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Synthesis of novel isoxazolo[2",3":1',2']pyrimido[4',5':4,5]pyrimido-[6,1-*b*]quinazoline-8-ones and their *in vitro* anticancer and antimicrobial activities

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Synthesis of novel isoxazolo[2",3":1',2']pyrimido[4',5':4,5]pyrimido[6,1-*b*]quinazoline-8-ones **6a-j,** has been achieved by reaction of 5-amino-2-methyl-7-phenyl-7*H*-isoxazolo[2,3-a] pyrimidin-6-yl cyanides **4**, with dimethyl formamide dimethylacetal followed by treatment with anthranilic acids *in situ* in one-pot. The key intermediate, *viz*., 5-amino-2-methyl-7-phenyl-7*H*-isoxazolo[2,3-*a*]pyrimidin-6-yl cyanide **4** is obtained by reaction of 3-amino-5-methyl isoxazole with aromatic aldehydes and malononitrile by a three-component one-pot process. The newly synthesized compounds **6a-j** have been evaluated for their *in vitro* anticancer and antimicrobial activity. Compounds **6e** and **6f** exhibit potent anticancer and antimicrobial activity comparable to that of standard drugs.

Keywords: Isoxazolo[2",3":1',2']pyrimido[4',5':4,5]pyrimido[6,1-*b*]quinazoline-8-ones, one-pot synthesis, three-component reaction, anticancer activity, antimicrobial activity

Quinazolines and condensed pyrimidines show a wide spectrum of biological activities and have been exhaustively reviewed. Pyrido [2,3-*d*] pyrimidines are considered to be bioisostreres of quinazoline. The concept of bioisosterism has been exploited by medicinal chemists as an approach to drug design. It is a strategy for rational design of new drugs, applied with a lead compound as special process of molecular modification[1]. The bioisosteres of quinazolines and pyridopyrimidines has given rise to a number of compounds exhibiting potent pharmacological actions like antibacterials[2], EGFr and C-erbB-2 inhibitory activity[3], kinase inhibitory activity[4], and phosphodiesterase-5-inhibitory activity[5-8].

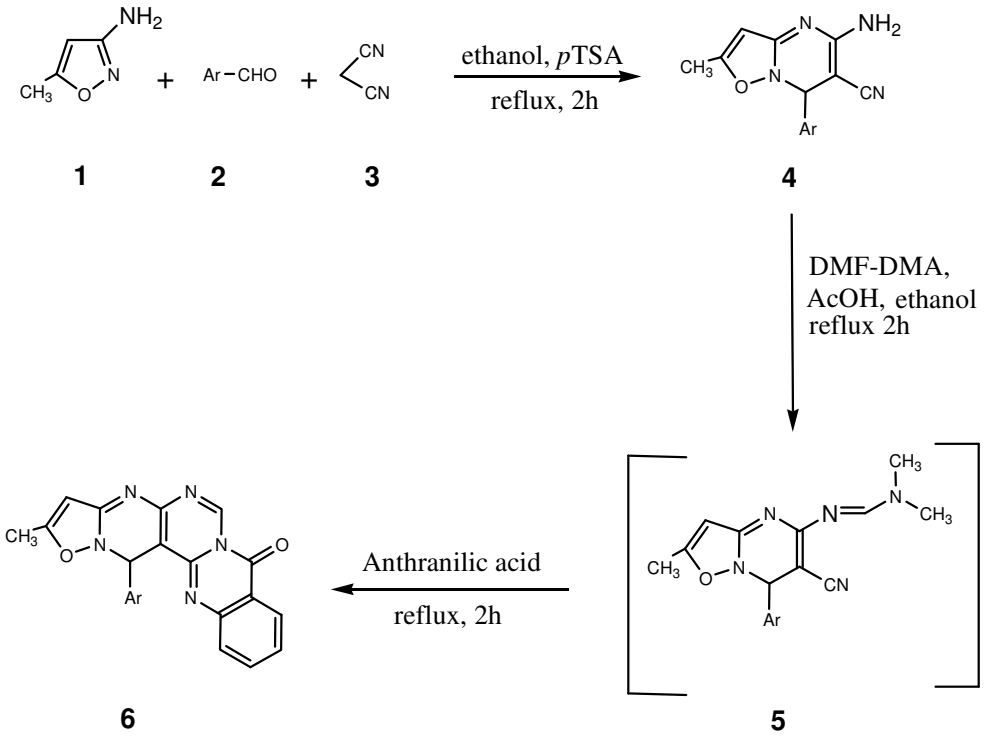
Similarly, isoxazole derivatives represent an interesting class of compounds possessing a wide spectrum of biological activities. A large number of isoxazole derivatives exhibited antibacterial[9], antifungal[10], anticonvulsant[11], analgesic[12], and anticancer[13] activities. Attracted by these impressive scaffolds *viz*., pyrimido quinazolines and isoxazole derivatives and their pharmacological properties, it was decided to set out to develop a synthetic route to prepare novel isoxazolo[2",3":1',2']pyrimido-[4',5':4,5]-pyrimido[6,1-*b*]quinazoline-8-ones. As a sequel to the work on the synthesis of pharmacologically active fused isoxazole derivatives[14-18], herein is reported the synthesis, *in vitro* anticancer and antimicrobial activity sof novel series of isoxazolo [2",3":1',2']pyrimido [4',5':4,5]-pyrimido[6,1-*b*]quinazoline-8-ones.

Results and Discussion

The synthesis of title compounds **6a-j** was accomplished by synthetic sequence shown in **Scheme I**. The three-component reaction of 3-amino-5-methylisoxazole **1** (purchased from Sigma-Aldrich), substituted aromatic aldehyde **2** and malononitrile **3** in presence of *p*-toluene sulphonic acid (*p*TSA), a Lewis acid catalyst, in ethanol furnished novel 5-amino-2-methyl-7-phenyl-7*H*-isoxazolo[2,3-*a*]pyrimidin-6-yl cyanides **4** in good yields. Compound **4** was treated with dimethyl formamide-dimethyl acetal (DMF-DMA) to produce *N*,*N*-dimethyl formamide derivative **5**, which was then treated with anthranilic acid *in situ* to furnish the corresponding novel isoxazolo [2",3":1',2']pyrimido[4',5':4,5]pyrimido[6,1-*b*]quinazo-line-8-ones **6**.

Ten new derivatives of isoxazolo[2",3":1',2']-pyrimido[4',5':4,5]pyrimido[6,1-*b*]quinazoline-8-ones **6a-j** were reported. The structures of the newly synthesized compounds **4a-j** and **6a-j** were confirmed by analytical and spectral data (IR, ^{1}H and ^{13}C NMR, and MS).

IR spectra of **4** exhibited absorption bands at 3430, 3410 cm^{-1} due to NH_2 functional group stretching vibration, whereas CN functional group shown



4&6 Ar			
a,	C_6H_5	**f,**	4-$OCH_3C_6H_4$
b,	2-Cl C_6H_4	**g,**	4-$N(CH_3)_2C_6H_4$
c,	2-BrC_6H_4	**h,**	2,6-$Cl_2C_6H_3$
d,	2-OHC_6H_4	**i,**	2,6-$Br_2C_6H_3$
e,	4-$CH_3C_6H_4$	**j,**	3,4-$OCH_2OC_6H_3$

Scheme I

absorption band at 2210 cm^{-1}. In ^1H NMR spectra of **4** pyrimidine ring CH proton appeared as a sharp singlet at δ 5.12, and isoxazole ring proton appeared as a singlet at δ 6.12. A broad peak at δ 8.21, which is D_2O exchangable, is assigned to NH_2 protons. ^{13}C NMR spectra of **4** exhibited CN and ArCH carbon signals at δ 82.07 and 50.29 confirming the cyclization. The mass spectra of **4a** displayed the molecular ion [M^+] peak at *m/z* 252 confirming cyclization. The IR spectra of **6** showed prominent absorption band at 1670 cm^{-1} due to C=O functional group. The absence of NH_2 and CN functional group absorption bands in **6**, which are present in its precursor **4**, clearly indicates the formation of title compound **6** by cyclization. ^1H NMR spectra of **6** exhibited a singlet at δ 7.95 due to the newly formed pyrimidine ring proton confirming cyclization. Rest of the signals are in agreement with the proposed structure. ^{13}C NMR spectra of **6** displayed C=O carbon at δ 191.43 confirming cyclization. The mass spectra of **6a** displayed the molecular ion [M^+] peak at *m/z* 415, which is in agreement with the assigned structure. Data from the elemental analyses further confirmed the assigned structures of **4a-j** and **6a-j**.

Anticancer activity

The newly synthesized isoxazolo[2",3":1',2']-pyrimido[4',5':4,5]pyrimido[6,1-*b*]quinazoline-8-ones **6a-j** were evaluated against human cancer cell lines Hela, MCF-**7** and NCI-H460 for their *in vitro* anti cancer activity, according to MTT assay method[19,20] using Cisplatin (DDP) as a reference drug. The human cell cultures HeLa (cervical cancer), MCF-**7** (breast cancer) and NCI-H460 (lung cancer) cell lines, were obtained from National Center for Cell Sciences (NCCS), Pune, India. These cells were grown in recommended media supplemented with10% FBS, 1% L-Glutamine and 1% penicillin-streptomycin amphotercin B in a 5% CO_2 humidified atmosphere at 37°C. Cells were seeded in 5 cm^2 tissue culture flasks (Tarsions, India) at 25,0000 cells/flask in a total volume of 9 mL. When confluent, all the cells were

trypsinized (using Trypsis-EDTA, Himedia, Mumbai, India) and seeded in 96-well plates (Tarsions, India). The cell suspension of 1×10^5cells/mL was prepared in complete growth medium. Stock solutions of the compounds **6a-j** were prepared in DMSO. The stock solutions were serially diluted with complete growth medium containing 50 mg/mL of gentamycin to obtain working test solution of required concentrations (having <1% DMSO). The 100 µL of cell suspension was added to each well of the 96-well plates. The test materials in complete growth medium (100 µL) were added after 4 h incubation to the wells containing cell suspension. After 48 h of treatment with different concentrations of the test compounds, the cells were incubated with MTT (2.5 mg/mL) for 2 h. The medium was then removed, and 100 µL of DMSO was added to each well to dissolve formazan crystals, which is the metabolite of MTT. After thoroughly mixing, the plate was read at 490 nm for optical density that is directly correlated with cell quantity. The cytotoxic effects of the compounds were calculated as percentage inhibition in cell growth as per the formula. % cytotoxicity = 1-[(O.D. in sample well)/ (O.D. in control well)] × 100.

The results are presented in **Table I**. IC_{50} values were based on dose-response curves (IC_{50} values, defined as the concentration corresponding to 50% growth inhibition). From **Table I**, it is clear that some of the compounds showed excellent activity against tumor cells. The compounds **6e** and **6f** are the most cytotoxic towards all cancer cell lines. This enhanced activity of **6e** and **6f** may be due to the presence of electron releasing methyl and methoxy substituents on the benzene ring, besides isoxazolo[2",3":1',2']-pyrimido [4',5':4,5]pyrimido[6,1-*b*] quinazoline-8-one nucleus. Compounds **6d**, **6g**, **6j** exhibited moderate to good anticancer activity against three different cell lines, and not selectively towards any particular cell line. The presence of electron withdrawing chloro and bromo groups on benzene ring (**6b**, **6c, 6h** and **6i**) did not influence the anticancer activity much, and the compounds showed only moderate activity. Compound **6a** did not show significant activity in all the tested cell lines. Among all the tested compounds **6a-j,** it is interesting to note that compounds **6e** and **6f** are most cytotoxic towards all the three cancer cell lines.

Table I — Anticancer activity of **6a-j** on human cancer cell lines

Compd[a]	*in vitro*[a] (IC_{50} µg/mL[b])		
	HeLa	MCF-7	NCI-H460
6a	62.30±2.6	61.34±2.5	61.34±2.5
6b	37.32±2.2	47.21±2.6	47.21±2.6
6c	39.46±3.4	38.29±2.8	38.29±2.8
6d	28.36±2.0	30.28±2.4	30.28±2.4
6e	8.61±1.2	10.93±1.3	10.93±1.3
6f	8.42±2.6	11.51±2.0	11.51±2.0
6g	21.60±2.3	20.22±2.5	20.22±2.5
6h	44.51±2.5	52.47±2.2	52.47±2.2
6i	49.57±2.1	32.17±2.4	32.17±2.4
6j	24.60±2.3	23.45±2.3	23.45±2.3
Cisplatin (DDP)	3.42±0.1	5.05±0.4	5.05±0.4

[a]Values are expressed as mean±SEM

Cytotoxicity as $IC_{50}$for each cell line, is the concentration of compound which reduced by 50% the optical density of treated cells with respect to untreated cells using the MTT assay.

[b]Data represent the mean ±SEM values of three independent determinations.

Antibacterial activity

Antibacterial activity of **6a-j** in acetone was performed by broth dilution method using nutrient agar against Gram-negative bacteria *Pseudomonas aeruginosa*, *Klebsiella aerogenes*, *Chormobacteium violaceum* and Gram-positive bacteria *Bacillus subtilis*, *Bacillus sphaericus* and *Staphylococcus aureus* at 100 µg/mL concentration. The minimum inhibitory concentration (MIC) study was carried out by broth dilution method[21], Ciprofloxacin was used as standard drug for comparison. The ready-made nutrient broth medium (Himedia, 24 g) was suspended in distilled water (100 mL) and heated until it dissolved completely. The medium and test tubes were autoclaved at pressure of 15 p.s.i. for 20 min. A set of sterilized test tubes with nutrient broth medium was capped with cotton plugs. The test compound was dissolved in acetone and concentration of 100 µg /mL of the test compound was added in the first test tube, which was then serially diluted. A fixed volume of 0.5 mL overnight culture was added in all the test tubes and was incubated at 37°C for 24 h. After 24 h, these tubes were taken out for turbidity measurement.

The results of antibacterial screening **(Table II)** reveal that the compounds **6a-j** displayed better activity and were more active than the standard drug Ciprofloxacin. Compounds **6e** and **6f** possessing methyl and methoxy groups as substituent on benzene ring showed better activity. However, the degree of inhibition varied both with the test compound as well as with the bacteria used in the present investigation. In conclusion, almost all the compounds **6a-j,** exhibited the maximum activity by inhibiting growth

Table II — Antibacterial activity of **6a-j**

Compd	Minimum Inhibitory Concentration (MIC)[a,b]					
	Gram positive			Gram negative		
	B. subtilis	*B. sphaericus*	*S. aureus*	*P. aeruginosa*	*K. aerogenes*	*C. violaceum*
6a	17	14	18	22	20	18
6b	18	16	15	21	18	16
6c	19	17	15	20	16	17
6d	18	19	20	16	21	20
6e	8	9	12	11	11	13
6f	9	8	10	8	10	9
6g	17	18	13	14	16	18
6h	15	16	19	21	18	19
6i	16	18	16	25	23	21
6j	14	15	22	20	12	20
Ciprofloxacin	20	20	25	30	25	25

[a]Negative control (acetone) – No activity
[b]Concentration 100µg/mL

Table III — Antifungal activity of **6a-j**

Compd	Zone of inhibition[a, b]				
	A. niger	*C. tropicum*	*R. oryzae*	*F. moniliformae*	*C. lunata*
6a	36	40	38	31	32
6b	40	45	32	40	45
6c	42	38	40	45	40
6d	38	41	45	30	33
6e	60	55	55	55	60
6f	65	58	57	60	62
6g	32	43	43	35	45
6h	35	45	32	28	28
6i	38	34	35	30	30
6j	45	35	43	27	35
Flucanazole	29	30	28	23	20

[a]Negative control (acetone) – No activity
[b]Concentration 100µg/mL

of all the six bacteria to a greater extent in comparison with standard drug Ciprofloxacin. The antibacterial activity of **6e** and **6f** is promising compared with standard drug Ciprofloxacin, and they can be exploited for the formulation of bacteriocide after detailed study.

Antifungal activity

Antifungal activity of **6a-j** was performed by the agar cup bioassay method[22] using Flucanazole as the standard. The compounds were tested for their antifungal activity against five test organisms, *Aspergillus niger*, *Chrysosporium tropicum*, *Rhizopus oryzae*, *Fusarium moniloformae* and *Curvularia lunata* using agar cup bioassay method at 100 µg/mL concentration. For the antifungal assay, the ready-made potato dextrose agar medium (Himedia, 39 g) was suspended in distilled water (1000 mL) and heated until it dissolved completely. The medium and Petri dishes were autoclaved at pressure of 15 p.s.i. for 20 min. The medium was poured into sterile petri dishes under aseptic conditions in a laminar flow chamber. When the medium in the plates solidified, 0.5 mL of (week old) culture of test organism was inoculated and uniformly spread over the agar surface with a sterile L-shaped rod. Solutions were prepared by dissolving plant extract in acetone (100 µg/mL). Agar inoculation cups were scooped out with 6 mm sterile cork borer and the lids of the dishes were replaced. To each cup, test solution 100 (µg/mL) was added. Controls were maintained with acetone and Flucanazole (100 µg/mL). The treated and the controls were kept at RT for 72-96 h. Inhibition zones were measured and diameter was calculated in millimeter. Three to four replicates were maintained for each treatment.

The antifungal activity results **(Table III)** indicated that compounds **6a-j** are significantly toxic towards

all the five fungi and they are lethal even at 100 μg/mL concentration. Compounds **6e** and **6f** exhibited high antifungal activity which may be due to presence of methyl and methoxy groups as substitutents on benzene ring, besides the presence of isoxazolo[2",3":1',2'] pyrimido[4',5':4,5]pyrimido[6,1-*b*]quinazoline-8-one nucleus. However, the degree of spore germination inhibition varied with the test compound as well as with the fungi. The antifungal activity of these compounds was compared with the standard drug Flucanazole, and they were found to be more toxic, when compared with standard drug. In conclusion, almost all the compounds, **6a-j** are highly toxic towards the fungi under investigation, and they are lethal even at 100 μg/mL concentration in comparison with standard drug Flucanazole at the same concentration. It is noteworthy that the compounds **6e** and **6f** exhibited maximum ativity, hence they may be exploited for control of wilt diseases of different crops as fungicides after detailed study.

Experimental Section

All the melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Analytical TLC was performed on Merck precoated 60 F_{254} silica gel plates. Visualization was carried out by exposure to iodine vapour. IR spectra (KBr pellet) were recorded on a Perkin-Elmer BX series FT-IR spectrometer. ^{1}H NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer. ^{13}C NMR spectra were recorded on a Bruker 75 MHz spectrometer. Chemical shift values are given in δ (ppm) with tetramethyl silane as an internal standard. Mass spectral measurements were carried out by EI method on a Jeol JMC-300 spectrometer at 70 eV. Elemental analyses were performed on a Carlo Erba 106 and Perkin-Elmer model 240 analyzers.

General procedure for the synthesis of 5-amino-2-methyl-7-phenyl-7*H*-isoxazolo[2,3-*a*] pyrimidin-6-yl cyanides, 4a-j.

To a vigrously stirred solution of aromatic aldehyde **2** (1 mmol) and malononitrile **3** (1 mmol) in ethanol (20 mL), 3-amino-5-methyisoxazole **1** (1 mmol) was added and the contents were refluxed while sitrring for 2 h. The progress of the reaction was monitored by TLC. After the completion of the reaction, the reaction mixture was poured on to crushed ice, the seperated solid was filtered, and purified by recrystallization from ethyl acetate to obtain pure compounds 5-amino-2-methyl-7-phenyl-7*H*-isoxazolo-[2, 3-*a*]pyrimidin-6-yl cyanides **4**.

5-Amino-2-methyl-7-phenyl-7*H*-isoxazolo[2, 3-*a*]-pyrimidin-6-yl cyanide, 4a. Orange solid; yield 65%, m.p.172-74°C. IR (KBr): 3430, 3410 (NH_2), 2210 cm^{-1} (CN); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.28 (s, 3H, CH_3), 5.12 (s, 1H, CH), 6.12 (s, 1H, isoxazole-CH), 6.98-7.53 (m, 5H, Ar-H), 8.21 (s, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.83, 50.29, 78.35, 82.07, 118.43, 126.63, 127.51, 127.85, 128.09, 128.21, 142.87, 165.11, 166.23, 187.29; EI-MS: *m/z* 253 $[M+H]^+$. Anal. Calcd for $C_{14}H_{12}N_4O$: C, 66.65; H, 4.79; N, 22.21. Found: C, 66.61; H, 4.76; N, 22.18%.

5-Amino-7-(2-chlorophenyl)-2-methyl-7*H*-isoxazolo-[2,3-*a*]pyrimidin-6-yl cyanide, 4b. Yellow solid; yield 68%, m.p. 168-70°C. IR (KBr): 3435, 3426 (NH_2), 2218 cm^{-1} (CN); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.24 (s, 3H, CH_3), 5.19 (s, 1H, CH), 6.09 (s, 1H, isoxazole-CH), 7.10-7.63 (m, 4H, Ar-H), 8.25 (s, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.91, 50.34, 78.41, 82.13, 119.02, 126.72, 127.42, 127.78, 128.29, 128.67, 143.13, 165.57, 166.71, 187.39; EI-MS: *m/z* 287 $[M+H]^+$. Anal. Calcd for $C_{14}H_{11}ClN_4O$: C, 58.65; H, 3.87; N, 19.54. Found: C, 58.61; H, 3.84; N, 19.50%.

5-Amino-7-(2-bromophenyl)-2-methyl-7*H*-isoxazolo[2,3-*a*]pyrimidin-6-yl cyanide, 4c. Brown solid, yield 73%, m.p. 175-77°C. IR (KBr): 3434, 3431 (NH_2), 2224 cm^{-1} (CN); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.27 (s, 3H, CH_3), 5.20 (s, 1H, CH), 6.16 (s, 1H, isoxazole-CH), 6.93-7.44 (m, 4H, Ar-H), 8.21 (s, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.76, 50.46, 78.64, 82.21, 119.38, 126.81, 127.24, 127.78, 128.11, 128.75, 142.87, 165.23, 166.56, 186.89; EI-MS: *m/z* 331$[M+H]^+$. Anal. Calcd for $C_{14}H_{11}BrN_4O$: C, 50.77; H, 3.35; N, 16.92. Found: C, 50.73; H, 3.32; N, 16.89%.

5-Amino-7-(2-hydroxyphenyl)-2-methyl-7*H*-isoxazolo[2,3-*a*]pyrimidin-6-yl cyanide, 4d. Yellow solid, yield 74%, m.p. 165-67°C. IR (KBr): 3445 (OH), 3428, 3422 (NH_2), 2216 cm^{-1} (CN); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.30 (s, 3H, CH_3), 5.28 (s, 1H, CH), 6.23 (s, 1H, isoxazole-CH), 7.11-7.64 (m, 4H, Ar-H), 8.29 (s, 2H, NH_2, D_2O exchangeable), 8.51 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.83, 49.67, 79.12, 82.56, 120.08, 126.62, 127.11, 127.92, 128.34, 128.87, 143.14, 166.29, 166.83, 187.25; EI-MS: *m/z* 269 $[M+H]^+$. Anal. Calcd for $C_{14}H_{12}N_4O_2$: C, 62.68; H, 4.51; N, 20.88. Found: C, 62.64; H, 4.48; N, 20.85%.

5-Amino-2-methyl-7-(4-methylphenyl)-7*H*-isoxazolo-[2,3-*a*]pyrimidin-6-yl cyanide, 4e. Pale yellow solid, yield 71%, m.p. 171-73°C. IR (KBr): 3445, 3430 (NH_2), 2229 cm^{-1} (CN); ^1H NMR (300 MHz, $CDCl_3$): δ 2.25 (s, 3H, CH_3), 2.54 (s, 3H, Ar-CH_3), 5.25 (s, 1H, CH), 6.21 (s, 1H, isoxazole-CH), 7.12-7.64 (m, 4H, Ar-H), 8.27 (s, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.91, 24.26, 51.13, 78.72, 82.36, 119.49, 126.72, 127.14, 127.67, 128.32, 129.12, 142.91, 165.45, 166.87, 187.09; EI-MS: *m/z* 267 $[M+H]^+$. Anal. Calcd for $C_{15}H_{14}N_4O$: C, 67.65; H, 5.30; N, 21.04. Found: C, 67.62; H, 5.27; N, 21.01%.

5-Amino-7-(4-methoxyphenyl)-2-methyl-7*H*-isoxazolo[2,3-*a*]pyrimidin-6-yl cyanide, 4f. Yellow solid, yield 78%, m.p. 180-82°C. IR (KBr): 3439, 3430 (NH_2), 2235 cm^{-1} (CN); ^1H NMR (300 MHz, $CDCl_3$): δ 2.28 (s, 3H, CH_3), 3.69 (s, 3H, Ar-OCH_3), 5.29 (s, 1H, CH), 6.28 (s, 1H, isoxazole-CH), 6.89-7.48 (m, 4H, Ar-H), 8.21 (s, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.82, 51.13, 64.47, 78.92, 82.29, 119.67, 126.82, 127.28, 127.88, 128.56, 130.13, 143.02, 165.78, 167.23, 187.56; EI-MS: *m/z* 283 $[M+H]^+$. Anal. Calcd for $C_{15}H_{14}N_4O_2$: C, 63.82; H, 5.00; N, 19.85. Found: C, 63.79; H, 4.97; N, 19.81%.

5-Amino-7-[4-(dimethylamino)phenyl]-2-methyl-7*H*-isoxazolo[2,3-*a*]pyrimidin-6-yl cyanide, 4g. Yellow solid, yield 80%, m.p. 166-68°C. IR (KBr): 3442, 3434 (NH_2), 2218 cm^{-1} (CN); ^1H NMR (300 MHz, $CDCl_3$): δ 2.24 (s, 3H, CH_3), 2.8 (s, 6H, Ar-N$(CH_3)_2$), 5.19 (s, 1H, CH), 6.21 (s, 1H, isoxazole-CH), 7.04-7.56 (m, 4H, Ar-H), 8.25 (s, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.92, 48.23, 51.32, 78.87, 82.34, 119.78, 126.67, 127.32, 128.05, 128.45, 130.56, 143.21, 165.86, 167.32, 187.67. ESI-MS: *m/z* 296 $[M+H]^+$. Anal. Calcd for $C_{16}H_{17}N_5O$: C, 65.07; H, 5.80; N, 23.71. Found: C, 65.04; H, 5.77; N, 23.68%.

5-Amino-7-(2, 6-dichlorophenyl)-2-methyl-7*H*-isoxazolo[2,3-*a*]pyrimidin-6-yl cyanide, 4h. Pale yellow solid, yield 75%, m.p. 185-87°C. IR (KBr): 3442, 3436 (NH_2), 2227 cm^{-1} (CN); ^1H NMR (300 MHz, $CDCl_3$): δ 2.28 (s, 3H, CH_3), 5.15 (s, 1H, CH), 6.18 (s, 1H, isoxazole-CH), 7.15-7.78 (m, 3H, Ar-H), 8.21 (s, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.81, 51.28, 79.13, 82.42, 120.12, 126.56, 127.48, 128.11, 128.67, 131.16, 143.36, 165.96, 167.58, 187.47. EI-MS: *m/z* 321 $[M+H]^+$. Anal. Calcd for $C_{14}H_{10}Cl_2N_4O$: C, 52.36; H, 3.14; N, 17.45. Found: C, 52.32; H, 3.11; N, 17.41%.

5-Amino-7-(2, 6-dibromophenyl)-2-methyl-7*H*-isoxazolo[2,3-*a*]pyrimidin-6-yl cyanide, 4i. Brown solid, yield 69%, m.p. 179-81°C. IR (KBr): 3441, 3438 (NH_2), 2211 cm^{-1} (CN); ^1H NMR (300 MHz, $CDCl_3$): δ 2.23 (s, 3H, CH_3), 5.17 (s, 1H, CH), 6.20 (s, 1H, isoxazole-CH), 7.04-7.53 (m, 3H, Ar-H), 8.29 (s, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.95, 51.43, 78.89, 82.49, 120.11, 126.72, 127.37, 128.35, 128.59, 129.89, 143.29, 166.32, 167.58, 187.69; ESI-MS: *m/z* 409 $[M+H]^+$. Anal. Calcd for $C_{14}H_{10}Br_2N_4O$: C, 41.01; H, 2.46; N, 13.66. Found: C, 41.00; H, 2.42; N, 13.63%.

5-Amino-7-(1, 3-benzodioxol-5-yl)-2-methyl-7*H*-isoxazolo[2,3-*a*]pyrimidin-6-yl cyanide, 4j. Yellow solid, yield 66%, m.p. 188-90°C. IR (KBr): 3425, 3420 (NH_2), 2220 cm^{-1} (CN); ^1H NMR (300 MHz, $CDCl_3$): δ 2.28 (s, 3H, CH_3), 5.12 (s, 2H, OCH_2O), 5.21 (s, 1H, CH), 6.23 (s, 1H, isoxazole-CH), 7.12-7.67 (m, 3H, Ar-H), 8.23 (s, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.91, 51.56, 78.78, 82.53, 101.58, 121.32, 126.87, 127.43, 128.57, 128.79, 130.12, 143.43, 166.54, 167.67, 187.79; EI-MS: *m/z* 297$[M+H]^+$. Anal. Calcd for $C_{15}H_{12}N_4O_3$: C, 60.81; H, 4.08; N, 18.91. Found: C, 60.79; H, 4.05; N, 18.87%.

General procedure for the synthesis of 2-methyl-14-phenyl-8*H*,14*H*-isoxazolo [2'', 3'':1', 2'] pyrimido[4',5':4,5]pyrimido[6,1-*b*] quinazolin-8-ones, 6a-j. To a stirred solution of 5-amino-2-methyl-7-phenyl-7*H*-isoxazolo[2,3-*a*]pyrimidin-6-yl cyanide **7** (1 mmol), in ethanol (20 mL), acetic acid (0.5 mL) and DMF-DMA (1.5 mmol) were added sequentially at ambient temperature. The contents were refluxed for 2 h and anthranilic acid (1 mmol) was then added to it, and the reaction was continued for another 2 h under reflux. After completion of the reaction (monitored by TLC), the reaction mixture was poured on to crushed ice, and the resulted precipitate was filtered, washed with cold ethanol and recrystallized from ethyl acetate.

2-Methyl-14-phenyl-8*H*,14*H*-isoxazolo-[2'',3'':1',2']-pyrimido[4',5':4,5]pyrimido[6,1-*b*] quinazolin-8-one, 6a. Brown solid, yield 60%, m.p. 210-12°C. IR (KBr): 1670 cm^{-1} (C=O); ^1H NMR (300 MHz, $CDCl_3$): δ 2.30 (s, 3H, CH_3), 4.98 (s, 1H, CH), 6.20 (s, 1H, isoxazole-CH), 6.98-7.50 (m, 9H, Ar-H), 7.95 (s, 1H, pyrimidine ring CH); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.61, 46.79, 82.11, 115.34, 119.45, 121.83, 126.51, 127.19, 127.34, 127.81, 128.55, 128.87, 129.09, 132.78, 133.38, 143.87, 144.11, 148.23, 163.75, 163.89, 182.21, 191.43; EI-MS: *m/z*

382 $[M+H]^+$. Anal. Calcd for $C_{22}H_{15}N_5O_2$: C, 69.28; H, 3.96; N, 18.36. Found: C, 69.24; H, 3.95; N, 18.33%.

14-(2-Chlorophenyl)-2-methyl-8*H*,14*H*-isoxazolo-[2",3":1',2']pyrimido[4',5':4,5]pyrimido [6,1-*b*]quinazolin-8-one, 6b. Yellow solid, yield 68%, m.p. 218-20°C. IR (KBr): 1680 cm^{-1} (C=O); ^1H NMR (300 MHz, $CDCl_3$): δ 2.31 (s, 3H, CH_3), 4.91 (s, 1H, CH), 6.26 (s, 1H, isoxazole-CH), 7.10-7.65 (m, 8H, Ar-H), 7.89 (s, 1H, pyrimidine ring CH); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.56, 46.87, 82.43, 115.67, 119.59, 122.45, 126.68, 127.34, 127.68, 128.11, 128.78, 129.05, 129.21, 132.86, 133.54, 143.91, 144.36, 148.47, 163.98, 164.23, 183.01, 192.56; EI-MS: *m/z* 416 $[M+H]^+$. Anal. Calcd for $C_{22}H_{14}ClN_5O_2$: C, 63.54; H, 3.39; N, 16.84. Found: C, 63.50; H, 3.36; N, 16.82%.

14-(2-Bromophenyl)-2-methyl-8*H*, 14*H*-isoxazolo-[2",3":1',2']pyrimido[4',5':4,5]pyrimido [6,1-*b*]-quinazolin-8-one, 6c. Yellow solid, yield 65%, m.p. 228-30°C. IR (KBr): 1685 cm^{-1} (C=O); ^1H NMR (300 MHz, $CDCl_3$): δ 2.28 (s, 3H, CH_3), 4.96 (s, 1H, CH), 6.29 (s, 1H, isoxazole-CH), 7.07-7.55 (m, 8H, Ar-H), 7.91 (s, 1H, pyrimidine ring CH); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.41, 46.81, 82.58, 115.79, 120.21, 122.58, 126.72, 127.59, 127.75, 128.32, 128.89, 129.17, 129.42, 132.94, 133.67, 144.13, 144.48, 148.79, 164.21, 164.57, 183.76, 193.32; EI-MS: *m/z* 460 $[M+H]^+$. Anal. Calcd for $C_{22}H_{14}BrN_5O_2$: C, 57.41; H, 3.07; N, 15.22. Found: C, 57.38; H, 3.03; N, 15.19%.

14-(2-Hydroxyphenyl)-2-methyl-8*H*, 14*H*-isoxazolo-[2",3":1',2']pyrimido[4',5':4,5]pyrimido [6,1-*b*]-quinazolin-8-one, 6d. Pale yellow solid, yield 61%, m.p. 237-39°C. IR (KBr): 3455 (OH), 1675 cm^{-1} (C=O); ^1H NMR (300 MHz, $CDCl_3$): δ 2.27 (s, 3H, CH_3), 4.91 (s, 1H, CH), 6.30 (s, 1H, isoxazole-CH), 6.97-7.49 (m, 8H, Ar-H), 7.90 (s, 1H, pyrimidine ring CH), 8.24 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.67, 46.81, 82.45, 115.56, 120.34, 122.71, 126.85, 127.69, 127.89, 128.21, 128.92, 129.26, 129.56, 133.12, 133.72, 144.21, 144.62, 148.82, 164.28, 164.68, 182.42, 192.87; EI-MS: *m/z* 398 $[M+H]^+$. Anal. Calcd for $C_{22}H_{15}N_5O_3$: C, 66.49; H, 3.80; N, 17.62. Found: C, 66.45; H, 3.77; N, 17.59%.

2-Methyl-14-(4-methylphenyl)-8*H*, 14*H*-isoxazolo-[2",3":1',2']pyrimido[4',5':4,5]pyrimido [6,1-*b*]-quinazolin-8-one, 6e. Pale yellow solid, yield 66%, m.p. 225-27°C. IR (KBr):1690 cm^{-1} (C=O); ^1H NMR (300 MHz, $CDCl_3$): δ 2.31 (s, 3H, CH_3), 2.35 (s, 3H, Ar-CH_3), 4.98 (s, 1H, CH), 6.28 (s, 1H, isoxazole-CH), 7.10-7.67 (m, 8H, Ar-H). 7.93 (s, 1H, pyrimidine ring CH); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.82, 24.23, 46.91, 82.58, 115.62, 120.41, 122.84, 126.91, 127.58, 127.93, 128.28, 129.02, 129.36, 129.69, 133.21, 133.82, 144.37, 144.72, 148.92, 164.42, 164.72, 183.55, 192.68; EI-MS: *m/z* 396 $[M+H]^+$. Anal. Calcd for $C_{23}H_{17}N_5O_2$: C, 69.86; H, 4.33; N, 17.71. Found: C, 69.82; H, 4.29; N, 17.68%.

14-(4-Methoxyphenyl)-2-methyl-8*H*, 14*H*-isoxazolo-[2",3":1',2']pyrimido[4',5':4,5]pyrimido [6,1-*b*]-quinazolin-8-one, 6f. Pale yellow solid, yield 62%, m.p. 242-44°C. IR (KBr): 1680 cm^{-1} (C=O); ^1H NMR (300 MHz, $CDCl_3$): δ 2.25 (s, 3H, CH_3), 3.68 (s, 3H, OCH_3), 4.98 (s, 1H, CH), 6.27 (s, 1H, isoxazole-CH), 6.99-7.54 (m, 8H, Ar-H), 7.86 (s, 1H, pyrimidine ring CH); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.76, 46.93, 61.30, 82.58, 115.69, 120.51, 122.69, 126.72, 127.72, 127.92, 128.38, 129.11, 129.41, 129.62, 133.24, 133.85, 144.34, 144.73, 148.93, 164.45, 164.85, 182.57, 193.59; EI-MS: *m/z* 412 $[M+H]^+$. Anal. Calcd for $C_{23}H_{17}N_5O_3$: C, 67.15; H, 4.16; N, 17.02. Found: C, 67.11; H, 4.13; N, 16.98%.

14-[4-(Dimethylamino)phenyl]-2-methyl-8*H*, 14*H*-isoxazolo[2",3":1',2']pyrimido[4',5':4,5] pyrimido-[6,1-*b*]quinazolin-8-one, 6g. Pale yellow solid, yield 69%, m.p. 246-48°C. IR (KBr): 1685 cm^{-1} (C=O); ^1H NMR (300 MHz, $CDCl_3$): δ 2.29 (s, 3H, CH_3), 2.8 (s, 6H, Ar-$N(CH_3)_2$), 4.92 (s, 1H, CH), 6.21 (s, 1H, isoxazole-CH), 7.12-7.67 (m, 8H, Ar-H), 7.94 (s, 1H, pyrimidine ring CH); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.91, 44.78, 46.78, 82.61, 115.56, 120.42, 122.72, 126.81, 127.66, 128.11, 128.41, 129.19, 129.54, 129.69, 133.37, 133.91, 144.53, 144.81, 149.11, 164.59, 164.74, 182.87, 193.65; EI-MS: *m/z* 425 $[M+H]^+$. Anal. Calcd for $C_{24}H_{20}N_6O_2$: C, 67.91; H, 4.75; N, 19.80. Found: C, 67.88; H, 4.72; N, 19.78%.

14-(2,6-Dichlorophenyl)-2-methyl-8*H*, 14*H*-isoxazolo[2",3":1',2']pyrimido[4',5':4,5]pyrimido-[6,1-*b*]-quinazolin-8-one, 6h. Pale yellow solid, yield 63%, m.p. 254-56°C. IR (KBr): 1682 cm^{-1} (C=O); ^1H NMR (300 MHz, $CDCl_3$): δ 2.27 (s, 3H, CH_3), 4.90 (s, 1H, CH), 6.22 (s, 1H, isoxazole-CH), 7.02-7.57 (m, 7H, Ar-H), 7.88 (s, 1H, pyrimidine ring CH); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.87, 46.83, 82.72, 115.79, 120.58, 122.61, 126.93, 127.58, 128.23, 128.57, 129.37, 129.61, 129.78, 133.41, 134.23, 144.62, 144.92, 149.17, 164.68, 164.81, 183.43, 193.83; EI-MS: *m/z* 450 $[M+H]^+$. Anal. Calcd for $C_{22}H_{13}Cl_2N_5O_2$: C, 58.68; H, 2.91; N, 15.55. Found: C, 58.65; H, 2.87; N, 15.53%.

14-(2,6-Dibromophenyl)-2-methyl-8*H*, 14*H*-isoxazolo[2'',3'':1',2']pyrimido[4',5':4,5] pyrimido-[6,1-*b*]quinazolin-8-one, 6i. Pale yellow solid, yield 64%, m.p. 248-50°C. IR (KBr): 1680 cm^{-1} (C=O); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.29 (s, 3H, CH_3), 4.95 (s, 1H, CH), 6.28 (s, 1H, isoxazole-CH), 7.13-7.72 (m, 7H, Ar-H). 7.92 (s, 1H, pyrimidine ring CH); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.91, 46.75, 81.43, 114.56, 121.78, 122.72, 127.23, 127.62, 128.35, 128.62, 130.21, 130.34, 130.59, 133.78, 134.23, 144.21, 145.45, 149.32, 164.72, 164.93, 183.22, 192.78; ESI-MS: *m/z* 538 $[M+H]^+$. Anal. Calcd for $C_{22}H_{13}Br_2N_5O_2$: C, 49.01; H, 2.43; N, 12.99. Found: C, 48.98; H, 2.40; N, 12.95%.

14-(1,3-Benzodioxol-5-yl)-2-methyl-8*H*, 14*H*-isoxazolo[2'',3'':1',2']pyrimido[4',5':4,5] pyrimido-[6,1-*b*]-quinazolin-8-one, 6j. Pale yellow solid, yield 70%, m.p. 258-60°C. IR (KBr): 1680 cm^{-1} (C=O); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.30 (s, 3H, CH_3), 4.90 (s, 1H, CH), 5.12 (s, 2H, OCH_2O), 6.21 (s, 1H, isoxazole-CH), 7.05-7.72 (m, 7H, Ar-H), 7.89 (s, 1H, pyrimidine ring CH); ^{13}C NMR (75MHz, $CDCl_3$): δ 12.95, 56.22, 82.11, 101.23, 115.32, 120.89, 122.56, 127.45, 127.76, 128.61, 128.96, 130.43, 131.11, 131.54, 133.82, 134.51, 144.19, 145.63, 149.41, 164.86, 165.20, 183.49, 193.74; EI-MS: *m/z* 426 $[M+H]^+$. Anal. Calcd for $C_{23}H_{15}N_5O_4$: C, 64.94; H, 3.55; N, 16.46. Found: C, 64.91; H, 3.52; N, 16.42%.

Conclusion

In conclusion, the synthesis of novel isoxazolo[2",3":1',2']pyrimido [4',5':4,5]pyrimido[6,1-*b*]quinazoline-8-ones has been achieved from readily accessible starting materials in moderate to good yields in one-pot. The newly synthesized novel isoxazolo[2",3":1',2'] pyrimido[4',5':4,5]pyrimido[6,1-*b*]quinazoline-8-ones have been evaluated for their *in vitro* anticancer and antimicrobial activity. Compounds **6e** and **6f** exhibited significant activity. Thus, they may be considered as future drug candidates and by doing a simple modification in the structure, a new potent analogue can be generated with desired activity with good efficacy. It also requires future attempts to reveal the exact mechanism of action and structure-activity relationship.

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