Current Chemistry Letters 12 (2023) 537–544

Contents lists available at GrowingScience

Current Chemistry Letters

homepage: www.GrowingScience.com

Synthesis, characterization and antibiotic evaluation of various biologically active derivatives of 4-Alkylpyrimidine-5-Carbonitrile

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C H R O N I C L E

Article history:
Received December 20, 2022
Received in revised form
January 28, 2023
Accepted March 4, 2023
Available online
March 4, 2023

Keywords:
Pyrimidine Analogs
Biologically active
Pharmacophoric Groups
Antibacterial
Antibiotic Evaluation

A B S T R A C T

Extensive research work has been published on Tetrahydro and Dihydropyrimidine derivatives. Pyrimidine-5-Carbonitrile and its analogs have demonstrated a large number of activities. Some 6-Halogenosubstituted pyrimidine analogs have also been reported to be biologically active to a certain extent, but the literature survey reveals not much report on 6-alkylated pyrimidine derivatives. Targeting enhancement in biologically useful properties of a lead molecule through the association of it with active pharmacophoric groups or molecules is a conventional method in pharmaceutical research. With an aim to explore a better useful Dihydropyrimidine derivative, a newer 4-Alkylated-1,6-dihydropyrimidine analog (1) has been prepared. The lead molecule (1) has further been converted to amine derivative (2), hydrazino derivative (3) tri substituted s-triazinyl derivative (4), sulphonamide (5), Schiff's base (6), a thiazolidinones (7), and 2-Azetidinones (8) respectively using various methods. Compounds 2, 4, 5, and 7 showed 50% and 8 showed 100% bacterial inhibition at 32µg/mL in single-point bacterial inhibition against various bacterial strains.

1. Introduction

The heterocyclic compounds occupy key positions in the area of drugs and pharmaceuticals because they have specific chemical reactivity. Almost 80% of the drugs in clinical use are based on the heterocyclic constitution and majority of the drugs introduced in pharmacopeias in recent years are also heterocyclic. A wide variety of modern drugs such as chlordiazepoxide (tranquillizer),[1,2] imipromine (antidepressant),[3] guanethidine (antihypertensive),[4] indapamide (diuretic and antihypertensive),[5] etc., contain heterocycles. Many non-steroidal drugs such as ketoprofen[6] are well known anti-inflammatory agents; these derivatives have been found to be potent with fewer side effects. Many antibiotics including cephalosporin,[7] norfloxacin[8], streptomycin[9] etc., also contain the heterocyclic ring.

Pyrimidine which is an integral part of DNA and RNA imparts diverse pharmacological properties. Pyrimidine has been isolated from hydrolysis of the nucleic acid and is a much weaker base than pyridine and soluble in water.[10] Pyrimidine and its derivatives have been reported to possess a wide range of biological potential i.e. anticancer,[11] antiviral,[12] antimicrobial,[13] anti-inflammatory,[14] analgesic[15] and antioxidant[16]. Pyrimidines derivatives have been used in coordination chemistry, metal-cage complexes and also act as CDK 4 inhibitors.[17] Pyrimidine nucleus is also a significant pharmacophore that exhibits excellent pharmacological activities.[18]

The growing health problems demand a search and synthesis of a new class of antimicrobial molecules that are effective against pathogenic microorganisms. Despite advances in antibacterial and antifungal therapies, many problems remain to be solved for most antimicrobial drugs available. The extensive use of antibiotics has led to the appearance of multidrug-resistant microbial pathogens which necessitated the search for new chemical entities for the treatment of microbial

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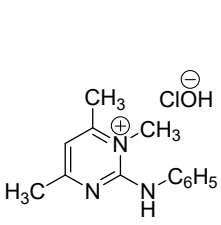
doi: 10.5267/j.ccl.2023.3.002

infections.[19] The combination of two biologically active constituents to explore for a better therapeutic agent is one of the important aspects of drug chemistry.



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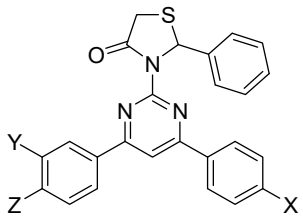

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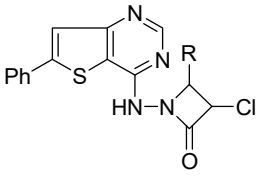

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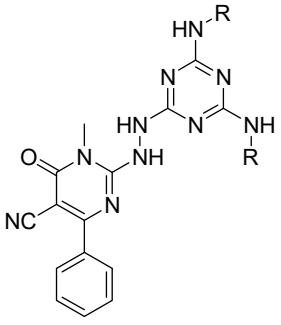

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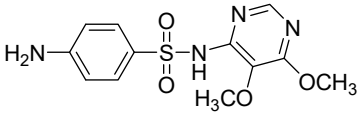


Sulfadoxine an antimalarial

Although various structurally important 6-Aryl pyrimidine analogs have been synthesized by reported methods, there has been a void in the literature for the 6-alkylated pyrimidine analogs. We have synthesized some new 2-(substituted)-4-isobuty-1,6-dihydro-1-methyl-6-oxopyrimidine-5-carbonitrile by associating 4-Isobuty-1,6-dihydro-1-methyl-2-(methylthio)-6-oxopyrimidine-5-carbonitrile with different pharmacologically active species using different methods.[20] Also, the 6-haloaryl derivatives have been synthesized by similar known and reported method[21] and are screened for antibiotic activity.

Although KINUGASA reaction[22] and conversion of 5-Nitroisoxazolidines to β-lactam are reported, [23] formation of β-lactam by [2+2] cycloaddition of ketene [24] to imine function is one of the most important and versatile route of synthesis.

4-thiazolidinones are well-studied by A. Verma et al.[25] The reaction proceeds by the attack of the mercapto acetic acid upon the C=N group, with the HS-CH_2-COOH adding to the carbon atom followed by the capture of a proton by nitrogen and subsequent cyclization.

2. Results and Discussion

The targeted dihydropyrimidines could be synthesized using conventional reported methods and also replacing organic solvents-based MCR with aqua-mediated MCR., for producing alkylsubstituted tetrahydropyrimidine analog. The obtained yields were reduced in the water-mediated process. Further steps required organic medium to produce compound-1 to 8. The overall yield ranged from 48% to 68% with color of the product ranging from pale yellow to brownish with poor solubility in alcohol. Compounds-1, 2 and 4 give fine needle-shaped crystals with sharp m.p, compound-5 and 6 also produced crystals in alcohol but 7, and 8 were obtained in poor yield and decomposed (**Table 1**).

Table. 1. Characterization Data of the Compounds 2-8

No.	Comp.	R'	M.F.	M.W.	M.P. ˚C	Yield %	N% Cal. (Found)
1.	2	-4-Cl-C_6H_4	$C_{16}H_{17}N_4OCl$	316	100	48	17.69 (17.67)
2.	4	-4-OCH_3-C_6H_4	$C_{27}H_{30}N_{10}O_3$	542	254	61	25.81 (25.79)
3.	5	-4-CH_3-C_6H_4	$C_{17}H_{21}N_5O_3S$	375	248	62	18.65 (18.62)
4.	7	-4-Cl-C_6H_4	$C_{20}H_{22}ClN_5O_2S$	432	158d	69	16.24 (16.17)
5.	8	-4-Cl-C_6H_4	$C_{19}H_{19}Cl_2N_5O_2$	420	164d	66	16.66 (16.65)

The spectroscopic data for compounds reveals characteristics absorptions in NMR and IR. In the illustrious compound-2, i.e., 2-(4-chloro phenylamino)-1,6-dihydro-4-isobutyl-1-methyl-6-oxopyrimidine-5-carbonitrile (1), the Mass M^+= 316; IR (KBr) ν(cm^{-1}), 3325 (-NH, secondary) str., 3035 (Aromatic C-H) str., 2964 (-CH_3, Asym.) str., 2872 (-CH_3, Sym.) str., 2216 (-C≡N) str., 1651 (-C=O) str., 1610 1585 1480 (C-C vibration in aromatic ring), 1346 (N-C) str., 777 (N-H) wag, 780 (C-Cl) str.; ^{1}H NMR (δ ppm) (400MHz, DMSO) δ 3.25 (s,3H,N-CH_3), δ 0.92 (d,6H, CH_2-CH-(CH_3)$_2$), δ 2.12 (m,1H, CH_2-CH-(CH_3)$_2$), δ 2.41 (d,1H, -CH-CH_2-(CH_3)$_2$), δ 7.99 (d,2H, C-H), δ 7.80 (d,2H, C-H).

The results of screening synthetic compounds for their antibiotic properties are performed by using the Broth dilution method. Broth micro- or macro-dilution is one of the most basic antimicrobial susceptibility testing methods. Compounds 2, 4, 5, and 7 have shown 50% bacterial inhibition at 32 µg/mL and compound 8 showed 100% bacterial inhibition at 32 µg/mL in single-point bacterial inhibition against five bacterial strains. Further Minimum Inhibitory Concentration Confirmation Assay of compound 8 was carried out and the results of this were compared with standard drugs like Colistin, Polymyxin B, Vancomycin, and Daptomycin. Compound 8 shows antibiotic activity against *Acinetobacter baumannii* ATCC 19606 (GN_034) bacteria at concentration of 8-32 µg/mL. (**Table 2** and **Table 3**).

Table. 2. Single-Point Bacterial Inhibition

100% inhibition at 32µg/ml	50% inhibition at 32µg/mL	> 32 µg/ml

No.	Compound	*E. coli (ATCC 25922)*	*K. pneumoniae (ATCC 700603)*	*A. buamanii (ATCC 19606)*	*P. aeruginosa (ATCC 27853)*	*S. aureus (ATCC 43300)*
1	2	>32	>32	>32	>32	32
2	4	>32	>32	>32	>32	32
3	5	>32	>32	>32	>32	32
4	7	>32	>32	>32	>32	32
5	8	>32	>32	32	>32	>32

Table. 3. MIC (Minimum Inhibitory Concentration) Confirmation Assay

≤ 0.015 - 0.06 µg/ml	0.125 - 0.5 µg/mL	1 - 4 µg/mL	8-32 µg/mL	>32 µg/mL

WADI Compound ID	Comp. ID	*E. coli (ATC 25922)*	*K. pneumoniae (ATCC700603)*	*A. buamanii (ATCC1966)*	*P. aeruginosa (ATCC27853)*	*S. aureus (ATCC 43300)*
MCC_000094	Colistin	0.06	0.015-0.03	0.03	0.025	-
MCC_000636	Polymyxin B	0.03	0.015	0.015	0.025	-
MCC_000095	Vancomycin	-	-	-	-	1
MCC_000561	Daptomycin	-	-	-	-	1
WADI_01332725	8	>32	>32	8	>32	>32

3. Conclusions

Alkyl substituted Pyrimidine-5-carbonitrile analogs can be prepared by condensing 4-Isobuty-1,6-dihydro-1-methyl-2-(methylthio)-6-oxopyrimidine-5-carbonitrile with different active pharmacophores (**Scheme 1**) in good yield (**Table 1**) Solubilities of these compounds range from moderate to poor in ionic solvents like water. All the compounds can be spectroscopically analyzed. The result of constitutional characterization of the obtained products by IR, ^{1}H-NMR, and Mass Spectroscopy show good agreement with the constitution of the targeted molecules. The spectroscopic properties also reveal

the stabilities of the N-alkyl and C-alkyl chains of the molecule. The 4-alkyl substituent pyrimidine derivatives depicted significant antibiotic activities against various strains of bacteria. (**Table 5**) Compound 8 showed the highest activity but the biological properties observed (**Table 2** and **Table 3**) are relatively poor compared to halogenated 4-aryl substituent in the pyrimidine analog (**Table 4**).[26,27]

Table. 4. Single-Point Bacterial Inhibition of halogenated 4-aryl substituent in the pyrimidine analog

100% inhibition at 32µg/ml	50% inhibition at 32µg/mL	> 32 µg/ml

No.	Compound	*E. coli (ATCC 25922)*	*K. pneumoniae (ATCC 700603)*	*A. buamanii (ATCC 19606)*	*P. aeruginosa (ATCC 27853)*	*S. aureus (ATCC 43300)*
1	2	>32	32	>32	>32	>32
2	4	>32	>32	>32	32	>32
3	5	32	>32	>32	>32	>32
4	6	>32	>32	>32	>32	32
5	7	>32	>32	>32	>32	32
6	8	>32	>32	>32	>32	32
7	9	>32	>32	>32	>32	32

Acknowledgements

The authors are thankful to Maharshi Dayanand Science College, Porbandar for providing Research facilities. We are grateful to the NFDD center, Saurashtra University, Rajkot for recording and providing ^{1}H-NMR, Mass, and IR Spectral data, and also thankful to CO-ADD for the biological screening of the synthesized compound..

4. Experimental Sections

4.1 Materials and Methods

Melting points were taken in open capillary and are not corrected. Progress of synthesized compounds was checked by TLC. Ethyl acetate and n-hexane in the ratio of 8:2 were used as mobile phase. Mass spectra were determined on Shimadzu-QP2010 spectrometer. IR spectra were recorded on Shimadzu-FTIR-8400 using KBr pallet. ^{1}H-NMR spectra were recorded in Bruker-Avance-II (400MHz) using DMSO-d6 as a solvent and TMS as an internal standard and the chemical shifts are reported as parts per million (ppm). The biological activity evaluation was carried out at CO-ADD, under open-access antimicrobial screening program at The University of Queensland, Australia using methodologies described for all the synthesized compounds.

4.2 General procedure

1. Synthesis of 1,6-dihydro-4-isobutyl-1-methyl-2-(methylthio)-6-oxopyrimidine-5-carbonitrile (1)[26]

A solution of 1,2,3,4-tetrahydro-6-isobutyl-4-oxo-2-thioxo pyrimidine-5-carbonitrile (0.05mol) in DMF (70ml) was stirred for 3 hrs with potassium carbonate (0.1mol) and methyl iodide (0.1mol). After completion of the reaction, the reaction mixture was poured into crushed ice and washed with water. Solid product was filtered, dried, and crystallized from DMF.

2. Synthesis of 2-(4-chloro phenylamino)-1,6-dihydro-4-isobutyl-1-methyl-6-oxopyrimidine-5-carbonitrile

A mixture of (1) (0.01 mol) and 4-chloro phenyl amine (5 ml) in absolute alcohol (30 ml) was refluxed for 10 hr. The progress of the reaction was monitored by thin-layer chromatography. After completion of the reaction, the reaction mixture was poured into crushed ice. The Product obtained was isolated and recrystallized from absolute alcohol.

3. Synthesis of 2-hydrazinyl-1,6-dihydro-4-isobutyl-1-methyl-6-oxo pyrimidine-5-carbonitrile

A mixture of (1) (0.01 mol) and hydrazine hydrate (4 ml) in absolute alcohol (30 ml) was refluxed for 8 hours. The progress of the reaction was monitored by thin-layer chromatography. The content was then diluted with ice water, and neutralized with gl. Acetic acid and kept overnight. The Product obtained was isolated and recrystallized from absolute alcohol.

4. Synthesis of 2-(2-(4,6-bis (4-methoxyphenylamino)-1,3,5-triazin-2-yl) hydrazinyl)-4-isobutyl-1-methyl-6-oxo-1,6-dihydropyrimidine-5-carbonitrile

A mixture of (3) (0.01 mol) and 2,4-bis (4-methoxy phenyl amino)-6-chloro-s-triazine (2.0 gm, 0.01mol) in dioxane (25 ml) was refluxed for 3 hrs. During which a solution of sodium bicarbonate (0.84 g, 0.01 mol) was added in fractions. The content was then poured into ice, a product isolated, and crystallized from ethanol.

5. Synthesis of N'-(5-cyano-4-isobutyl-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl) 4-methyl benzene sulfonohydrazide

A mixture of (3) (0.01 mol) and 4-methyl benzene sulphonyl chloride (1.90 gm, 0.01 M) in dry pyridine (8 ml) was refluxed for 4 hrs. The excess pyridine was distilled off and the content was poured into crushed ice. After neutralization, the solid separated was filtered, washed, and crystallized from ethanol.

6. Synthesis of 4-Isobuty-1-methyl-2-(2-(3-nitrobenzylidene) hydrazinyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile

Compound (3) (0.01 mol), and 3-nitro benzaldehyde (1.18 ml, 0.01 mol) in absolute alcohol (20 ml) were taken and 2-3 drops of gl. acetic acid was added. The reaction mixture was refluxed for 3 hours. The progress of the reaction was monitored by thin-layer chromatography. The content was then diluted with ice water and neutralized. The Product obtained was isolated and Crystalized from absolute alcohol.

7. Synthesis of 2-(2-(4-chlorophenyl)-5-methyl-4-oxothiazolidin-3-ylamino)-1,6-dihydro-4-isobutyl-1-methyl-6-oxopyrimidine-5-carbonitrile

A mixture of (6) (0.01 mol) and mercapto lactic acid (1.59 g, 0.015 M) was fused at 120°C for 10-12 hrs. The reaction mixture was cooled and triturated with a 10% sodium bicarbonate solution. The solid product was isolated and crystallized from absolute alcohol.

8. Synthesis of 2-(3-chloro-2-(4-chlorophenyl)-4-oxoazetidin-1-ylamino)-1,6-dihydro-4-isobutyl-1-methyl-6-oxopyrimidine-5-carbonitrile

To a well-stirred mixture of chloroacetyl chloride (0.95 ml, 0.012 mol) and triethylamine (1.65 ml, 0.012 mol) in dry dioxane (15ml), was added a solution of (6) (0.01mol) in dry dioxane at 0°C. The reaction mixture was then stirred a room temperature for 20-22 hrs. and kept at R.T for 2 days. The product was isolated and crystallized from ethanol.

4.3 Physical Constants and Spectral Data

2. Synthesis of 2-(4-chloro phenylamino)-1,6-dihydro-4-isobutyl-1-methyl-6-oxopyrimidine-5-carbonitrile (1)

Mass M^+= 316; IR (KBr) ν(cm^{-1}), 3325 (-NH, secondary) str., 3035 (Aromatic C-H) str., 2964 (-CH_3, Asym.) str., 2872 (-CH_3, Sym.) str., 2216 (-CN) str., 1651 (-CO) str., 1610 1585 1480 (C-C vibration in aromatic ring), 1346 (N-C) str., 777 (N-H) wag, 780 (C-Cl) str. ; ^{1}H NMR (δ ppm) (400MHz, DMSO) δ 3.25 (s,3H,N-CH_3), δ 0.92 (d,6H, CH_2-CH-$(CH_3)_2$), δ 2.12 (m,1H, CH_2-CH-$(CH_3)_2$), δ 2.41 (d,1H, -CH-CH_2-$(CH_3)_2$), δ 7.99 (d,2H, C-H), δ 7.80 (d,2H, C-H)

4. Synthesis of 2-(2-(4,6-bis (4-methoxy phenylamino)-1,3,5-triazin-2-yl) hydrazinyl)-4-isobutyl-1-methyl-6-oxo-1,6-dihydropyrimidine-5-carbonitrile

Mass M^+= 542; IR (KBr) ν(cm^{-1}), 3196 (-NH, secondary) str., 3037 (Aromatic C-H) str., 2954 (-CH_3, Asym.) str., 2850 (C-H vibration of -OCH_3) str., 2822 (-CH_3, Asym.) str., 2225 (-CN) str., 1658 (-CO) str., 1615 1580 1485 (C-C vibration in aromatic ring) str. , 1602, 1529 (C=C + C=N) str., 1090 (C-O vibration of -OCH_3) str. ; ^{1}H NMR (δ ppm) (400MHz, DMSO) δ 3.43 (s,3H,N-CH_3), δ 0.74 (d,6H, CH_2-CH-$(CH_3)_2$), δ 1.92 (m,1H, CH_2-CH-$(CH_3)_2$), δ 2.38 (d,1H, -CH-CH_2-$(CH_3)_2$), δ 2.43 (s,3H,O-CH_3), δ 7.01-7.77 (d,4H, Ar-H), δ 7.11-7.67 (d,4H, Ar-H)

5. Synthesis of N'-(5-cyano-4-isobutyl-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl) 4-methyl benzene sulfonohydrazide

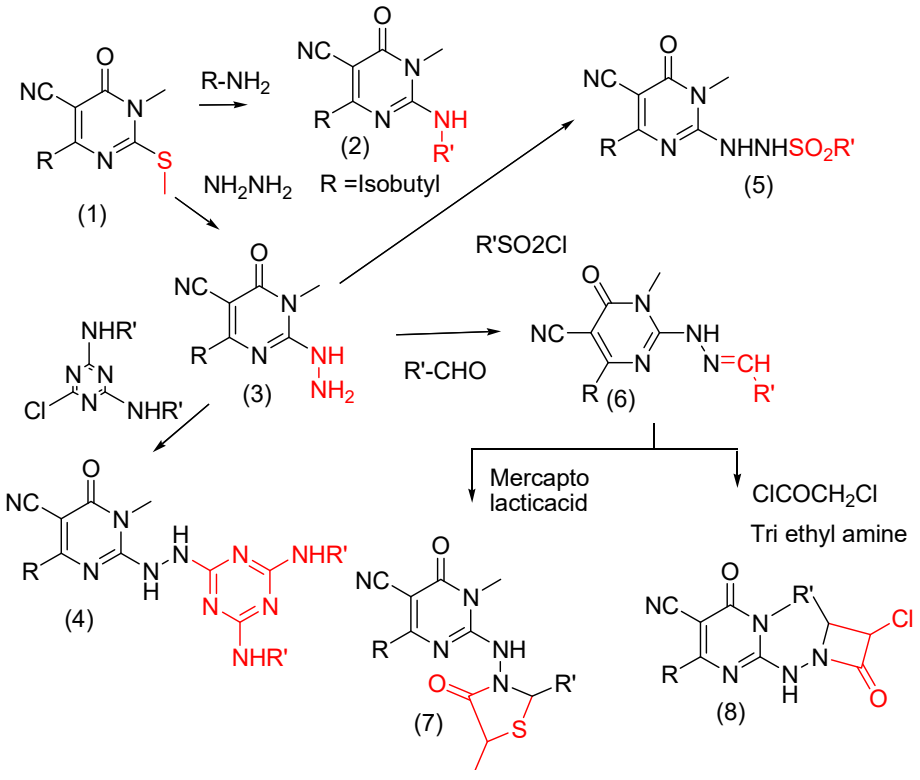
Mass M^+= 375; IR (KBr) ν(cm^{-1}), 3196 (-NH, secondary) str., 3039 (Aromatic C-H) str., 2954 (-CH_3, Asym.) str., 2822 (-CH_3, Sym.) str., 2225 (-CN) str., 1658 (-CO) str., 1620 1588 1490 (C-C vibration in aromatic ring) str., 1328 (S = O Str. Asym.), 813 (N – SO_2) str.; ^{1}H NMR (δ ppm) (400MHz, DMSO) δ 3.37 (s,3H,N-CH_3), δ 2.22 (s,3H, -CH_3) δ 0.77-0.96 (d,6H, CH_2-CH-$(CH_3)_2$), δ 1.80 (m,1H, CH_2-CH-$(CH_3)_2$), δ 2.06 (d,1H, -CH-CH_2-$(CH_3)_2$), δ 7.42-7.85 (d,2H, Ar-H), δ 7.40-7.80 (d,2H, Ar-H)

7. Synthesis of 2-(2-(4-chlorophenyl)-5-methyl-4-oxothiazolidin-3-ylamino)-1,6-dihydro-4-isobutyl-1-methyl-6-oxopyrimidine-5-carbonitrile

Mass M^+= 432; IR (KBr) ν(cm^{-1}), 3278 (-NH, secondary) str., 3036 (Aromatic C-H) str., 2970 (-CH_3, Asym.) str., 2868 (-CH_3, Sym.) str., 2225 (-CN) str., 1658 (-CO) str., 1601 1580 1490 (C-C vibration in aromatic ring) str., 1251 (N-C) str., 709 (N-H) wag, 785 (C-Cl) str. ,1562 (N-H) def., 669 (C-S-C) str. 1722 (-CO) str. ; ^{1}H NMR (δ ppm) (400MHz, DMSO) δ 3.44 (s,3H,N-CH_3), δ 0.93 (d,6H, CH_2-CH-(CH_3)$_2$), δ 2.01 (m,1H, CH_2-CH-(CH_3)$_2$), δ 2.44 (d,1H, -CH-CH_2-(CH_3)$_2$), δ 13.01 (s,1H, N-H), δ 5.75 (s,1H, Ar-CH-N), δ 1.51 (m,1H, CH-CH_3) δ 7.34-7.47 (m,4H, Ar-H)

8. Synthesis of 2-(3-chloro-2-(4-chlorophenyl)-4-oxoazetidin-1-ylamino)-1,6-dihydro-4-isobutyl-1-methyl-6-oxopyrimidine-5-carbonitrile

Mass M^+= 420; IR (KBr) ν(cm^{-1}), 3269 (-NH, secondary) str., 3038 (Aromatic C-H) str., 2968 (-CH_3, Asym.) str., 2870 (-CH_3, Sym.) str., 2223 (-CN) str., 1636 (-CO) str., 1600 1590 1495 (C-C vibration in aromatic ring) str., 1365 (N-C) str., 761 (N-H) wag, 1606 (N-H) def., 783 (C-Cl) str., 1697 (-CO) str.; ^{1}H NMR (δ ppm) (400MHz, DMSO) δ 3.23 (s,3H,N-CH_3), δ 0.91 (d,6H, CH_2-CH-(CH_3)$_2$), δ 1.73 (m,1H, CH_2-CH-(CH_3)$_2$), δ 2.39 (d,1H, -CH-CH_2-(CH_3)$_2$), δ 6.09 (s,1H, N-CH), δ 7.01 (s,1H, -CH-Cl), δ 7.20-7.70 (d,2H, Ar-H), δ 7.25-7.75 (d,2H, Ar-H)



Reaction Scheme 1.

4.4 Biological Evaluations using Broth dilution method[28]

The compounds were tested for bacterial growth inhibition activity against a primary panel including *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Staphylococcus aureus*. (Details are given in **Table 5**)

Table 5. Tested bacteria strains

ID	*Organism*	Strain	Description	Type
GN_001:02	*Escherichia coli*	ATCC 25922	control strain	G-ve
GN_003:02	*Klebsiella pneumoniae*	ATCC 700603	MDR	G-ve
GN_034:02	*Acinetobacter baumannii*	ATCC 19606	type strain	G-ve
GN_042:02	*Pseudomonas aeruginosa*	ATCC 27853	type strain	G-ve
GP_020:02	*Staphylococcus aureus*	ATCC 43300	MRSA	G+ve

4.4.1 Antibiotic activity of compounds:

Compound preparation:

Stock solutions were prepared at 10 mg/mL in DMSO, according to the weight of each compound. Gentle heating and sonication were required to solubilize the compounds.

Single-point bacterial inhibition assay:

The primary bacteria panel, including *Escherichia coli ATCC 25922 (GN_001), Klebsiella pneumonia ATCC 700603 (GN_003), Acinetobacter baumannii ATCC 19606 (GN_034), Pseudomonas aeruginosa ATCC 27853 (GN_042), and Staphylococcus aureus ATCC 43300 (MRSA) (GP_020)* were cultured in Muller Hinton broth (MHB) at 37 °C overnight. A sample of each culture was then diluted 40-fold in fresh MHB broth and incubated at 37°C for 1.5-3 hrs. The compounds were plated at a single test concentration of 64 μg/mL. Colistin, Polymyxin B, Vancomycin, and Daptomycin were serially diluted two-fold across the wells, with compound concentrations ranging from 0.5 to 64 μg/mL, as controls of bacterial inhibitors. The resultant mid-log phase cultures were diluted to the final concentration of 5×105 CFU/mL, then 50 μL was added to each well of the compound containing 96-well plates (Corning; Cat. No 3641, NBS), giving a final compound concentration range of 0.25 μg/mL to 32 μg/mL for control inhibitors and 32 μg/mL for test compounds. All the plates were covered and incubated at 37 °C for 24 h.

Inhibition of bacterial growth was determined visually and was recorded at 32 μg/mL where 100% inhibition was identified. (Details **Table 2**)

MIC (Minimum Inhibitory Concentration) assay:

The primary bacteria panel, including *Escherichia coli ATCC 25922 (GN_001) and Staphylococcus aureus ATCC 43300 (MRSA) (GP_020)* were cultured in Muller Hinton broth (MHB) at 37°C overnight. A sample of each culture was then diluted 40-fold in fresh MHB broth and incubated at 37°C for 1.5-3 h. The compounds were serially diluted twofold across the wells of non-binding surface 96-well plates (Corning; Cat. No 3641, NBS), with compound concentrations ranging from 0.03 μg/mL to 64 μg/mL, plated in duplicate. The resultant mid-log phase cultures were diluted to the final concentration of 5×105 CFU/mL, then 50 μL was added to each well of the compound-containing 96-well plates, giving a final compound concentration range of 0.015 μg/mL to 32 μg/mL. All the plates were covered and incubated at 37 °C for 24 h.

Inhibition of bacterial growth was determined visually after 24 h, where the MIC is recorded as the lowest compound concentration with no visible growth. (Details are given in **Table 3**)

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