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ARTICLE

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Synthesis of chloro, fluoro, and nitro derivatives of 7-amino-5-aryl-6-cyano-5*H*-pyrano pyrimidin-2,4-diones using organic catalysts and their antimicrobial and anticancer activities

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Abstract

Chloro, fluoro, and nitro derivatives of 7-amino-5-aryl-6-cyano-5*H*-pyrano pyrimidin-2,4-diones were produced by reacting malononitrile, barbituric acid, and aromatic aldehydes together with a DABCO catalyst in an aqueous one-pot reaction. This is the first report of these compounds being synthesized with DABCO as a catalyst, which produced the compounds in yields in excess of 90%. The 2,4-difluoro derivative (**11**) was novel. The structures of the synthesized compounds were elucidated by means of ^{1}H, ^{13}C, and 2D NMR spectroscopy. Compound **2** (2-Cl derivative) had MBC values of <200μM against both *Staphylococcus aureus* and MRSA, and the 2-nitro derivative **5** had an MBC of 191μM against the Gram–ve *Escherichia coli*. The synthesized compounds were also tested for their anticancer activity against a HeLa cell line, where all the compounds showed better activity (IC_{50} values between 129μM and 340μM) than 5-fluorouracil, a commonly known anticancer drug.

1 | INTRODUCTION

Pyrimidines and its analogues are an important class of heterocyclic compounds, and their structural skeleton is a key constituent of nucleic acids, alkaloids, and numerous other pharmacophores with a variety of potent biological activities.[1] Pyrano[2,3-*d*]pyrimidines consists of a pyran ring fused together with pyrimidine. As such, their basic framework consists of both nitrogen and oxygen in their carbocyclic structure. This pyrimidine-annulated derivative is known to possess good pharmacological activity such as antioxidant, antidiabetic,[2] antimicrobial,[3,4] anti-inflammatory, analgesic,[5] moderate anticancer,[3] antimycobacterial,[6] and antimalarial activity.[7]

Pyrimidines are generally synthesized by a Biginelli multicomponent reaction (MCR), a method considered green since they are fast, solvent free, produces good yields, and avoids exposing the environment to environmentally harmful intermediates.[8] Pyrano[2,3-*d*]pyrimidines are commonly formed from barbituric acid, malonitrile, and aldehdyes with a catalyst such as 1,4-diazabicyclo[2,2,2]octane (DABCO), an organic catalyst,[9] a mesoporous solid acid catalyst (SBA-15-Pr-SO_3H),[10] a $ZnFe_2O_4$ nanocatalyst,[11] a nano-sawdust-OSO_3H catalyst,[12] a Mn-doped ZrO_2 catalyst,[13] or triethylammonium acetate (a green catalyst).[4] The same compounds were made using arylidenemalonitrile with barbituric acid under microwave irradiation without a catalyst[14] or using ionic liquid catalysts such as *N*-butyl-*N*-methyl imidazole tetrafluoroborate $[BMIm]BF_4$, *N*-ethyl-*N*-methyl imidazole tetrafluoroborate $[EMIm]BF_4$, or *N*-butyl pyridinium tetrafluoroborate $[BPy]BF_4$.[15] The resultant pyrimidine diones usually have a substituted phenyl group at position 5 and reactive nitrile and amino groups at C-6 and C-7 on the pyrano[2,3-*d*]pyrimidine skeleton.

By reacting ethyl 2-cyanoacetate with barbituric acid and substituted benzaldehydes in the presence of

DABCO, an ethyl ester group was placed at C-6 instead of the nitrile group.[16] Pyrano[2,3-*d*]pyrimidines with phenyl groups at both positions 5 and 7 were synthesized by the reaction of chalcones with barbituric acid in the presence of acetic acid and P_2O_5 as a catalyst.[17]

Other pyrimidines with substituted phenyl groups were synthesized in a one-pot reaction with 1-nitroguanidine, malonitrile, and substituted benzaldehydes under basic conditions in a one-pot reaction with short reaction times, mild reaction conditions, and excellent yields[18] or with guanidine nitrate, ethylcyanoacetate, and aldehydes using piperidine as a catalyst.[19] Spiropyrimidinones were synthesized using urea instead of malonitrile together with barbituric acid and benzaldehydes in the presence of a nanoporous solid acid catalyst (SBA-Pr-SO_3H).[20] A totally different synthesis to pyrano[2,3-*d*]pyrimidines was achieved using a one-pot three-component reaction of 2-amino-7-methyl-5-oxo-4-phenyl-4,5-dihydropyrano[4,3-*b*]pyran-3-carbonitriles, *N*,*N*-dimethylacetaldehyde dimethyl acetal, and aromatic amines in the presence of 1-butyl-3-methylimidazole hydrogen sulphate (an ionic liquid) as a catalyst.[21] Another synthetic report makes use of *N*,*N*-dimethyl-5-formylbarbituric acid with maleimide and phenyl isocyanate under microwave irradiation to afford pyrano[2,3-*d*]pyrimidines in good yields.[22]

We herein report the synthesis of a small library of chloro, nitro, and fluoro 7-amino-5-aryl-6-cyano-5*H*-pyrano[2,3-*d*]pyrimidin-(1*H*,3*H*)-2,4-diones using DABCO as a catalyst together with their antibacterial and anticancer activity. This paper follows our earlier report on the synthesis, anticancer, and antibacterial activity of oxygenated pyrimidin-2,4-diones.[23]

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

The pyrimidin-2,4-diones (**1–11**) were synthesized using malonitrile, barbituric acid, and chloro, fluoro, and nitro benzaldehydes in ethanol (Figure 1). DABCO was used as an organic catalyst, producing yields of 90% to 96% (Table 1). NMR spectroscopy was used to confirm the structures of the compounds, and the structural elucidation was discussed in our previous publication.[23]

With the exception of the novel, 2,4-difluoro derivative **11**, all compounds were synthesized previously in comparable yields using a variety of methods or catalysts (Table 1), including microwave synthesis without the use of a catalyst,[14] with the ionic liquids 1-n-butyl-3-methylimidazolium tetrafluoroborate and 1-butylpyridinium tetrafluoroborate,[15] a Zn [(L) proline] catalyst,[24] with glycerol without the use of a catalyst,[25] nanocatalysts Fe_3O_4[26] and SBA-15-Pr-SO_3H (a mesoporous solid acid catalyst),[10] and ethyl cyanoacetate.[27] DABCO has been used previously for the synthesis of furan-2-yl, pyrrol-2-yl and thiophen-2-yl derivatives of pyrano pyrimidin-2,4-diones in a one pot reaction.

Plausible mechanisms for the reaction are provided in Bhat et al[16] and Jain et al[9]; however, these mechanisms are either unclear or have missing steps. A more comprehensive mechanism is presented in Figure 2, involving formation of an arylidene intermediate, which is subject to a Michael reaction by barbituric acid. Cyclisation involving the hydroxy and cyano groups ultimately lead to the products. In order to test the mechanism, we performed several experiments with compound **4**, keeping the reaction time constant at 1 hour. This produced a yield of 94% in the one-pot reaction. With anhydrous ethanol, the reaction occurred, but in a very low yield (23%), presumably because the anhydrous ethanol is not as good a proton donor as water. We then tried THF and water as a solvent, and the reaction proceeded similarly to the original reaction in a yield of 92%. We then added the aldehyde to the reaction, 30 minutes after mixing the malonitrile, DABCO, and barbituric acid. The yield was lower, 58%. Finally, we added the malonitrile 30 minutes after mixing the aldehyde, DABCO, and barbituric acid. Again, the yield was lower (42%) than the original reported method.

We had previously investigated four basic organic catalysts, together with K_2CO_3 to identify the catalyst among them that produced the greatest yield in the shortest amount of time.[23] We studied L-proline, dibutylamine (DBA), triethylamine (Et_3N), and 1,4-diazabicyclo [2.2.2] octane (DABCO) and found that DBA, Et_3N, and DABCO all produced yields of over 70% in 1 hour; however, DABCO was the best of the three, resulting in 94% in 38 minutes.

3 | ANTIMICROBIAL ACTIVITY

The synthesized compounds were tested for their antimicrobial activity against two Gram +ve and three Gram–ve

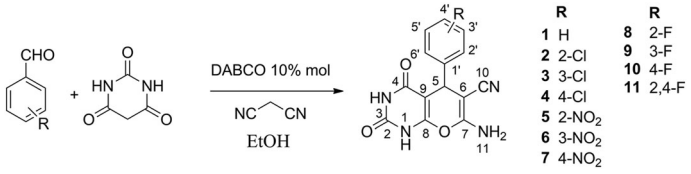

FIGURE 1 Synthetic scheme for chloro, fluoro, and nitro pyrano pyrimidines

TABLE 1 Yields and melting points of aromatic pyrano [2,3-*d*] pyrimidines catalyzed by DABCO[a] in comparison with other reported catalysts and methods

Entry	R	Yield	mp, °C	Reference	Yield	Catalyst/method
1	H	90	205-208	Gao et al[14]	92	Microwave[c]
				Yu and Wang[15]	84	ILs[d]
				Heravi et al[24]	85	Zn [(L) proline]
				Ziarani et al[20]	65	SBA-Pr-SO_3H[e]
2	2-Cl	93	212-215	Gao et al[14]	89	Microwave[c]
				Yu and Wang[15]	94	ILs[d]
				Safaei et al[25]	90	Glycerol[c]
3	3-Cl	95	223-225	Safaei et al[25]	89	Glycerol[c]
				Kidwai et al[26]	94	Fe_3O_4[e]
4	4-Cl	94	235-237	Gao et al[14]	92	Microwave[c]
				Yu and Wang[15]	92	ILs[d]
				Heravi et al[24]	90	Zn [(L) proline]
				Ziarani et al[20]	30	SBA-Pr-SO_3H[e]
				Safaei et al[25]	91	Glycerol[c]
				Kidwai et al[26]	97	Fe_3O_4[e]
5	2-NO_2	96	258-259	Safaei et al[25]	92	Glycerol[c]
6	3-NO_2	90	256-258	Heravi et al[24]	90	Zn [(L) proline]
				Ziarani et al[20]	80	SBA-Pr-SO_3H[e]
				Safaei et al[25]	94	Glycerol[c]
7	4-NO_2	93	225-228	Gao et al[14]	86	Microwave[c]
				Heravi et al[24]	92	Zn [(L) proline]
				Ziarani et al[20]	90	SBA-Pr-SO_3H[e]
				Safaei et al[25]	95	Glycerol[c]
				Kidwai et al[26]	95	Fe_3O_4[e]
8	2-F	90	226-229	Commercially available[b]		
9	3-F	96	224-226	Sharanin and Klokol[27]	90	Ethyl cyanoacetate
10	4-F	92	228-230	Gao et al[14]	91	Microwave[c]
				Yu and Wang[15]	90	ILs[d]
11	2,4-diF	92	250-252	Novel	-	

[a]Reaction conditions: barbituric acid (1.0 mmol), substituted benzaldehydes (1.0 mmol), and malononitrile (1.1 mmol), EtOH:water (1:1 v/v, 10 mL), room temp.;

[b]Akos Consulting and Solutions; Sigma Aldrich;

[c]no catalyst;

[d]ionic liquids – 1-n-butyl-3-methylimidazolium tetrafluoroborate and 1-butylpyridinium tetrafluoroborate;

[e]nanocatalyst

strains of bacteria. The compounds were initially screened for their antimicrobial potential using the disk diffusion assay, where compounds showing a mean inhibition zone of greater than 9 mm were selected to determine their minimum bactericidal concentrations (MBCs). In general, the compounds were more active against the Gram +ve *Staphylococcus aureus* and MRSA than the Gram –ve strains (Table 2). Compound **2**, the 2-chloro derivative, was active at <200μM for both the Gram +ve strains. Several of the compounds were also active at <200μM against MRSA, showing better activity than ampicillin. Only compound **5**, the 2-nitro derivative showed activity <200μM against *E coli*. None of the other compounds showed any appreciable activity to the Gram –ve strains.

4 | MTT CYTOTOXICITY ASSAY

The in vitro cytotoxicity levels of pyrimidinones **1-11** against HeLa cells are summarized in Table 3 below. Most of the compounds tested showed a dose-dependent cytotoxicity profile. Based on the IC_{50} values, it can be deduced that all compounds are most active at

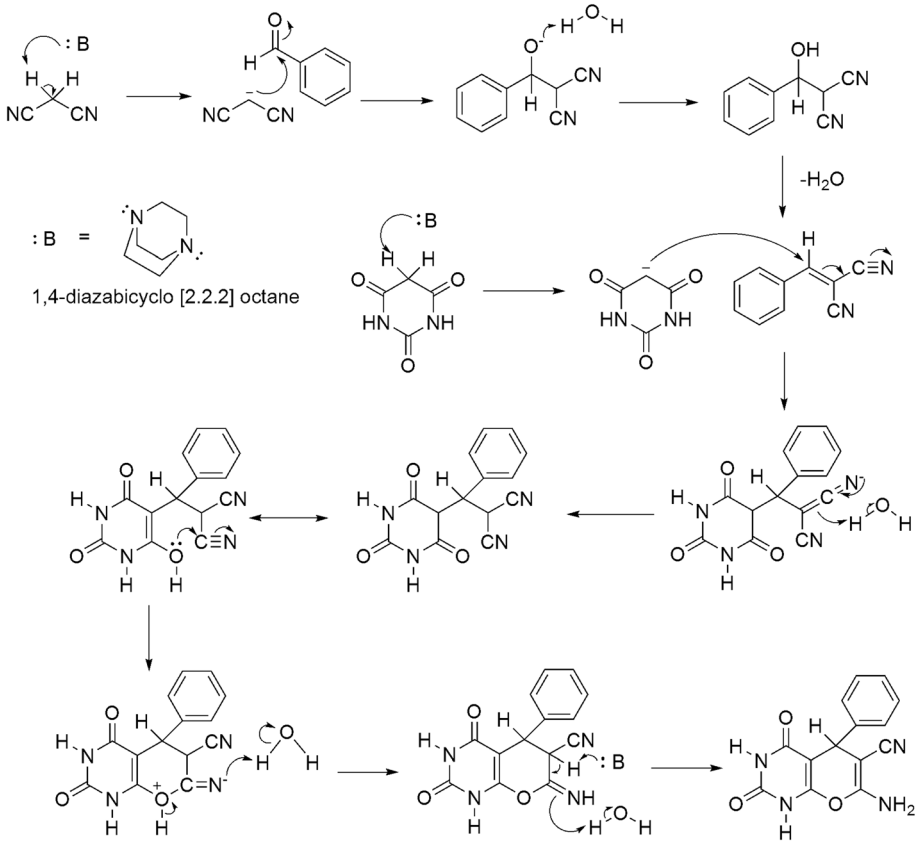

FIGURE 2 A plausible mechanism for the one pot reaction of malonitrile, barbituric acid, and benzaldehydes in the presence of DABCO

TABLE 2 Antibacterial activity of the synthesized compounds

Bacterial strains	Minimum Bactericidal Concentration, μM							
Compounds	**2**	**3**	**4**	**5**	**8**	**11**	**AMP**	**CIP**
Gram + ve								
Staphylococcus aureus	197	394	394	382	208	196	56	1.84
MRSA	197	197	394	191	1665	393	894	7.36
Gram −ve								
Escherichia coli	1579	1579	789	191	1665	786	447	1.84
Pseudomonas aeruginosa	1579	1579	789	1528	1665	1571	1788	1.84
Klebsiella pneumoniae	394	789	1579	382	1665	1571	447	3.68

Note. Data are reported as the average of duplicate readings

concentrations greater than 100 μg mL^{-1}. The activities of all compounds were better than 5-fluorouracil (5-FU), a commonly used anticancer drug, with IC_{50} values ranging from 129 to 340μM. 5-FU had an IC_{50} in the same assay at 480μM. The activities of the compounds could be due to the pyranopyrimidinone core structure, with aryl groups at C-5, to which electron withdrawing groups were attached. Compounds **3**, **4**, **7**, and **8** were active at lower concentrations, indicating promise as anticancer agents. Further studies are however needed before this conclusion can be made.

5 | MOLECULAR DOCKING STUDIES

In order to support the experimental anticancer activity of the synthesized compounds and to predict their

TABLE 3 Viabilities (%) of the HeLa cell lines at different concentrations of compounds **1**-**11**

Cpd	10 μg mL^{-1}	25 μg mL^{-1}	50 μg mL^{-1}	100 μg mL^{-1}	IC_{50} μg mL^{-1}	IC_{50} μM
1	95.58 ± 0.02	85.82 ± 0.0005	70.10 ± 0.01	41.35 ± 0.02	84.92	301
2	96.72 ± 0.01	83.06 ± 0.01	65.40 ± 0.02	40.48 ± 0.01	81.34	257
3	79.11 ± 0.01	65.64 ± 0.001	53.20 ± 0.02	43.33 ± 0.04	74.02	234
4	72.08 ± 0.02	52.13 ± 0.02	37.99 ± 0.02	27.84 ± 0.02	40.67	129
5	98.22 ± 0.03	82.46 ± 0.01	69.79 ± 0.02	46.68 ± 0.02	90.86	278
6	95.81 ± 0.02	78.36 ± 0.01	69.31 ± 0.02	57.15 ± 0.02	111.27	340
7	86.65 ± 0.02	73.82 ± 0.02	62.80 ± 0.01	40.64 ± 0.02	78.81	241
8	81.04 ± 0.02	68.92 ± 0.01	50.87 ± 0.03	42.54 ± 0.03	72.54	242
9	87.88 ± 0.02	83.25 ± 0.01	69.91 ± 0.01	50.91 ± 0.03	101.11	337
10	88.55 ± 0.02	69.79 ± 0.01	57.86 ± 0.01	41.19 ± 0.01	96.51	322
11	84.79 ± 0.01	77.37 ± 0.02	63.67 ± 0.02	52.41 ± 0.02	101.2	318
12	90.28 ± 0.01	85.15 ± 0.01	71.56 ± 0.01	71.56 ± 0.02	108.75	339
5-FU	78.40 ± 0.03	58.89 ± 0.03	50.47 ± 0.02	38.00 ± 0.02	62.41	480

Note. Data are reported as ± the standard deviation of triplicate readings.

mechanism of action, we docked two representative compounds (RCs) **4** (4-Cl) and **8** (2F), observed to be potent under in vitro conditions, into the binding site of human kinesin protein, Eg5. Inhibitors of human kinesin protein, through cancer cell line screening resulted in the development of new anticancer therapeutic agents.[28,29] Thus, this enzyme was used in our docking studies. The CDocker docking method embedded in the Discovery Studio (DS) was used for all docking simulations. The docking results obtained suggested both compounds to be strong inhibitors of Eg5 based on the computed binding energy (BE) data. The most active compound **4** with BE of value −184.4 kcal mol^{-1} exhibited a stronger interaction than its structural analogue **8** (BE = −150.1 kcal mol^{-1}). Both the RCs exhibited stronger binding affinity for Eg5 relative to the standard drug, 5-FU (BE = −116.7 kcal mol^{-1}).

To understand the host-guest relationship between ligand and receptor, the docked complexes of both RCs were further visualized using DS visualizer and are diagrammatically represented in Figures 3 and 4. Compound **4** (Figure 3) exhibited two concurrent hydrogen bond interactions through its protonated amine functionality (NH_2) with Glu116 (1.81 Å) and Gly117 (1.98 Å) amino acid residues of Eg5. Additionally, an electrostatic interaction between **4** and Glu116 including several hydrophobic forces (with Pro137, Trp127, Arg119, Tyr211, and Ala133) were also observed. Similarly, **8** (Figure 4) interacted with Eg5 through two hydrogen bonds; one conventional with Arg221 (1.17 Å) through its nitrile group and another nonconventional with Arg119 (2.80 Å) through the nitrogen atom of the pyrimidine ring. In addition, two electrostatic interactions (with

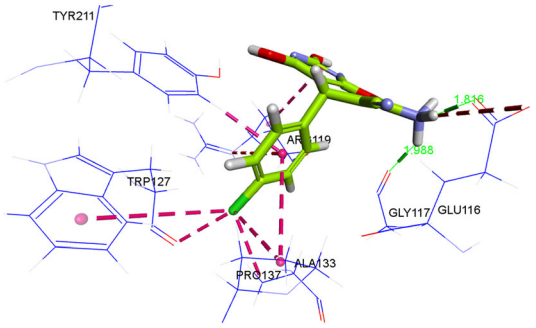

FIGURE 3 Docked pose of **4** in the binding site of Eg5. Hydrophobic and electrostatic interactions are shown as magenta and red dotted lines, respectively. Conventional hydrogen bonds are shown as green [Color figure can be viewed at wileyonlinelibrary.com]

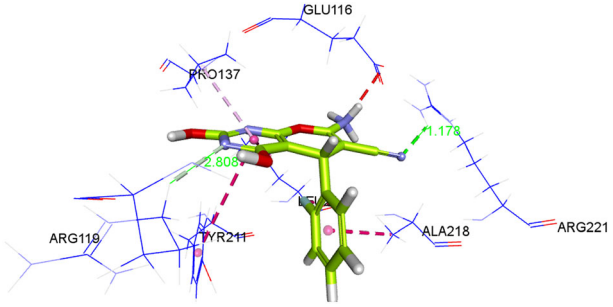

FIGURE 4 Docked pose of **8** in the binding site of Eg5. Hydrophobic and electrostatic interactions are shown as magenta and red dotted lines, respectively. Conventional hydrogen bonds are shown as green, and nonconventional hydrogen bonds are shown as gray [Color figure can be viewed at wileyonlinelibrary.com]

Glu116 and Arg221) and hydrophobic forces (with Tyr211, Ala218, and Pro137) were also observed.

6 | CONCLUSION

An environmentally friendly synthesis of pyrano [2,3-*d*] pyrimidinone derivatives in a one-pot reaction using an organic catalyst, DABCO, produced chloro, fluoro, and nitro pyrimidin-2,4-diones in high yields (>90%). The synthesized compounds showed good anticancer activity against HeLa cells. The most active compounds also showed good binding affinity to the human kinesin protein, Eg5, which supported our experimental findings.

7 | EXPERIMENTAL

7.1 | General

Chemicals and reagents were purchased from Sigma Aldrich. Organic solvents were redistilled and dried according to standard procedures. NMR spectra were recorded using a Bruker AvanceIII 400-MHz spectrometer. Chemical shifts (δ) were reported against tetramethylsilane (TMS), the internal standard. IR spectra were obtained on a Perkin Elmer Spectrum 100 FTIR spectrometer with universal ATR sampling accessory. An Agilent GC-MSD apparatus fitted with a DB-5SIL MS (30 m × 0.25 mm) fused-silica capillary column was used for GC-MS analysis using helium (at 2 mL min^{-1}) as a carrier gas. The MS was operated in the EI mode at 70 eV. An ErnstLeitzWetzlar micro hot stage melting point apparatus was used to record melting points and are uncorrected.

7.1.1 | Synthesis of aromatic substituted pyrano [2,3-*d*] pyrimidinones (1-11)

Substituted aromatic benzaldehydes (1.0 mmol each), malononitrile (396 mg, 1.0 mmol), barbituric acid (640 mg, 1.0 mmol each), and 10 mol% DABCO (30.45 mg, 0.271 mmol) were added to 20-mL aqueous ethanol and the reaction mixture stirred for 1 hour at room temperature. The progress of the reaction was monitored by TLC. Upon precipitation, the products were filtered and, thereafter, recrystallized in ethanol before being dried at the vacuum pump. The NMR data of 7-amino-5-phenyl-6-cyano-5*H*-pyrano[2,3-*d*]pyrimidin -(1*H*,3*H*)-2,4-dione **(1)** is reported in Aremu et al.[23]

7.1.2 | 7-Amino-5-(2-chlorophenyl)-6-cyano-5*H*-pyrano[2,3-*d*]pyrimidin-(1*H*,3*H*)-2,4-dione (2)

White powder (93%); mp 212°C-215°C; UV λ_{max} (DMSO) nm (log ε) 259 (2.74); IR (KBr) υ_{max}: 3013 (NH), 2192 (CN), 1714 (C=O), 1673 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.07 (brs, NH), 11.04 (s, NH), 7.36 (1H, d, J = 8.0 Hz, H-6′), 7.21-7.27 (3H, m, H-3′,4′,5′), 7.12 (s, NH_2-11), 4.72 (1H, s, H-5); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 162.2 (C-4), 157.8 (C-7), 152.7 (C-8), 149.5 (C-2), 140.7 (C-1′), 132.2 (C-2′), (130.4, 129.5, 128.4, 127.4 (4CH, C-3′,4′,5′,6′)), 118.7 (C-10), 87.5 (C-9), 56.0 (C-6), 30.6 (CH, C-5); EIMS (*m/z*, rel. int.) 316 (M^+) (14), 281 (31), 207 (100), 273 (14), 189 (15); *Anal*. calcd. for $C_{14}H_9ClN_4O_3$: C: 53.09, H: 2.86, N: 17.69, Found: C: 53.00, H: 2.79, N: 17.60.

7.1.3 | 7-Amino-5-(3-chlorophenyl)-6-cyano-5*H*-pyrano[2,3-*d*]pyrimidin-(1*H*,3*H*)-2,4-dione (3)

White powder (95%); mp 223°C-225°C; UV λ_{max} (DMSO) nm (log ε) 259 (2.69); IR (KBr) υ_{max}: 3417 (NH), 3317 (NH), 2192 (CN), 1706 (C=O), 1660 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.07 (brs, NH), 11.07 (s, NH), 7.32 (1H, t, J = 8.4 Hz, H-5′), 7.26-7.28 (2H, m, H-4′,6′), 7.19 (1H, s, H-2′), 7.16 (s, NH_2-11), 4.26 (1H, s, H-5); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 162.4 (C-4), 157.6 (C-7), 152.4 (C-8), 149.5 (C-2), 146.6 (C-1′), 132.8 (C-3′), 130.1 (C-4′), 127.2 (CH, C-6′), 126.7 (CH, C-2′), 126.1 (CH, C-5′), 118.9 (C-10), 87.7 (C-9), 58.1 (C-6), 35.4 (CH, C-5); EIMS (*m/z*, rel. int.) 316 (M^+) (6), 281 (11), 207 (100), 188 (22), 153 (33); *Anal*. calcd. for $C_{14}H_9ClN_4O_3$: C: 53.09, H: 2.86, N: 17.69, Found: C: 53.10, H: 2.59, N: 17.70.

7.1.4 | 7-Amino-5-(4-chlorophenyl)-6-cyano-5*H*-pyrano[2,3-*d*]pyrimidin-(1*H*,3*H*)-2,4-dione (4)

White powder (94%); mp 235°C-237°C; UV λ_{max} (DMSO) nm (log ε) 258 (2.68); IR (KBr) υ_{max}: 3383 (NH_2), 3186 (NH), 2197 (CN), 1717 (C=O), 1672 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.06 (brs, NH), 11.05 (s, NH), 7.33 (2H, d J = 8.4 Hz, H-3′/5′), 7.23 (2H, d, J = 8.4 Hz, H-2′/6′), 7.13 (s, NH_2-11), 4.24 (1H, s, H-5); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 162.4 (C-4), 157.6 (C-7), 152.3 (C-8), 149.5 (C-2), 143.1 (C-1′), 131.2 (C-4′), 129.3 (2CH, C-2′/6′), 128.2 (2CH, C-3′/5′), 119.0 (C-10), 88.0 (C-9), 58.3 (C-6), 35.1 (CH, C-5); EIMS (*m/z*, rel. int.) 316 (M^+) (15), 281 (33), 207 (100), 188 (25), 153

(23); *Anal*. calcd. for $C_{14}H_9ClN_4O_3$: C: 53.09, H: 2.66, N: 17.65, Found: C: 53.09, H: 2.86, N: 17.69.

7.1.5 | 7-Amino-5-(2-nitrophenyl)-6-cyano-5*H*-pyrano[2,3-*d*]pyrimidin-(1*H*,3*H*)-2,4-dione (5)

Off-white powder (96%); mp 258°C-259°C; UV λ_{max} (DMSO) nm (log ε) 260 (2.71); IR (KBr) υ_{max}: 3365 (NH_2), 2198 (CN), 1697 (C=O), 1618 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 12.14 (brs, NH), 11.06 (s, NH), 7.82 (1H, d, J = 8.3 Hz, H-6′), 7.65 (1H, dd, J = 8.1, 7.6 Hz, H-4′), 7.49 (1H, d, J = 7.6 Hz, H-3′), 7.45 (1H, dd, J = 8.3, 8.1 Hz, H-5′), 7.27 (s, NH_2-11), 5.04 (1H, s, H-5); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 162.4 (C-4), 158.4 (C-7), 152.3 (C-8), 149.4 (C-2), 149.2 (C-2′), 138.1 (C-1′), 133.3 (CH, C-6′), 130.7 (CH, C-4′), 128.0 (CH, C-3′), 123.7 (CH, C-5′), 118.6 (C-10), 87.9 (C-9), 56.7 (C-6), 30.3 (CH, C-5); EIMS (*m/z*, rel. int.) 327 (M^+) (14), 298 (32), 207 (100); *Anal*. calcd. for $C_{14}H_9N_5O_5$: C: 51.38, H: 2.77, N: 21.40, Found: C: 51.04, H: 2.47, N: 21.36.

7.1.6 | 7-Amino-5-(3-nitrophenyl)-6-cyano-5*H*-pyrano[2,3-*d*]pyrimidin-(1*H*,3*H*)-2,4-dione (6)

Off-white powder (90%); mp 256°C-258°C; UV λ_{max} (DMSO) nm (log ε) 262 (2.77); IR (KBr) υ_{max}: 3414 (NH), 3202 (NH), 2192 (CN), 1707 (C=O), 1687 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 12.15 (brs, NH), 11.09 (s, NH), 8.08 (1H, d, J = 8.1 Hz, H-6′), 8.06 (1H, brs, H-2′), 7.74 (1H, d, J = 7.7, H-4′), 7.60 (1H, dd, J = 8.1, 7.7 Hz, H-5′), 7.26 (s, NH_2-11), 4.47 (1H, s, H-5); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 162.5 (C-4), 157.8 (C-7), 152.5 (C-8), 149.5 (C-2), 147.7 (C-1′), 146.4 (C-3′), 134.7 (C-2′), 129.8 (CH, C-6′), 122.0 (CH, C-4′), 121.9 (CH, C-5′), 118.8 (C-10), 87.4 (C-9), 57.6 (C-6), 35.4 (CH, C-5); EIMS (*m/z*, rel. int.): 327 (M^+) (15), 281 (40), 207 (100); *Anal*. calcd. For $C_{14}H_9N_5O_5$: C: 51.38, H: 2.77, N: 21.40, Found: C: 51.01, H: 2.46, N: 21.24.

7.1.7 | 7-Amino-5-(4-nitrophenyl)-6-cyano-5*H*-pyrano[2,3-*d*]pyrimidin-(1*H*,3*H*)-2,4-dione (7)

White powder (93%); mp 225–228°C; UV λ_{max} (DMSO) nm (log ε) 260 (2.84); IR (KBr) υ_{max}: 3186 (NH), 2196 (CN), 1720 (C=O), 1671 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 12.15 (brs, NH), 11.10 (s, NH), 8.15 (2H, d, J = 8.7 Hz, H-3′/5′), 7.52 (2H, d, J = 8.7 Hz, H-2′/6′), 7.25 (s, NH_2-11), 4.41 (1H, s, H-5); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 162.4 (C-4), 157.7 (C-7), 152.6 (C-8), 151.7 (C-4′), 149.5 (C-2), 146.4 (C-1′), 128.8 (2CH, C-2′/6′), 123.5 (2CH, C-3′/5′), 118.8 (C-10), 87.4 (C-9), 57.4 (C-6), 35.6 (CH, C-5); EIMS (*m/z*, rel. int.): 327 (M^+) (27), 207 (100); *Anal*. calcd. for $C_{14}H_9N_5O_5$: C: 51.38, H: 2.77, N: 21.40, Found: C: 50.93, H: 2.37, N: 21.16.

7.1.8 | 7-Amino-5-(2-fluorophenyl)-6-cyano-5*H*-pyrano[2,3-*d*]pyrimidin-(1*H*,3*H*)-2,4-dione (8)

White powder (90%); mp 226-229°C; UV λ_{max} (DMSO) nm (log ε) 260 (2.93); IR (KBr) υ_{max}: 3421 (NH), 3303 (NH), 2203 (CN), 1716 (C=O), 1692 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 12.11 (brs, NH), 11.07 (s, NH), 7.23-7.28 (2H, m, H-4′,6′), 7.14 (2H, s, NH_2-11), 7.07-7.12 (2H, m H-3′,5′), 4.50 (1H, s, H-5); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 162.3 (C-4), 160.1 (d, J = 243.8 Hz, C-2′), 157.9 (C-7), 152.6 (C-8), 149.5 (C-2), 130.6 (d, J = 11.8 Hz, CH, C-4′), 129.8 (d, J = 3.8 Hz, C-1′), 128.7 (d, J = 8.4 Hz, CH, C-6′), 124.4 (d, J = 3.0 Hz, CH, C-5′), 118.9 (C-10), 115.3 (d, J = 21.7 Hz, CH, C-3′), 87.2 (C-9), 57.3 (C-6), 30.6 (CH, C-5); EIMS (*m/z*, rel. int.): 300 (M^+) (25), 281 (35), 226 (35), 207 (100), 167 (40), 159 (42); *Anal*. calcd. for $C_{14}H_9FN_4O_3$: C: 56.00, H: 3.02, N: 18.66, Found: C: 56.10, H: 2.97, N: 18.02.

7.1.9 | 7-Amino-5-(3-fluorophenyl)-6-cyano-5*H*-pyrano[2,3-*d*]pyrimidin-(1*H*,3*H*)-2,4-dione (9)

White powder (96% yield); mp 224°C-226°C; UV λ_{max} (DMSO) nm (log ε) 270 (2.93); IR (KBr) υ_{max}: 3376 (NH), 3186 (NH), 2197 (CN), 1717 (C=O), 1674 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 12.06 (brs, NH), 11.06 (s, NH), 7.30-7.36 (1H, m, H-5′), 7.14 (s, NH_2), 7.01-7.07 (3H, m, H-2′,4′,6′), 4.27 (1H, s, H-5); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 162.4 (C-4), 162.1 (d, J = 241.9 Hz, C-3′), 157.6 (C-7), 152.4 (C-8), 149.5 (C-2), 147.0 (d, J = 6.3 Hz, C-1′), 130.1 (d, J = 8.1 Hz, CH, C-5′), 123.4 (CH, C-6′), 119.0 (C-10), 114.0 (d, J = 21.5 Hz, CH, C-2′), 113.5 (d, J = 20.9 Hz, CH, C-4′), 87.8 (C-9), 58.2 (C-6), 35.4 (CH, C-5); EIMS (*m/z*, rel. int.): 300 (M^+) (18), 281 (41), 207 (100), 172 (74); *Anal*. calcd. for $C_{14}H_9FN_4O_3$: C: 56.00, H: 3.02, N: 18.66, Found: C: 55.97, H: 2.85, N: 18.39.

7.1.10 | 7-Amino-5-(4-fluorophenyl)-6-cyano-5*H*-pyrano[2,3-d]pyrimidin-(1*H*,3*H*)-2,4-dione (10)

White powder (92% yield); mp 228°C-230°C; UV λ_{max} (DMSO) nm (log ε) 260 (2.93); IR (KBr) υ_{max}: 3194 (NH), 2197 (CN), 1723 (C=O), 1677 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 12.06 (brs, NH), 11.05 (s, NH), 7.25 (2H, dd, J = 8.6, 5.6 Hz, H-2′/6′), 7.10 (2H, t, J = 8.6 Hz, H-3′/5′), 7.10 (s, NH), 4.26 (1H, s, H-5); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 162.4 (C-4), 161.0 (d, J = 240.8 Hz, C-4′), 157.5 (C-7), 152.2 (C-8), 149.5 (C-2), 140.1 (d, J = 2.8 Hz, C-1′), 129.1 (d, J = 8.1 Hz, 2CH, C-2′/6′), 119.1 (C-10), 114.8 (d, J = 21.2 Hz, 2CH, C-3′/5′), 88.3 (C-9), 58.7 (C-6), 35.0 (CH, C-5); EIMS (*m/z*, rel. int.): 300 (M^+) (10), 281 (13), 266 (19), 233 (19), 207 (100); *Anal*. calcd. for $C_{14}H_9FN_4O_3$: C: 56.00, H: 3.02, N: 18.66, Found C: 56.22, H: 2.65, N: 18.14.

7.1.11 | 7-Amino-5-(2,4-difluorophenyl)-6-cyano-5*H*-pyrano[2,3-*d*]pyrimidin-(1*H*,3*H*)-2,4-dione (11)

White powder (92% yield); mp 250°C-252°C; UV λ_{max} (DMSO) nm (log ε) 265 (2.98); IR (KBr) υ_{max}: 3395 (NH), 3306 (NH), 2195 (CN), 1718 (C=O), 1674 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 12.10 (brs, NH), 11.08 (s, NH), 7.32 (ddd, J = 8.6, 8.6, 6.3, H-6′), 7.15 (1H, ddd, J = 11.8, 9.4, 2.6, H-3′), 6.99 (1H, ddd, J = 8.4, 8.4, 2.4 Hz, H-5′), 7.18 (s, NH_2), 4.49 (s, H-5); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 162.3 (C-4), 161.1 (dd, J = 243.0, 12.4 Hz, C-2′*), 160.1 (dd, J = 246.5, 12.3 Hz, C-4′*), 157.9 (C-7), 152.6 (C-8), 149.5 (C-2), 131.1(dd, J = 9.6, 5.6 Hz, C-6′), 127.1 (dd, J = 12.3, 3.6 Hz, C-1′), 118.9 (C-10), 111.4 (d, J = 20.8 Hz, CH, C-5′), 103.6 (t, J = 25.9 Hz, CH, C-3′), 87.0 (C-9), 57.0 (C-6), 29.6 (CH, C-5); EIMS (*m/z*, rel. int.): 318 (M^+) (15), 207 (100); *Anal*. calcd. for $C_{14}H_8F_2N_4O_3$: C: 52.84, H: 2.53, N: 17.61, Found: C: 51.98, H: 2.39, N: 17.47. * assignments can be interchanged.

7.2 | Antibacterial assay

The following bacterial strains were used: Gram +ve *S aureus* ATCC 25923 and methicillin resistant *S aureus* ATCC BAA-1683 (MRSA), Gram −ve *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumonia* ATCC 314588. The standard antibiotics ciprofloxacin and ampicillin were used as controls for comparison. The method in Andrews[30] was adapted with modification according to Aremu et al.[23] Compounds **2**-**5**, **8**, and **11** showed zones of inhibition >9 mm and their MBC values determined. To calculate the MBC values, the compounds were dissolved in DMSO (4.0 mg mL^{-1}) and serially diluted in Mueller-Hinton broth (Oxoid, England) in Eppendorf tubes. The method according to Moodley et al[31] was used.

7.3 | Cytotoxicity tests by the MTT assay

Human cervical cancer (HeLa) cells were purchased from Highveld Biological (Pty) Ltd. (Lyndhurst, RSA). Cells were grown, seeded, and incubated according to our previous method.[23] Compounds **1**-**11** (initially dissolved in DMSO at a concentration of 1 mg mL^{-1}) were then added in triplicate to the cells (containing 100 μL of fresh medium) to a final concentration of 10, 25, 50, and 100 μg mL^{-1} and incubated for 48 hours at 37°C. 5-FU was used as a standard positive control. The MTT assay was adapted from Mosmann et al[32] and Gichumbi et al.[33] Tests were conducted in triplicate, and the IC_{50} values (concentration at which 50% cell death was achieved) were determined using Microsoft Excel 2010.

7.4 | Molecular docking

Different isomers of the RCs at physiological pH were generated using "Prepare Ligands" and energetically minimized in DS using CHARMm force field. The isomer with the lowest CHARMm energy was selected for docking. The crystal structure of Human Eg5 protein (pdb id: 2X7C) was downloaded from the protein data bank (http://www.rcsb.org). Only the B-chain of the protein was considered while the native ligand, (s)-Enastron (KZ91367), and associated water molecules were removed. Initially, the protonation state of the protein was determined at physiological pH followed by its minimization. The "Prepare Protein" module in DS was used to build any missing loops/chains and determine the protonation state of each amino acid of the protein.

The shake algorithm was used to constrain the hydrogen atoms of the protein during minimization. Before docking, a binding sphere (diameter 6.34 Å) with coordinates 16.8 (X), 14.4 (Y), and − 30.9 (Z) was generated using DS. Docking simulations were conducted using the CDocker docking program[34] by keeping the position of the protein fixed while allowing the ligand to flex. A total of 10 poses were generated for each compound and ranked according to the scoring function (-CDocker energy). The best pose was selected for BE calculations.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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