Investigation Of Synthesis Methods For Novel Pyrrolo[2,3-D]Pyrimidine Derivatives As Kinase Inhibitor Compounds

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ABSTRACT
The current study has been planned to synthesize and assess the pharmacological characteristics of pyrrolo[2,3-d]pyrimidine derivatives as kinase inhibitors. The findings of the study indicated that different structural modifications, especially at critical cyclic sites, significantly influenced the development of inhibitory potency and specificity against the target enzymes. Compound 5k was identified as a highly potent multi-target inhibitor with an IC50 value in the nanomolar scale and is capable of inducing apoptosis and facilitating cell cycle arrest in HepG2 cells. These results not only highlight the significance of rational compound design based on efficient chemical structures but also reveal new avenues for the design of anticancer drugs with enhanced efficacy. One of the major accomplishments of this study is the establishment of optimized and effective synthetic procedures for the synthesis of pyrrolopyrimidine derivatives with significant yield and sufficient levels of purity. The application of new reaction conditions and advanced catalytic systems, including microwave-assisted reactions and DBU-catalyzed cyclization, allowed for the circumvention of the drawbacks associated with conventional methodologies. From the pharmacological perspective, the study showed that the pyrrolopyrimidine derivatives synthesized were able to interact effectively with the ATP-binding site of kinase enzymes. Moreover, data about compounds 14a and 17 showed that the derivatives can cause cell cycle arrest at the G1/S phase, which is especially useful for the treatment of cancers that manifest with increased cell growth. The 2-thioalkyl-6-amino-4-oxo pyrimidine compounds were able to engage effectively in a Michael reaction with functionalized nitroalkenes. In addition, the substitution of the thioalkyl group at the C2 position of pyrrolo[3,2-d]pyrimidines was successfully realized.

Keywords: Anticancer agents, Kinase, Pyrrolopyrimidine, Thioalkyl.

INTRODUCTION

The synthesis and investigation of the pharmacological properties of heterocyclic compound synthesis systems is one of the objectives in heterocyclic compound synthesis. Among these, the synthesis of pyridolopyrimidine derivatives holds special significance due to their isoelectronic structure with purine bases. Extensive research has been conducted on the synthesis and pharmacological properties evaluation of pyridolo[2,3-d]pyrimidine derivatives. Notable among these investigations is the inhibitory effect of some of these derivatives on kinase enzymes.

The kinase enzyme acts as a control mechanism for many cellular activities, from growth to death. At any moment, more than 500 different kinases are functioning in each cell. Kinases are involved in almost all physiological aspects of the body. Protein kinases act on proteins and phosphorylate them. These reactions primarily occur on nucleophilic amino acids such as serine, threonine, tyrosine, and histidine. Protein kinase inhibition is crucial in treating certain cancers and inflammatory diseases. Therefore, protein kinase inhibitors can function as therapeutic agents. (1)

Previous studies have shown that pyrrolopyrimidine derivatives are extensively effective in inhibiting key kinases such as EGFR, VEGFR-2, CDK2, and Her2. Al-Tayyebi et al. (2023) investigated the discovery of novel pyrrolo[2,3-d]pyrimidine derivatives as multi-target kinase inhibitors and effective apoptosis inducers. Mechanistic studies of compound 5k demonstrated its ability to induce cell cycle arrest and apoptosis in HepG2 cells, accompanied by a significant increase in pro-apoptotic proteins caspase-3 and Bax, as well as decreased Bcl-2 activity [2]. Xia et al. (2021) studied the synthesis and biological activity of pyrrolo[2,3-d]pyrimidine derivatives as tyrosine kinase inhibitors for NSCLC cells

with EGFR mutations. Theoretical simulations provided structural evidence for selective kinase inhibitory activity. Thus, this series of pyrrolo[2,3-d]pyrimidine derivatives could serve as a starting point for developing new EGFR-TKI inhibitors [3].
Methodologically, this study utilizes modern synthesis methods such as C–N coupling reaction optimization and spectroscopic techniques (NMR, FTIR, GC-MS) for compound structure determination. This approach is similar to methods used in studies such as Sarwar et al. (2023), who investigated the design, synthesis, in vitro anticancer evaluation, molecular docking studies, and structure-activity relationship analysis of a new series of pyrrolo[2,3-d]pyrimidine derivatives. Results showed that compounds 14a, 16b and 18b exhibited the highest activity against the MCF7 cell line with IC50 values of 1.7, 5.7, and 3.4 μg/mL, respectively, compared to doxorubicin (26.1 μg/mL). Additionally, compound 17 showed promising cytotoxic effects against HePG2 and PACA2 cell lines with IC50 values of 8.7 and 6.4 μg/mL, respectively, compared to doxorubicin (21.6 and 28.3 μg/mL, respectively) [4].
From a pharmacological perspective, this study aims to evaluate the inhibitory activity of synthesized compounds against cancer-related kinases and investigate mechanisms of apoptosis induction and cell cycle arrest. Previous studies, such as Li et al. (2018), examined the synthesis and evaluation of novel 5,6-disubstituted pyrrolo[2,3-d]pyrimidine derivatives as broad antiproliferative agents: molecular docking studies and kinase profiling. Structure-activity relationship (SAR) studies revealed that the presence of an octamide terminal moiety at the C2 position and disubstitution with fluorobenzyl piperazine at C5 and C6 positions of pyrrolo[2,3-d]pyrimidine are key structural features for achieving optimal antiproliferative activity [5].
Encouraged by these findings, the current study is aimed at synthesizing compounds that not only target the ATP binding domain of kinases but also enable non-competitive inhibition modes through binding to allosteric sites. Moreover, assessment of the cytotoxicity of these compounds against various cell lines, such as MCF-7, HepG2, and HCT116, and comparison with standard drugs like doxorubicin enables the identification of lead candidates for optimization. Lastly, this study is also in agreement with earlier seminal works, including those reported by Mawsomsy et al. (2017), highlighting the applicability of the pyrrolopyrimidine scaffold as the core scaffold of kinase inhibitors [6]. Nevertheless, the initial uniqueness of this study is that it highlights the design of derivatives that enhance selectivity and inhibitory activity by employing structural manipulation of functional groups at desired ring positions. Findings from this study may play a part in creating a new generation of multi-target anticancer drugs with the ability to reverse drug resistance and minimize side effects. Not only do these findings enhance basic knowledge regarding structure-activity relationships of heterocyclic compounds, but they also reveal new avenues for the design of targeted drugs in cancer therapy.
Some pyridolopyrimidine derivatives possess antibacterial properties, analgesic effects, anti-inflammatory, antidepressant, antidiabetic, and antiallergic properties. In this study, we aim to synthesize new derivatives of this heterocyclic system for future evaluation of their pharmacological properties as kinase enzyme inhibitors or other pharmacological properties. In this project, pyridole derivatives are initially obtained through a three-molecular reaction from amine-active compounds and thiourea. These compounds then form pyridolo[2,3-d]pyrimidine through a reaction with Markovnikov amides.

MATERIALS AND METHODS

NMR spectra were recorded using a Bruker Spectrospin spectrometer at frequencies of 400 MHz for H^1 spectra and 100 MHz for C^{13} spectra. Chemical shifts are reported in ppm relative to residual solvent signals: DMSO-d6 (2.50 ppm for H^1 and 39.5 ppm for C^{13}), CDCl3 (7.27 ppm for H^1 and 77.0 ppm for C^{13}), or TFA-d (164.2 ppm for C^{13}). The following multiplicities are indicated: s (singlet), brs (broad singlet), d (doublet), dd (doublet of doublets), dt (doublet of triplets), dq (doublet of quartets), t (triplet), q (quartet), m (multiplet). Coupling constants (J values) are given in Hz.
IR spectra were recorded on a Nicolet Impact 400D FT instrument as KBr discs or neat samples on sodium chloride plates for liquids. Melting points, where measurable, were determined using a Reichert hot stage apparatus and were uncorrected. Newly synthesized compounds were also characterized by HRMS. Data for electron impact (EI), chemical ionization (CI), and fast atom bombardment (FAB) modes were obtained on a JEOL JMS-700 High-Resolution Mass Spectrometer. Electron spray ionization (ESI) data were recorded on a ThermoFinnigan LCQ instrument.
Elemental analysis data, where obtainable, were determined using a Perkin Elmer series II CHN analyzer 2400. However, obtaining elemental analysis data from polyaza heterocycles is very difficult, even using tungstate as a combustion catalyst. For many synthesized compounds, results within the acceptable range (±0.4%) of analysis were not achieved. Microwave reactions were performed using an Initiator Unit

(Biotage, Uppsala, Sweden) with a stirring option. When necessary, HPLC performed separation. A Waters 1525 binary pump system, Waters 2487 dual absorbance detector, and Breeze software (column: Phenomenex Luna 5 micron C18, dimensions 60 × 21 mm, wavelength = 254 nm) were used with a gradient elution system according to Table 1.

Table 1: Gradient elution conditions for HPLC separation

%B Acetonitrile containing 0.1% TFA	%A Water containing 0.1% TFA	Flow rate (mL/min)	Time (min)
10	90	6.00	0
60	40	6.00	28.00
100	0	6.00	33.00
10	90	6.00	38.00
10	90	6.00	39.00
10	90	0.00	40.00
10	90	0.00	40.10

TLC was conducted on silica plates (Merck 0.25 mm 60 F254). Column chromatography was performed using silica gel (230-400 mesh; 40-60 micrometers) according to the procedure of W. C. Still et al. Chemical reagents were obtained from commercial suppliers and used as is without purification.
According to standard procedures, solvents were dried before use with the respective drying agents ($Mg(OMe)_2$ for methanol, $Mg(OEt)_2$ for ethanol, and calcium hydride for dichloromethane and acetonitrile) or were purchased anhydrous (e.g., dimethylformamide from Aldrich). Tetrahydrofuran, diethyl ether, and dichloromethane were dried according to the standard procedure of the Innovative Technology Solvent Purification System (Pure-Solv® 400 instrument).

FINDINGS

2-Thiobenzyl-containing Pyrrolo[2,3-d]pyrimidines

2-Thiobenzyl-5-substituted pyrrolo[2,3-d]pyrimidine compounds (2.5) are important precursors for the production of a library of 2,6-functionalized compounds via C2 substitution with amino nucleophiles. Compounds 2.5 can be prepared by the reaction of 2-thiobenzyl-6-aminopyrimidine (2.4) with nitroalkene (2.3). The reaction occurs via a Michael reaction followed by a Nef reaction. To achieve the important intermediate pyrimidine (2.4), benzyl bromide (2.1a) was used to benzylate commercially available 2-mercapto-6-amino-4-pyrimidinone (2.2). The benzylation was carried out using a procedure devised by our study group. Compound 2.4 was isolated as a white solid (Figure 1).

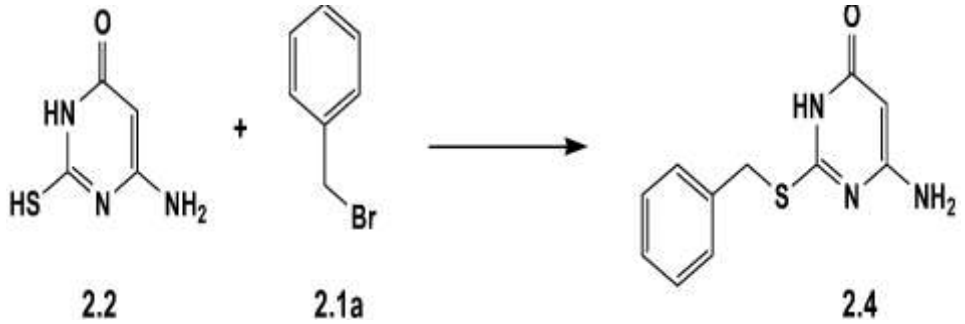


Figure 1: Reagents, conditions, and yield: water/ethanol (3:5), triethylamine, room temperature, 88%.

Scheme 2.4 illustrates two plausible mechanisms for the Michael addition reaction of a 6-amino-4-pyrimidinone with nitrostyrene, in accordance with the representations presented in Scheme 2.3:

1. path A
Activation of carbon 5 (C5) occurs through the amino group at position 6.
- Electron-donating amino group at position 6 donates electron density via the aromatic pyrimidine ring system to carbon 5
- This electron transfer facilitates nucleophilic attack by carbon 5 on nitrostyrene

2. Path B
- Activation of carbon 5 (C5) by nitrogen 3 (N3)
If an anion is created on nitrogen 3, the electrons in the lone pair can migrate to carbon 5 via the

conjugated system.
This phenomenon causes electron density to rise at carbon 5, thus improving its nucleophilic nature. Both processes result in the creation of a carbon-carbon bond between the β-carbon of nitrostyrene and the 5-position carbon of pyrimidine. The reaction is the most important step in the synthesis of pyrrolo[2,3-d]pyrimidine derivatives.

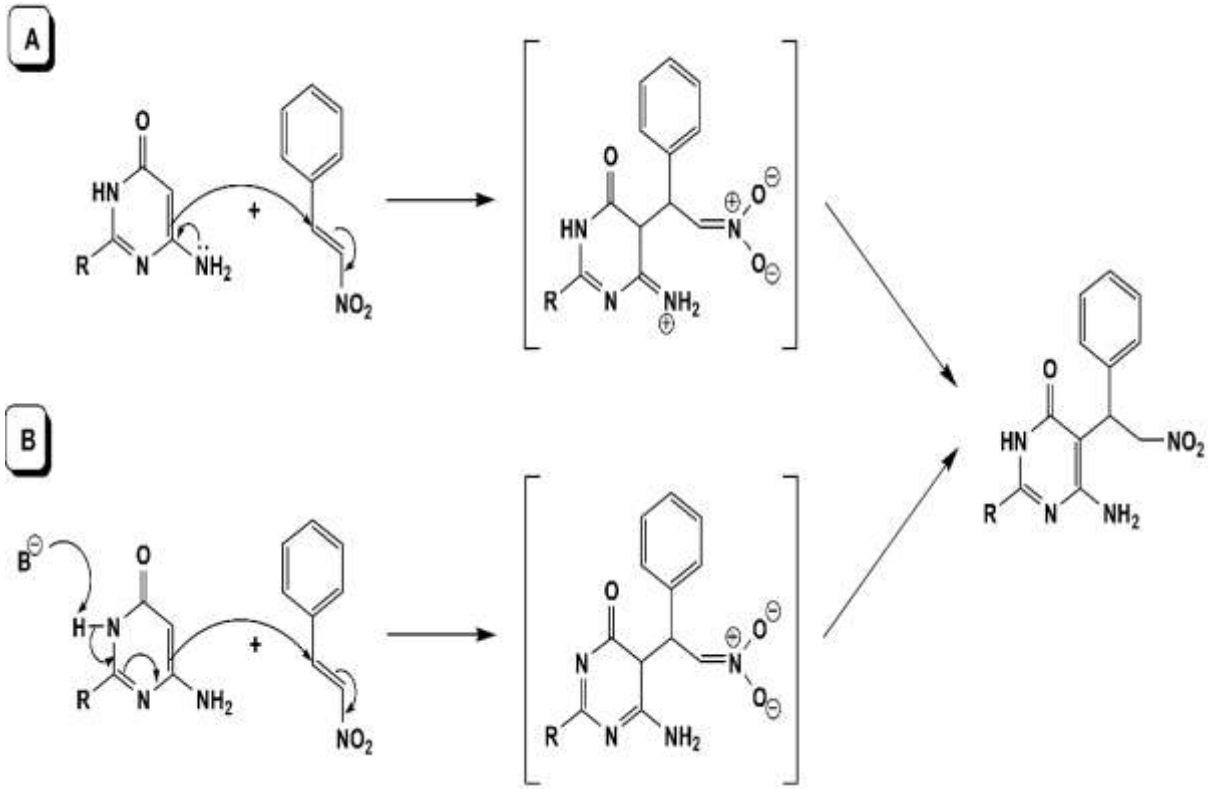


Figure 2: Scheme 2.4. Two probable mechanisms for the Michael reaction. (A: Activation by 6-NH2, B: Activation by N3).

Based on Taylor's satisfactory results using the Michael reaction in a neutral biphasic solvent system (50% water in ethyl acetate, v/v), we applied these reaction conditions to the reaction between 2-thiobenzyl-6-aminopyrimidine 2.4 and nitrostyrene 2.3a.

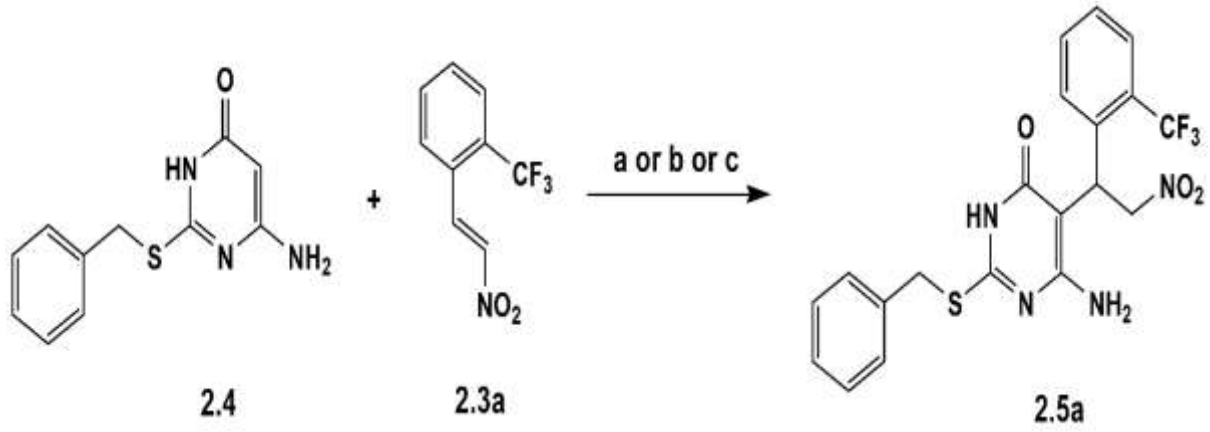


Figure 3: Reagents, conditions, and yields:
(a) EtOAc/H2O (1:1), reflux, 3 days, 35%
(b) EtOAc/H2O (1:1)، NaOH , reflux, 5 days, 38%
(c) EtOAc/H2O (1:1),NaHCO3, reflux, 5 days, 33%

Table 2: Michael reaction results using phase transfer catalyst in a biphasic solvent system

Compound	Reaction time (hours)	Temperature (°C)	Yield (%)
2.5a	72	80	55
2.5b	4	70	72
2.5c	48	60	96
2.5d	4	60	81

The general successful pathway for the formation of 2-thiobenzyl-6-substituted pyrrolo[2,3-d]pyrimidines is shown in Scheme 2.16.

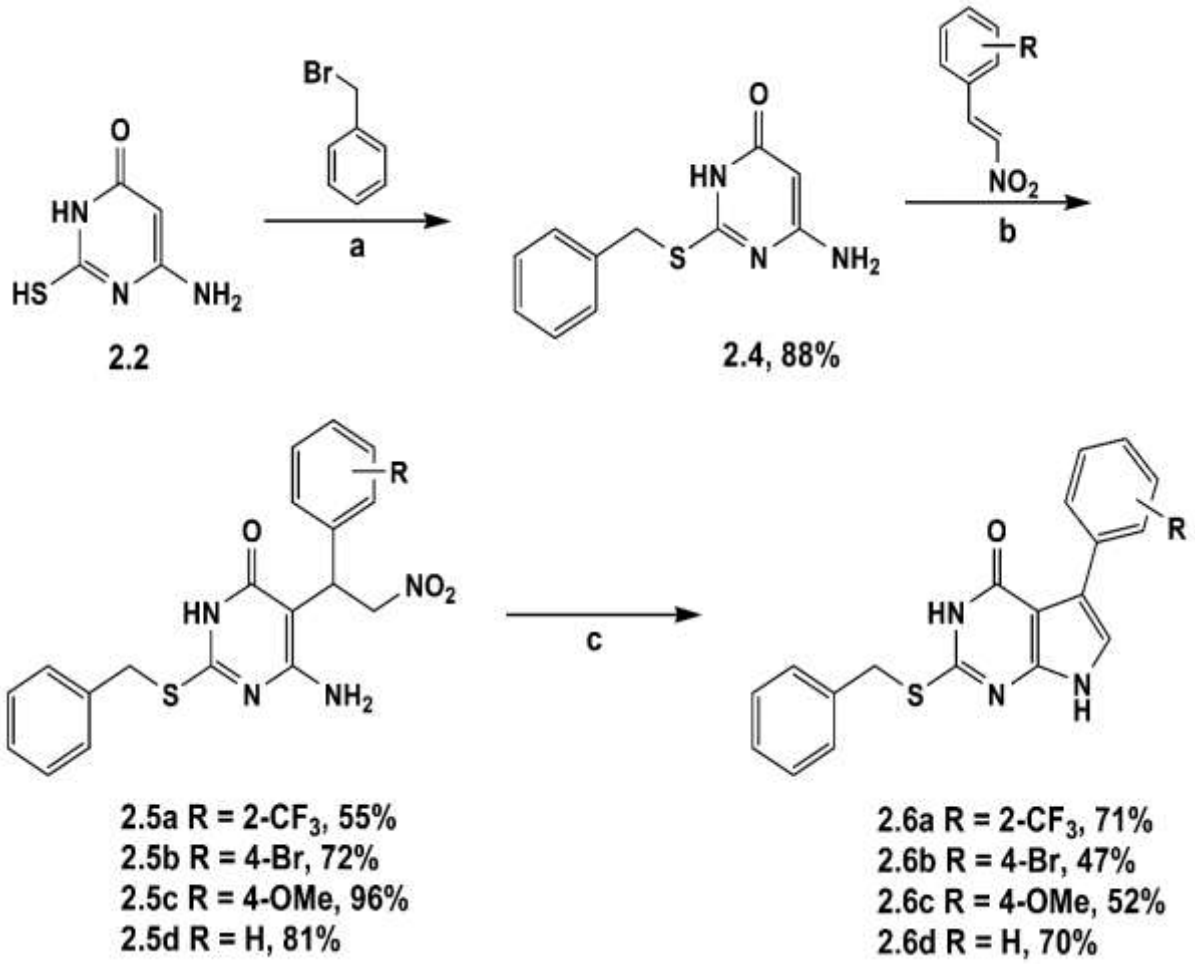


Figure 4: Scheme 2.16. Reagents and conditions:
(a) $H_2O/EtOH$ (3: 5)، Et_3N
(b) $EtOAc/H_2O$ (1: 1)، $PhCH_2N^+Me_3HO^-$,60 − 90 ℃ , 4-72 hours
(c) MeOH, NaOMe, $TiCl_3$ (4-6 equiv.), NH_4OAc , room temperature to 60°C, overnight

2-Thiomethyl-containing Pyrrolo[2,3-d]pyrimidines

The pyrrolo[2,3-d]pyrimidine scaffold containing a 2-thiomethyl group (2.10) was synthesized (Scheme 2.17). Methylation was successfully carried out under conditions similar to benzylation. However, in the subsequent step, using a biphasic system with a phase transfer catalyst led to the simultaneous formation of three products:

1. Non-cyclized product (2.8)
2. Oxime intermediate (2.9)
3. Cyclized product (2.10)

It appears that the thiomethyl group confers less hydrophobicity to the pyrimidine scaffold compared to thiobenzyl. This characteristic may provide a greater opportunity for nitrosalt formation through deprotonation by the catalyst's hydroxyl anion in the aqueous layer. The quaternary ammonium cation can then lead to the formation of an iminium ion, which is attacked by water to produce the

corresponding aldehyde and oxime intermediate (2.9). Finally, these intermediates are converted to the cyclized product (2.10) through intramolecular condensation.

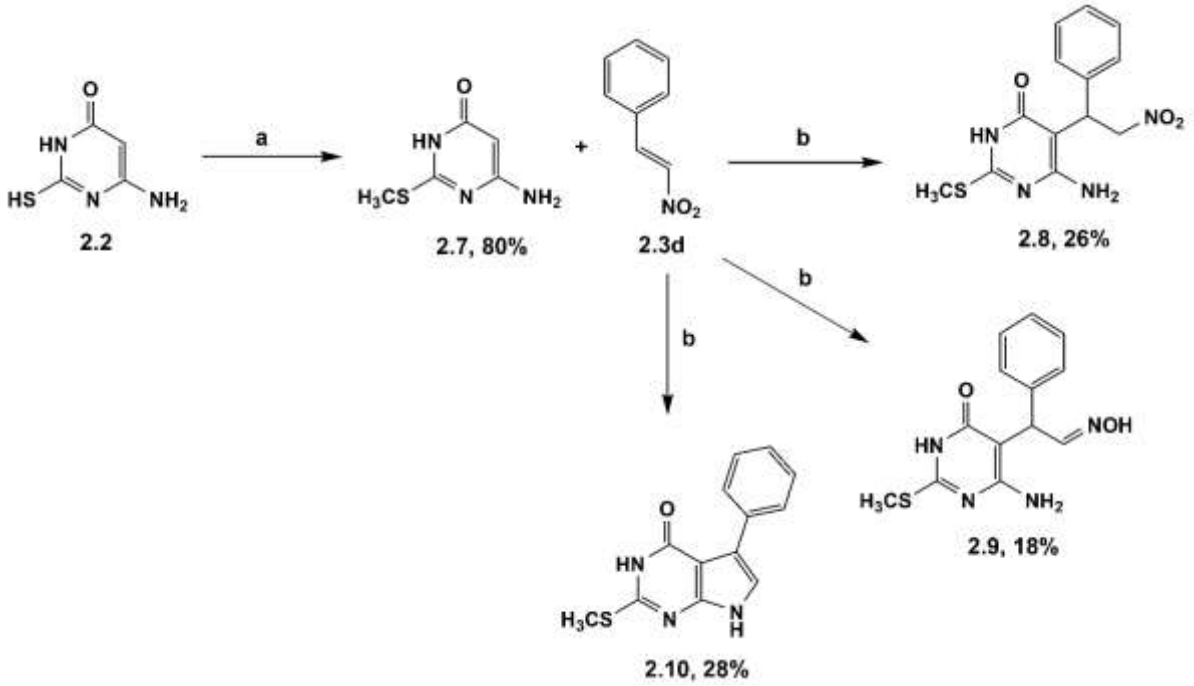
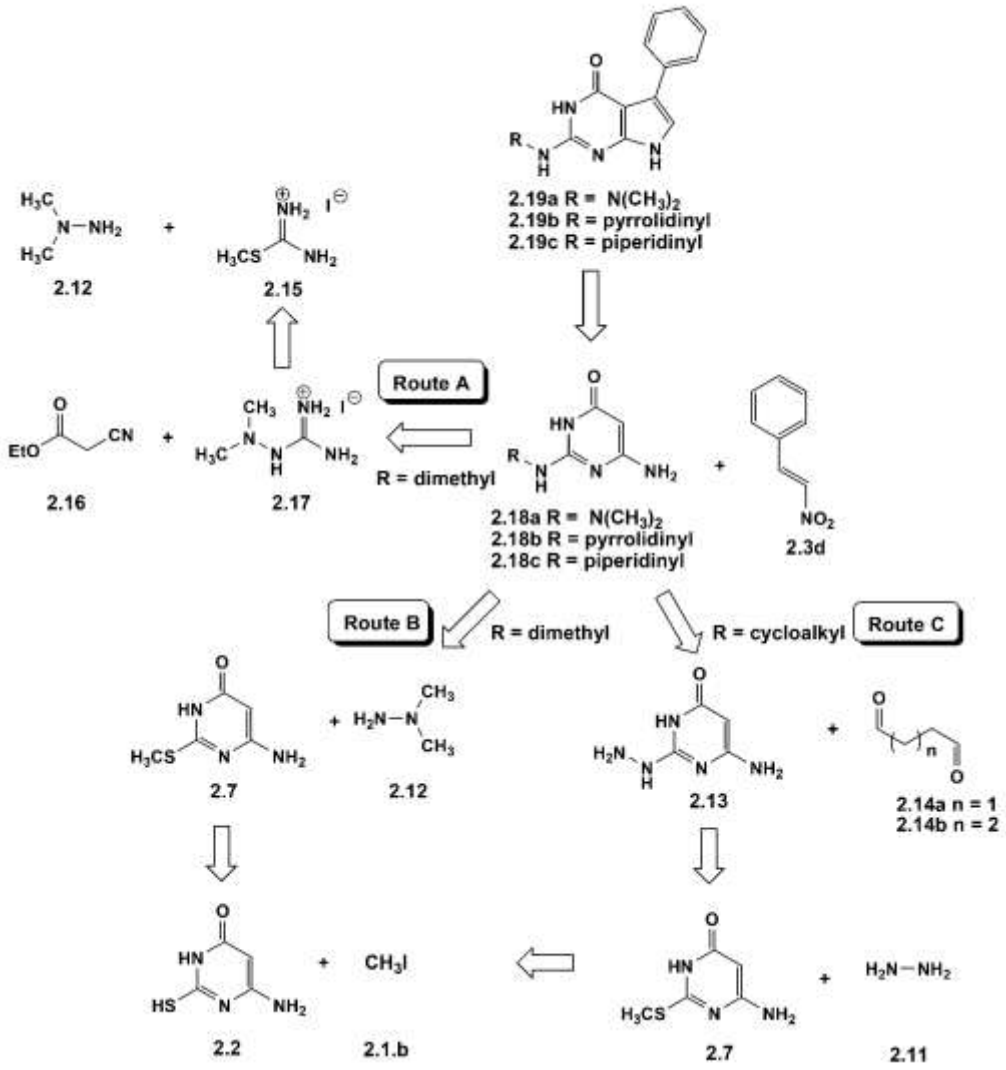


Figure 5: Reagents and conditions:
(a) $EtOH/H_2O$ (2: 1)، Et_3N، MeI, room temperature, 5 hours
(b) $EtOAc/H_2O$ (1: 1)، $PhCH_2N^+Me_3HO^-$,60℃ , 3 hours

2-Alkylhydrazino-containing Pyrrolo[2,3-d]pyrimidines
To investigate an alternative approach for C2 position substitution, 2-alkylhydrazino-5-substituted pyrrolo[2,3-d]pyrimidine compounds (2.19a-2.19c) were designed. The hydrazine group, after protonation at N1 nitrogen, can be substituted by amino nucleophiles. In this section, the synthesis of these compounds is reported, and the results of C2 substitution (including cases similar to 2-thioalkyl derivatives) will be discussed.

Retrosynthetic analysis (Scheme 2.18):
Three different pathways were investigated for synthesizing derivatives containing 2,2-dialkylhydrazino at the C2 position:
1. Synthesis of the main scaffold 2.19 via Michael-Nef reaction pathway (Section 2.1.1.1)
2. Formation of 2-(2,2-dimethylhydrazino)pyrimidine (2.18a) from the reaction of amino(2,2-dimethylhydrazino)methaniminium iodide (2.17) with ethyl 2-cyanoacetate (2.16) (Path A)
3. Synthesis of 2.17 from the reaction of amino(methylsulfanyl)methaniminium iodide (2.15) with 1,1-dimethylhydrazine (2.12)



Scheme 6: Retrosynthetic Analysis of 2-Alkylhydrazino-5-Substituted Pyrrolo[2,3-d]pyrimidines

Alternative method (Path B):
Compound 2.18a may be prepared by the reaction between 1,1-dimethylhydrazine (2.12) and 2-thiomethylpyrimidine (2.7) via C2 substitution. Starting material 2-thiomethylpyrimidine (2.7) may be easily obtained by the reaction of 2-thiopyrimidine (2.2) and iodomethane, as illustrated in section 2.1.1.2.

Alternative method (Path C):
2-Cycloalkylhydrazino pyrimidine compounds (2.18b and 2.18c) can be synthesized through reductive amination between 2-hydrazinopyrimidine (2.13) and a dialdehyde (2.14). 2-Hydrazinopyrimidine (2.13) can also be prepared from the reaction of 2-thiomethylpyrimidine (2.7) with hydrazine (2.11).

Failure of Path A
Path A was a ring-formation strategy for producing the key intermediate 2.18a (Scheme 2.18). Although the displacement of amino(methylsulfanyl)methaniminium iodide (2.15) by 1,1-dimethylhydrazine (2.12) was successful and led to the formation of 2.17, the ring-formation step failed under several different conditions (Scheme 2.19, b). Therefore, it was decided to test the C2 position substitution of 2-thiomethylpyrimidine (2.7) with 1,1-dimethylhydrazine (2.12) (Path B).

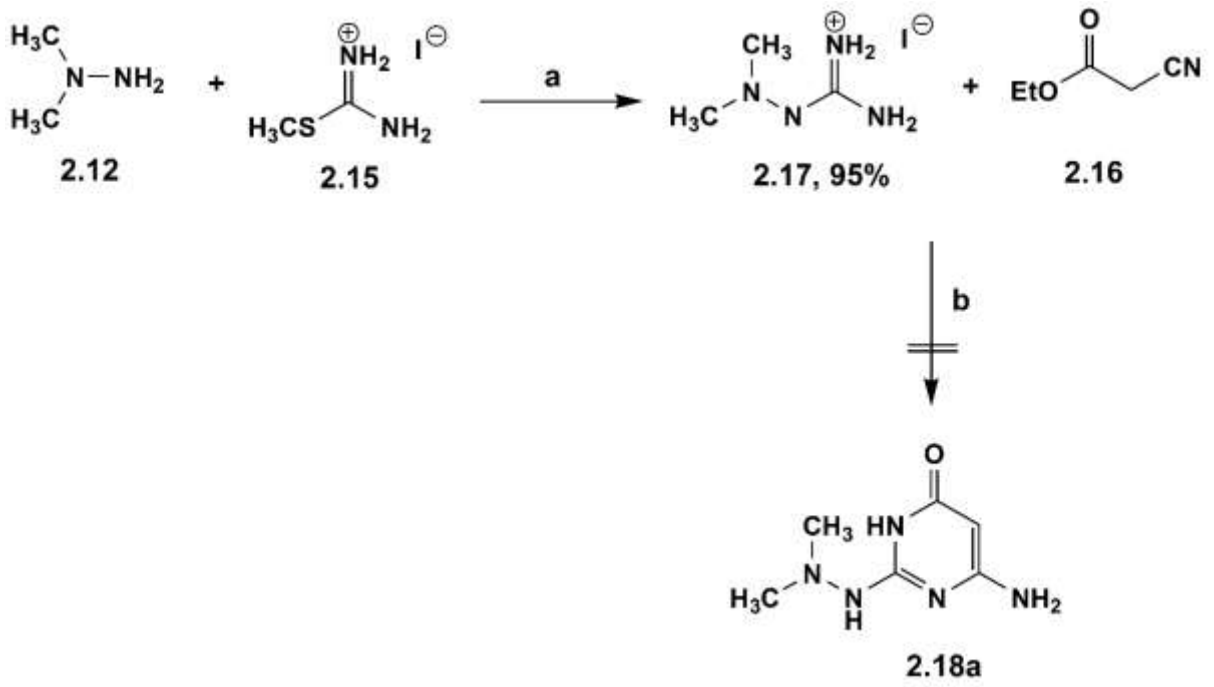


Figure 7: Reagents, Conditions, and Yields
(a) 1,1-dimethylhydrazine, reflux, 20 hours
(b) EtOH, NaOEt, reflux, 15 hours or Et_3N, EtOH, reflux, 15 hours or DBU, EtOH, reflux, 15 hours

C2 Position Replacement by Hydrazine and 1,1-Dimethylhydrazine
In the first trial, we were attempting to synthesize 2-pyrrolidinylaminopyrimidine 2.18b, but the necessary precursor 2.14a was not accessible through commercial means (Scheme 2.18).
Our prediction was that butane-1,4-diol (2.20) would be oxidized to give dialdehyde 2.14a through the application of a comparatively mild oxidizing agent, in this case, pyridinium chlorochromate (PCC), since PCC will not fully oxidize alcohols to carboxylic acids. But intramolecular ring closure came into prominence, and consequently, the only product that was formed was lactone 2.23 (Scheme 2.21).

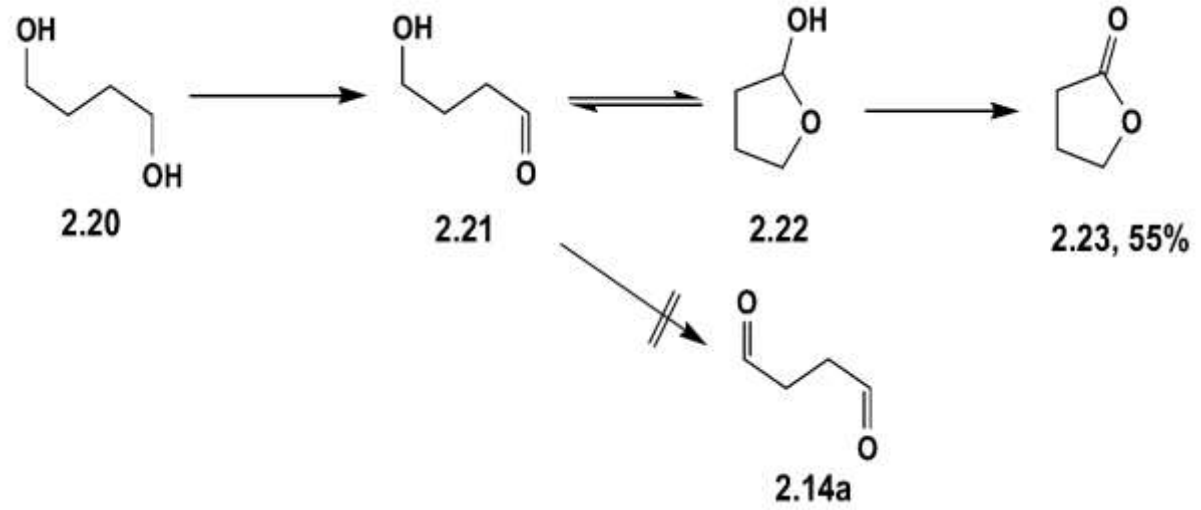


Figure 8: Reagents and Conditions
Pyridinium chlorochromate (PCC), dichloromethane (DCM), room temperature, 20 hour duration.

Luckily, glutaraldehyde (2.14b) was easily available in the market, thus providing ease for the reductive amination required for synthesizing 2-piperidinylaminopyrimidine (2.18c) (Figure 8).
Reaction with an aldehyde or ketone and ammonia or primary or secondary amines, at conditions of pH 7, and in the presence of $[BH_3CN]^-$ results in primary, secondary, or tertiary amines. The transformation takes place by reductive amination with the carbonyl group (Figure 9).

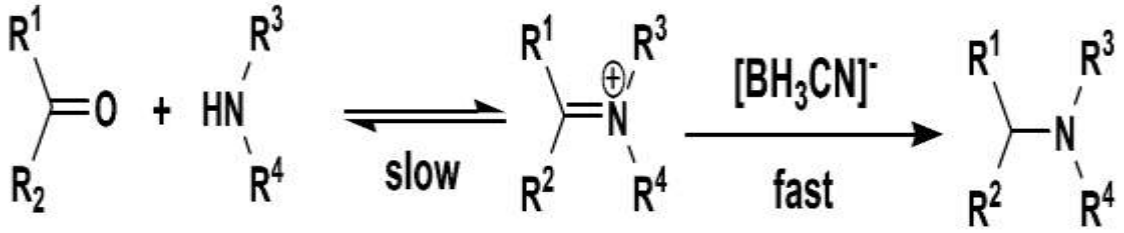


Figure 9: Reductive Amination Pathway

In order to synthesize compound 2.18c, it was required that the monoalkylated intermediate undergo subsequent intramolecular alkylation. Excess of substrate 2.13 was thus not required. Likewise, an excess of dialdehyde 2.14b was also not required. In practice, we could obtain the desired product (2.18c) in moderate yield under standard reductive amination conditions using just 1.2 molar equivalents of glutaraldehyde (2.14b) (Scheme 2.24).

The likely mechanism of reaction can be discussed based on Scheme 2.25, wherein:

1. The slow, rate-determining pre-equilibrium step forms the iminium intermediate 2.26, which then undergoes rapid reduction to 2.27.

Aminocarbinol (hemiaminal) 2.24 can also be transformed into iminium salt 2.26 via the enamine 2.25 formation.

- This conversion takes place by facile and reversible protonation of the enamine to the critical intermediate 2.26.

3. Since the reduction time of enamine (~15 minutes) is shorter than that of reductive amination (several hours), the enamine reduction pathway can also be involved.

The following steps of reaction occur as follows

1. Hemiaminal (2.24) is formed first when the reaction of the amine with glutaraldehyde yields the hemiaminal intermediate compound.

2. Conversion to enamine (2.25): The hemiaminal can lose water under reaction conditions and form an enamine.

3. Iminium formation (2.26): Rapid protonation of the enamine converts it into the reactive iminium species.

4. Reduction to final product (2.27): The reducing agent ($NaBH_3CN$) attacks the iminium species and then reduces it to yield the final amine product (2.18c). The resulting intermediate compound 2.18c was utilized in the synthesis of 2-piperidinylaminopyrrolo[2,3-d]pyrimidine (2.19c), as indicated in Figure 10.

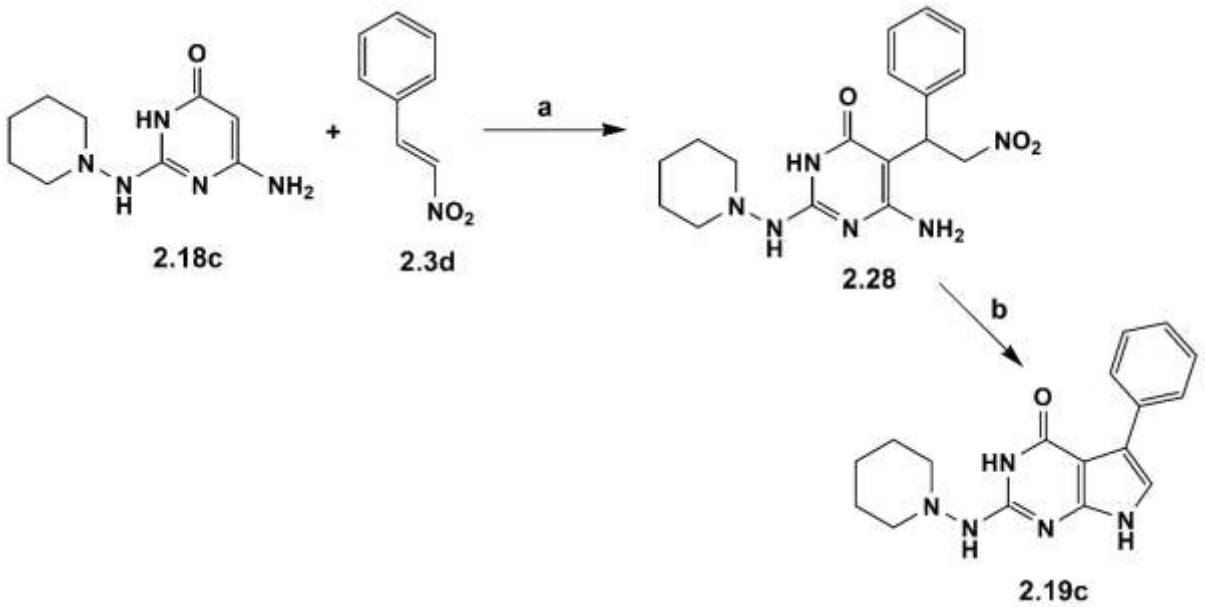
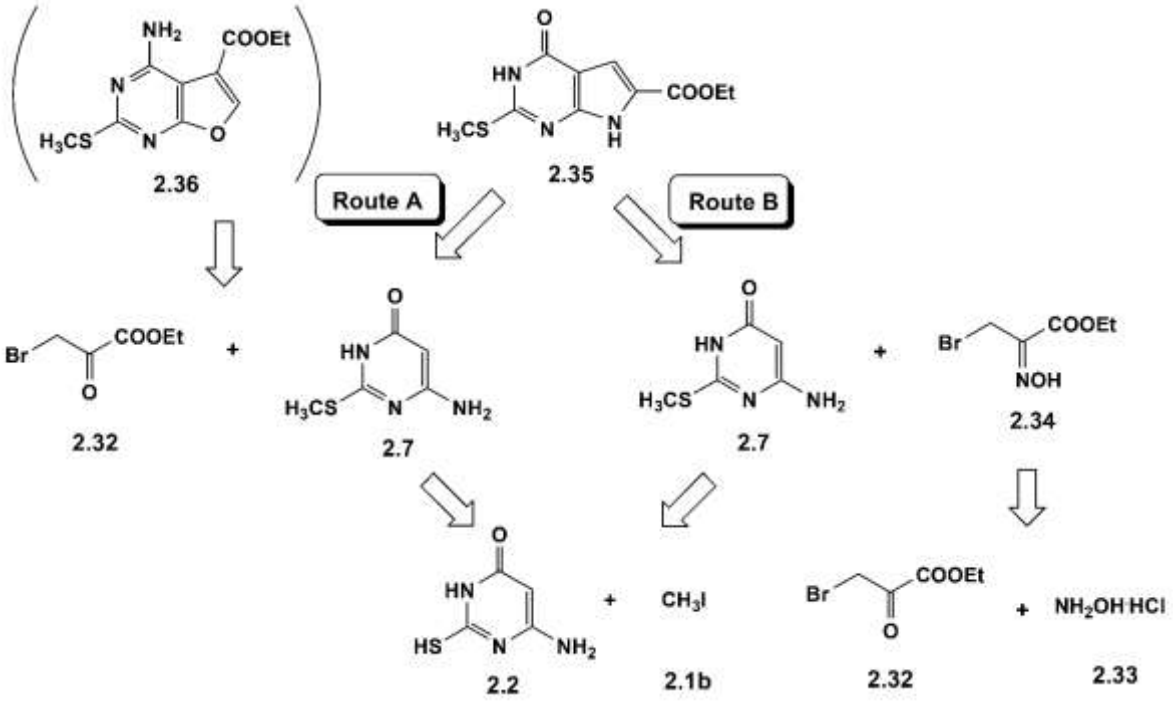


Figure 10: Reagents, Conditions, and Yields

(a) Ethyl acetate/water (1:1), benzyltrimethylammonium hydroxide, room temperature, 19 hours, 58%.
(b) Tin(II) chloride dihydrate (3 equivalents), ethyl acetate, 90°C, 24 hours, 48%.

2-Thiomethyl-6-carboxyethyl pyrrolo[2,3-d]pyrimidine 2.35 was prepared to examine the reactivity of substitution at C2, as the carboxyl group at position 6 can reduce the electron density of the fused ring as it is an electron-withdrawing functional group. Substitution reaction conditions are provided in the section to follow. The synthesis pathway of this compound is provided below.

Retrosynthetic Analysis (3-28)
Path A is a ring-formation pathway through α-halogenated ketones, as discussed in section 1.3.2.3. Despite the fact that the potential for the formation of furopyrimidine 2.36 is present, it is still worthwhile to explore, given that the reaction steps involved are not complicated. Path B has been utilized by J. K. Huggan already. The compound ethyl bromopyruvate oxime 2.34 can be synthesized through the reaction of ethyl bromopyruvate 2.32 and hydroxylamine hydrochloride 2.33.



Scheme 11: Retrosynthetic Analysis of 2-Thiomethyl-6-carboxyethyl pyrrolo[2,3-d]pyrimidine 2.35

Initially, it was expected that a mixture of two products would form from path A (Scheme 2.29), but furopyrimidine 2.36 was obtained as the sole product. Although this method could be a suitable starting point for the synthesis of C5-functionalized furopyrimidines, it is not appropriate for obtaining the desired 2-thioalkyl pyrimidine with an extended chain at the C6 position.
Path B was reported by J. K. Huggan (Figure 12).[117] The partner substrate 2.34 was synthesized from hydroxylamine hydrochloride 2.33 and ethyl bromopyruvate 2.32 via the method reported by H. C. J. Ottenheijm et al.[133] 2-Thiomethylpyrimidine was reacted with oxime 2.34 in DMF to obtain the desired product 2.37. However, in this work, Dowex-50 (H^+ form) was used as the acid catalyst instead of hydrochloric acid for the subsequent cyclization. The H^+-catalyzed reaction was more effective in terms of yield (85% versus 54%).

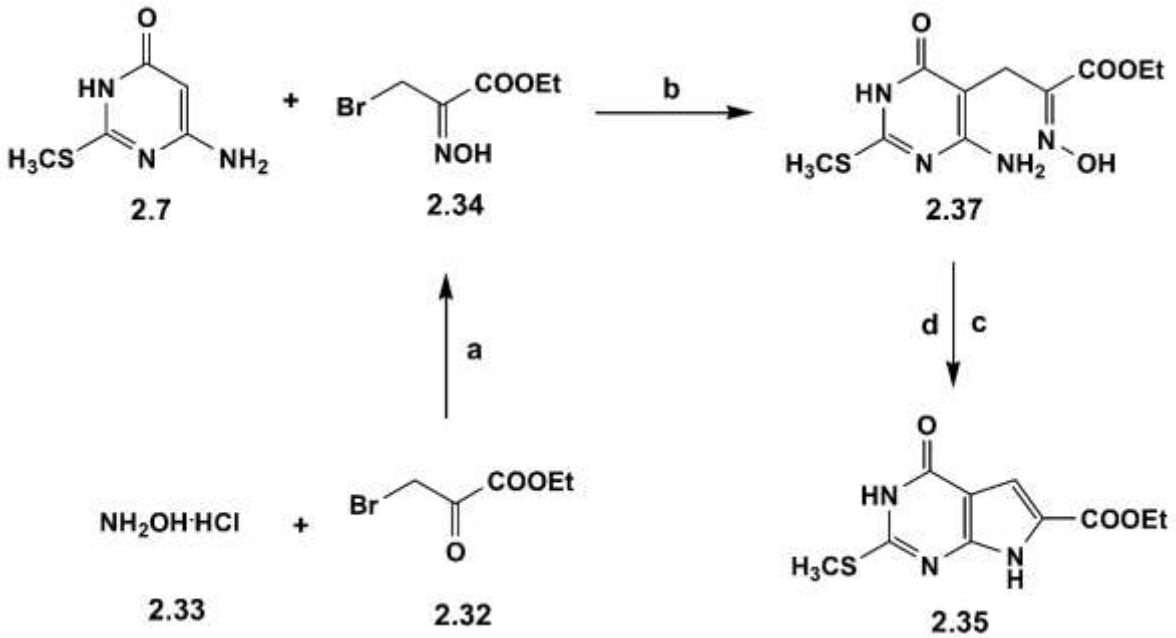
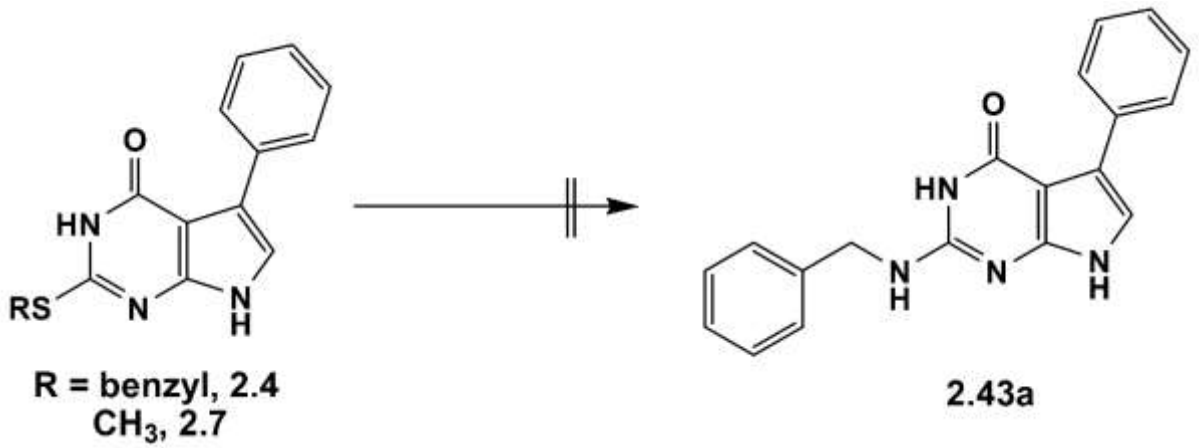


Figure 12: Reagents, Conditions, and Yields
(a) $CHCl_3$، MeOH, room temperature, 24 hours, 84%.
(b) DMF، Et_3N, room temperature, 2 days, 21%.
(c) $H_2O/EtOH$، HCl, benzaldehyde, reflux, 54%.[117]
(d) $Dowex - 50\ (H^+)$، H_2O، reflux, 15 hours, 85%.

In the present study, benzylamine was selected as a test nucleophilic amine for C2 position substitution in pyrrolo[2,3-d]pyrimidines via the direct displacement method (Figure 13). By varying the temperature, we observed that above 130°C, an inseparable mixture was formed (confirmed by ^{1}H NMR spectroscopy). Although the ratio of side product decreased at 115°C, isolation of the pure product was very difficult, and a large amount of starting material remained. At a lower temperature (103°C), no side product was formed, but the reaction did not proceed well, and the majority of the starting material remained unchanged.



Scheme 13: Reagent and Conditions: Benzylamine in neat, 103 to 150°C, several days, no pure product was isolated.

Throughout the present study, sulfone 2.46 isolation failure was faced while utilizing a number of oxidizing agents, i.e., m-chloroperbenzoic acid, Oxone®, H_2O_2, Na_2WO_4, H_2O_2 and $(NH_4)_6Mo_7O_{24}$, and H_2O_2 and SeO_2 in varied reaction conditions. In certain instances, H_2O_2 oxidation afforded a low-yielding sulfoxide and sulfone mixture (yield below 30%).
A plausible explanation for not being able to decouple sulfone 2.46 could involve nucleophilic attack by water in the aqueous workup step with subsequent formation of the water-soluble species 2.47 (see Figure 14).

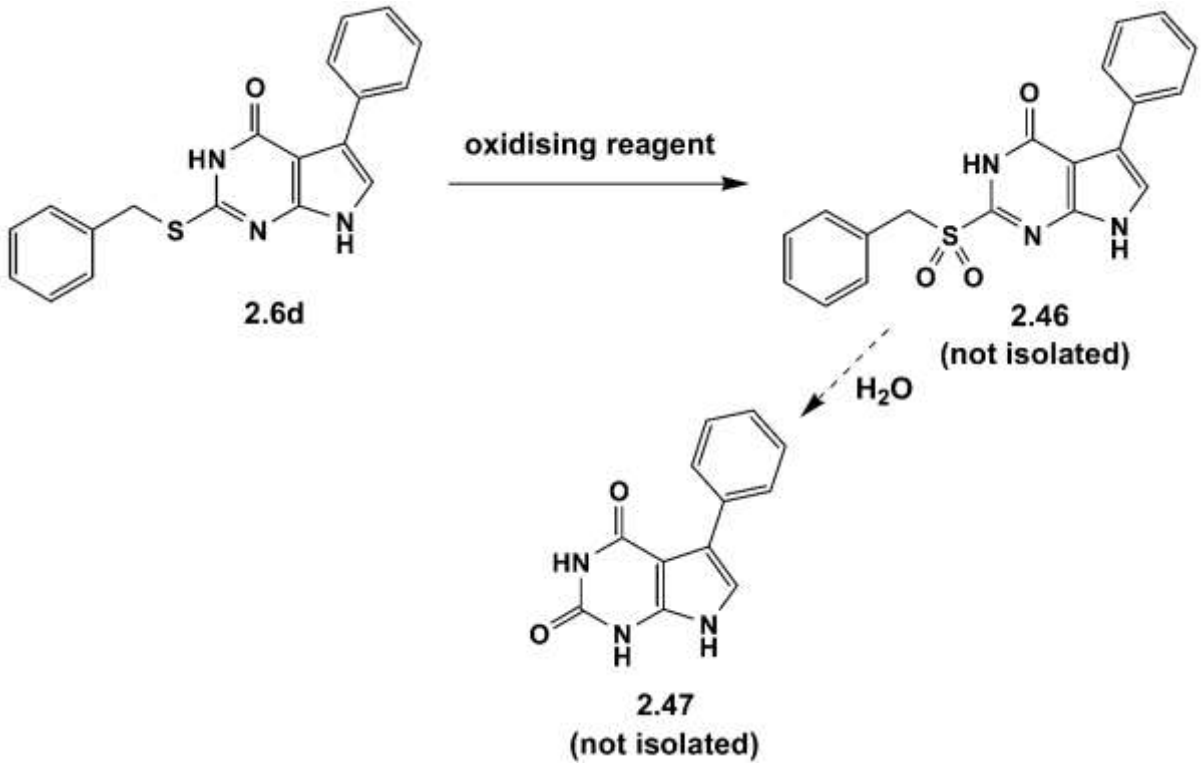
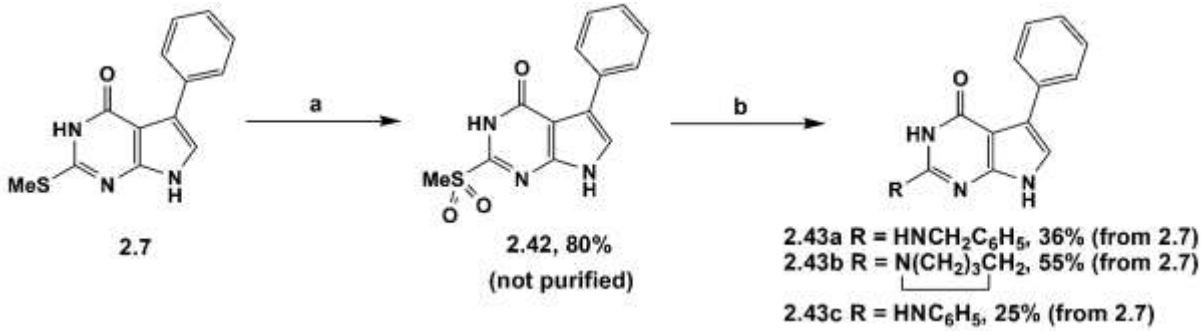


Figure 14. Unsuccessful isolation of sulfone 2.46

After the unsuccessful attempts at direct thermal reaction and one-pot oxidation-substitution reaction, we devised a successful strategy for the C2 position substitution (Figure 15). The oxidation reaction in m-chloroperbenzoic acid in DMF at room temperature was accomplished within four hours. On a straightforward workup procedure, involving vacuum evaporation of DMF followed by diethyl ether washing, crude sulfone 2.42 was isolated in an acceptable yield of 80%. The product identity was confirmed by low-resolution mass spectrometry, which showed a strong molecular ion peak (MH^+ = 290.1). The crude sulfone was reacted with selected amines in the absence of any solvent, and upon overnight stirring at 100°C, C2 position substitution products in modest yields (25 to 55%) were realized.



Scheme 15. Reagents and Conditions: (a) m-CPBA (3 equiv.), DMF, room temperature, 4 hours. (b) Respective amines, 100°C (in sealed tube), 15 hours.

Although oxidation followed by treatment with amines are standard methods for this type of chemistry, two points are important in our case: (1) simple non-aqueous workup gives crude sulfone, (2) high concentration of amines without solvent.
Interestingly, there are no reported cases of using neat amines as nucleophiles for cleaving the sulfone moiety at the C2 position in purine/pyrrolopyrimidine scaffolds. Therefore, we propose that our successful C2 displacement method could be applied to similar frameworks in library synthesis in medicinal chemistry fields.

Two Unexpected Results
During the development of C2 position displacement reaction methods, we encountered two unexpected results. The first case was the reaction between cyanogen bromide and 2-thiobenzylpyrrolo[2,3-d]pyrimidine 2.6d. Initially, we thought that cyanation on sulfur would form a more unstable leaving

group in 2.6d and lead to compound 2.53 (Figure 16). However, intermediate compound 2.53 was not formed, and instead, the brominated product 2.55 was obtained in 93% yield.
The probable mechanism for this reaction is presented in Figure (17). The first step is nucleophilic attack by the pyrrole ring on the carbon atom in cyanogen bromide, followed by bromination at the C6 position. Then, rearomatization occurs with elimination of HCN, giving the 6-bromo product 2.55. The obtained product 2.55 was fully characterized by high-resolution mass spectrometry (HRMS), ^{1}H and ^{13}C NMR, and infrared (IR) spectroscopy.

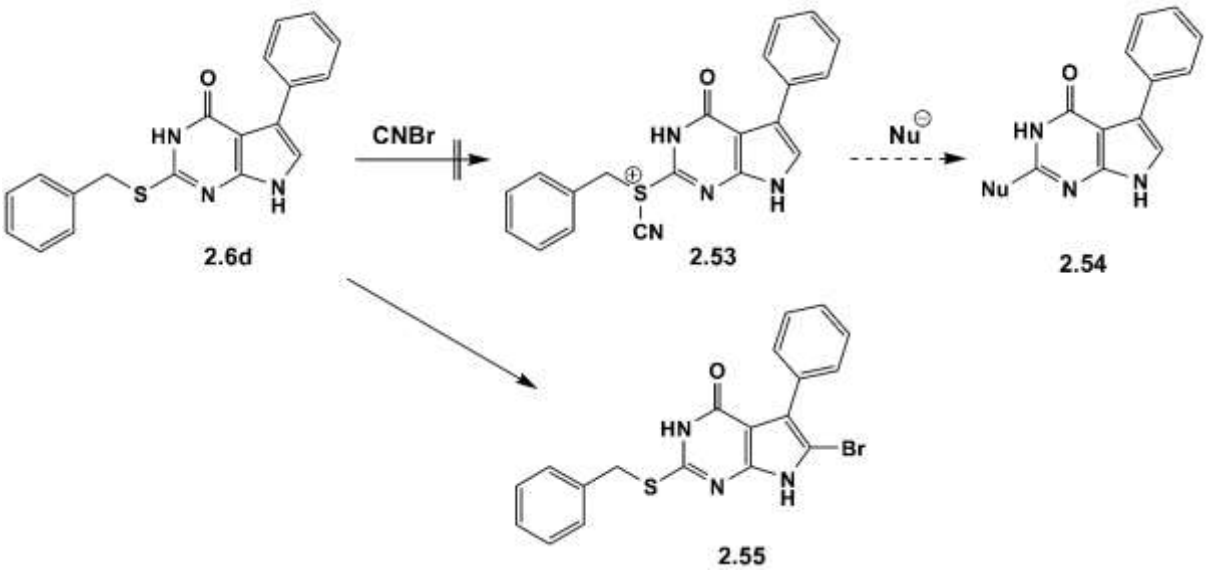


Figure 16: Reagents, Conditions, and Yield: CNBr, THF, 40°C, 15 hours, 93%

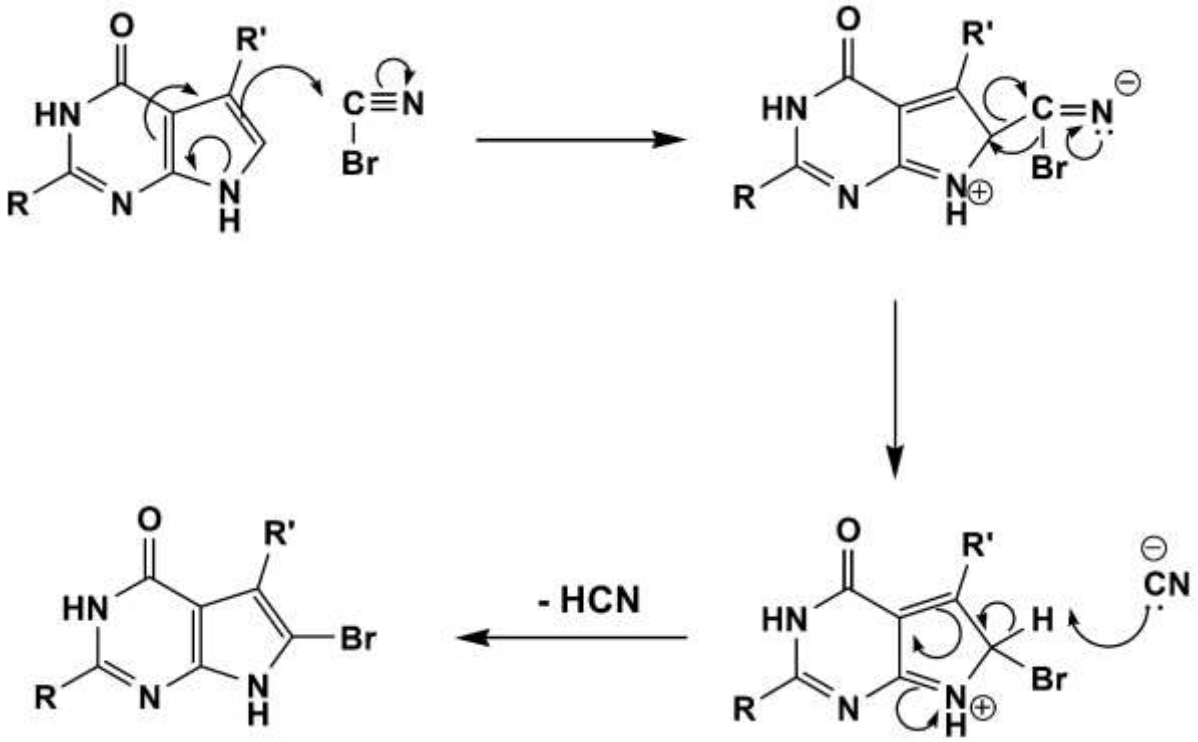


Figure 17: Probable mechanism for the formation of 6-bromopyrrolo[2,3-d]pyrimidine product.

The second case is the reaction between N,O-dimethylhydroxylamine and 2-thiobenzylpyrrolo[2,3-d]pyrimidine 2.6d (Figure 18). In this reaction, the C6-substituted product (compound 2.57) was obtained instead of the C2-substituted product (compound 2.56).
Figure 19 presents a possible mechanism for this reaction. The non-bonding electron pair of the free amine nitrogen abstracts one of the O-methyl amine salt protons, producing methylamine and formaldehyde. Then, the non-bonding electron pair of the free amine nitrogen can attack the carbonyl carbon of the aldehyde, forming an iminium cation. Finally, nucleophilic attack by the pyrrole ring on the iminium cation carbon yields the final product 2.57.

The C6-substituted product (2.57) was fully characterized, and its connectivity pattern was also confirmed by HMBC NMR spectroscopy (Heteronuclear Multiple Bond Correlation) (long-range H-C correlations, Appendix II).

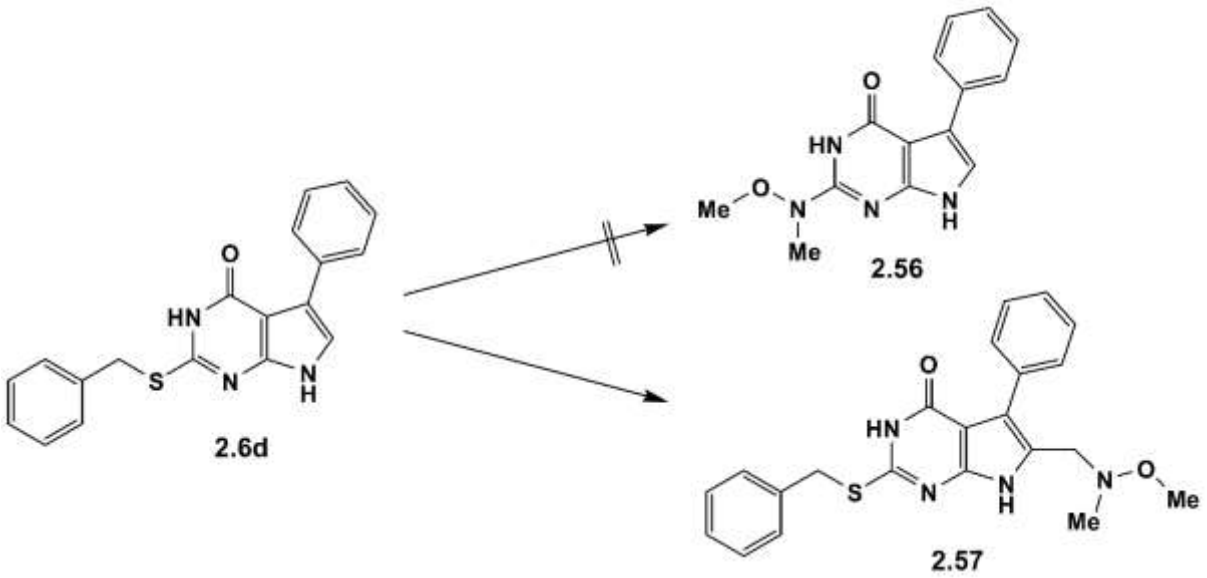


Figure 18: Reagents, Conditions, and Yield: N,O-dimethylhydroxylamine, N,O-dimethylhydroxylamine hydrochloride, ethanol, 100°C, 48 hours, 15%.

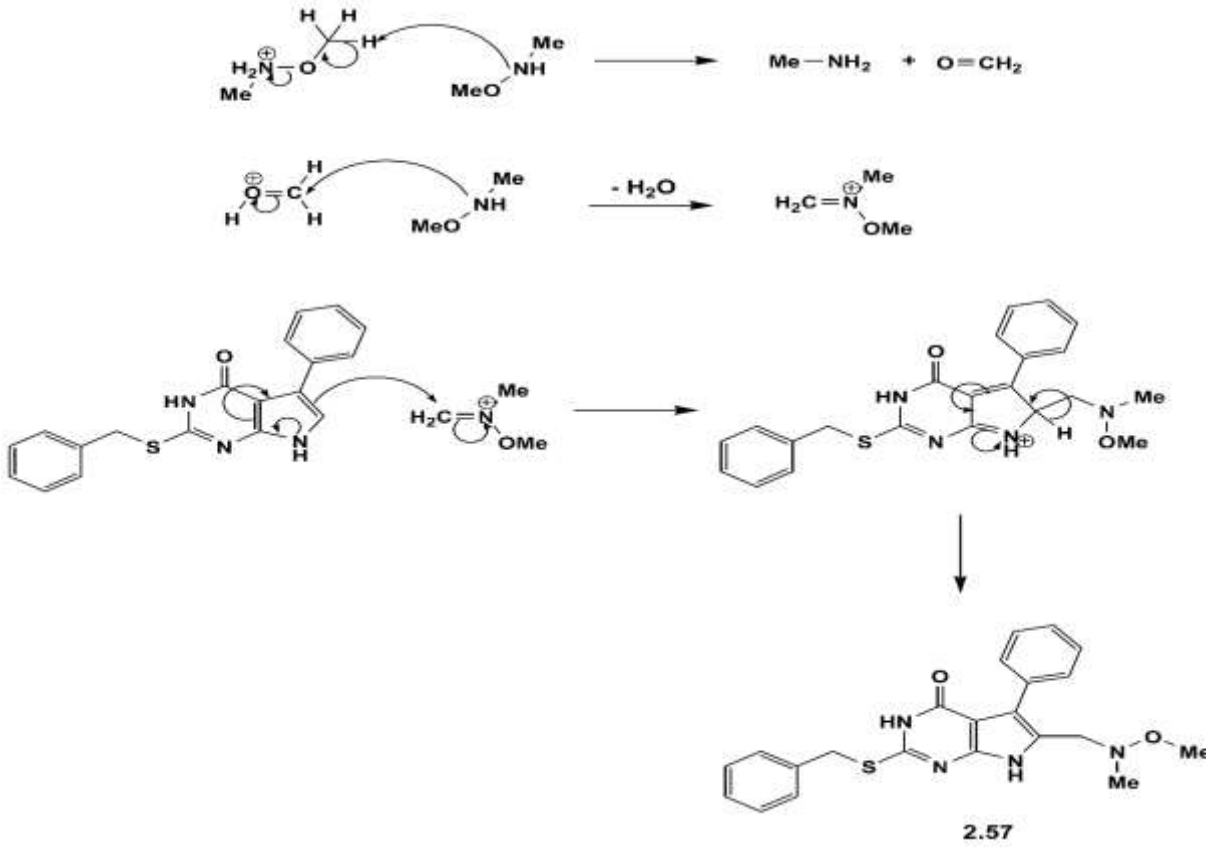


Figure 19: Probable mechanism for the formation of compound 2.57.

CONCLUSION

The present study was designed to synthesize and evaluate the pharmacological properties of pyrrolo[2,3-d]pyrimidine derivatives as kinase inhibitors. The results demonstrated that various structural modifications, particularly at key cyclic positions, significantly improved inhibitory potency and selectivity against target enzymes. Specifically, compound 5k was identified as a potent multi-target

inhibitor with IC50 values in the nanomolar range, capable of inducing apoptosis and cell cycle arrest in HepG2 cells. These findings emphasize the importance of rational compound design based on effective chemical structures and present new opportunities for developing improved anticancer drugs.

One of this study's significant achievements is the development of effective and optimized synthetic methods for producing pyrrolopyrimidine derivatives with high yield and appropriate purity. Using novel reaction conditions and advanced catalysts, such as microwave reactions and DBU-mediated cyclization, helped overcome limitations of traditional methods. These advances reduced laboratory time and costs and enabled larger-scale production of targeted compounds, which is particularly important in developing anticancer drugs with clinical potential.

From a pharmacological perspective, the results demonstrated that the designed pyrrolopyrimidine derivatives can effectively interact with the ATP region of kinase enzymes. These interactions occur through hydrogen bonding and van der Waals interactions with amino acid residues in the kinase active site. Molecular docking studies confirmed that compounds such as 5k and 6f exhibit binding patterns similar to well-known examples like sunitinib. These similarities not only validate the inhibitory capability of these compounds but also indicate their potential as new drug candidates for treating cancers resistant to existing drugs. Furthermore, mechanistic studies showed that the synthesized compounds can induce apoptosis in cancer cells by increasing the expression of pro-apoptotic proteins such as caspase-3 and Bax while decreasing the activity of anti-apoptotic protein Bcl-2. These findings demonstrate the compounds' ability to target key signaling pathways in cancer and disrupt tumor cell growth and survival.

Moreover, results related to compounds 14a and 17 showed that these derivatives can induce cell cycle arrest in the G1/S phase, which is particularly beneficial in treating cancers with rapid cell cycles. Another important aspect of this study is the emphasis on developing multi-target drugs. Results showed that pyrrolopyrimidine derivatives can simultaneously inhibit multiple kinases, which could help overcome drug resistance and reduce side effects. For example, compound 6f, with sub-micromolar GI50 and selective inhibition of FGFR4, Tie2, and TrkA, demonstrates the potential of these compounds in designing targeted drugs with a broad spectrum of activity. This multi-target approach could lead to a new generation of drugs capable of treating various types of cancer through different mechanisms.

2-Thioalkyl-6-amino-4-oxo pyrimidine compounds successfully underwent Michael reaction with functionalized nitroalkenes. The use of a biphasic system (water/ethyl acetate, 1:1) along with a phase transfer catalyst ($PhCH_2N^+Me_3HO^-$) proved highly effective in these Michael reactions. The subsequent cyclization reaction was carried out via the Nef reaction. Systems containing titanium(III) chloride or tin(II) chloride dihydrate produced cyclized products with better yields compared to the reaction with DBU.

Additionally, the substitution of the thioalkyl group at the C2 position of pyrrolo[3,2-d]pyrimidines was successfully accomplished. This was achieved using a modified oxidation-substitution method in which the crude sulfone was first obtained through a simple work-up and then reacted in neat amine medium. This achievement will be particularly valuable for diversification at the C2 position of pyrrolo[3,2-d]pyrimidines in synthesizing combinatorial libraries.

REFERENCES

1. Alotaibi AA, Asiri HH ,Rahman AFMM, Alanazi MM. Novel pyrrolo[2,3-d]pyrimidine derivatives as multi-kinase inhibitors with VEGFR-2 selectivity. Journal of Saudi Chemical Society. 2023;27(5):101712.
2. Alotaibi AA, Alanazi MM, Rahman AM. Discovery of new pyrrolo [2, 3-d] pyrimidine derivatives as potential multi-targeted kinase inhibitors and apoptosis inducers. Pharmaceuticals. 2023;16(9):1324.
3. Xia Z, Huang R, Zhou X, Chai Y, Chen H, Ma L, et al .The synthesis and bioactivity of pyrrolo[2,3-d]pyrimidine derivatives as tyrosine kinase inhibitors for NSCLC cells with EGFR mutations. European Journal of Medicinal Chemistry. 2021;224:113711.
4. Sroor FM, Tohamy WM, Zoheir KM, Abdelazeem NM, Mahrous KF, Ibrahim NS. Design, synthesis, in vitro anticancer, molecular docking and SAR studies of new series of pyrrolo [2, 3-d] pyrimidine derivatives. BMC chemistry. 2023;17(1):106.
5. Lee J-H, El-Damasy AK, Seo SH, Gadhe CG, Pae AN, Jeong N, et al. Novel 5,6-disubstituted pyrrolo[2,3-d]pyrimidine derivatives as broad spectrum antiproliferative agents: Synthesis, cell based assays, kinase profile and molecular docking study. Bioorganic & Medicinal Chemistry. 2018;26(21):5596-611.
6. Musumeci F, Sanna M, Grossi G, Brullo C, Fallacara AL, Schenone S. Pyrrolo [2, 3-d] pyrimidines as kinase inhibitors. Current Medicinal Chemistry. 2017;24(19):2059-85.