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RESEARCH ARTICLE

Synthesis and Antimicrobial Evaluation of Novel Oxopyrimido[1,2–a]pyrimidine Derivatives

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ABSTRACT: This study details the efficient synthesis and antimicrobial evaluation of a novel series of oxopyrimido[1,2-a]pyrimidine derivatives. The synthetic pathway commenced with the preparation of a key chalcone intermediate, 3-(4-chlorophenyl)-1-phenylprop-2-en-1-one, via a Claisen-Schmidt condensation of 4-chlorobenzaldehyde and acetophenone. This chalcone was subsequently cyclized with guanidine nitrate under basic conditions to yield the pivotal dihydropyrimidin-2-amine precursor. The core pyrimidopyrimidine scaffold was constructed through a Michael addition reaction, where the dihydropyrimidin-2-amine was reacted with ethyl 2-cyano-3,3-bis(methylthio)acrylate. This crucial step afforded the central intermediate, 2-(4-chlorophenyl)-8-(methylthio)-6-oxo-4-phenyl-1,6,9,9a-tetrahydro-2H-pyrimido[1,2-a]pyrimidine-7-carbonitrile, which features a reactive methylthio group. This group served as a versatile handle for further functionalization, allowing for the synthesis of a diverse library of ten new analogues through nucleophilic substitution with various aromatic amines, phenols, and active methylene compounds. The structures of all synthesized compounds were unequivocally confirmed using modern spectroscopic techniques, including IR, ^{1}H NMR, ^{13}C NMR, and mass spectrometry. The entire compound library was screened for in vitro antibacterial and antifungal activities. The results were promising, with several derivatives exhibiting significant growth inhibition against strains of Escherichia coli, Bacillus subtilis, Aspergillus niger, and Aspergillus flavus, showcasing their potential as leads for new antimicrobial agents.

Keywords: Pyrimidopyrimidine, Antimicrobial Activity, Nucleophilic Substitution, Chalcone, Heterocyclic Synthesis, Structure-Activity Relationship

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1. INTRODUCTION

The pyrimidine nucleus represents a cornerstone of medicinal chemistry, extensively present within a vast spectrum of biologically active natural products and synthetic therapeutic agents. The profound pharmacological significance of pyrimidine derivatives is exceptionally well-documented in scientific literature, showcasing a remarkable diversity of biological activities [1]. These compounds are recognized for their potent antiviral, antibacterial, antitumor, anti-inflammatory, antifungal, and anti-leishmanial properties, making them indispensable scaffolds in modern drug discovery campaigns [2, 3]. The exploration of fused heterocyclic systems containing the pyrimidine motif has further expanded this therapeutic potential. Several reports detail the preparation of such complex structures, with their derivatives demonstrating significant utility as bronchodilators, vasodilators, anti-allergic agents, anti-hypertensive drugs, and anti-cancer agents [4, 5]. A fundamental reason underpinning the widespread biological relevance of pyrimidines can be traced to their essential role in life processes; the presence of the pyrimidine system in fundamental nucleobases like thymine, cytosine, and uracil, which are the critical building blocks of nucleic acids (DNA and RNA), provides a plausible explanation for their inherent

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bioactivity and ability to interact with enzymatic targets [6]. This intrinsic biological compatibility continues to drive research into more complex pyrimidine-based structures.

The strategic fusion of the pyrimidine ring with other heterocycles has proven to be a highly successful approach for enhancing and modulating biological activity. For instance, fused derivatives of pyrimidine with thiazole have emerged as particularly bioactive compounds. Such molecular architectures have been developed and utilized as potent and selective inhibitors of key enzymatic targets including acetyl-CoA carboxylase 2 and vascular endothelial growth factor (VEGF) receptors I and II, which are critical in metabolic disorders and angiogenesis-dependent diseases like cancer [7]. Beyond this, these fused systems have also demonstrated potent and selective antagonism for the human adenosine A3 receptor, implicating them in neurological and inflammatory pathways, as well as vanilloid receptor I (TRPV1) antagonism, which is relevant in pain management [8, 9]. The synthesis of these complex, polycyclic systems often presents a significant challenge to chemists, requiring efficient and sustainable methodologies.

In recent years, one-pot multicomponent reactions (MCRs) have gained substantial importance within synthetic organic chemistry, primarily due to their compelling environmental implications and superior atom economy [10]. MCRs are economically and environmentally highly advantageous because traditional multi-step syntheses typically produce considerable amounts of chemical waste. This waste generation is mainly attributable to complex isolation and purification procedures that often involve the use of expensive, toxic, and hazardous solvents after each individual step. Due to these inherent advantages, MCRs are perfectly suited for the rapid assembly of combinatorial libraries, and thus are finding increasing use in the streamlined discovery process for new pharmaceutical compounds and agrochemicals [11-13]. A large proportion of these MCR strategies are based on the condensation reactions of carbonyl groups, which offer a versatile entry point for forming new carbon-carbon and carbon-heteroatom bonds [14]. A quintessential example is the Biginelli reaction, which has been effectively employed for the synthesis of pyrimido[4,5-d]pyrimidine derivatives via a one-pot condensation reaction between acetophenone, urea, and various aldehydes [15]. Numerous methodological refinements of the classic Biginelli reaction have been reported, including condensations of aldehydes, urea, and alkyl aryl ketones in acetic acid utilizing catalytic amounts of potassium hydrogen sulfate ($KHSO_4$) to facilitate the transformation [16]. Furthermore, the application of the Biginelli reaction for constructing pyrimido[4,5-d]pyrimidine cores has been enhanced through the use of basic catalysts and modern techniques like microwave-assisted synthesis, which offer improved reaction rates and yields [17, 18].

Beyond MCR approaches, other promising synthetic routes for accessing pyrimido[4,5-d]pyrimidines exist, though they often involve more elaborate multistep syntheses. These alternative pathways may start from advanced intermediates like 1,3-disubstituted cyanouracils, employ polymer-bound aminopyrimidine derivatives to facilitate purification, utilize aza-Wittig-type reactions for nitrogen heterocycle formation, or involve the reaction of aminouracils with various heterocumulenes [19-21]. While these methods are valuable, many synthetic alternatives resort to harsh reaction conditions that limit their practicality and scalability. Common examples include the use of strong acid catalysts like p-toluene sulfonic acid (PTSA) or the application of aggressive reagents such as phosphorus oxychloride ($POCl_3$) in solvents like dimethylformamide (DMF) [22]. Additionally, a significant drawback of some specialized routes is that the required starting reagents are not always readily or commercially available, posing a barrier to widespread adoption. Considering all these factors—the established importance of pyrimido[4,5-d]pyrimidine derivatives and the limitations of existing synthetic methods—it is considered highly worthwhile to develop new methodologies for synthesizing these valuable compounds. The ideal approach would utilize green chemistry protocols, such as employing eco-friendly reagents and catalysts, operating under solvent-free conditions, or using non-hazardous solvents. Such a strategy offers the dual benefit of enhanced chemical process economics concomitant with a significantly reduced environmental burden [23, 24]. The structures of all novel compounds synthesized in this work were rigorously assigned on the basis of comprehensive spectroscopic data, including IR, ^{1}H NMR, ^{13}C NMR, and Mass spectrometry.

In view of these observations and as a direct extension of our earlier work, we have designed and executed the synthesis of a novel molecular framework, 2-(4-chlorophenyl)-8-(methylthio)-6-oxo-4-phenyl-1,6,9,9a-tetra-hydro-2H-pyrimido[1,2-a]pyrimidine-7-carbonitrile. This was achieved using a convergent strategy that couples two key building blocks: 6-(4-chlorophenyl)-4-phenyl-1,6-dihydropyrimidin-2-amine [23-24] and ethyl 2-cyano-3,3-bis(methylthio)acrylate. The requisite oxo-dihydro-pyrimidine precursor was itself prepared via the reaction of a custom-synthesized chalcone [25, 26] with guanidine nitrate in the presence of ethanol and potassium hydroxide under reflux conditions, establishing a robust and efficient route to this important intermediate.

The distinct novelty of this work lies in the innovative synthetic pathway developed to access a new class of functionally diverse oxopyrimido[1,2-a]pyrimidine derivatives, which centers on the strategic incorporation of a reactive methylthio ($-SCH_3$) group at a critical position on the complex tetracyclic core. This deliberate design feature is not merely a structural embellishment but serves as a versatile chemical handle, enabling an efficient and facile post-cyclization functionalization strategy through nucleophilic substitution reactions with a wide array of amines, phenols, and active methylene compounds. This approach effectively bypasses the common reliance on harsh reagents or complex multi-step protocols reported in prior literature, offering instead a more modular and adaptable route for generating a focused library of novel analogues that would be difficult to

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access by other means. Furthermore, the biological evaluation of these specific molecular architectures, particularly those bearing the 7-cyano and 8-substituted functionalities, against a panel of bacterial and fungal pathogens represents a significant and original contribution to the structure-activity relationship (SAR) of this underexplored fused heterocyclic system, providing valuable insights for the future development of new antimicrobial agents.

2. EXPERIMENTAL DETAILS

2.1. General Information

All reagents and solvents were procured from commercial suppliers (Sigma-Aldrich, Spectrochem, and others) and were used without further purification unless otherwise stated. Reactions were monitored by thin-layer chromatography (TLC) performed on pre-coated silica gel 60 F254 plates (Merck), and visualization was achieved using UV light (254 nm and 365 nm) or iodine vapor. Melting points were determined in open capillary tubes on a Buchi melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a PerkinElmer FT-IR Spectrometer using KBr pellets, and frequencies are reported in reciprocal centimeters (cm^{-1}). ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were acquired on a Bruker Avance (400 MHz for ^{1}H, 100 MHz for ^{13}C) spectrometer using DMSO-d6 as the solvent and tetramethylsilane (TMS) as an internal standard; chemical shifts are reported in parts per million (δ scale) and coupling constants (*J*) in Hertz (Hz). Mass spectra were recorded on a Waters Xevo G2-XS QToF mass spectrometer using electrospray ionization (ESI) techniques. Elemental analyses (C, H, N) were performed on a Thermo Scientific Flash 2000 CHNS/O Analyzer, and the results were within ±0.4% of the theoretical values.

2.2. Synthetic Procedures

2.2.1. Synthesis of 3-(4-Chlorophenyl)-1-phenylprop-2-en-1-one (Chalcone, 1)

The synthesis of the key chalcone intermediate was accomplished via a Claisen-Schmidt condensation reaction, following a modified literature procedure [25, 26]. To an equimolar solution of acetophenone (1.20 g, 0.01 mol) and 4-chlorobenzaldehyde (1.40 g, 0.01 mol) in 95% ethanol (30 mL), a 50% (w/v) aqueous potassium hydroxide (KOH) solution (15 mL) was added dropwise under vigorous mechanical stirring at an ambient temperature of 0-5 °C. The addition was controlled to maintain the reaction temperature below 10 °C. Upon complete addition, the resulting deep reddish-brown reaction mixture was allowed to stir at room temperature for 12 hours. The progress of the reaction was monitored by TLC (n-hexane/ethyl acetate, 8:2). After completion, the mixture was carefully poured into crushed ice (100 g) and neutralized by slow addition of dilute hydrochloric acid (1N HCl) until the solution was distinctly acidic (pH ~5-6). The resulting yellow precipitate was isolated by vacuum filtration, washed copiously with cold distilled water to remove inorganic salts, and air-dried. The crude solid was subsequently recrystallized from hot ethanol to afford pure chalcone **(1)** as pale yellow crystalline flakes. Yield: 2.35 g (87%); m.p. 118-120 °C (Lit. [25] 119-121 °C).

2.2.2. Synthesis of 6-(4-Chlorophenyl)-4-phenyl-1,6-dihydropyrimidin-2-amine (2)

The dihydropyrimidine core was synthesized by cyclocondensation of the chalcone with guanidine nitrate [23, 24]. A mixture of the synthesized chalcone **1** (2.42 g, 0.01 mol) and guanidine nitrate (1.22 g, 0.01 mol) was suspended in a solution of potassium hydroxide (0.56 g, 0.01 mol) in absolute ethanol (20 mL). The reaction flask was fitted with a reflux condenser, and the mixture was heated under reflux with constant stirring for 4 hours. The reaction progress was checked by TLC (n-hexane/ethyl acetate, 7:3). Upon completion, the hot reaction mixture was carefully poured into a beaker containing ice-cold water (100 mL) with stirring. The precipitated solid was collected by filtration, washed with cold water, and dried under reduced pressure. The crude product was purified by recrystallization from a mixture of ethanol and water (3:1) to yield the title compound **(2)** as a white solid. Yield: 2.15 g (78%); m.p. 192-194 °C.

2.2.3. Synthesis of 2-(4-Chlorophenyl)-8-(methylthio)-6-oxo-4-phenyl-1,6,9,9a-tetrahydro-2H-pyrimido[1,2-a]pyrimidine-7-carbonitrile (3)

The pivotal Michael addition and cyclization step was performed to construct the complex pyrimidopyrimidine scaffold. A mixture of dihydropyrimidin-2-amine **2** (2.76 g, 0.01 mol) and ethyl 2-cyano-3,3-bis(methylthio)acrylate (2.32 g, 0.01 mol) was dissolved in anhydrous *N*,*N*-dimethylformamide (DMF, 25 mL). Anhydrous potassium carbonate (K_2CO_3, 10 mg) was added as a catalytic base. The reaction mixture was heated under reflux at 110-115 °C for 6 hours, with the course of the reaction being monitored by TLC (n-hexane/ethyl acetate, 6:4). After completion, the mixture was allowed to cool to room temperature and then poured into ice-cold water (150 mL) with vigorous stirring. The aqueous mixture was extracted with ethyl acetate (3 × 50 mL). The combined organic extracts were washed with brine (50 mL), dried over anhydrous sodium sulfate (Na_2SO_4), and concentrated under reduced pressure using a rotary evaporator to afford a viscous brown residue. The crude product was purified by flash column chromatography on silica gel (60-120 mesh) using a gradient elution of n-hexane and ethyl acetate (from 8:2 to 6:4) to isolate the desired product **(3)** as a light-yellow solid. Yield: 3.25 g (80%); m.p. 228-230 °C.

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Spectroscopic Data for Compound (3): IR (KBr, cm^{-1}): 3330 (N-H stretch), 2210 (C≡N stretch), 1650 (C=O stretch), 748 (C-Cl stretch).
^{1}H NMR (400 MHz, DMSO-d6): δ 2.41 (s, 3H, SCH_3), 4.50 (d, 1H, *J* = 4.0 Hz, CH), 5.05 (s, 1H, N-H), 5.80 (s, 1H, CH), 5.84 (d, 1H, *J* = 10.0 Hz, =CH), 1.80 (s, 1H, N-H), 7.25 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.30 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.60 (s, 5H, Ar-H).
^{13}C NMR (100 MHz, DMSO-*d*6): δ 14.5 (SCH_3), 55.8, 57.2, 98.5, 116.8 (CN), 119.5, 127.8, 128.1, 128.5, 128.9, 132.5, 133.8, 135.2, 140.5, 153.2 (C=O), 161.5.
ESI-MS: m/z 408 $[M]^+$, 410 $[M+2]^+$.
Elemental Analysis: Calcd. for $C_{21}H_{17}ClN_4OS$: C, 61.68; H, 4.19; Cl, 8.67; N, 13.70; S, 7.84. Found: C, 61.56; H, 4.13; Cl, 8.75; N, 13.76; S, 7.95.

2.2.4. General Procedure for the Synthesis of Derivatives (3a-3j)

A general nucleophilic substitution protocol was employed to synthesize the library of derivatives. A mixture of the key intermediate **3** (1 mmol, 0.408 g) and the appropriate nucleophile (1 mmol)—such as an aromatic amine, phenol, heterocyclic amine, or active methylene compound—was dissolved in anhydrous DMF (10 mL). Anhydrous potassium carbonate (K_2CO_3, 10 mg, catalytic amount) was added to the solution. The reaction mixture was heated under reflux at 90-95 °C for 4-6 hours, with progress monitored by TLC (n-hexane/ethyl acetate, 7:3). After completion, the mixture was cooled to room temperature and poured into vigorously stirred ice-cold water (50 mL). The precipitated solid was collected by vacuum filtration, washed thoroughly with cold water to remove residual DMF, and dried. The crude product was recrystallized from hot ethanol to afford the pure title derivatives **(3a-3j)**. The physical and spectroscopic data for all novel compounds are provided below.

2.3. Characterization Data for Synthesized Derivatives

3a. 2-(4-Chlorophenyl)-6-oxo-8-phenoxy-4-phenyl-1,6,9, 9a -tetrahydro-2H-pyrimido[1,2-a]pyrimidine-7-carbonitrile

Yield: 82%; m.p. 245-247 °C.
IR (KBr, cm^{-1}): 3330, 2210, 1650, 748.
^{1}H NMR (400 MHz, DMSO-d6): δ 4.52 (d, 1H, *J* = 4.0 Hz, CH), 5.01 (s, 1H, N-H), 5.82 (s, 1H, CH; d, 1H, *J* = 10.0 Hz, =CH), 1.83 (s, 1H, N-H), 7.10 (s, 5H, OAr-H), 7.27 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.32 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.64 (s, 5H, Ar-H).
ESI-MS: m/z 454 $[M]^+$, 456 $[M+2]^+$.
Elemental Analysis: Calcd. for $C_{26}H_{19}ClN_4O_2$: C, 68.65; H, 4.21; Cl, 7.79; N, 12.32. Found: C, 68.77; H, 4.15; Cl, 7.75; N, 12.28.

3b. 8-(4-Bromophenoxy)-2-(4-chlorophenyl)-6-oxo-4-phenyl-1,6,9,9a-tetrahydro-2H-pyrimido[1,2-a]pyrimidine-7-carbonitrile

Yield: 79%; m.p. 260-262 °C.
IR (KBr, cm^{-1}): 3330, 2210, 1650, 748, 650.
^{1}H NMR (400 MHz, DMSO-d6): δ 4.55 (d, 1H, *J* = 4.0 Hz, CH), 5.03 (s, 1H, N-H), 5.84 (s, 1H, CH), 5.87 (d, 1H, *J* = 10.0 Hz, =CH), 1.86 (s, 1H, N-H), 6.74 (dd, 2H, *J* = 2.0, 6.8 Hz, OAr-H), 7.22 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.30 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.32 (dd, 2H, *J* = 2.0, 6.8 Hz, OAr-H), 7.69 (s, 5H, Ar-H).
ESI-MS: m/z 532 $[M]^+$, 534 $[M+2]^+$.
Elemental Analysis: Calcd. for $C_{26}H_{18}BrClN_4O_2$: C, 58.50; H, 3.40; Br, 14.97; Cl, 6.64; N, 10.50. Found: C, 58.54; H, 3.43; Br, 14.90; Cl, 6.68; N, 10.56.

3c. 8-(4-Chlorophenyl)-2-(4-nitrophenoxy)-4-oxo-6-phenyl-1,6,9,9a-tetrahydro-4H-pyrimido[1,2-a]pyrimidine-3-carbonitrile

Yield: 75%; m.p. 275-277 °C.
IR (KBr, cm^{-1}): 3330, 2210, 1650, 1510, 748.
^{1}H NMR (400 MHz, DMSO-d6): δ 4.58 (d, 1H, *J* = 4.0 Hz, CH), 5.07 (s, 1H, N-H), 5.81 (s, 1H, CH), 5.90 (d, 1H, *J* = 10.0 Hz, =CH), 1.89 (s, 1H, N-H), 7.10 (dd, 2H, *J* = 2.0, 6.8 Hz, OAr-H), 7.25 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.28 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.35 (dd, 2H, *J* = 2.0, 6.8 Hz, OAr-H), 7.66 (s, 5H, Ar-H).
ESI-MS: m/z 499 $[M]^+$, 501 $[M+2]^+$.
Elemental Analysis: Calcd. for $C_{26}H_{18}ClN_5O_4$: C, 62.47; H, 3.63; Cl, 7.09; N, 14.01. Found: C, 62.41; H, 3.69; Cl, 7.15; N, 14.06.

3d. 2-(4-Chlorophenyl)-6-oxo-4-phenyl-8-(phenylamino)-1,6,9,9a-tetrahydro-2H-pyrimido[1,2-a]pyrimidine-7-carbonitrile

Yield: 85%; m.p. 285-287 °C.
IR (KBr, cm^{-1}): 3330, 3250 (N-H), 2210, 1650, 748.
^{1}H NMR (400 MHz, DMSO-d6): δ 4.53 (d, 1H, *J* = 4.0 Hz, CH), 5.02 (s, 1H, N-H), 5.80 (s, 1H, CH), 5.84 (d, 1H, *J* = 10.0 Hz, =CH), 1.84 (s, 1H, N-H), 6.80 (s, 5H, NAr-H), 7.22 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.26 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.63 (s, 5H, Ar-H), 10.66 (s, 1H, N-H).
ESI-MS: m/z 452 $[M]^+$, 454 $[M+2]^+$.
Elemental Analysis: Calcd. for $C_{26}H_{20}ClN_5O$: C, 68.80; H, 4.44; Cl, 7.81; N, 15.43. Found: C, 68.75; H, 4.50; Cl, 7.91; N, 15.38.

3e. 8-((4-Bromophenyl)amino)-2-(4-chlorophenyl)-6-oxo-4-phenyl-1,6,9,9a-tetrahydro-2H-pyrimido[1,2-a]pyrimidine-7-carbonitrile

Yield: 81%; m.p. 290-292 °C.
IR (KBr, cm^{-1}): 3330, 3250, 2210, 1650, 748, 640.
^{1}H NMR (400 MHz, DMSO-d6): δ 4.55 (d, 1H, *J* = 4.0 Hz, CH), 5.04 (s, 1H, N-H), 5.82 (s, 1H, CH), 5.89 (d, 1H, *J* = 10.0 Hz, =CH), 1.86 (s, 1H, N-H), 6.72 (dd, 2H, *J* = 2.0, 6.8 Hz, NAr-H), 7.16 (dd, 2H, *J* = 2.0, 6.8 Hz, NAr-H), 7.25 (dd,

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2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.29 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.66 (s, 5H, Ar-H), 10.69 (s, 1H, N-H).
ESI-MS: m/z 531 $[M]^+$, 533 $[M+2]^+$.
Elemental Analysis: Calcd. for $C_{26}H_{19}BrClN_5O$: C, 58.61; H, 3.59; Br, 15.00; Cl, 6.65; N, 13.14. Found: C, 58.48; H, 3.65; Br, 15.17; Cl, 6.57; N, 13.10.

3f. 2-(4-Chlorophenyl)-8-((4-nitrophenyl)amino)-6-oxo-4-phenyl-1,6,9,9a-tetrahydro-2H-pyrimido[1,2-a]pyrimidine-7-carbonitrile

Yield: 77%; m.p. >300 °C (dec.).
IR (KBr, cm^{-1}): 3330, 3250, 2210, 1650, 1520, 1480, 748.
^{1}H NMR (400 MHz, DMSO-d6): δ 4.58 (d, 1H, *J* = 4.0 Hz, CH), 5.06 (s, 1H, N-H), 5.84 (s, 1H, CH), 5.91 (d, 1H, *J* = 10.0 Hz, =CH), 1.89 (s, 1H, N-H), 6.85 (dd, 2H, *J* = 2.0, 6.8 Hz, NAr-H), 7.27 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.31 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.69 (s, 5H, Ar-H), 7.81 (dd, 2H, *J* = 2.0, 6.8 Hz, NAr-H), 11.10 (s, 1H, N-H).
ESI-MS: m/z 498 $[M]^+$, 500 $[M+2]^+$.
Elemental Analysis: Calcd. for $C_{26}H_{19}ClN_6O_3$: C, 62.50; H, 3.84; Cl, 7.11; N, 16.84. Found: C, 62.59; H, 3.90; Cl, 7.06; N, 16.79.

3g. 2-(4-Chlorophenyl)-6-oxo-4-phenyl-8-(pyrrolidin-1-yl)-1,6,9,9a-tetrahydro-2H-pyrimido[1,2-a]pyrimidine-7-carbonitrile

Yield: 88%; m.p. 255-257 °C.
IR (KBr, cm^{-1}): 3330, 2210, 1650, 748.
^{1}H NMR (400 MHz, DMSO-d6): δ 1.52 (quintet, 4H, *J* = 6.8 Hz, 2xCH_2-pyrrolidine), 1.82 (s, 1H, N-H), 2.44 (t, 4H, *J* = 6.8 Hz, 2xNCH_2-pyrrolidine), 4.44 (d, 1H, *J* = 4.0 Hz, CH), 5.04 (s, 1H, N-H), 5.80 (s, 1H, CH), 5.82 (d, 1H, *J* = 10.0 Hz, =CH), 7.21 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.23 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.55 (s, 5H, Ar-H).
ESI-MS: m/z 431 $[M]^+$, 433 $[M+2]^+$.
Elemental Analysis: Calcd. for $C_{24}H_{22}ClN_5O$: C, 66.74; H, 5.13; Cl, 8.21; N, 16.21. Found: C, 66.70; H, 5.18; Cl, 8.21; N, 16.21.

3h. 2-(4-Chlorophenyl)-6-oxo-4-phenyl-8-(piperidin-1-yl)-1,6,9,9a-tetrahydro-2H-pyrimido[1,2-a]pyrimidine-7-carbonitrile

Yield: 86%; m.p. 250-252 °C.
IR (KBr, cm^{-1}): 3330, 2210, 1650, 748.
^{1}H NMR (400 MHz, DMSO-d6): δ 1.50 (m, 6H, 3xCH_2-piperidine), 1.86 (s, 1H, N-H), 3.10 (t, 4H, *J* = 5.2 Hz, 2xNCH_2-piperidine), 4.51 (d, 1H, *J* = 4.0 Hz, CH), 5.07 (s, 1H, N-H), 5.83 (s, 1H, CH), 5.87 (d, 1H, *J* = 10.0 Hz, =CH), 7.24 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.28 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.65 (s, 5H, Ar-H).
ESI-MS: m/z 445 $[M]^+$, 447 $[M+2]^+$.
Elemental Analysis: Calcd. for $C_{25}H_{24}ClN_5O$: C, 67.33; H, 5.42; Cl, 7.95; N, 15.70. Found: C, 67.21; H, 5.48; Cl, 7.89; N, 15.76.

3i. 2-(2-(4-Chlorophenyl)-7-cyano-6-oxo-4-phenyl-1,6,9,9a-tetrahydro-2H-pyrimido[1,2-a]pyrimidin-8-yl)malononitrile

Yield: 80%; m.p. 265-267 °C.
IR (KBr, cm^{-1}): 3330, 2950, 2210 (C≡N), 1650, 748.
^{1}H NMR (400 MHz, DMSO-d6): δ 1.84 (s, 1H, N-H), 4.08 (s, 1H, CH-malononitrile), 4.49 (d, 1H, *J* = 4.0 Hz, CH), 5.01 (s, 1H, N-H), 5.78 (s, 1H, CH), 5.83 (d, 1H, *J* = 10.0 Hz, =CH), 7.18 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.23 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.61 (s, 5H, Ar-H).
ESI-MS: m/z 425 $[M]^+$, 427 $[M+2]^+$.
Elemental Analysis: Calcd. for $C_{23}H_{15}ClN_6O$: C, 64.72; H, 3.54; Cl, 8.30; N, 19.69. Found: C, 64.67; H, 3.48; Cl, 8.40; N, 19.70.

3j. Ethyl 2-(2-(4-chlorophenyl)-7-cyano-6-oxo-4-phenyl-1,6,9,9a-tetrahydro-2H-pyrimido[1,2-a]pyrimidin-8-yl)-2-cyanoacetate

Yield: 83%; m.p. 235-237 °C.
IR (KBr, cm^{-1}): 3330, 2210, 1710 (C=O ester), 1650 (C=O amide), 748.
^{1}H NMR (400 MHz, DMSO-d6): δ 1.16 (t, 3H, *J* = 7.2 Hz, CH_3-CH_2), 1.88 (s, 1H, N-H), 3.95 (s, 1H, CH-cyanoacetate), 4.14 (q, 2H, *J* = 7.2 Hz, CH_2-CH_3), 4.54 (d, 1H, *J* = 4.0 Hz, CH), 5.06 (s, 1H, N-H), 5.83 (s, 1H, CH), 5.88 (d, 1H, *J* = 10.0 Hz, =CH), 7.24 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.30 (dd, 2H, *J* = 2.0, 6.8 Hz, Ar-H), 7.68 (s, 5H, Ar-H).
ESI-MS: m/z 473 $[M]^+$, 475 $[M+2]^+$.
Elemental Analysis: Calcd. for $C_{25}H_{20}ClN_5O_3$: C, 63.36; H, 4.25; Cl, 7.48; N, 14.78. Found: C, 63.31; H, 4.30; Cl, 7.53; N, 14.68.

2.4. Explanation of Reaction Schemes

Reaction Scheme 1 illustrates the three-step synthetic pathway to the key intermediate **(3)**. Step I involves the base-catalyzed Claisen-Schmidt condensation between acetophenone and 4-chlorobenzaldehyde to form the α,β-unsaturated ketone (chalcone) **1**. Step II depicts the cyclocondensation of chalcone **1** with guanidine nitrate in an ethanolic KOH medium. This reaction proceeds via a Michael addition of the guanidine nitrogen to the enone system, followed by intramolecular cyclization and dehydration to afford the 1,6-dihydropyrimidin-2-amine **2**. Step III is the pivotal cyclization step where compound **2** reacts with ethyl 2-cyano-3,3-bis(methylthio)acrylate. This reaction is a Michael addition followed by an intramolecular cyclization and elimination, facilitated by the catalytic base (K_2CO_3), leading to the formation of the complex tetracyclic pyrimidopyrimidine system **3**. The methylthio (-SCH_3) group at the 8-position is installed in this step and serves as an excellent leaving group for subsequent nucleophilic substitution.

Reaction Schemes 2 through 5 conceptually represent the general nucleophilic aromatic substitution (SNAr)

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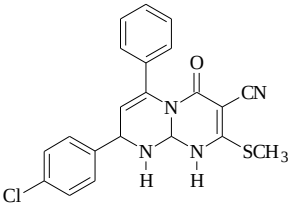
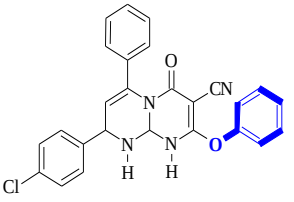
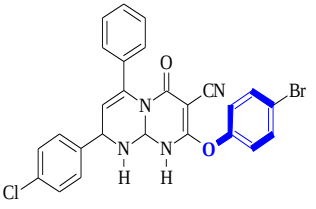
reactions performed on intermediate **3** to generate the diverse library of derivatives **(3a-3j)**. The electron-withdrawing nature of the adjacent cyano and carbonyl groups activates the carbon at the 8-position towards nucleophilic attack. Scheme 2 represents substitution with various phenols (e.g., phenol, 4-bromophenol), Scheme 3 with aromatic amines (e.g., aniline, 4-bromoaniline, 4-nitroaniline), Scheme 4 with heterocyclic amines (e.g., pyrrolidine, piperidine), and Scheme 5 with active methylene compounds (e.g., malononitrile, ethyl cyanoacetate). In each case, the methylthio group is displaced by the nucleophile (Nu-H) in the presence of a base, yielding the final products with significantly altered electronic and steric properties, which were then evaluated for their biological activity.

3. RESULTS AND DISCUSSION

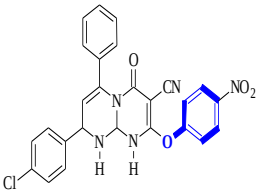
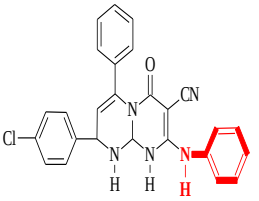
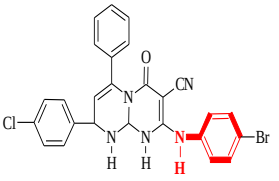
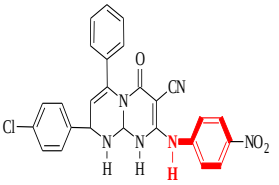
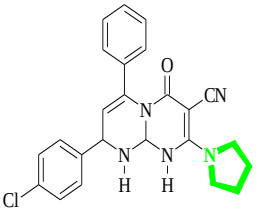
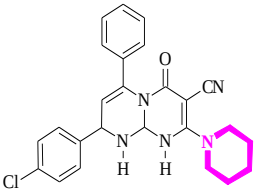
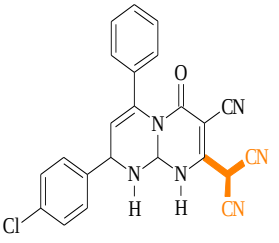
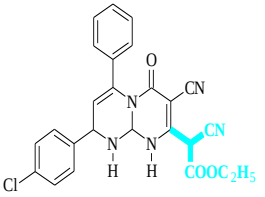
3.1. Antimicrobial Activity Evaluation: Overview and Methodology

The escalating global threat of antimicrobial resistance necessitates the continuous development of novel chemotherapeutic agents with unique mechanisms of action. In this context, all newly synthesized oxopyrimido[1,2-a]pyrimidine derivatives **(3, 3a-3j)** were subjected to a comprehensive *in vitro* antimicrobial screening to assess their potential efficacy. The evaluation was conducted against two representative bacterial strains, encompassing both Gram-negative (*Escherichia coli* MTCC-443) and Gram-positive (*Bacillus subtilis* MTCC-441) bacteria, and two fungal strains (*Aspergillus flavus* MTCC-871 and *Aspergillus niger* MTCC-404). The agar well diffusion method was employed for this assessment, a standard technique that measures the zone of inhibition (ZOI) around a well containing the test compound, which correlates with the compound's ability to diffuse through the agar and inhibit microbial growth [1, 2]. The antibacterial and antifungal activities of the test compounds were quantitatively compared against standard reference drugs, streptomycin (15 µg/disc) and fluconazole (15 µg/disc), respectively. All assays were performed in triplicate to ensure reproducibility, and the results are presented as mean zone of inhibition (in mm) ± standard deviation (SD). The biological data for all eleven target compounds are systematically compiled in Table 1.

Table 1. *In vitro* antimicrobial activity of synthesized oxopyrimido[1,2-a]pyrimidine derivatives (3, 3a-3j).

Sr. No.	Comp. No.	R Group	Zone of Inhibition (mm) ± Standard Deviation			
			Fungal Species		**Bacterial Species**	
			A. flavus (MTCC-871)	*A.niger* (MTCC-404)	*E.coli* (MTCC-443)	*B. Subtilis* (MTCC-441)
01	3		12 ± 0.28	09 ± 1.32	12 ± 0.51	12 ± 0.81
02	3a		13 ± 0.22	11 ± 1.42	13 ± 0.52	13 ± 1.37
03	3b		13± 1.42	10 ± 0.47	09± 0.12	12 ± 1.48

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04	3c		----	----	---	-----
05	3d		13 ± 0.58	12 ±1.54	10 ± 0.12	13 ± 1.85
06	3e		13 ± 1.58	11 ±1.29	13 ± 1.57	12 ± 1.24
07	3f		15 ± 1.49	12 ± 0.52	15 ± 1.21	14 ± 0.45
08	3g		15 ± 0.89	15± 0.70	15 ± 0.48	15 ± 0.51
09	3h		15 ± 0.40	15 ± 1.20	13 ± 0.32	14± 0.88
10	3i		13 ± 0.96	11± 1.42	13 ± 1.69	12± 1.34
11	3j		13 ± 1.60	13 ± 0.40	12 ± 0.96	12 ± 0.48
		Standard	15 ± 1.38	15± 1.38	15 ± 0.29	15 ± 0.43

Antimicrobial activity expressed as mean zone of inhibition (mm) ± standard deviation. (-) indicates no observable activity. *Ec- Escherichia coli, An- Aspergillus Niger, Bs- Bacillus subtillis, Af- Aspergillus flavus, --No activity, NA- Not Applicable.*

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3.2. Analysis of Antifungal Activity

The antifungal screening results against *Aspergillus flavus* and *Aspergillus niger* revealed a compelling spectrum of activity across the synthesized series. The overall zones of inhibition for the active compounds ranged from 9.0 ± 1.32 mm to 15.0 ± 1.20 mm, which were compared against the standard drug fluconazole (15.0 ± 1.38 mm). A critical observation from Table 1 is the complete inactivity of compound **3c** against both fungal species. The structure of **3c** features a 4-nitrophenoxy group at the 8-position. It is postulated that the strong electron-withdrawing nature of the nitro group, while potentially aiding in the SNAr reaction, might adversely affect the molecule's ability to interact with the fungal biological targets, possibly by impeding cellular uptake or altering its binding affinity to crucial enzymes like CYP51 (lanosterol 14α-demethylase), which is a primary target for azole antifungals like fluconazole [3].

In striking contrast, the majority of the analogues demonstrated significant to excellent antifungal potency. Notably, the parent methylthio compound **3** itself exhibited moderate activity (ZOI: 12.0 mm against *A. flavus* and 9.0 mm against *A. niger*), establishing the inherent bioactivity of the core pyrimidopyrimidine scaffold. The replacement of the methylthio group with various nucleophiles led to a marked enhancement in activity. Compounds bearing anilino groups (**3d**, ZOI: 13.0 mm vs *A. flavus*, 12.0 mm vs *A. niger*; **3e**, ZOI: 13.0 mm vs *A. flavus*, 11.0 mm vs *A. niger*) and particularly the 4-nitroanilino group (**3f**, ZOI: 15.0 mm vs *A. flavus*, 12.0 mm vs *A. niger*) showed remarkable inhibition, with **3f** matching the activity of fluconazole against *A. flavus*. The most profound antifungal effects, however, were observed with the heterocyclic amine substitutions. Both the pyrrolidin-1-yl (**3g**) and piperidin-1-yl (**3h**) derivatives displayed zones of inhibition identical to the fluconazole standard against both fungal strains (15.0 mm). This superior activity can be rationalized by the enhanced lipophilicity and potential for hydrogen bonding offered by the aliphatic amine groups, which may facilitate better penetration through the fungal cell membrane and stronger interaction with the target site [4]. The active methylene derivatives **3i** and **3j** also maintained very good activity, further underscoring the versatility of the 8-position for optimizing antifungal properties.

3.3. Analysis of Antibacterial Activity

The antibacterial assessment against *Escherichia coli* (Gram-negative) and *Bacillus subtilis* (Gram-positive) unveiled a distinct structure-activity relationship and varying degrees of efficacy. Similar to the antifungal results, compound **3c** was entirely inactive, reinforcing the notion that its specific substituent renders it biologically inert in these assays. The parent compound **3** again displayed a baseline moderate activity against both bacterial types (ZOI: 12.0 mm), suggesting a broad-spectrum potential for the core structure. The phenoxy derivative **3a** showed a slight improvement in activity (ZOI: 13.0 mm against both strains), while its brominated analogue **3b** exhibited a curious selectivity, showing reduced activity against *E. coli* (9.0 mm) but maintained good activity against *B. subtilis* (12.0 mm). This differential activity could be attributed to the increased hydrophobicity and larger size of the bromine atom, which might hinder its passage through the complex outer membrane of Gram-negative bacteria [5].

The anilino-based compounds displayed a mixed profile. While **3d** was less potent against *E. coli* (10.0 mm), it was effective against *B. subtilis* (13.0 mm). Conversely, the 4-bromoanilino derivative **3e** was potent against both (ZOI: 13.0 mm). The most potent antibacterial compound among the aromatic amines was unequivocally the 4-nitroanilino derivative **3f**, which exhibited zones of inhibition of 15.0 mm against *E. coli* and 14.0 mm against *B. subtilis*, values that are nearly equivalent to the streptomycin standard. The electron-withdrawing nitro group, detrimental in **3c**, appears to be beneficial for antibacterial activity when attached via an amino linker, potentially influencing the molecule's electronic distribution and interaction with bacterial enzymes like DNA gyrase or topoisomerase IV [6]. The pinnacle of antibacterial potency was once again achieved with the heterocyclic amines. Compound **3g** (pyrrolidin-1-yl) demonstrated outstanding, broad-spectrum antibacterial activity, matching the streptomycin standard against both bacterial strains with ZOIs of 15.0 mm. Its homologue **3h** (piperidin-1-yl) was also highly active, particularly against the Gram-positive *B. subtilis* (14.0 mm). The exceptional performance of these aliphatic amine derivatives suggests they may interfere with fundamental bacterial processes, possibly cell wall synthesis or membrane integrity, in a highly effective manner [7].

3.4. Structure-Activity Relationship (SAR) Considerations

A coherent structure-activity relationship emerges from the biological data, primarily governed by the nature of the substituent at the 8-position of the pyrimidopyrimidine scaffold.

The methylthio group in the lead compound **3** provides a foundation for activity but is suboptimal. Its replacement is crucial for enhancing potency.

Aromatic ethers (e.g., **3a, 3b**) generally confer good activity, but can lead to selectivity issues, particularly against Gram-negative bacteria, as seen with the bulky bromo substituent in **3b**.

Aromatic amines (e.g., **3d, 3e, 3f**) significantly boost activity, especially against fungi. The incorporation of strong electron-withdrawing groups like nitro (in **3f**) on the anilino ring dramatically enhances antibacterial potency, making it a dual-acting agent.

Heteroaliphatic amines (3g, 3h**)** represent the most effective modification. The pyrrolidine and piperidine rings consistently deliver the highest levels of activity against all tested microbial strains, matching or approaching the

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efficacy of the standard drugs. This is likely due to a combination of optimal lipophilicity, basicity, and hydrogen-bonding capacity, which favor absorption and target engagement. Active methylene compounds (3i, 3j) maintain respectable activity, indicating that this position can tolerate significant steric and electronic variation while retaining biological function.

The present study successfully demonstrates that the strategic structural modification of the 8-position on the novel oxopyrimido[1,2-a]pyrimidine nucleus is a highly effective strategy for developing potent antimicrobial agents. The synthesis of a focused library of derivatives and their subsequent biological evaluation has yielded several promising candidates, most notably compounds **3f**, **3g**, and **3h**, which exhibit broad-spectrum activity against both fungal and bacterial pathogens at levels comparable to established standard drugs. The complete inactivity of compound **3c** provides a valuable negative control and highlights the critical influence of substituent choice. The resistance of many pathogens to existing therapeutics is a pressing concern [8], and these new molecular entities, with their unique fused heterocyclic structure, could serve as valuable leads for the development of alternative control agents. The insights gleaned from the initial SAR are invaluable; however, they underscore the necessity for more profound investigation. Future work must focus on determining the minimum inhibitory concentrations (MICs) of the most active compounds, evaluating their cytotoxicity against mammalian cell lines to establish a preliminary safety profile, and ultimately elucidating their precise mechanism of action through targeted biochemical assays. Such studies are essential to translate these promising *in vitro* results into potential therapeutic applications.

4. CONCLUSION

In conclusion, this research successfully established a robust and efficient synthetic route for generating a novel series of oxopyrimido[1,2-a]pyrimidine derivatives. The strategic approach began with the construction of a chalcone building block, progressed through the formation of a dihydropyrimidine ring, and culminated in the key Michael addition step that built the complex fused tetracyclic system. The deliberate incorporation of a replaceable methylthio group at the 8th position of the core scaffold was a critical design feature, enabling straightforward diversification into a wide array of ten new analogues with varied electronic and steric properties. The structural integrity of every synthesized compound was rigorously verified using comprehensive analytical and spectral data. The primary objective of this work was to explore the antimicrobial potential of these novel heterocyclic systems. The biological screening data revealed that the majority of the tested compounds possess moderate to excellent antimicrobial properties against both bacterial and fungal pathogens. Notably, compounds **3f**, **3g**, and **3h** emerged as broad-spectrum agents, demonstrating considerable activity against all tested microbial strains, including Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria, as well as the fungi *Aspergillus niger* and *Aspergillus flavus*. Their performance was comparable to the standard reference drugs streptomycin and fluconazole in some cases. Conversely, compound **3c** was found to be largely inactive, providing a valuable point of comparison for establishing a structure-activity relationship (SAR). The significant bioactivity observed, particularly in the piperidin-1-yl and pyrrolidin-1-yl substituted derivatives (**3g**, **3h**), suggests that the nature of the substituent at the 8th position profoundly influences antimicrobial potency. This indicates that the pyrimidopyrimidine nucleus is a highly promising pharmacophore for antimicrobial development. The findings of this study provide a strong foundation for future work. Further investigation into the mechanism of action, in vivo efficacy, and more extensive SAR studies through the synthesis of even more targeted analogues is highly encouraged to optimize the potency and selectivity of this compelling class of compounds.

DECLARATIONS

Ethical Approval

We affirm that this manuscript is an original work, has not been previously published, and is not currently under consideration for publication in any other journal or conference proceedings. All authors have reviewed and approved the manuscript, and the order of authorship has been mutually agreed upon.

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Availability of data and material

All of the data obtained or analyzed during this study is included in the report that was submitted.

Conflicts of Interest

The authors declare that they have no financial or personal interests that could have influenced the research and findings presented in this paper. The authors alone are responsible for the content and writing of this article.

Authors' contributions

All authors contributed equally to this work.

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