14H, Ar–H+NH_2), 8.68 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 21.4, 27.8, 58.1, 114.4, 115.1, 116.4, 116.9, 120.5, 122.4, 123.6, 125.5, 127.8, 128.7, 129.5, 131.5, 131.4, 133.4, 133.7, 135.6, 149.1, 152.5, 159.2, 160.4, 161.5, 163.1, 176.3, 181.4 ppm; MS (ESI): *m/z* = 542.05 ($[M+1]^+$).

2-Amino-4-[3-(4-fluorophenyl)-1-(6-methylbenzo[d]thiazol-2-yl)-1H-pyrazol-4-yl]-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile
(**7k**, $C_{29}H_{24}FN_5O_2S$)
White powder; yield 78%; m.p.: 228 °C; IR (KBr): $\bar{\nu}$ = 3455, 3294 (asym., sym. str. of NH_2), 2207 (C≡N str.), 1682 (C=O str.), 1232 (Ar–C–F str.) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.02 (s, 3H, CH_3), 1.09 (s, 3H, CH_3), 2.20 (s, 3H, CH_3), 2.23–2.57 (m, 4H, 2 × CH_2), 4.45 (s, 1H, H4), 7.02 (s, 2H, NH_2), 7.33–7.94 (m, 7H, Ar–H), 8.52 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 21.3, 25.9, 28.0, 28.8, 32.1, 50.5, 58.3, 112.2, 121.9, 122.4, 128.1, 128.5, 128.8, 129.0, 129.2, 129.4, 129.5, 133.1, 135.1, 138.2, 148.8, 154.0, 158.4, 159.4, 162.7, 196.5 ppm; MS (ESI): *m/z* = 526.15 ($[M+1]^+$).

2-Amino-4-[3-(4-fluorophenyl)-1-(6-methylbenzo[d]thiazol-2-yl)-1H-pyrazol-4-yl]-7-methyl-5-oxo-4,5-dihydropyrano[4,3-b]pyran-3-carbonitrile (**7l**, $C_{27}H_{18}FN_5O_3S$)

Yellow powder; yield 75%; m.p.: 209 °C; IR (KBr): $\bar{\nu}$ = 3448, 3290 (asym., sym. str. of NH_2), 2201 (C≡N str.), 1678 (C=O str.), 1230 (Ar–C–F str.) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 2.40 (s, 3H, CH_3), 2.45 (s, 3H, CH_3-benzothiazole), 4.45 (s, 1H, H4), 6.98 (s, 2H, NH_2), 7.09–8.62 (m, 7H, Ar–H), 8.75 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 21.4, 27.1, 57.8, 105.2, 112.7, 115.2, 117.0, 122.0, 122.7, 123.1, 125.0, 127.8, 128.8, 129.6, 131.4, 133.3, 133.4, 136.1, 149.0, 152.6, 154.0, 159.7, 160.7, 161.3 ppm; MS (ESI): *m/z* = 512.20 ($[M+1]^+$).

2-Amino-4-[3-(4-fluorophenyl)-1-(6-methylbenzo[d]thiazol-2-yl)-1H-pyrazol-4-yl]-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile (**7m**, $C_{30}H_{18}FN_5O_3S$)
Yellow powder; yield 81%; m.p.: 196 °C; IR (KBr): $\bar{\nu}$ = 3456, 3292 (asym., sym. str. of NH_2), 2194 (C≡N str.), 1682 (C=O str.), 1229 (Ar–C–F str.) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 2.43 (s, 3H, CH_3-benzothiazole), 4.71 (s, 1H, H4), 7.29–7.87 (m, 13H, Ar–H+NH_2), 8.82 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 21.3, 27.2, 57.8, 103.2, 113.7, 115.8, 116.0, 116.9, 121.9, 122.6, 123.0, 124.9, 127.6, 128.6, 129.5, 131.1, 131.2, 133.1, 133.2, 135.2, 148.8, 152.5, 153.5, 153.9, 158.0, 159.2, 160.3, 161.6 ppm; MS (ESI): *m/z* = 548.05 ($[M+1]^+$).

6-Amino-4-[3-(4-fluorophenyl)-1-(6-methylbenzo[d]thiazol-2-yl)-1H-pyrazol-4-yl]-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**7n**, $C_{25}H_{18}FN_7OS$)
Light yellow powder; yield 75%; m.p.: 220 °C; IR (KBr): $\bar{\nu}$ = 3449, 3296 (asym., sym. str. of NH_2), 2210 (C≡N str.), 1230 (Ar–C–F str.) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 1.84 (s, 3H, CH_3-pyran), 2.41 (s, 3H, CH_3-benzothiazole), 4.96 (s, 1H, H4), 6.87 (s, 2H, NH_2), 7.21–8.34 (m, 7H, Ar–H), 8.67 (s, 1H, pyrazole H5), 12.07 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 10.2, 21.4, 26.7, 56.3, 96.7, 115.5, 115.7, 122.0, 122.4, 122.6, 126.3, 128.5, 128.9, 130.6, 130.7, 130.8, 132.9, 133.1, 135.0, 135.2, 148.9, 148.9, 161.3 ppm; MS (ESI): *m/z* = 484.10 ($[M+1]^+$).

2-Amino-4-[3-(4-fluorophenyl)-1-(6-methylbenzo[d]thiazol-2-yl)-1H-pyrazol-4-yl]-5,10-dioxo-5,10-dihydro-4H-benzo[g]chromene-3-carbonitrile (**7o**, $C_{31}H_{18}FN_5O_3S$)
White powder; yield 70%; m.p.: 225 °C; IR (KBr): $\bar{\nu}$ = 3457, 3288 (asym., sym. str. of NH_2), 2203 (C≡N str.), 1682 (C=O str.), 1230 (Ar–C–F str.) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 2.40 (s, 3H, CH_3-benzothiazole), 4.70 (s, 1H, H4), 7.28–7.98 (m, 13H, Ar–H+NH_2), 8.56 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 21.3, 27.9, 57.9, 114.2, 115.0, 116.3, 116.7, 120.2, 122.2, 123.4, 125.4, 127.7, 128.7, 129.6, 131.4, 131.3, 133.3, 133.5, 135.4, 149.0, 152.5, 159.2, 160.4, 161.6, 163.1, 176.3, 181.4 ppm; MS (ESI): *m/z* = 560.15 ($[M+1]^+$).

2-Amino-7,7-dimethyl-4-[1-(6-methylbenzo[d]thiazol-2-yl)-3-(p-tolyl)-1H-pyrazol-4-yl]-5-oxo-5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile (**7p**, $C_{30}H_{27}N_5O_2S$)
Yellow powder; yield 79%; m.p.: 226 °C; IR (KBr): $\bar{\nu}$ = 3453, 3291 (asym., sym. str. of NH_2), 2194 (C≡N str.), 1676 (C=O str.) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 1.08 (s, 3H, CH_3), 1.12 (s, 3H, CH_3), 2.20 (s, 3H, CH_3), 2.25–2.54 (7H, m, 2 × CH_2, 3H), 4.47 (s, 1H, H4), 7.01 (s, 2H, NH_2), 7.31–7.93 (m, 7H, Ar–H), 8.48 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 21.4, 21.4, 25.9, 28.0, 28.7, 32.1, 50.5, 58.3, 112.3, 121.9, 122.5, 128.2, 128.6, 128.9, 129.0, 129.3, 129.4, 129.6, 133.1, 135.1, 138.5, 148.9, 154.2, 158.6, 159.3, 162.8, 196.5 ppm; MS (ESI): *m/z* = 522.20 ($[M+1]^+$).

2-Amino-7-methyl-4-[1-(6-methylbenzo[d]thiazol-2-yl)-3-(p-tolyl)-1H-pyrazol-4-yl]-5-oxo-4,5-dihydropyrano[4,3-b]pyran-3-carbonitrile (**7q**, $C_{28}H_{21}N_5O_3S$)
White powder; yield 70%; m.p.: 215 °C; IR (KBr): $\bar{\nu}$ = 3455, 3287 (asym., sym. str. of NH_2), 2201 (C≡N str.), 1680 (C=O str.) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 2.34 (s, 3H, CH_3), 2.41 (s, 3H, CH_3), 2.46 (s, 3H, CH_3-benzothiazole), 4.48 (s, 1H, H4), 6.99 (s, 2H, NH_2), 7.11-8.20 (m, 7H, Ar–H), 8.78 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 21.3, 21.4, 27.1, 57.8, 104.8, 112.1, 114.9, 116.9, 121.8, 122.2, 123.1, 124.9, 127.6, 128.4, 129.3, 131.1, 133.1, 133.1, 135.9, 148.8, 151.7, 153.9, 159.6, 160.7, 161.3 ppm; MS (ESI): *m/z* = 508.15 ($[M+1]^+$).

2-Amino-4-[1-(6-methylbenzo[d]thiazol-2-yl)-3-(p-tolyl)-1H-pyrazol-4-yl]-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile (**7r**, $C_{31}H_{21}N_5O_3S$)
Light yellow powder; yield 84%; m.p.: 223 °C; IR (KBr): $\bar{\nu}$ = 3450, 3294 (asym., sym. str. of NH_2), 2209 (C≡N str.), 1684 (C=O str.) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 2.36 (s, 3H, CH_3), 2.43 (s, 3H, CH_3-benzothiazole), 4.71 (s, 1H, H4), 7.28–7.87 (m, 13H, Ar–H+NH_2), 8.83 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 21.4, 27.2, 57.8, 103.2, 113.7, 115.8, 116.0, 116.9, 119.6, 121.9, 122.6, 123.0, 124.9, 127.6, 128.6, 129.5, 131.1, 131.2, 133.1, 133.2, 135.2, 148.8, 152.5, 153.5, 153.9, 158.0, 159.2, 160.3, 161.6 ppm; MS (ESI): *m/z* = 544.10 ($[M+1]^+$).

6-Amino-3-methyl-4-[1-(6-methylbenzo[d]thiazol-2-yl)-3-(p-tolyl)-1H-pyrazol-4-yl]-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**7s**, $C_{26}H_{21}N_7OS$)
Yellow powder; yield 76%; m.p.: 225 °C; IR (KBr): $\bar{\nu}$ = 3440, 3288 (asym., sym. str. of NH_2), 2200 (C≡N str.) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 1.80 (s, 3H, CH_3-pyran), 2.35 (s, 3H, CH_3), 2.45(s, 3H, CH_3-

benzothiazole), 4.95 (s, 1H, H4), 6.87 (s, 2H, NH_2), 7.20–7.89 (m, 7H, Ar–H), 8.61 (s, 1H, pyrazole H5), 12.04 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 10.2, 21.3, 21.5, 27.6, 56.5, 97.0, 121.2, 122., 122.6, 126.7, 128.5, 128.6, 129.3, 129.4, 133.1, 135.2, 135.9, 138.4, 148.9, 154.2, 154.9, 159.3, 161.3$ ppm; MS (ESI): $m/z = 480.10$ ($[M+1]^+$).

2-Amino-4-[1-(6-methylbenzo[d]thiazol-2-yl)-3-(p-tolyl)-1H-pyrazol-4-yl]-5,10-dioxo-5,10-dihydro-4H-benzo[g]-chromene-3-carbonitrile (**7t**, $C_{32}H_{21}N_5O_3S$)
Yellow powder; yield 82%; m.p.: 213 °C; IR (KBr): $\bar{\nu} = 3455, 3290$ (asym., sym. str. of NH_2), 2190 (C≡N str.), 1680 (C=O str.) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): $\delta = 2.37$ (s, 3H, CH_3), 2.42 (s, 3H, CH_3-benzothiazole), 4.66 (s, 1H, H4), 7.30–8.03 (m, 13H, Ar–H+NH_2), 8.61 (s, 1H, pyrazole H5) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 21.3, 21.4, 27.8, 58.0, 114.1, 114.9, 116.3, 116.7, 120.1, 122.2, 123.4, 125.4, 127.7, 128.6, 129.5, 131.4, 131.3, 133.2, 133.4, 135.4, 148.9, 152.5, 159.2, 160.3, 161.5, 163.0, 176.2, 181.4$ ppm; MS (ESI): $m/z = 556.15$ ($[M+1]^+$).

(E)-3-[[1-(Benzo[d]thiazol-2-yl)-3-(p-tolyl)-1H-pyrazol-4-yl]methylene]naphthalene-1,2,4(3H)-trione
(**8**, $C_{28}H_{17}N_3O_3S$)
White powder; yield 79%; m.p.: 206 °C; ^1H NMR (400 MHz, DMSO-d_6): $\delta = 2.36$ (s, 3H, CH_3), 7.31–7.87 (m, 13H, Ar–H), 8.83 (s, 1H, pyrazole H5), 9.18 (s, 1H, H4) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 21.4, 112.2, 115.7, 116.1, 116.9, 119.6, 121.5, 122.4, 123.2, 125.1, 126.2, 127.8, 128.6, 129.4, 131.1, 131.2, 132.5, 133.3, 133.7, 135.4, 149.0, 151.8, 159.2, 160.3, 161.6, 176.3, 178.5, 181.4$ ppm; MS (ESI): $m/z = 476.10$ ($[M+1]^+$).

In vitro antimicrobial assay

The in vitro antimicrobial activity of newly synthesized compounds **7a**–**7t** was carried out by the broth microdilution method [37, 38]. Mueller-Hinton broth was used as nutrient medium to grow and dilute the compound suspension for the test bacteria; 2% DMSO in water was used as the diluent to obtain the desired concentration of compounds to test upon standard bacterial strains. Sabouraud dextrose broth was used for fungal nutrition. The inoculum size for the test strain was adjusted to 10^8 CFU cm^{-3} by comparing the turbidity. Serial dilutions were prepared in primary and secondary screening. Each synthesized compound and the standard drugs were diluted obtaining 2000 μg/cm^3 concentration as a stock solution. The drugs, which were found to be active in primary screening (i.e., 500, 250, and 200 μg/cm^3 concentrations), were further screened in their second set of dilutions at 100, 50, 25, and 12.5 μg/cm^3 concentration against all microorganisms; 10 mm^3 suspension was further inoculated on appropriate media, and growth was noted after 24 and 48 h. The control tube containing no antibiotic was instantaneously subcultured by consistent spreading over an area of the plate of medium fitting for the growth of the test organism. The tubes were then incubated at 37 °C overnight. The maximum dilution preventing the appearance of turbidity after spot subculture was considered the minimal inhibitory concentration (MIC). All the tubes showing no visible growth (same as the control tube) were subcultured and incubated overnight at 37 °C. The amount of growth in the control tube before incubation was compared. In this study ampicillin, norfloxacin, and ciprofloxacin were used as the standard antibacterial drugs, while nystatin and griseofulvin were used as standard antifungal drugs.

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