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Development of Pyrimidinon Derivative Compounds from Methoxy Bis-Chalcone Analogs as Potential Anticancer Candidates

Nia Tussholiha
Department of Chemistry, Science and Technology, Airlangga University, Indonesia

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ABSTRACT

Purpose of the study: This study aims to evaluate its bioactivity as a candidate anticancer compound through inhibition testing of dihydrofolate reductase enzyme activity.

Methodology: Organic synthesis methods through condensation and cyclization, reflux, recrystallization, preparative thin layer chromatography, Fisher John Melting Point Apparatus, ultraviolet visible spectrometer, nuclear magnetic resonance spectrometer, mass spectrometer, infrared spectrometer, centrifugation, in vitro dihydrofolate reductase enzyme inhibition test, probit analysis, methotrexate as a positive control, and evaluation of anticancer bioactivity.

Main Findings: The pyrimidinone derivative compound was successfully synthesized and confirmed through physical and spectroscopic characterization. The test results showed significant inhibitory activity against the dihydrofolate reductase enzyme. The synthesized compound had a fifty percent inhibitory concentration of 25.086 micrograms per milliliter, lower than the positive control, indicating better anticancer potential and high inhibitory effectiveness.

Novelty/Originality of this study: The novelty of this research lies in the synthesis of pyrimidinone derivatives based on bis-chalcone analogs containing methoxy groups, which have not been widely studied as anticancer candidates. This study provides new insights into the relationship between structure and biological activity, while also expanding the development of heterocyclic compounds as dihydrofolate reductase inhibitors for anticancer therapy.

Corresponding Author:
Nia Tussholiha,
Department of Chemistry, Science and Technology, Airlangga University, Airlangga Street No. 4-6, Surabaya, Surabaya City, East Java, Indonesia
Email: nialihaa93@gmail.com

1. INTRODUCTION

Cancer is one of the leading causes of death worldwide and poses a major challenge to global health [1], [2]. According to various international health reports, the incidence of cancer continues to increase annually due to changes in lifestyle, environmental exposure, and genetic factors that influence the development of this disease [3], [4]. Although various therapeutic methods such as chemotherapy, radiotherapy, and immunotherapy have developed rapidly, the effectiveness of cancer treatment is often hampered by high side effects, cancer cell resistance to drugs, and low selectivity of therapeutic compounds for target cells [5], [6]. This situation drives the need to explore new compounds with more selective, effective, and safe anticancer activity.

In modern anticancer drug development, the synthesis of heterocyclic compounds is one widely developed approach. Heterocyclic compounds are known to possess broad structural diversity and significant

biological activities, including anticancer, antibacterial, anti-inflammatory, and antiviral activities [7], [8]. One class of heterocyclic compounds that has attracted considerable attention is pyrimidinone derivatives [9], [10]. The pyrimidinone skeleton is known to possess important pharmacological activities due to its ability to interact with various biological targets, such as enzymes, receptors, and cell growth regulatory proteins. This structure also has the potential to be modified to enhance its biological activity through variations in specific substituents.

Pyrimidinone derivatives have been widely reported to exhibit anticancer activity against various types of cancer cells [11], [12]. This activity is generally related to their ability to inhibit cell proliferation, induce apoptosis, and disrupt the cancer cell division cycle [13], [14]. Several studies have shown that modifying the pyrimidinone ring by adding electron donor or electron acceptor groups can affect the compound's electronic properties, thereby increasing its biological activity [15], [16]. Therefore, the development of pyrimidinone derivatives through organic synthesis approaches continues to be pursued in an effort to discover new anticancer candidate compounds.

One potential precursor in the synthesis of pyrimidinone derivatives is bis-chalcone analogs. Bis-chalcone compounds possess an α,β-unsaturated carbonyl conjugation system known to play a key role in biological activity, particularly as anticancer agents through the inhibition of cell proliferation and induction of apoptosis [17], [18]. Bis-chalcone analogs with methoxy substituents on the aromatic ring are known to increase the compound's lipophilicity and ability to interact with cell membranes and molecular targets. The compound (3E,5E)-3,5-bis-(4-methoxybenzylidine)-N-methylpiperidine-4-one is a bis-chalcone analog that has high potential to be further developed into a pyrimidinone-derived heterocyclic compound through a cyclocondensation reaction.

Transforming bis-chalcone structures into pyrimidinone derivatives is a promising synthetic strategy because it can produce compounds with higher structural complexity and enhanced biological activity potential [19], [20]. This cyclization process allows for the formation of stable and bioactive heterocyclic frameworks. Furthermore, the presence of methoxy groups in the precursor structure is expected to provide a synergistic effect on anticancer activity by enhancing hydrophobic interactions with biological targets [21], [22]. Therefore, the synthesis of pyrimidinone derivatives from bis-chalcone analogs is a rational approach in the development of new anticancer compound candidates.

Although various studies have reported the synthesis of pyrimidinone derivatives and the evaluation of their bioactivity, studies on the synthesis of pyrimidinone derivatives derived from a specific bis-chalcone analog, (3E,5E)-3,5-bis-(4-methoxybenzylidine)-N-methylpiperidine-4-one, remain very limited. Most previous studies have focused on optimizing synthesis methods or characterizing the compound's structure without in-depth evaluation of its potential anticancer bioactivity [23]-[25]. This research gap indicates an opportunity to explore the structure-activity relationship of pyrimidinone derivatives synthesized from these analogs. The novelty of this research lies in the synthesis of pyrimidinone derivatives using bis-chalcone analogs with methoxy groups as primary precursors and the direct evaluation of their anticancer activity. This research is important and urgent given the need for new anticancer candidate compounds with high efficacy and minimal side effects.

Based on this analysis, this study was conducted to synthesize pyrimidinone derivatives from the bis-chalcone analog (3E,5E)-3,5-bis-(4-methoxybenzylidine)-N-methylpiperidine-4-one and to characterize the structure of the synthesized compounds. The primary objective of this study is to test the bioactivity of the synthesized compounds as anticancer agents, which is expected to contribute to the development of new anticancer drug candidates with the potential for further development in advanced research.

2. RESEARCH METHOD

2.1. Place and Time of Research

This research was conducted at the Organic Chemistry Laboratory, Department of Chemistry, Faculty of Science and Technology, Airlangga University, Surabaya. The research activities were carried out over a period of seven months, starting at the beginning of the research period until the final stage of completion of the entire series of experiments. Analysis using ultraviolet-visible spectroscopy was carried out at the Research Laboratory, Department of Chemistry, Faculty of Science and Technology, Airlangga University. Analysis using nuclear magnetic resonance spectroscopy was carried out at the Phytochemistry Laboratory, Faculty of Science and Food, University of Technology Malaysia. Meanwhile, mass spectroscopy analysis was carried out at the Research Laboratory, Bandung University of Technology. Characterization using infrared spectroscopy was carried out at the Research Laboratory, State University of Surabaya. The in vitro anticancer activity test was carried out at the Organic Chemistry and Biochemistry Laboratory, Department of Chemistry, Faculty of Science and Technology, Airlangga University, as a stage of evaluating the bioactivity of the synthesized compounds.

2.2. Research Tools and Materials

The equipment used in this study consisted of a set of laboratory glassware commonly used in organic chemical synthesis, such as round-bottom flasks, beakers, graduated cylinders, separating funnels, droppers, stirring rods, and other supporting equipment. In addition, a set of thin-layer chromatography equipment was used

to monitor the reaction progress and evaluate the purity of the synthesized compounds [26], [27]. Compound characterization was performed using several analytical instruments, namely a Fisher John Melting Point Apparatus, an ultraviolet-visible spectrometer, a nuclear magnetic resonance spectrometer, a mass spectrometer, and an infrared spectrometer. All of these instruments were used to support structural identification and confirm the physicochemical characteristics of the synthesized compounds.

The organic materials used in this study included N-methylpiperidine-4-one, para-anisaldehyde, sodium hydroxide, one-(four-chlorophenyl)-three-(three,four-dichlorophenyl)-urea, hydrochloric acid, ethyl acetate, ethanol, chloroform, dichloromethane, methanol, and n-hexane. These materials are used as primary reagents in the synthesis process and as solvents in the purification and analysis stages of the compounds [28]. The solvents used in this study are technical-grade solvents that were purified through a distillation process before use to remove impurities that could affect the reaction and the results of the compound analysis.

2.3. Research Stages

2.3.1. Synthesis of Chalcone Derivative Compounds

The synthesis of chalcone derivative compounds was carried out through a condensation reaction between para-anisaldehyde, N-methylpiperidin-4-one, sodium hydroxide solution, and ethanol as a solvent. The initial stage was carried out by dissolving para-anisaldehyde in ethanol while stirring until homogeneous, then the solution was put into a round-bottom flask with N-methylpiperidin-4-one. The reaction mixture was then cooled using an ice bath with a low temperature. After that, sodium hydroxide solution was added slowly while maintaining a stable temperature. The mixture was then reacted through a reflux process under cooling conditions, continued without cooling until the reaction was complete. After the reaction process was complete, the mixture was poured into ice water until a solid precipitate formed. The precipitate obtained was separated by filtration using a Buchner funnel, then purified by the recrystallization method using a mixture of ethanol and water to obtain a chalcone derivative compound with a good level of purity.

2.3.2. Synthesis of Target Compounds

The synthesis of the target pyrimidinone derivative compound was carried out by reacting the bis-chalcone analogue (3E,5E)-3,5-bis-(4-methoxybenzylidine)-N-methylpiperidine-4-one with one-(four-chlorophenyl)-three-(three,four-dichlorophenyl)-urea in ethanol with the addition of concentrated hydrochloric acid as a catalyst. The reaction mixture was refluxed for a certain time until the cyclization product was formed. After the reaction was complete, the mixture was concentrated until its volume was reduced to about half of the initial volume. The purification stage was carried out using preparative thin layer chromatography. The silica gel portion containing the eluted compound was scraped and redissolved in ethanol. The solution was then left to stand until the silica precipitated, filtered, and concentrated. The synthesized compound obtained was then weighed, tested for purity, and analyzed for its bioactivity as an anticancer compound.

2.3.3. Testing the Physical Properties of Synthesized Compounds

Melting point determination was performed using a Fisher John Melting Point Apparatus. Measurements were made by observing the temperature range from the time the compound begins to melt until the entire sample has completely melted. A compound is considered to have a good level of purity if its melting point range is narrow. The purity of the synthesized compound was tested using thin layer chromatography with various eluent compositions. The sample was spotted onto a silica gel plate using a capillary tube, then eluted until the solvent reached the upper limit of the plate. After the plate was dry, the separated spots were observed using ultraviolet light because the tested compound was colorless. The retardation factor value was determined based on the ratio of the distance traveled by the compound to the distance traveled by the eluent. A compound was declared pure if it produced a single spot in various eluent systems.

2.3.4. Spectroscopic Test

The synthesized compounds were dissolved in methanol and then analyzed using an ultraviolet-visible spectrometer to determine the maximum absorption wavelength in the appropriate spectral range. Nuclear magnetic resonance analysis was performed by dissolving the sample in deuterated chloroform solvent and then placing it in an analysis tube. The obtained spectrum was interpreted to identify the environment of hydrogen and carbon atoms in the compound structure. Mass spectroscopic analysis was performed to determine the molecular mass of the synthesized compounds. The samples were ionized to produce molecular ions, then the mass spectrum was analyzed based on the relationship between ion abundance and mass-to-charge ratio. The samples were ground with dry potassium bromide until homogeneous and free of water, then formed into pellets. The pellets were then analyzed using an infrared spectrophotometer to identify functional groups based on absorption patterns at certain wavenumber regions.

2.3.5. Anticancer Activity Test

Dihydrofolate reductase enzyme extraction was performed using cleaned and frozen mouse liver tissue. The sample was then homogenized in cold water and centrifuged to separate the supernatant. The resulting supernatant was acidified with acetic acid to a specific acidity level and then allowed to stand overnight. After further centrifugation, the supernatant was separated as an enzyme extract ready for use in activity testing. Enzyme activity was tested by mixing the enzyme extract, two-mercaptoethanol, dihydrofolic acid, reduced nicotinamide adenine dinucleotide phosphate, and phosphate buffer solution to a specified volume. The mixture was incubated at a controlled temperature, and the absorbance was measured at the appropriate wavelength. Control measurements were performed without the addition of enzyme extract or substrate. Inhibition testing was performed by mixing the enzyme extract with the synthesized compound at varying concentrations in a phosphate buffer solution. The mixture was incubated at room temperature for several minutes. Substrate and reaction cofactors were then added, and the mixture was incubated again at the specified temperature. The absorbance value obtained was measured to determine the ability of the synthesized compound to inhibit the activity of the dihydrofolate reductase enzyme as an indicator of potential anticancer activity.

3. RESULTS AND DISCUSSION

3.1. DHFR enzyme extraction

To extract the DHFR enzyme, mouse livers were washed thoroughly and then frozen. This was done to ensure the blood coagulates and doesn't affect the absorbance measurement. Water (1:1) was then added and ground in an ice bath to prevent damage to the enzyme. This treatment yielded 5 ml of DHFR enzyme extract.

3.2. DHFR enzyme extract activity test

To test its activity, DHFR enzyme extract, 2 mercaptoethanol, dihydrofolic acid, NADPH were added and phosphate buffer (pH 7.0) was added to a total volume of 1500 µL. The mixture was incubated at 37°C for 5 minutes and then the absorbance was measured at λ 340 nm. As a control, the same measurement was carried out but without the addition of enzyme extract and without the addition of substrate. Enzyme activity was calculated using the formula:

NADPH absorption decrease (λ 340 nm) = NADPH initial–NADPH final
$= (A_{(-E)}) - (A_{(E+S)} - A_{(-S)})$

$$\text{Enzyme activity} = \frac{1\ \mu mol/mL.minute\ x\ reduction\ A\lambda 340\ x\ vol.Assay\ x\ P}{6{,}22\ x\ vol.enzim\ x\ time}$$

3.3. Inhibition of DHFR enzyme extract

To inhibit the DHFR enzyme extract, 2 mercaptoethanol, enzyme extract, and synthesized compounds were required with varying concentrations of 20 ppm, 40 ppm, 50 ppm, 80 ppm, and 100 ppm. Phosphate buffer (pH 7.0) was added, adjusted to the concentration of the synthesized compound. The mixture was incubated for 5 minutes at room temperature. After incubation, DHFA and NADPH were added. Then, it was incubated again for 5 minutes and then the absorbance was measured at λ 340 nm. The percentage of sample inhibition of enzyme activity was calculated using the formula:

$$\%\ Inhibition\ = \frac{activity\ (E) - activity\ (E{+}1)}{activity\ (E)}\ \text{x}\ 100\%$$

DHFR (dihydrofolate reductase) is an enzyme that catalyzes a reaction essential for DNA base biosynthesis. DHFR (Dihydrofolate reductase) is an enzyme that reduces dihydrofolic acid to tetrahydrofolic acid. The positive control used in this study was methotrexate because it is an antimetabolite agent that competitively inhibits the dihydrofolate reductase enzyme. Methotrexate is a drug that can stop the growth of cells that can interfere with the immune system. Methotrexate is used to treat cancer and autoimmune diseases.

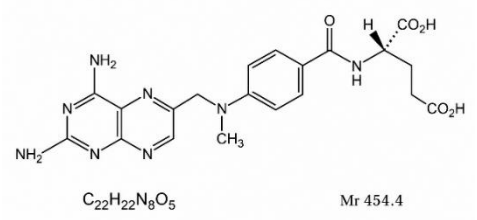

Figure 1. Structure of Methotrexate

Methotrexate will be compared with the synthesized compound. The anticancer activity test was conducted in duplicate, and the IC50 (Inhibitory Concentration) value was determined using a probit analysis. After obtaining the anticancer analysis data listed in Appendix 5, the % inhibition value was calculated. Below is a graph of % inhibition vs. concentration (µg/mL):

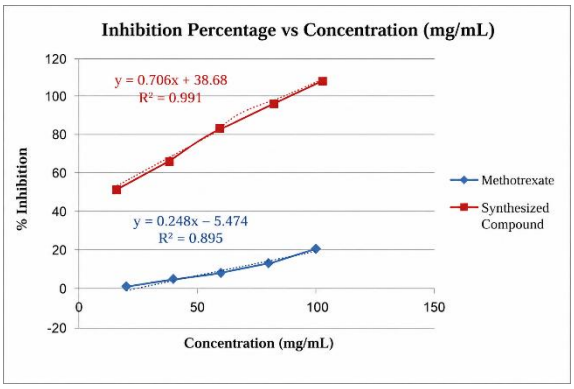

Figure 2. Graph of % Inhibition vs Concentration (µg/mL)

The synthesized pyrimidinone derivative demonstrated promising anticancer potential through its inhibitory activity against the dihydrofolate reductase (DHFR) enzyme. The observed inhibitory effect indicates that the synthesized compound was able to interfere with the folate metabolic pathway, which plays an essential role in DNA synthesis and cell proliferation [29], [30]. Cancer cells generally exhibit rapid and uncontrolled growth, requiring high amounts of nucleic acid precursors. Therefore, inhibition of DHFR activity can suppress the formation of tetrahydrofolate needed for purine and pyrimidine biosynthesis, ultimately inhibiting cancer cell growth. The lower IC50 value of the synthesized compound compared to methotrexate suggests that the compound possesses strong inhibitory efficiency and may serve as a potential alternative anticancer candidate.

The anticancer activity observed in this study is strongly related to the structural characteristics of the synthesized pyrimidinone derivative. The presence of methoxy substituents on the aromatic rings is predicted to enhance the lipophilicity of the compound, thereby improving its interaction with biological membranes and active sites of enzymes. In addition, the pyrimidinone heterocyclic framework is known to contribute significantly to biological activity because it can facilitate hydrogen bonding and electronic interactions with amino acid residues in target proteins [31], [32]. The cyclization process from bis-chalcone analogs to pyrimidinone derivatives may also increase molecular stability and optimize the electronic distribution of the compound, which contributes to stronger enzyme inhibition activity.

The findings of this study are in agreement with previous reports stating that pyrimidinone derivatives possess broad pharmacological activities, especially as anticancer agents. Several studies have demonstrated that pyrimidine-based compounds are capable of inhibiting enzymes involved in nucleotide biosynthesis and disrupting cancer cell proliferation pathways [33], [34]. The results obtained in this research further strengthen the evidence that structural modification of chalcone analogs into heterocyclic pyrimidinone derivatives can enhance biological activity. Furthermore, the incorporation of methoxy groups may provide synergistic effects by increasing electron donation and stabilizing interactions between the synthesized compound and the DHFR enzyme target [35], [36].

The successful synthesis and characterization of the target compound also indicate that the applied synthetic strategy was effective in producing pyrimidinone derivatives with good purity and structural conformity. The use of spectroscopic analyses such as UV-Vis, FTIR, NMR, and mass spectroscopy provided comprehensive confirmation regarding the molecular structure of the synthesized compound [37], [38]. These characterization results are important because the biological activity of heterocyclic compounds is highly influenced by their structural accuracy and purity level. The formation of a single spot in thin layer chromatography analysis further supports the successful purification of the synthesized product.

This study provides important implications for the development of new anticancer agents based on heterocyclic compounds. The promising inhibitory activity against DHFR suggests that pyrimidinone derivatives derived from methoxy bis-chalcone analogs have the potential to be further developed as lead compounds in anticancer drug discovery. In addition, this research contributes to the understanding of structure–activity relationships in pyrimidinone-based compounds, particularly regarding the role of methoxy substituents in enhancing biological activity [39], [40]. The findings may also encourage further exploration of chalcone-derived heterocyclic compounds for broader pharmaceutical applications.

Despite the promising findings, this study still has several limitations. The anticancer evaluation was limited to in vitro DHFR enzyme inhibition assays and did not include cytotoxicity testing against specific cancer cell lines. Therefore, the direct effect of the synthesized compound on cancer cell viability and selectivity toward

normal cells remains unclear. In addition, molecular docking studies and mechanistic investigations were not conducted, so the interaction pattern between the synthesized compound and the active site of the DHFR enzyme has not been fully elucidated. Another limitation is the absence of in vivo studies to evaluate pharmacokinetic properties, toxicity, and therapeutic efficacy in biological systems.

4. CONCLUSION

From this study, it can be concluded that the compound 4-(4-methoxyphenyl)-6-methyl-5,6-dihydro-1H-pyrido[4,3-d]pyrimidine-2 on has activity as an anticancer compound because it has an IC50 value of 25.086 µg/mL which is smaller than the positive control. Future studies are recommended to conduct cytotoxicity assays against various cancer cell lines, molecular docking simulations, and in vivo evaluations to further validate the anticancer potential of the synthesized compound. Structural modification of the pyrimidinone framework may also be explored to optimize biological activity and reduce possible toxicity. Through these advanced studies, the synthesized pyrimidinone derivative may contribute significantly to the development of safer and more effective anticancer therapies in the future.

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AUTHOR CONTRIBUTIONS

Conceptualization, N.T.; Methodology, N.T.; Software, N.T.; Validation, N.T.; Formal Analysis, N.T.; Investigation, N.T.; Resources, N.T.; Data Curation, N.T.; Writing – Original Draft Preparation, N.T.; Writing – Review and Editing, N.T.; Visualization, N.T.; Supervision, N.T.; Project Administration, N.T.; Funding Acquisition, N.T.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

Not applicable.

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