

Study In Silico, Synthesis and Analgesic Activity Test of N-Benzoyl-N'-4-Methoxyphenylthiourea Compound in Mus musculus

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Pain is an unpleasant sensory and emotional experience resulting from actual or potential tissue damage and is the most common reason individuals seek health care. Nonsteroidal anti-inflammatory drugs (NSAIDs), which act through cyclooxygenase inhibition, provide good analgesic effects but carry a risk of gastrointestinal toxicity. This study aimed to develop a thiourea derivative compound, namely N-benzoyl-N'-4-methoxyphenylthiourea (MFT), as an alternative analgesic candidate with lower side effects. The research stages included silico testing, synthesis, structure confirmation, and in vivo analgesic activity testing. In silico testing was performed by molecular docking using the Molecular Operating Environment (MOE) program against the COX-2 receptor (PDB code 5IKR), validated through redocking (RMSD 0.135-0.136 Å). Synthesis was carried out via the Schotten-Baumann reaction between 4-methoxyphenylthiourea and benzoyl chloride, yielding the MFT compound with a yield of 34.80%, single-spot purity on TLC testing, and a consistent melting point of 171-173°C. Structure confirmation using UV, IR, ¹H-NMR, ¹³C-NMR, and GC-MS spectrophotometry showed that the synthesized compound matched the target structure. In vivo analgesic activity testing in mice (*Mus musculus*) using the writhing method showed that at a dose of 25 mg/kgBW, the analgesic activity of MFT was not significantly different from mefenamic acid ($p>0.05$), whereas at a dose of 100 mg/kgBW the two differed significantly ($p<0.05$). The N-benzoyl-N'-4-methoxyphenylthiourea compound has potential to be developed as a new analgesic drug candidate.



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Pain is an unpleasant sensory and emotional experience, often associated with actual or potential tissue damage, and is the most common reason individuals seek health care because it disrupts daily activities [3]. Pain is a major global health problem; it is estimated that one in five adults experiences pain

and one in ten adults is diagnosed with chronic pain every year, with the main causes including cancer, osteoarthritis, rheumatoid arthritis, post-surgical conditions, trauma, and spinal disease [2]. Pain is also subjective and can only be described by the individual experiencing it [12,14].

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The sensation of pain drives the use of analgesic drugs. Nonsteroidal anti-inflammatory drugs (NSAIDs), which act through inhibition of cyclooxygenase-1 (COX-1), provide good analgesic effects, but carry a considerable risk of gastrointestinal toxicity, including impaired kidney function and post-surgical bleeding [11]. The most frequently reported side effect is the induction of gastric ulcers or peptic ulcers, sometimes accompanied by secondary anemia due to gastrointestinal bleeding.

The discovery of new drugs aims to treat specific diseases while increasing drug activity, reducing side effects or toxicity, and improving selectivity. Thiourea and urea derivative compounds are known to have broad biological activity, including as antiviral, anti-HIV, antibacterial, analgesic, and anti-inflammatory agents [6,10]. Based on this, the present study was designed to develop a parent thiourea derivative compound, namely N-benzoyl-N'-4-methoxyphenylthiourea (hereinafter referred to as MFT), as an analgesic candidate.

The MFT compound was synthesized through a reaction between benzoyl chloride and its derivatives with the primary amine group of N-4-methoxyphenylthiourea (acylation reaction), following the principle of the Schotten-Baumann reaction, in which the nucleophilic primary amine group attacks the carbon atom of the carbonyl group on benzoyl chloride. The choice of substituent on the aromatic ring of the benzoyl group was made using the Topliss approach, namely introducing groups with specific lipophilic, electronic, and steric properties at specific positions of the parent compound structure [19]. Prior to synthesis, the compound was first tested in silico against the cyclooxygenase-2 (COX-2) receptor with code 5IKR in the Protein Data Bank, which was selected because it has native ligand similarity to mefenamic acid as the comparator.

Previous studies on allylthiourea and substituted benzoylthiourea derivatives reported better analgesic activity compared to sodium diclofenac, as well as potent anti-inflammatory activity in substituted benzoylthiourea compounds,

while modification of the benzoylurea structure was also reported to increase activity on the central nervous system compared to the parent compound [21]. These findings support the feasibility of developing the MFT compound as a new analgesic candidate.

This study aimed to: (1) obtain the N-benzoyl-N'-4-methoxyphenylthiourea compound that, in silico, has higher analgesic activity than mefenamic acid; and (2) obtain a compound that, in vivo, has higher analgesic activity in mice (*Mus musculus*) than mefenamic acid.

Results and Discussion

Validation of the In Silico Testing Method

Docking method validation was performed through redocking of the native ligand (mefenamic acid) against the COX-2 receptor (5IKR) in three replicates. The results showed RMSD values of 0.136 Å; 0.135 Å; and 0.136 Å (Table 1), all of which were below the 2 Å thresholds, so the docking method was declared valid and usable for the subsequent docking stage [cited based on Ruswanto's criteria, 2015].

Table 1. RMSD values from the native ligand (mefenamic acid) validation test on the COX-2 protein (5IKR)

Replicate	RMSD (Å)	S Value (kcal/mol)
1	0.136	-7.837
2	0.135	-7.879
3	0.136	-7.837

In Silico Testing of Mefenamic Acid and the MFT Compound

In silico testing of the MFT compound and mefenamic acid on the COX-2 receptor showed that the MFT compound had a lower binding energy value (S) than mefenamic acid, indicating better binding affinity for the target receptor. A lower binding energy value correlates with higher ligand-receptor complex stability, so the MFT compound potentially has analgesic activity equivalent to or better than mefenamic acid in silico.

ADMET Profile

Prediction of absorption, distribution, metabolism, excretion, and toxicity (ADMET) of the MFT compound showed a good profile and no indication of toxicity (Table 2). Mefenamic acid the reference compound also has a good ADMET. MFT compound The LD₅₀ value and the absence of indications of hepatotoxicity or CYP2D6 metabolism inhibition support the feasibility of further developing this compound as a drug candidate.

Table 2. ADMET profile of the N-benzoyl-N'-4-methoxyphenylthiourea compound

Parameter	Value
Water solubility	-4.176 mol/L
Gastrointestinal solubility (%)	93.219 %
Blood-brain barrier (BBB) permeability	0.127
CNS activity	-2.059
CYP2D6 metabolism inhibitor	No
Potential excretion issues	No
LD50	2.495 mk/kg
Hepatotoxicity	No

Compound Interaction with the COX-2 Receptor

The interactions of the MFT compound and mefenamic acid with the COX-2 receptor showed a similar binding pattern, namely conventional hydrogen bonds at the Tyr 385 and Ser 530 residues, as well as van der Waals, alkyl, and pi-alkyl interactions at the Val 349, Ala 527, and Leu 352 residues. The similarity of this interaction pattern supports the notion that the MFT compound binds to the same active site as mefenamic acid on the COX-2 receptor, potentially providing an analgesic effect through a comparable inhibition mechanism.

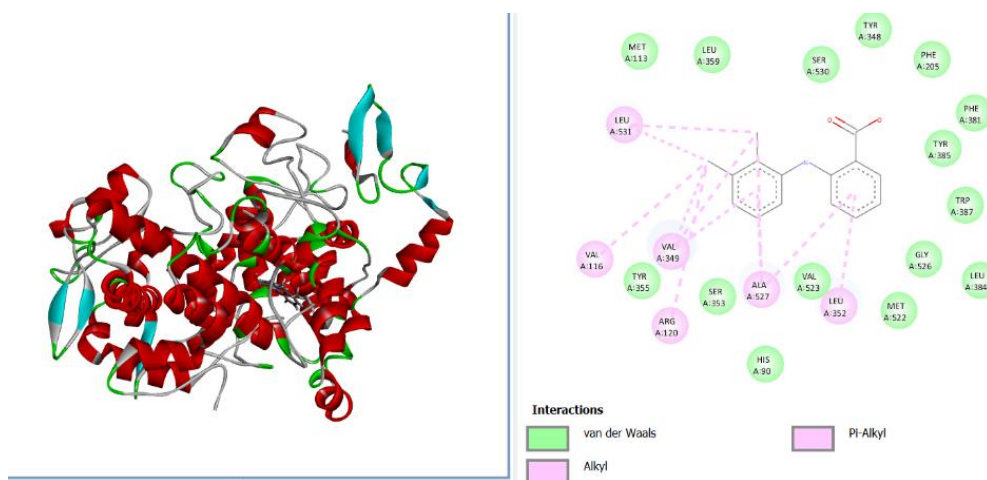


Figure 1. Interaction of MFT compound with COX-2 receptor

Synthesis Results and Structure Confirmation

The synthesized MFT compound was obtained as a fine, odorless white powder, with a yield of 34.80%.

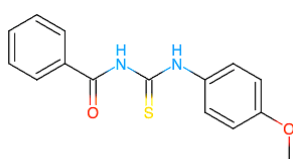


Figure 2. Compounds resulting from MFT synthesis

Purity testing showed a single spot on thin layer chromatography (TLC) and a consistent melting point of 171-173°C across three replicates (Table 3), indicating that the synthesized compound was pure.

Table 3. Melting point of the synthesized MFT compound

Compound	Replicate 1	Replicate 2	Replicate 3
MFT	172°C	172°C	172°C

The UV spectrum of the MFT compound in ethanol solvent showed three absorption peaks at wavelengths of 206 nm, 237 nm, and 271 nm.

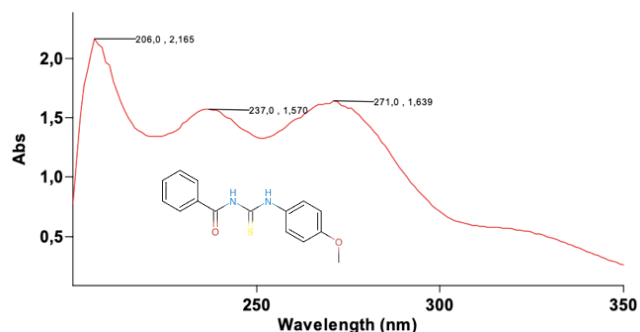


Figure 3. UV Spectrum of MFT compound in ethanol water

The IR spectrum showed absorption bands consistent with the theoretical functional groups of the target compound (Table 4), including the secondary -NH, imide -C=O, and -C-S groups that characterize the substituted thiourea structure.

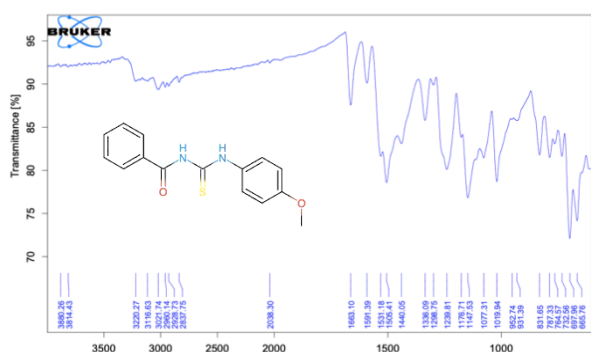


Figure 4. IR Spectrum of MFT compound

The ^1H -NMR spectrum showed a total of 14 hydrogen atoms, comprising two singlet signals at 12.45 and 11.50 ppm (1 H atom each from the NH groups), a doublet signal at 7.98-7.96 ppm (2 H atoms, benzoyl ortho position), a triplet at 7.68-7.64 ppm (1 H atom, benzoyl para position), a multiplet at 7.57-7.52 ppm (4 H atoms, combined benzoyl meta position and amino-benzene ortho position), a doublet at 6.99-6.96 ppm (2 H atoms, amino-benzene meta position), and a singlet at 3.90 ppm (3 H atoms, methoxy group) (Pavia et al, 2009).

Table 4. Infrared (IR) spectrum interpretation of the MFT compound

Functional Group	Theoretical Wavenumber (cm ⁻¹)	Synthesis Result (cm ⁻¹)
-NH secondary	3500-3100	3220
-C-H stretching	3000-2850	2960
-C=O imide	1700-1640	1663
-NH bend secondary amine	1640-1550	1531
-C=C aromatic	1600-1475	1591 and 1336
-C-N	1350-1000	1147
-C-S	1175-750	831 and 787
-C-O	1300-1000	1019
-CH ₃	1450 and 1375	1440

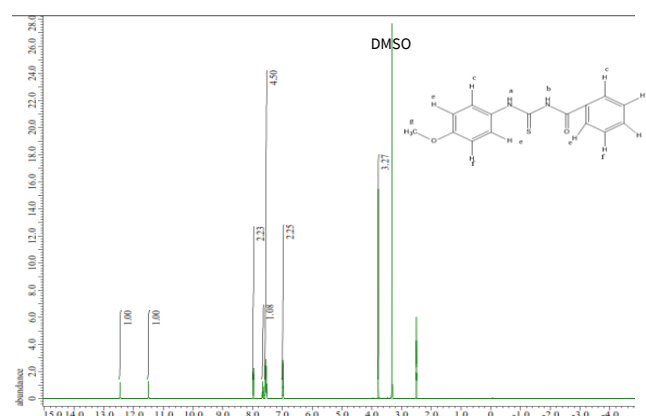


Figure 5. ^1H -NMR Spectrum of MFT compound

The ^{13}C -NMR spectrum showed 15 carbon signals, with the thiourea carbonyl signal at a chemical shift of 179.69 ppm and the benzoyl carbonyl at 168.77 ppm as the main markers of the target structure.

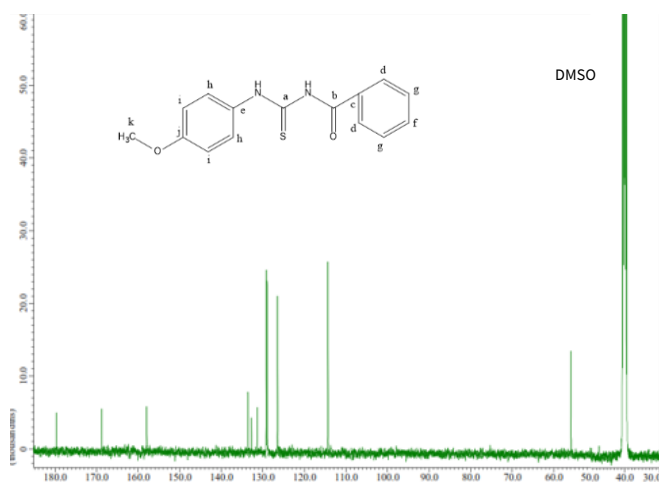


Figure 6. ^{13}C -NMR Spectrum of MFT compound

GC-MS analysis showed two fragments with molecular masses of 121 and 165, giving a total observed molecular mass of 286.00, close to the theoretical molecular mass of the compound $\text{C}_{15}\text{H}_{14}\text{N}_2\text{O}_2\text{S}$ of 286.24.

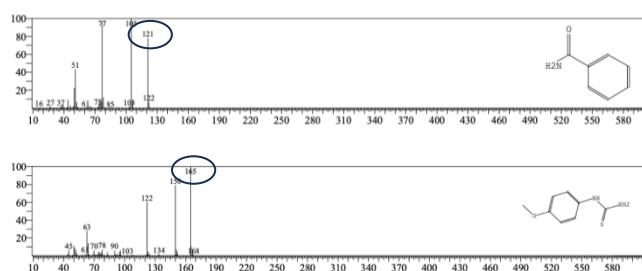


Figure 6. GC-MS Spectrum of MFT compound

The UV, IR, ^1H -NMR, ^{13}C -NMR, and GC-MS spectroscopic data consistently confirm that the synthesized compound is N-benzoyl-N'-4-methoxyphenylthiourea, in accordance with the expected structure.

Analgesic Activity Test

Analgesic activity testing was performed using the writhing test method in mice induced with acetic acid, compared against a negative control (0.5% CMC-Na) and a positive control (mefenamic acid) at doses of 25 and 100 mg/kgBW (Table 5). The mean percentage of pain inhibition (PPI) for the mefenamic acid group was 42.22% (25 mg/kgBW dose) and 64.45% (100 mg/kgBW dose), while for the MFT group it was 34.22% (25 mg/kgBW dose) and 68.44% (100 mg/kgBW dose).

Table 5. Percentage of pain inhibition (PPI) of the MFT compound and mefenamic acid

Group	Dose (mg/kgBW)	Mean PPI (%)	SD
Negative control (0.5% CMC-Na)	-	-	-
Mefenamic acid	25	42.22	2.55
Mefenamic acid	100	64.45	1.87
MFT	25	34.22	3.36
MFT	100	68.44	2.77

The homogeneity test showed a Levene statistic of 1.215 with a significance of 0.333 ($p > 0.05$), so the variance between groups was declared homogeneous and the analysis was continued with a one-way ANOVA test. The ANOVA results at a 95% confidence level showed a significance value of 0.000 ($p < 0.05$) at both the 25 and 100 mg/kgBW doses, indicating a meaningful difference in mean PPI between groups. The Post Hoc Least Significant Difference (LSD) test showed that at a dose of 25 mg/kgBW, the analgesic activity of MFT was not significantly different from mefenamic acid ($p > 0.05$), whereas at a dose of 100 mg/kgBW, the analgesic activity of the two differed significantly ($p < 0.05$). These findings show that at a low dose (25 mg/kgBW), the MFT compound has analgesic activity equivalent to mefenamic acid, in line with the *in silico* results showing good binding affinity of MFT for the COX-2 receptor. This result is consistent with reports that modification of the benzoylurea/benzoylthiourea structure can increase pharmacological activity compared to the parent compound [6,10,20]. At higher doses, the significant difference in activity indicates that the dose-response relationship of MFT differs from that of mefenamic acid, so further dose optimization and pharmacokinetic studies are needed in the next development stage, including acute toxicity testing, anti-inflammatory testing, and molecular dynamics simulation. From the percentage value of pain inhibition obtained in the analgesic activity test (which is calculated based on the number of mice writhing), the ED₅₀ value of the N-benzoyl-N'-4-methoxyphenyl compound and its three derivatives

can be calculated using probit analysis using the SPSS computer program.

Table 6. Value of ED₅₀ and Log 1/ED₅₀ MFT Compound

Compound	Value of ED ₅₀	Log 1/ED ₅₀
MFT	59.58 mg	-1.77
Mefenamic acid	51.25 mg	-1.70

Conclusion

The compound N-benzoyl-N'-4-methoxyphenylthiourea was successfully obtained through in silico tests which showed a higher binding affinity than mefenamic acid to the COX-2 receptor, and was successfully synthesized with purity and structure confirmed through UV, IR, 1H-NMR, 13C-NMR, and GC-MS spectrophotometry. In vivo analgesic activity tests in mice (*Mus musculus*) showed that this compound had analgesic activity at a dose of 25 mg/kgBW equivalent to mefenamic acid and at a dose of 100 mg/kgBW significantly higher than mefenamic acid.

Materials and Methods

This study is a laboratory experimental study of the N-benzoyl-N'-4-methoxyphenylthiourea compound, consisting of three stages, namely: (1) in silico testing, (2) synthesis and structure confirmation, and (3) in vivo analgesic activity testing. The study was conducted at the Chemistry Laboratory of the Institute of Technology, Science and Health, dr. Soepraoen Hospital, Malang and Pharmacology Laboratory of the University of Muhammadiyah Malang. The use of mice (*Mus musculus*) as test animals in the analgesic activity test followed the research ethical clearance (E.5.a/058/KEPK-UMM/III/2024) issued by the institution's research ethics authority.

Materials and Equipment

The materials used included 4-methoxyphenylthiourea (Aldrich); p.s. grade benzoyl chloride and its derivatives, namely 3-trifluoromethyl benzoyl chloride, 4-trifluoromethyl benzoyl chloride, 4-bromo benzoyl chloride, and tert-butyl benzoyl

chloride (Aldrich); p.a. grade tetrahydrofuran, pyridine, sodium bicarbonate, chloroform, ethyl acetate, n-hexane, methanol, and acetone (Merck); glacial acetic acid (Aldrich); distilled water; and white mice (*Mus musculus*) as test animals. The equipment used included a reflux apparatus set, an analytical balance, a magnetic stirrer with heater, and a personal computer with ChemDraw 20.0 and Molecular Operating Environment (MOE) software for in silico testing.

In Silico Testing

In silico testing was performed using the MOE program to determine the interaction energy (S value) between the test compound and the receptor. The first stage was the preparation of 20 derivative compounds in .sdf format obtained from the MOE database. The second stage was preparation of the COX-2 receptor with code 5IKR from the Protein Data Bank (<http://www.rcsb.org>), selected because it has a resolution of 2.34 Å and a stable three-dimensional structure. The third stage was internal validation of the docking method through redocking of the native ligand (mefenamic acid) against the receptor, with an acceptance criterion of RMSD value ≤ 2 Å. The fourth stage was docking of mefenamic acid and the MFT compound along with its derivatives after each compound was optimized to its most stable conformation. The ADMET profile was also analyzed for the selected compound. The MFT compound was chosen because it has as much as the parent compound and mefenamic acid as a comparison because it has similar ligands.

Synthesis

Synthesis was carried out through a nucleophilic acyl substitution reaction on the free amine group of 4-methoxyphenylthiourea with benzoyl chloride in tetrahydrofuran solvent under basic conditions using pyridine, referring to a modified Schotten-Baumann reaction. The purity of the synthesized compound was tested by thin layer chromatography (TLC) using several mobile phases of different polarity, and its melting point was determined.

Structure Confirmation

Structure confirmation of the synthesized compound was carried out using ultraviolet (UV) spectrophotometry, infrared (IR) spectrophotometry, proton nuclear magnetic resonance spectrometry (¹H-NMR) and carbon (¹³C-NMR), as well as gas chromatography-mass spectrometry (GC-MS).

Analgesic Activity Test

Analgesic activity testing was performed in vivo in white mice (*Mus musculus*) using the writhing test method through chemical pain induction with acetic acid solution administered intraperitoneally. This method was chosen because it is reproducible, simple, and easy to observe. Testing was carried out on three groups, namely the test group (synthesized compound), the comparator group (mefenamic acid at doses of 25 and 100 mg/kgBW), this dose was chosen because it is below the effective dose of 50 and above the effective dose of 50 and the negative control group (0.5% CMC-Na suspension), each with five replicates. The percentage of pain inhibition (PPI) was calculated using the formula:

$$\text{PPI} = \left[\frac{\text{number of writhes, negative control} - \text{number of writhes, test compound}}{\text{number of writhes, negative control}} \right] \times 100\%$$

Writhing frequency data were analyzed using a one-way ANOVA statistical test with SPSS software to determine differences in analgesic activity between groups, followed by a Post Hoc Least Significant Difference (LSD) test where applicable. The effective dose value (ED₅₀), i.e. the dose producing a 50% effect, was obtained by substitution into the linear regression equation for the relationship between log dose and % PPI for each compound.

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