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LETTERS

Synthesis and biological evaluation of new fused Isoxazolo[4',5':4,5] pyrano[2,3-d] Pyrimidines as potent anticancer agents

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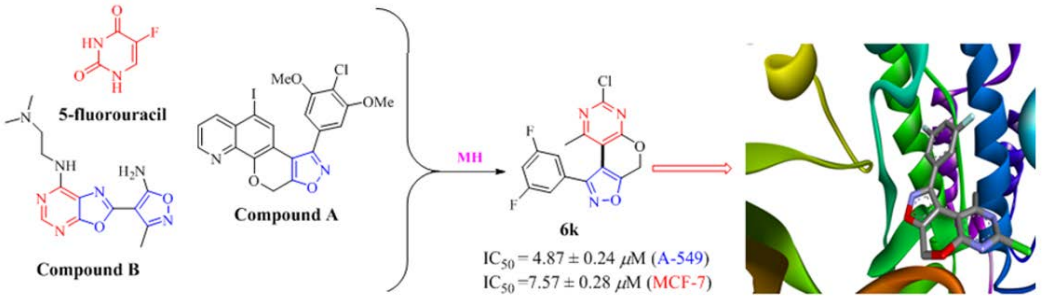
Article

ABSTRACT



Synthetic chemists have developed efficient and straightforward methods for optimal synthesis to meet the demand for scaffolds critical for medicinal applications. The synthesis of fused isoxazoles, both with and without microwave irradiation, was investigated through the [3 + 2]cycloaddition reaction, followed by C–C bond formation. This was achieved using 2-chloro-4-((3-iodoprop-2-yn-1-yl) oxy)-6-methylpyrimidine and substituted nitrile oxides under optimized conditions. The anticancer activity of the synthesized compounds was subsequently assessed in vitro against two cancer cell lines, MCF-7 and A-549. The majority of the tested compounds, specifically 6i, 6j, and 6k, showed notable activity, with compounds 6j and 6k surpassing the standard drug in both cell lines, while 6i displayed comparable activity against the tested cell lines. In silico analyses of more potent compounds (6i, 6j, and 6k) and erlotinib on the EGFR protein indicated that compounds 6i and 6k demonstrate significantly higher binding energies and inhibition constants in comparison to erlotinib.

Keywords: Isoxazole, Pyrimidine, MWI, Anticancer activity, Molecular docking

INTRODUCTION

Cancer is one of the leading causes of death, responsible for around 9 million fatalities annually.[1,2] Anticancer agents play a crucial role in cancer treatment, with more than 100 drugs approved for this indication.[3,4] The emergence of drug resistance and the notable side effects of existing anticancer drugs present significant challenges to effective chemotherapy, underscoring the urgent need to explore new agents with low toxicity and high efficacy.[5-7] Among the numerous anticancer therapeutic targets identified, protein kinases have been the subject of extensive research.[8-11] Hybrid compounds that incorporate two or more distinct pharmacophores may exhibit multiple action mechanisms.[12,13] Consequently, hybrid molecules have the potential to reduce the severity of adverse effects and effectively address drug resistance. It is essential to acknowledge that several hybrid molecules are presently in clinical trials across different phases for the treatment of various diseases, including those caused by medication-resistant organisms. This illustrates that hybridization serves as an effective method for the advancement of innovative pharmaceuticals.[14,15]

In medicinal chemistry, nitrogen and oxygen-containing heterocycles are thought to be a good combination because of their various pharmacological actions, especially when it comes to fused heterocyclic molecules.[16–23] Isoxazole is a five-membered azole found in ibotenic acid and is a crucial constituent of many therapeutically approved pharmaceuticals, including danazol, cloxacillin, valdecoxib, flucloxacillin, dicloxacillin, risperidone, and zonisamide. Fused isoxazole and its derivatives are acknowledged pharmacophores in drug development and medicinal chemistry.[24] Fused heterocyclic compounds containing pyrimidine derivatives have shown considerable biological potential, especially in anticancer research.[25-29] Recently, Karthik *et al*. reported novel fused isoxazole derivatives as potent EGFR-targeting anticancer agents, and compound A demonstrated significant activity against human breast cancer cell lines MCF-7, MDA-MB-468, and MDA-MB-231.[30] In 2022, Aleksandra and colleagues synthesized and evaluated the anticancer activity of oxazolo[5,4-d]pyrimidine containing isoxazoles (Compound B).[31]

In the past two decades, several methods have emerged as effective tools for heterocyclic synthesis, particularly the

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following: Ionic liquids (ILs) function as catalysts and reaction mediums, in conjunction with energy sources like electrosynthesis, electromagnetic (microwave) irradiation, and ultrasonic irradiation, which have recently gained importance in chemical synthesis.[32-36] Ultrasound, unlike traditional methods, is a crucial technique for improving organic synthesis in green chemistry, as it promotes higher yields, selectivity, and shorter reaction times.[37] Polyethylene glycols (PEGs) have attracted considerable attention as a reaction medium for organic transformations due to their cost-effectiveness, thermal stability, recyclability, and biodegradability.[38,39]

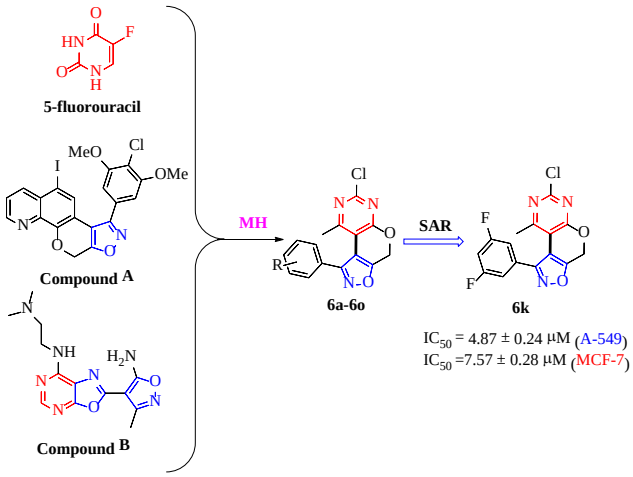
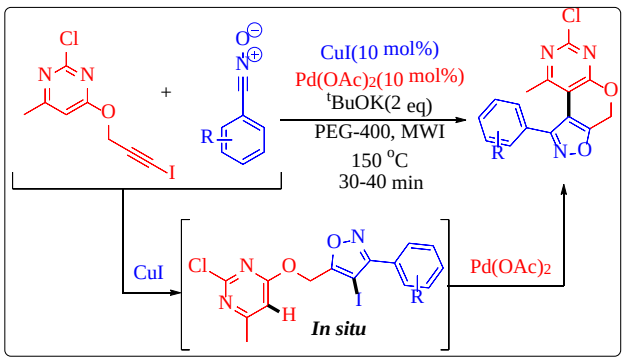


Figure 1. Design strategy for new fused isoxazolo[4',5':4,5] pyrano[2,3-*d*]pyrimidines *via* molecular hybridization approach.

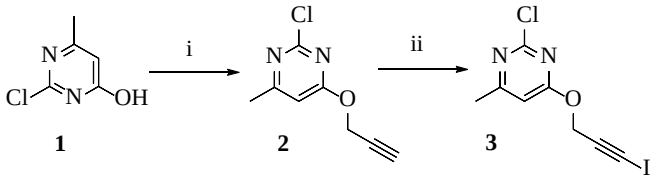
Because of (i) the important roles that pyrimidine and isoxazole play in the development of various anticancer drugs (Figure 1) and (ii) the application of green chemistry concepts through the use of MW energy and environmentally friendly PEG-400 for sustainable organic synthesis, we synthesize some novel fused isoxazolo[4',5':4,5]pyrano[2,3-*d*]pyrimidines using Cu(I)-catalyzed 1,3-dipolar cycloaddition followed by Pd(II)-catalyzed the intramolecular C-C bond coupling (Scheme 1).[40] Each freshly synthesized chemical's anticancer activity was examined using the A-549 and MCF-7 cancer cell lines. The binding affinity and mechanism of interactions with the selected enzymes' essential amino acids were then assessed using molecular docking.



Scheme 1. One-pot synthesis of isoxazolo[4',5':4,5]pyrano[2,3-d]pyrimidines (6a-6o).

RESULTS AND DISCUSSION

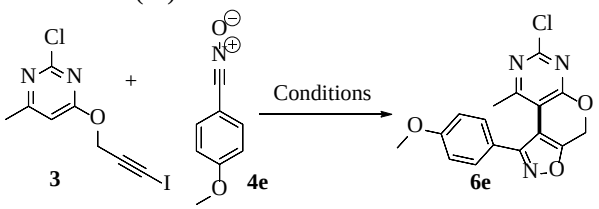
Initially, commercially available 2-chloro-6-methyl pyrimidin-4-ol (1) was treated with propargyl bromide using K_2CO_3 in DMF at 80 ℃temperature for 6h to obtain 2-chloro-4-methyl-6-(prop-2-yn-1-yloxy)pyrimidine (2).[41] Later, the key intermediate 2-chloro-4-((3-iodoprop-2-yn-1-yl)oxy)-6-methyl pyrimidine (3) was obtained from the CuI catalyzed reaction between 2 and N-iodomorpholine in THF at room temperature for 2h (Scheme 2).[42]



Scheme 2. Reagents & conditions: (i) Propargyl bromide, K_2CO_3, DMF, 80 °C, 6h. (ii) N-iodomorpholine, CuI, THF, RT, 2h.

Our initial investigation began with the intramolecular cyclization of in situ generated 3,5-disustituted isoxazole (5) from 2-chloro-4-((3-iodoprop-2-yn-1-yl)oxy)-6-methyl pyrimidine (3) with 4-methoxyphenyl nitrile oxide by following the previously reported Cu(I) catalyzed C-C bond coupling reactions.[17,18] Accordingly, at first, we have concentrated on the optimization of reaction conditions using 2-chloro-4-((3-iodoprop-2-yn-1-yl)oxy)-6-methylpyrimidine (3) and freshly prepared 4-methoxy phenyl nitrile oxide as model reactants and the results are presented in Table 1. We did not get the desired product 7-chloro-1-(4-methoxyphenyl)-9-methyl-4H-isoxazolo[4',5':4,5]pyrano[2,3-d]pyrimidine (6e), in the presence of 5 and 10 mol% of CuI catalyst and 2 equivalent ᵗBuOK in PEG-400 at 100 °C even after 24h (Table 1 , entry 1-2). Since it was known that the use of palladium catalyst substantially improves the yields of many cross-coupling reactions,[43,44] we decided to explore the application of such catalyst to our reaction.

Table 1. Optimization of the Cu/Pd-catalyzed reaction to access fused isoxazole (6e).



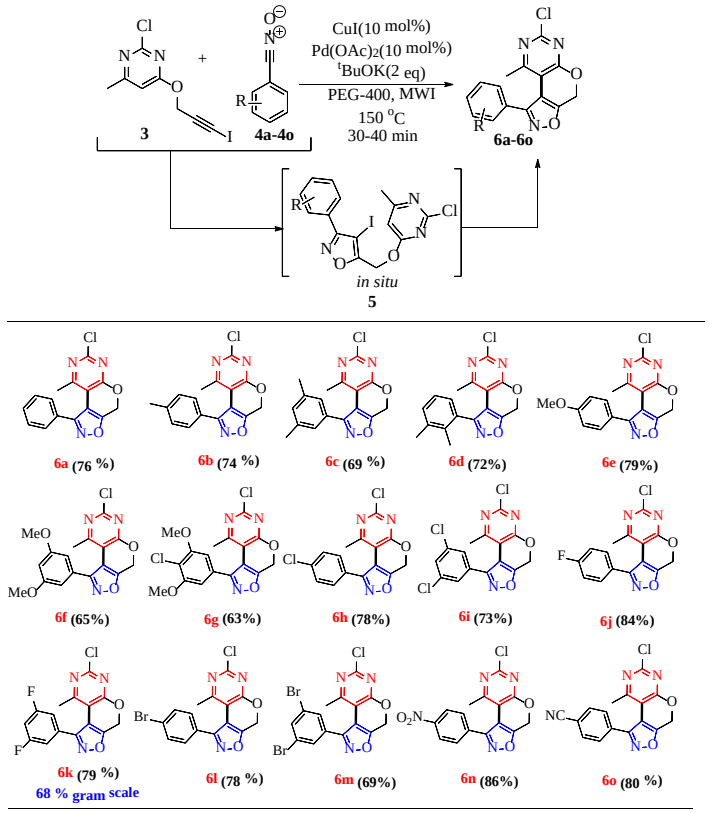
Entry	Catalyst (mol%)	Solvent	Temp (ºC)	Time (h)	Yield (%)
1	CuI(5)	DMF	100	24	-
2	CuI(10)	DMF	100	24	-
3	CuI(5)/Pd(PPh$_3$)$_4$(5)	DMF	100	24	18
4	CuI(10)/Pd(PPh$_3$)$_4$(5)	DMF	100	24	21
5	CuI(10)/Pd(PPh$_3$)$_4$(10)	DMF	100	24	25
6	CuI(10)/Pd(OAc)$_2$(5)	PEG-400	100	24	53
7	CuI(10)/Pd(OAc)$_2$(10)	PEG-400	100	24	59

8	CuI(15)/Pd(OAc)$_2$(15)	PEG-400	100W	30	68
9	**CuI(10)/Pd(OAc)$_2$(10)**	**PEG-400**	**150W**	**30 min**	**79**
10	CuI(10)/Pd(OAc)$_2$(10)	PEG-400	200W	35 min	77
11	CuI(15)/Pd(OAc)$_2$(15)	PEG-400	150W	40 min	77

[a]=Reactions were carried out in solvent (10 ml) with 1.0 equiv. of **3** and 1.2 equiv of 1-azido-4-methoxybenzene. ^{t}BuOK (2 eq). [b]=Yields are given for isolated products.

When the reaction was carried out with of CuI(10 m0l%) and $Pd(PPh_3)_4$ in DMF the desired compound 6e was produced in below 24 % yield (Table 1, entrie 3-5). Surprisingly, the reaction of 3 with Pd(OAc)$_2$ (5 mol%) and CuI (10 mol%) in the presence of ^{t}BuOK in PEG-400 produced the desired compound 6e in a 51 % yield (Table 1, entry 6). The results encouraged us to investigate further promising conditions to achieve better reaction yields. To this end we have carried out the reaction with 10 mol% CuI and 10 mol% Pd(OAc)$_2$ in PEG-400, in the presence of ^{t}BuOK, and we have found that the yield of the desired product 6e was 59% (Table 1, entry 7). Again the same reaction was carried out 10 mol% CuI and 10 mol% Pd(OAc)$_2$ in PEG-400 under microwave irradiation in 30 min, and we have found that the yield of the desired product 6e was 68% (Table 1, entry 8). Based on the above results, we investigated the effect of other reaction parameters under microwave irradiation (MWI). When the same reaction was carried out under MWI at 150W and 200W in PEG-400 medium for about 30–45 min, the desired product was obtained in excellent yield (Table 1, entries 9, 10). Finally the same reaction was carried out by using 10 mol% CuI and 10 mol% Pd(OAc)$_2$ in PEG-400 under microwave irradiation in 40 min the desired product was obtained 77 % yield and there was not much affect of the increasing catalytic ratio of CuI and Pd(OAc)$_2$ on the yield of the product (Table 1, entry 11). The PEG-400 was recycled and reused for the remaining analogous.

Following the completion of optimization experiments, it was obvious that using CuI (10 mol%) and Pd(OAc)$_2$ (10 mol%) catalytic loading with 2 equivalents of ^{t}BuOK in PEG-400 under microwave irradiation at 150W was the ideal reaction condition to generate the final desired product in a good yield. To further simplify the procedure, the intramolecular C-H arylation of in situ produced 4-iodo-isoxazole (5) was carried out employing different nitrile oxides under the above hopeful conditions (Scheme 3).



Scheme 3. Cu/Pd catalyzed one pot synthesis of fused isoxazole. The isolated yields are given as percentages.

In vitro cytotoxicity

Recently synthesized fused isoxazoles (6a-6o) were examined for their in vitro cytotoxicity against human cancer cell lines A-549 and MCF-7, with 5-FU serving as the positive control.[45-47] The cytotoxicity data (Table 2) indicate that the compounds exhibited varying degrees of cytotoxic activity against the tested cancer cell lines, categorized as strong, good, moderate, and weak. Among all compounds, those containing a 3,5-difluorophenyl group on the isoxazole ring exhibited superior cytotoxic activity against both tested cancer cell lines, with IC_{50} values of 4.87 ± 0.24 μM (A-549) and 7.57 ± 0.28 μM (MCF-7), compared to the standard 5-fluorouracil, which had IC_{50} values of 6.35 ± 0.21 μM (A-549) and 10.32 ± 0.87 μM (MCF-7). Similarly, compound 6j which contains 5-fluorophenyl on fused isoxazole ring have shown potent activity against tested cancer cell lines as compared to standard drug with IC_{50} values 5.19 ± 0.37 and 9.86 ± 0.23 μM, and also compound 6i, which contains 3,5-difluorophenyl on isoxazole ring have shown equipotent activity against testes cancer cell lines with IC_{50} values 6.43 ± 0.28 and 10.36 ± 0.31 μM. Compound 6m (which contains 3,5-dibromophenyl group on isoxazole) and compound 6h (which contains 4-chlorophenyl group on isoxazole) have shown good activity against tested cell lines with IC_{50} values ranging from 7.43 ± 0.38 μM to 12.20 ± 0.35 μM. In specific, compounds 6i, 6j, and 6k showed superior cytotoxic results against tested cell lines than the 5-FU with IC_{50} values ranging from 4.87 ± 0.24 μM to 10.02 ± 0.31 μM, respectively. In addition, compounds 6h, 6m, and 6o showed good cytotoxicity against both tested cell lines with IC_{50} values ranging from 7.43 ± 0.38 μM to 16.86 ± 0.17 μM. Similarly, compounds 6m, 6n and 6o have shown good activity against A-549 cell line with IC_{50} values 11.64 ± 0.41 μM, 15.88 ± 0.26 μM and 14.90 ± 0.12 μM. In addition, the remaining compounds exhibited moderate to poor activity against tested cell lines with IC_{50} values 16.63 ± 0.23 μM to 50.21 ± 0.26 μM. The more potent compounds further evaluated for their cytotoxicity against human normal cancer cell line HEK-293, and the compounds 6i, 6j, and 6k have shown less cytotoxicity against HEK-293 as compared to standard 5-FU.

Table 2: *In vitro* cytotoxicity of fused isoxazole derivatives (6a-6o) with IC_{50} in μM.

Compd	R	IC_{50} (μM)[a]		
		A-549	MCF-7	HEK-293
6a	H	50.21 ± 0.89	43.04 ± 1.08	NT
6b	4-Me	44.95 ± 0.72	50.80 ± 0.89	NT
6c	3,5-diMe	36.52 ± 0.55	48.26 ± 1.02	NT
6d	2,3-diMe	31.23 ± 0.63	42.29 ± 0.76	NT
6e	4-OMe	26.07 ± 0.85	29.32 ± 0.47	NT
6f	3,5-diOMe	20.65 ± 0.59	25.57 ± 0.67	NT
6g	3,5-diOMe, 4-Cl	12.68 ± 0.33	16.01 ± 0.59	NT
6h	4-Cl	9.65 ± 0.41	12.20 ± 0.35	10.44 ± 0.38
6i	3,5-diCl	6.43 ± 0.28	10.36 ± 0.31	13.67 ± 0.51
6j	4-F	5.19 ± 0.37	9.86 ± 0.23	14.73 ± 0.64
6k	3,5-diF	4.87 ± 0.24	7.57 ± 0.28	13.91 ± 0.38
6l	4-Br	13.18 ± 0.36	14.62 ± 0.39	NT
6m	3,5-diBr	7.43 ± 0.38	11.64 ± 0.47	9.37 ± 0.27
6n	4-NO_2	14.65 ± 0.44	15.89 ± 0.56	NT
6o	4-CN	10.63 ± 0.35	12.32 ± 0.62	NT
5-FU	-	**6.35 ± 0.21**	**10.32 ± 0.87**	**12.22 ± 0.19**

[a]= Values are expressed as Mean±SD. NT= Not tested.

Molecular docking studies

The epidermal growth factor receptor, often known as EGFR, is a cell surface receptor that plays a significant role in the formation of ducts in mammary glands.[48] Additionally, it has been linked to the overexpression that is associated with a number of different malignancies. On account of this, EGFR is an important target in the process of therapeutic development. Molecular docking experiments were carried out on epidermal growth factor receptors that were obtained from the protein data bank (PDB id-4HJO).[49] These studies were carried out with the assistance of auto documentation tools.

The compounds 6i, 6j, and 6k which have shown potential anticancer and EGFR activity *in vitro*, were evaluated for binding interactions with the EGFR. According to the findings (Table 3), the compound 6i has the highest binding energy (-9.02 Kcal/mol) and forms two hydrogen bonds with ALA698 and PHE699 with bond lengths of 2.017 Å and 1.798 Å (Fig. 2). Similarly, compounds 6k has shown second highest binding energy (-7.86 Kcal/mol) and forms three hydrogen bonds with ALA698, PHE699, and ALA835 with bond lengths of 1.986 Å, 1.948 Å and 2.411 Å (Fig. 3) and these results are better than standard erlotinib (-7.69 Kcal/mol). Finally compound 6j has shown comparable binding energy compared to standard with binding energy (-7.59 Kcal/mol).

Table 3: Binding energy and hydrogen binding interactions of compounds with EGFR (PDB ID:4HJO).

Compounds	B.E (kcal/mol)	No. of Hydrogen bonds	Residues involved in the hydrogen bonding
6i	-9.02	2	ALA698, PHE699
6j	-7.59	3	LYS721, GLY700, ALA698
6k	-7.86	3	ALA698, PHE699, ALA835
Erlotinib	-7.69	1	THR830

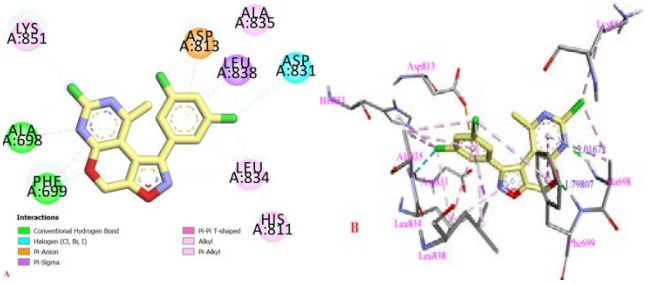


Figure 2. 2D and 3D interactions of compound 6i with EGFR-protein.

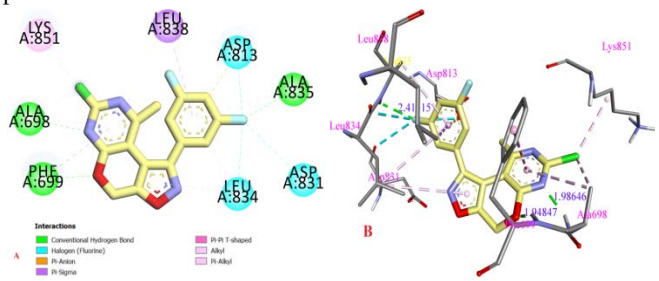


Figure 3. 2D and 3D interactions of compound 6k with EGFR-protein.

EXPERIMENTAL SECTION

All the commercially available chemicals were utilized without further purification. The purity of compounds was analyzed on Merck 60F254 silica gel TLC plates. Melting points were recorded on a hot-stage melting point apparatus in Ernst Leitz Wetzlar, Germany, and were uncorrected. The ^{1}H and ^{13}C NMR spectra were recorded using the Mercuryplus spectrometer (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C), and the chemical shifts were referenced to TMS. The ESI (electrospray ionization) mass spectra at an ionizing voltage of 70 eV were obtained with the help of a Shimadzu QP5050A quadrupole-based mass spectrometer. Elemental analyses were obtained with an Elemental Analyzer Perkin-Elmer 240 C apparatus.

Synthesis of 2-chloro-4-methyl-6-(prop-2-yn-1-yloxy) pyrimidine (2)

A mixture of 2-chloro-6-methylpyrimidin-4-ol (1) (5g, 0.035 mol), K_2CO_3 (0.01 mol) and Propargyl bromide (0.04 mol) in DMF (50 mL) was stirred at 80 ^{o}C temperature for 6h. The completion of the reaction as monitored by TLC, and the mixture was diluted with ice-water (50 mL) and extracted with ethyl acetate (3 ×50 mL). The combined organic layer was washed with brine (2×50 mL), then dried under anhydrous Na_2SO_4 and finally concentrated under vacuum to afford compound (2). White solid (Yield 83%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 6.69 (s, 1H, -CH), 4.36 (d, J=8.0 Hz, 2H, O-CH_2), 2.31 (s, 3H, -CH_3), 2.03 (t, J= 4.0 Hz, CH-alkyne); ESI-MS: 183 $[M+H]^+$.

Synthesis of 2-chloro-4-((3-iodoprop-2-yn-1-yl)oxy)-6-methylpyrimidine (3).

To 2-chloro-4-methyl-6-(prop-2-yn-1-yloxy)pyrimidine (2) (0.016 mol) in THF (30 ml), CuI (2 mmol) and N-iodomorpholine (0.024 mmol) were added and the resulting reaction mixture was stirred at 0 oC to room temperature for 2h to get a fine white precipitate. The suspension was poured onto a

pad of activated neutral alumina (100 ml) and the filtrate was collected. The solid phase was washed with CH_2Cl_2 (3 ×30 ml) and the combined organic fractions were concentrated by evaporation under reduced pressure to afford compound 3. White solid. Yield 69%. 1H-NMR (400 MHz, DMSO-d6) δ 6.63 (s, 1H, -CH), 4.31 (s, 2H, O-CH_2), 2.29 (s, 3H, -CH3); ESI-MS: 309 $[M+H]^+$.

Synthesis of novel fused Isoxazolo[4',5':4,5]pyrano[2,3-d] pyrimidines (6a-6o).

To a mixture of 2-chloro-4-((3-iodoprop-2-yn-1-yl)oxy)-6-methylpyrimidine (3) (0.001 mol), nitrile oxide (4) (0.001 mmol), ^{t}BuOK (2.0 mmol), CuI (10 mol%) and $Pd(OAc)_2$ (10 mol%) in PEG-400 (10 mL) under microwave irradiation at 100 W for about 30-40 min, and the process was monitored by TLC. After completion of the reaction, the reaction mixture was extracted with ether (PEG being insoluble in ether). Ether layer was transfer, dried, and concentrated under reduced pressure. The crude products were purified by column chromatography, eluent EtOAc–hexane, 2.5:7.5. The PEG 400, after the extraction with dry ether, can be reused for a number of cycles without significant loss of activity.

7-chloro-9-methyl-1-phenyl-4H-isoxazolo[4',5':4,5]pyrano[2,3-d]pyrimidine (6a): White solid. M.p: 110-112 ºC; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.63 (d, J = 8.0 Hz, 2H, Ar-H), 7.43-7.37 (m, 3H, Ar-H), 5.32 (s, 2H, O-CH_2), 2.31 (s, 3H, -CH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.61, 165.60, 158.38, 155.23, 144.86, 131.95, 129.23, 128.66 (2C), 126.62(2C), 119.19, 116.28, 54.32, 23.79.; ESI-MS: 300 $[M+H]^+$. Anal. Calcd for $C_{15}H_{10}ClN_3O_2$: C, 60.11; H, 3.36; N, 14.02. Found: C, 60.08; H, 3.33; N, 13.98.

7-chloro-9-methyl-1-(p-tolyl)-4H-isoxazolo[4',5':4,5]pyrano[2,3-d]pyrimidine (6b): Pale red solid. M.p: 116-118 ºC; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.59 (d, J = 8.0 Hz, 2H, Ar-H), 7.14 (d, J = 8.0 Hz, 2H, Ar-H), 5.31 (s, 2H, O-CH_2), 2.32 (s, 3H, -CH_3), 2.23 (s, 3H, -CH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.61, 165.60, 158.38, 155.23, 144.86, 139.32, 129.60, 129.00(2C), 126.89(2C), 119.19, 116.28, 54.42, 23.79, 21.31; ESI-MS: 314 $[M+H]^+$. Anal. Calcd for $C_{16}H_{12}ClN_3O_2$: C, 61.25; H, 3.86; N, 13.39. Found: C, 61.23; H, 3.84; N, 13.36.

7-chloro-1-(3,5-dimethylphenyl)-9-methyl-4H-isoxazolo[4',5': 4,5]pyrano[2,3-d]pyrimidine (6c): Pale red solid. M.p: 121-123 ºC; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.48 (s, 1H, Ar-H), 7.12 (s, 2H, Ar-H), 5.31 (s, 2H, O-CH_2), 2.34 (s, 3H, -CH_3), 2.18 (s, 6H, 2-CH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.61, 165.60, 158.38, 155.23, 144.78, 136.02(2C), 132.11, 130.18, 127.12(2C), 119.19, 116.28, 55.42, 23.79, 21.03(2C); ESI-MS: 328 $[M+H]^+$. Anal. Calcd for $C_{17}H_{14}ClN_3O_2$: C, 62.30; H, 4.31; N, 12.82. Found: C, 62.33; H, 4.35; N, 12.79.

7-chloro-1-(2,3-dimethylphenyl)-9-methyl-4H-isoxazolo[4',5' :4,5]pyrano[2,3-d]pyrimidine (6d): White solid. M.p: 128-130 ºC; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.42-7.36 (m, 3H, Ar-H), 5.32 (s, 2H, O-CH_2), 2.35 (s, 3H, -CH_3), 2.17(s, 3H, -CH_3), 1.97 (s, 3H, -CH3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.61, 165.60, 158.55, 155.23, 144.23, 137.74, 135.80, 134.42, 130.85, 127.49, 125.17, 119.22, 117.19, 54.42, 23.79, 19.95, 16.53; ESI-MS: 328 $[M+H]^+$. Anal. Calcd for $C_{17}H_{14}ClN_3O_2$: C, 62.30; H, 4.31; N, 12.82. Found: C, 62.27; H, 4.35; N, 12.79.

7-chloro-1-(4-methoxyphenyl)-9-methyl-4H-isoxazolo[4',5':4,5] pyrano[2,3-d]pyrimidine (6e): Pale yellow solid. M.p: 125-127 ºC; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.69 (d, J = 8.0 Hz, 2H, Ar-H), 7.00 (d, J = 8.0 Hz, 2H, Ar-H), 5.32 (s, 2H, O-CH_2), 3.86 (s, 3H, -OCH_3), 2.32 (s, 3H, -CH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.69, 165.22, 159.77, 158.42, 155.55, 144.67, 131.41(2C), 123.61, 119.56, 116.34, 114.16(2C), 55.65, 54.30, 23.53; ESI-MS: 330 $[M+H]^+$. Anal. Calcd for $C_{16}H_{12}ClN_3O_3$: C, 58.28; H, 3.67; N, 12.74. Found: C, 58.25; H, 3.69; N, 12.71.

7-chloro-1-(3,5-dimethoxyphenyl)-9-methyl-4H-isoxazolo[4',5':4,5]pyrano[2,3-d]pyrimidine (6f): Pale yellow solid. M.p: 139-131 ºC; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.66 (s, 2H, Ar-H), 6.96 (s, 1H, Ar-H), 5.34 (s, 2H, O-CH_2), 3.83 (s, 6H, 2-OCH_3), 2.33 (s, 3H, -CH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.61, 165.60, 160.36 (2C), 158.38, 155.23, 144.78, 135.28, 119.19, 116.28, 108.96 (2C), 104.09, 56.04 (2C), 54.42, 23.79; ESI-MS: 360 $[M+H]^+$. Anal. Calcd for $C_{17}H_{14}ClN_3O_4$: C, 56.76; H, 3.92; N, 11.68. Found: C, 56.73; H, 3.95; N, 11.65.

7-chloro-1-(4-chloro-3,5-dimethoxyphenyl)-9-methyl-4H-isoxazolo[4',5':4,5]pyrano[2,3-d]pyrimidine (6g): Pale red solid. M.p: 149-151 ºC; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 6.96 (s, 2H, Ar-H), 5.35 (s, 2H, O-CH_2), 3.84 (s, 6H, 2-OCH_3), 2.33 (s, 3H, -CH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.61, 165.60, 159.60(2C), 158.38, 155.23, 144.78, 133.62, 121.10, 119.19, 116.28, 108.55(2C), 56.79(2C), 54.42, 23.79.; ESI-MS: 394 $[M+H]^+$. Anal. Calcd for $C_{17}H_{13}Cl_2N_3O_4$: C, 51.80; H, 3.32; N, 10.66. Found: C, 51.78; H, 3.36; N, 10.64.

7-chloro-1-(4-chlorophenyl)-9-methyl-4H-isoxazolo[4',5':4,5] pyrano[2,3-d]pyrimidine (6h): Red solid. M.p: 122-124 ºC; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.73 (d, J = 8.0 Hz, 2H, Ar-H), 7.39 (d, J = 8.0 Hz, 2H, Ar-H), 5.35 (s, 2H, O-CH_2), 2.34 (s, 3H, -CH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.29, 165.48, 158.63, 155.39, 144.67, 134.62, 130.45(2C), 129.23, 126.71(2C), 119.80, 116.25, 54.30, 23.58; ESI-MS: 334 $[M+H]^+$. Anal. Calcd for $C_{15}H_9Cl_2N_3O_2$: C, 53.92; H, 2.71; N, 12.58. Found: C, 53.89; H, 2.68; N, 12.56.

7-chloro-1-(3,5-dichlorophenyl)-9-methyl-4H-isoxazolo[4',5': 4,5]pyrano[2,3-d]pyrimidine (6i): White solid. M.p: 139-141 ºC; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.71 (s, 2H, Ar-H), 7.30 (s, 1H, Ar-H), 5.34 (s, 2H, O-CH_2), 2.36 (s, 3H, -CH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.49, 165.42, 158.57, 155.53, 144.76, 132.84 (2C), 131.19, 130.47, 126.44 (2C), 119.62, 116.59, 54.72, 23.58; ESI-MS: 369 $[M+H]^+$. Anal. Calcd for $C_{15}H_8Cl_3N_3O_2$: C, 48.88; H, 2.19; N, 11.40. Found: C, 48.86; H, 2.16; N, 11.37.

7-chloro-1-(4-fluorophenyl)-9-methyl-4H-isoxazolo[4',5':4,5] pyrano[2,3-d]pyrimidine (6j): Yellow solid. M.p: 123-125 ºC; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.18 (d, J = 8.0 Hz, 2H, Ar-H), 7.94 (d, J = 8.0 Hz, 2H, Ar-H), 5.35 (s, 2H, O-CH_2), 2.35 (s, 3H, -CH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.38, 165.48, 162.54, 160.84, 158.64, 155.66, 144.57, 131.42 (2C), 127.85, 118.16, 116.69, 114.64, 114.40, 54.49, 23.75; ESI-MS: 318 $[M+H]^+$. Anal. Calcd for $C_{15}H_9ClFN_3O_2$: C, 56.71; H, 2.86; N, 13.23. Found: C, 56.67; H, 2.84; N, 13.20.

7-chloro-1-(3,5-difluorophenyl)-9-methyl-4H-isoxazolo[4',5': 4,5]pyrano[2,3-d]pyrimidine (6k): Pale red solid. M.p: 145-147 ºC; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.18(s, 2H, Ar-H), 7.97 (s, 1H, Ar-H), 5.35 (s, 2H, O-CH_2), 2.37 (s, 3H, -CH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.61, 166.78, 166.39, 165.60,

163.87, 163.45, 158.38, 155.23, 144.78, 134.56, 119.19, 116.28, 110.88, 110.56, 110.37, 110.512, 106.88, 106.65, 106.22, 54.42, 23.79.; ESI-MS: 336 $[M+H]^+$. Anal. Calcd for $C_{15}H_8ClF_2N_3O_2$: C, 53.67; H, 2.40; N, 12.52. Found: C, 53.64; H, 2.37; N, 12.48.

1-(4-bromophenyl)-7-chloro-9-methyl-4H-isoxazolo[4',5':4,5] pyrano[2,3-*d*]pyrimidine (6l): Yellow solid. M.p: 140-142 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.55-7.49 (m, 4H, Ar-H), 5.34 (s, 2H, O-CH_2), 2.35 (s, 3H, -CH3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.40, 165.84, 158.60, 155.25, 144.71, 130.45 (2C), 129.34, 127.86 (2C), 124.80, 119.76, 116.40, 54.33, 23.63; ESI-MS: 379 $[M+H]^+$. Anal. Calcd for $C_{15}H_9BrClN_3O_2$: C, 47.59; H, 2.40; N, 11.10. Found: C, 47.56; H, 2.37; N, 11.07.

7-chloro-1-(3,5-dibromophenyl)-9-methyl-4H-isoxazolo[4',5': 4,5] pyrano[2,3-d]pyrimidine (6m): Yellow solid. M.p: 159-161 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.50 (s, 2H, Ar-H), 7.29 (s, 1H, Ar-H), 5.34 (s, 2H, O-CH_2), 2.33 (s, 3H, -CH3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.61, 165.60, 158.38, 155.23, 144.86, 130.46(2C), 129.05, 128.59(2C), 124.91, 119.19, 116.28, 54.42, 23.79.; ESI-MS: 458 $[M+H]^+$. Anal. Calcd for $C_{15}H_8Br_2ClN_3O_2$: C, 39.38; H, 1.76; N, 9.18. Found: C, 39.35; H, 1.74; N, 9.15.

7-chloro-9-methyl-1-(4-nitrophenyl)-4H-isoxazolo[4',5':4,5] pyrano[2,3-d]pyrimidine (6n): Pale red solid. M.p: 133-135 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.28 (d, J = 8.0Hz, 2H, Ar-H), 8.12 (d, J = 8.0 Hz, 2H, Ar-H), 5.37 (s, 2H, O-CH_2), 2.37 (s, 3H, -CH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.54, 165.62, 158.38, 155.53, 148.53, 144.78, 135.29, 128.21(2C), 123.88(2C), 119.71, 116.62, 54.44, 23.60; ESI-MS: 345 $[M+H]^+$.Anal. Calcd for $C_{15}H_9ClN_4O_4$: C, 52.27; H, 2.63; N, 16.25. Found: C, 52.29; H, 2.60; N, 16.22.

4-(7-chloro-9-methyl-4H-isoxazolo[4',5':4,5]pyrano[2,3-d] pyrimidin-1-yl)benzonitrile (6o): Yellow solid. M.p: 132-134 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.89 (d, J = 8.0 Hz, 2H, Ar-H), 7.46 (d, J = 8.0 Hz, 2H, Ar-H), 5.35 (s, 2H, O-CH_2), 2.34 (s, 3H, -CH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.61, 165.60, 158.38, 155.23, 144.86, 136.39, 131.81(2C), 131.12(2C), 119.78, 117.19, 116.28, 115.70, 54.42, 23.79; ESI-MS: 325 $[M+H]^+$. Anal. Calcd for $C_{16}H_9ClN_4O_2$: C, 59.18; H, 2.79; N, 17.25. Found: C, 59.21; H, 2.75; N, 17.22.

CONCLUSION

In conclusion, we have designed a simple and effective protocol for the production of fused isoxazolo[4',5':4,5] pyrano[2,3-*d*]pyrimidines (6a-6o) in PEG-400 medium during sustainable MWI. The reaction is characterized by a broad substrate range, a brief reaction time, operational simplicity, and the ability to be applied to gram-scale synthesis from the perspective of synthetic chemistry. The corresponding products were obtained in high purity and excellent yields following brief reaction times. In addition, the in vitro anticancer activity of all of these synthesized derivatives was assessed against MCF-7 and A-549. The compounds 6j and 6k demonstrated superior activity in comparison to the standard 5-FU and the remaining compounds, with IC_{50} values ranging from 4.87 ± 0.24 μM to 9.86 ± 0.23 μM. Surprisingly, *in silico* analyses such as molecular docking of more potent compounds 6i, 6j, and 6k were demonstrated to be consistent with the corresponding *in vitro* activity IC_{50} data.

Supplementary materials

Supplementary material associated with this article can be downloaded from the online article page.

Conflict of Interests

The authors declare no conflict of interest.

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Authorship contribution:

T Gopikishan: Methodology. **V. Muralimohanarao:** Software. **H. Singh:** Writing – review & editing.

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