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Original Article

Synthesis and *in vitro* activity tests of N-benzoyl-N'-phenylthiourea derivatives as macrophage migration inhibitory factor

[Síntesis y pruebas de actividad *in vitro* de derivados de N-benzoil-N'-feniltiourea como factor inhibidor de la migración de macrófagos]

Dini Kesuma[1*], Galih S. Putra[2], Tegar A. Yuniarta[1], Farida Suhud[1], I G.A. Sumartha[1], Sawitri Boengas[3], Melanny I. Sulistyowaty[4], Tjie Kok[5]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Surabaya, Surabaya, Indonesia.
[2]Department of Chemistry, Faculty of Mathematics and Natural Sciences, State University of Malang, Malang Indonesia.
[3]Faculty of Medicine, University of Surabaya, Surabaya, Indonesia.
[4]Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya 60115, Indonesia.
[5] Faculty of Biotechnology, University of Surabaya, Surabaya, Indonesia.
*E-mail: dini_kesuma@staff.ubaya.ac.id

Abstract

Context: The COVID-19 pandemic in 2020 resulted in widespread mortalities due to cytokine storms in the affected patients. Macrophage migration inhibitory factor (MIF) is one of the most interesting targets in developing anti-COVID-19 drugs. Some thiourea compounds have been identified as having potential as MIF inhibitors.

Aims: To investigate MIF inhibitory activity of N-benzoyl-N'-phenylthiourea derivatives.

Methods: The study consists of in-silico activity prediction of designed compounds using a molecular docking approach against MIF protein (PDB ID: 1LJT). Afterwards, the designed compounds were synthesized and tested *in vitro* using the tautomerase activity approach.

Results: The molecular docking study showed that all designed compounds possess comparable docking scores to the native ligand of the protein. MIF Assay performed on compounds (1) and (2) indicated a decrease in tautomerase activity of the MIF target protein of only 10.1 and 6.2%, respectively, compared to the positive control.

Conclusions: *In silico* results predicted better bioactivity against MIF protein, but the result does not translate to the *in vitro* assay, where two of the designed compounds possess only low inhibitory activity.

Keywords: 1LJT; MIF assay; tautomerase activity; thiourea derivatives.

Resumen

Contexto: La pandemia de COVID-19 en 2020 provocó mortalidades generalizadas debido a las tormentas de citocinas en los pacientes afectados. El factor inhibidor de la migración de macrófagos (MIF) es una de las dianas más interesantes en el desarrollo de fármacos anti-COVID-19. Se han identificado algunos compuestos de tiourea con potencial como inhibidores de MIF.

Objetivos: Investigar la actividad inhibidora de MIF de derivados de N-benzoil-N'-feniltiourea.

Métodos: El estudio consiste en la predicción *in silico* de la actividad de los compuestos diseñados utilizando un enfoque de acoplamiento molecular frente a la proteína MIF (PDB ID: 1LJT). Posteriormente, los compuestos diseñados se sintetizaron y probaron *in vitro* mediante el método de actividad tautomerasa.

Resultados: El estudio de acoplamiento molecular mostró que todos los compuestos diseñados poseen puntuaciones de acoplamiento comparables al ligando nativo de la proteína. El ensayo MIF realizado con los compuestos (1) y (2) indicó una disminución de la actividad tautomerasa de la proteína diana MIF de sólo el 10,1 y el 6,2%, respectivamente, en comparación con el control positivo.

Conclusiones: Los resultados *in silico* predijeron una mejor bioactividad frente a la proteína MIF, pero el resultado no se traslada al ensayo *in vitro*, donde dos de los compuestos diseñados sólo poseen una baja actividad inhibitoria.

Palabras Clave: actividad tautomerasa; derivados tiourea; ensayo MIF; 1LJT.

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AUTHOR INFO
ORCID:
0000-0002-6612-372X (DK)
0000-0003-4192-3621 (GSP)
0000-0002-5326-7636 (TAY)
0000-0002-6950-3795 (IGAS)
0000-0002-9510-6822 (MIS)

INTRODUCTION

The COVID-19 virus pandemic at the end of 2019 still holds many scientific mysteries. The high morbidity and mortality rates of COVID-19 patients have significantly increased due to the presence of the biomarker Macrophage Migration factor (MMF) (Bleilevens et al., 2021). MMF has become one of the indicators of cytokine storms in patients and has a high potential to predict critical conditions of COVID-19 patients and acute respiratory distress syndrome (ARDS) (Aslan et al., 2021). One of the strategies employed by many researchers worldwide is to reduce the levels of MMF in plasma using drugs of the macrophage migration inhibitory factor (MIF) group.

Until now, a specific drug to inhibit MIF (Macrophage migration inhibitory factor) has not been found. The therapy options used in COVID-19 patients with ARDS status due to cytokine storms are the administration of neuromuscular blocking agents and high-dose corticosteroids based on WHO guidelines (NIH, 2021; WHO, 2020). The use of antivirals (lopinavir-ritonavir, remdesivir) in ARDS conditions is not recommended as it will worsen the patient's condition (NIH, 2021; WHO, 2020). One of the steps intended to highlight the management of intensive care unit patients with COVID-19-related ARDS is the use of high-dose corticosteroids and interleukin-6 receptor inhibitors (Tocilizumab) to overcome cytokine storm conditions (Aslan et al., 2021).

MMF is a process that initiates cytokine storms (Donnelly et al., 1997; Gao et al., 2007). Based on statistical data, the use of drugs that lower MIF can reduce morbidity and mortality rates in COVID-19 patients (Bleilevens et al., 2021). Currently, no drug has been found with a mechanism specifically targeting MIF. The use of immunomodulators through suppress inflammatory reaction mechanisms such as corticosteroids (dexamethasone and methylprednisolone), interleukin-6 receptor inhibitors prevent cytokine storm and dampen the inflammatory response (tocilizumab), and JAK inhibitors, prevent cytokine storm and dampen the inflammatory response (baricitinib and ruxolitinib) can reduce mortality rates by reducing cytokine storms through indirect mechanisms, not direct mechanisms through MIF (Bleilevens et al., 2021). Drugs that inhibit the direct mechanism through MIF are mostly still in the prototype development stage. Development has been ongoing for the last two decades, not aimed at addressing cytokine storms in COVID-19 patients but for anti-inflammatory purposes (Donnelly et al., 1997; Gao et al., 2007; Turk et al., 2010). The COVID-19 pandemic has prompted researchers to re-examine the development of MIF drugs.

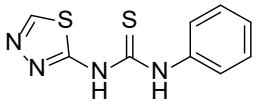
One compound that has strong potential for development as a MIF drug is N-phenyl-N-1,3,4-thiadiazol-2-yl-thiourea, which has excellent *in vitro* activity with an IC_{50} value of 0.3 µM, and N-(2-chlorophenyl)-N'-2-pyridinylthiourea with an IC_{50} value of 1.04 µM (Fig. 1). Thiourea compounds can be used as lead compounds to find and develop other more potent MIF drugs. In our previous research, we successfully synthesized N-benzoyl-N'-phenylthiourea derivatives, which were investigated as anticancer agents for T47D, MCF-7, and HeLa cell lines through *in silico* and *in vitro* testing (Kesuma et al., 2020; 2022a; 2022b; 2022c; 2023). This study aimed to test the N-benzoyl-N'-phenylthiourea derivative compounds for their ability to inhibit macrophages (MIF), preceded by an *in silico* test comparing the N-benzoyl-N'-phenylthiourea derivative compound to the native ligand (GDP: 1LJT).

MATERIAL AND METHODS

Tools and material

Tools for synthesis and structure analysis: glassware for synthesis, Sartorius Scales AG Gontingen, Germany, Corning Hot Plate P351, Fisher-John Electrothermal Mel-Temp. Spectrophotometer Jasco FT-IR 5300, Spectrometers ^{1}H-NMR and ^{13}C-NMR Agilent, Waters Mass Spectrometer

Ingredients for synthesis included: Feniltiourea (Merck); 3,4-dichlorobenzoylchloride; 2,4-dichlorobenzoyl chloride; (Sigma Aldrick); THF (Merck); distiller water, TEA (Merck), ethanol 70% (Merck); n-hexane (Merck); ethyl acetate (Merck); chloroform (Merck); acetone (E. Merck), Whatman 41 filter paper and KLT plate of 60 GF254 silica gel (Merck).



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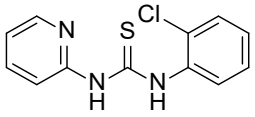

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Figure 1. Thiourea derivatives as macrophage migration inhibitory factor (MIF).
Compound 1: N-Phenyl-N'-1,3,4-thiadiazol-2-ylthiourea; Compound 2: N-(2-Chlorophenyl)-N'-2-pyridinylthiourea.

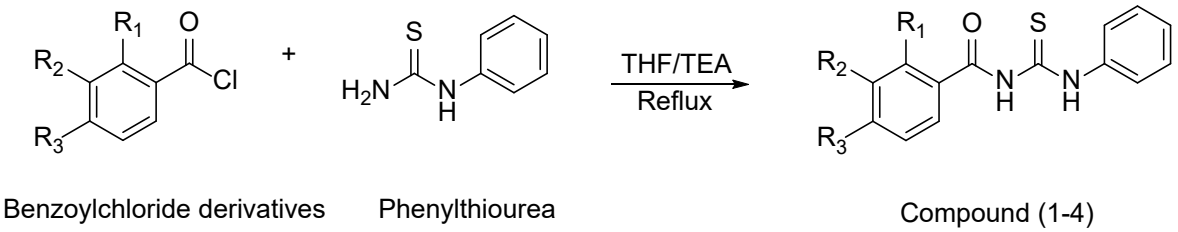


Figure 2. Synthesis of compounds (1-4).

Compound 1: R_1 = H; R_2 = Cl; R_3 = Cl. Compound 2: R_1 = Cl; R_2 = H; R_3 = Cl. Compound 3: R_1 = H; R_2 = H; R_3 = CN. Compound 4: R_1 = H; R_2 = H; R_3 = NO_2.

Molecular modeling was used in Windows 11 operating system-based Intel Core i5 processor computers with the program ChemBioDraw Ultra 15.0, MVD (Molegro Virtual Docker) version 5.5.

In silico study (molecular docking)

Prediction of activity by molecular modeling of N-benzoyl-N'-feniltiourea derivatives through docking with MIF with coded PDB ID: 1LJT downloaded from protein data bank (https://www.wwpdb.org/) (Lubetsky et al., 2002). MVD 5.5 software was used in the *in silico* process.

Derivative synthesis of N-benzoyl-N'-phenylthiourea

In a round-bed flask, phenylthiourea (1 mol) was dissolved in THF and catalyzed with TEA and then added benzoyl chloride derivatives (1 mol) little by little at 0°C, then refluxed at 80°C for two hours (Fig. 2). To monitor the perfection of the reaction, thin-layer chromatography was performed using 1:1 hexane/ethyl acetate eluent. After a perfect reaction characterized by the loss of phenylthiourea stains and benzoyl chloride derivatives in the TLC process, the reaction results were evaporated by rotavapor to remove THF until crystals were formed, the crystals obtained were treated in the recrystallization process (Kesuma et al., 2020).

The purity test was carried out by determining the melting point and thin layer chromatography (TLC) with three eluent systems. The structures were identified using FT-IR, UV-Vis, ^{1}H-NMR, ^{13}C-NMR, and MS spectroscopy.

MIF activity test

Production of MIF proteins

Luria Bertani (LB) media preparation -ampicillin agar: The process of making LB-ampicillin agar was started by dissolving 20 g of LB media into 500 ml of purified water. Then, the medium was stirred until homogeneous and sealed tightly using cotton and coated with aluminum foil. The medium was sterilized using an autoclave with a temperature of 121°C for 20 min with a pressure of 1 atm. The medium was left to warm the nails, added ampicillin until reaching a final concentration of 100 µg/mL, and then poured into sterile Petri dishes. The media was left to form agar and then stored in a cold room (Kok et al., 2018).

Media preparation 2×YT broth: The preparation of 2×YT broth media was started by mixing tryptone 10 g, yeast 5 g, and NaCl 2.5 g into 500 mL of purified water at Erlenmeyer 1 L. The medium was stirred until homogeneous and pH 7.0. Erlenmeyer was covered with cotton and coated with aluminum foil and then sterilized with an autoclave at 121°C for 20 min with a pressure of 1 atm. After autoclaving, the medium was left to warm the nails to add 500 µL of 0.1 g/mL ampicillin antibiotic to obtain a final 100 µg/mL concentration. Then, the media was stored in the cold room (Kok et al., 2018).

E. coli BL21 cell rejuvenation (DE3) pET20b (+)-MIF

One swab inoculum of glycerol stock culture into 2×YT broth medium in a small culture tube was performed. A small culture was incubated using an incubator shaker at 37°C for 18 h at 175 rpm. Then, one swab of small culture was inoculated on LB-Amp media and incubated at 37°C for 18 h. Finally, the cells were stored in a cold room (Kok et al., 2018).

Preparation of *E. coli* BL21(DE3) pET20b (+) -MIF cells

It was observed in a small culture to see bacterial growth. The medium was turbid if the bacteria grew successfully. If bacterial growth was observed, 5 mL of 2×YT broth medium was transferred into 500 mL of 2×YT broth medium aseptically. Then, it was incubated with an incubator shaker for 3 h at 37°C with a speed of 175 rpm until it reached a value of OD600 0.5-0.6. If the value of OD600 has been reached, the culture was induced using 31.5 µL of IPTG 800 nM until a final concentration of 0.05 mM was obtained. Finally, the culture was incubated overnight with an

incubator shaker at 20°C with a speed of 175 rpm (Kok et al., 2018).

E. coli BL21 cell harvesting (DE3) pET20b (+)-MIF

E. coli BL21(DE3) pET20b (+)-MIF cultures that IPTG induced were introduced into centrifugation tubes. Then, centrifugation was carried out at 4,500 rpm for 15 min at 4°C. Centrifugation was repeated until the entire culture medium had been centrifuged. The supernatant was removed, and the pellet was washed with 25 mL of 0.9% NaCl three times using a 4°C centrifuge at 4500 rpm for 15 min. The pellet was weighed and immersed in pH 7.4 Tris-HCl washing buffer (Tris 50 mM, 10% glycerol, and purified water) at a ratio of 2:3 (pellet:buffer) for overnight at 4°C (Kok et al., 2018).

Isolation of MIF proteins from cultures

Sonication was conducted at 20 kHz with 30% amplitude for 10 cycles in cold conditions. One cycle ran for 15 seconds and then was given a 45 s pause. Then, the mixture was centrifuged at 4°C with a speed of 13,000 rpm for 1 h. The supernatant was taken and stored at -80°C (Kok et al., 2018).

Purification of MIF proteins by affinity chromatography

0.5 mL of homogenized resin was prepared in an affinity chromatography column, to which were added ethanol solvent and washing buffer up to five times. Then, MIF protein isolation samples can be put into a resin column that has been perfectly packed. After mixing, it was homogenized with a rotator at a speed of 10 rpm at a temperature of 4°C overnight.

The process is continued by quieting the resin first until the resin was packed before flowing through the flowthrough phase. Then, 25 mL of washing buffer were added slowly, and eluted MIF protein by adding 0.5 mL of pH 7.4 elution buffer (Tris-HCl 50 mM, glycerol 50%, imidazole 500 mM, and purified water) in increments of four times. The fraction results were spliced into 1.5 mL microtubes and stored in a -80°C freezer (Kok et al., 2018).

Analysis of MIF protein levels by Bradford method

25 µL of the MIF protein sample was reacted with 1.25 mL of Bradford reagent. It was then incubated at room temperature for 10 min. Then, the solution was read out at 595 nm. Finally, the protein content by entering the absorbance value into the standard curve was calculated (Kruger, 2002)

SDS PAGE

Sample preparation

The preparation of fraction 1 was carried out by mixing 5 µL of protein samples from the elution of the first fraction with 10 µL of loading buffer 2×. Then, for fraction 2, 15 µL of the second fraction elution protein sample with 5 µL of 4× loading buffer were mixed. For fractions 3 and 4, 25 µL of protein sample with 5 µL of 4× loading buffer were mixed. Finally, the mixture was heated for 5 min before being stored in the polyacrylamide gel (Kok et al., 2018).

Preparation of polyacrylamide separating and stacking gel

The preparation of the separating gel was carried out by mixing 2.25 mL of acrylamide/bis-acrylamide 30%; 0.9375 mL of Tris-HCl (1 M; pH 8.8); 0.5075 mL of purified water; 37 µL of SDS 10%; 1.875 µL of TEMED, and 18.75 µL of APS 10% in sequence and each addition of solution, Erlenmeyer was shaken slowly. After the whole was added, it was immediately poured into the plate. Then, purified water was added on top of the gel solution so that the gel surface became flat. The purified water could be removed when the gel had solidified.

The preparation of gel stacking was carried out by mixing 0.225 mL of acrylamide/bis-acrylamide 30%; 0.19 mL of Tris-HCl (1 M; pH 6.8); 1.055 mL of purified water, 15 µL of SDS 10%; 2.5 µL of TEMED, and 15 µL of APS 10% in sequence and each addition of solution, Erlenmeyer was shaken slowly. The mixture was poured on a gel separator that had solidified on a plate, then placed on a gel comb and allowed to solidify (Kok et al., 2018).

Preparation of polyacrylamide separating and stacking gel

Sample running

Plate containing gel that had been pulled from the gel comb was placed into the electrophoresis chamber, then poured running buffer pH 8.3 until the entire gel was submerged. Then, the sample was put into the well, and electrophoresis was performed at a constant current of 80 V until the tracking dye reached a distance of ± 0.5 cm from the gel base. When the electrophoresis process had been completed, the gel was taken, and gel staining was carried out (Kok et al., 2018).

Polyacrylamide gel staining

Prepare a *staining* solution by mixing 1 g of Coomasie Brilliant Blue G-250; 450 ml of purified water; 450 mL of methanol, and 100 mL of glacial acetic acid. The destaining solution was discharged by mixing

100 mL of glacial acetic acid, 100 mL of methanol, and 800 mL of aqua. The gel coloring process was carried out by immersing the gel in a staining solution and placed on the shaker for 15 min. The staining solution could be poured into separate containers, and the gel washed with water several times. Finally, the gel was soaked in destaining solution and placed on the shaker for 30 min until the blue ribbon was clearly visible (Kok et al., 2018).

Inhibition test on MIF tautomerase activity

Sample preparation ready for test

The inhibitor candidate sample was dissolved in DMSO according to the required concentration and, if necessary, diluted with serial dilution using DMSO on microtube (Xio et al., 2020)

4-HPP substrate preparation

The substrate was prepared with a concentration of 10 mM, incubated at room temperature for 24 h and stored at 4°C. Before testing, 4-HPP stock was diluted to a concentration of 1 mM with ammonium acetate pH 6 and incubated at room temperature for 24 h (Xio et al., 2020).

Negative control preparation, MIF inhibitor positive control, and MIF positive control

The negative control used was 45 µL of MIF protein diluted to 1000× in boric acid and reacted with 4-HPPsubstrate. The positive control of the MIF inhibitor was carried out by mixing MIF protein with 1 MM boric acid and 4-HPPsubstrate. The positive control of the MIF inhibitor was carried out by reacting MIF with 2 µM Cu^{2+} solution and 4-HPPsubstrate (Xio et al., 2020).

Absorption reading with microplate-reader

The software was set to a temperature of 35°C, a wavelength of 306 nm, and 20 cycles by adjusting the cycle time. The first cycle was carried out by shaking at a speed of 300 rpm for 2 seconds. The reading process was carried out by mixing 50 µL of the sample (5 µL of the sample that had been dissolved with DMSO + 45 µL of MIF protein 1000× dilution) and 50 µL of 4-HPP just before the absorption reading. The absorption readings were observed with current state view (Xio et al., 2020).

Statistical analysis

In this research, the synthesis, molecular docking, and MIF assay results were subjected to three experimental repetitions (n = 3). Accuracy calculations were derived from the averages, while precision was determined from the standard deviation (SD) values.

RESULTS AND DISCUSSION

Molecular docking study

The grid box binding site was set X = -40.67 Å; Y = 40.65 Å; Z= 7.51 Å with a cavity 183 Å3 surrounded by 12 amino acids, namely Pro 1; Met 2; Lys 32; Tyr 36; His 62; Ile 64; Tyr 95; Asn 97; Met 101; Val 106; Phe 113. The result of the redocking validation process with RMSD 0.73Å is shown in Fig. 3. Hence, these results indicate that the method is valid for the docking test of the tested compound since the RMSD obtained is less than 2 Å.

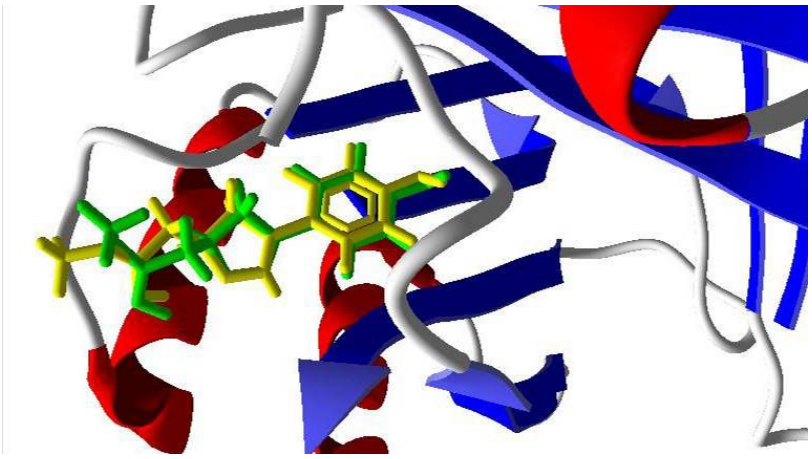
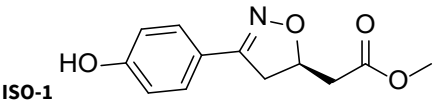
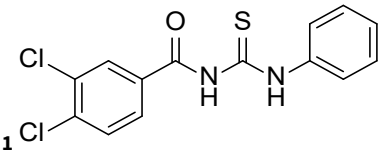
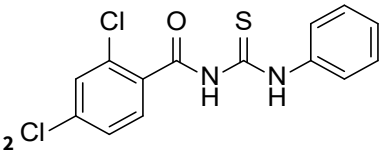
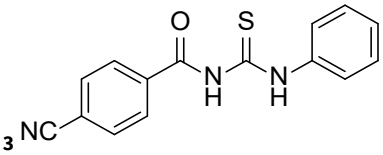
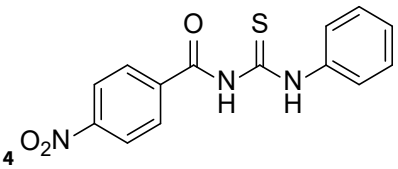


Figure 3. Comparison of the native ligand (ISO-1) (yellow) with the docking results from 193 simulations (green) by Molegro Virtual Docker (MVD) ver.5.5 with RMSD is 0.73 Å.

Table 1. The Molecular docking result of ISO-1 and compounds 1-4 into active site macrophage migration inhibitory factor (MIF).

Compound	Moldock score (Kcal/mol) (n = 3)	Hydrogen bond	Steric interaction
 ISO-1	-127.57 ± 0.35	Pro 1, Lys 32, Asn 97	Pro 1, Lys 32, Asn 97
 1	-127.08 ± 0.13	Lys 32, Ser 63, Ile 64	Lys 32, Tyr 36, His 62, Ser 63, Ile 64
 2	-121.88 ± 0.10	Lys 32, Ser 63, Ile 64	Lys 32, Ser 63, Ile 64, Tyr 95
 3	-127.24 ± 0.15	Lys 32, Asn 97	Lys 32, Ile 64, Asn 97
 4	-133.52 ± 0.44	Lys 32, Ser 63, Ile 64, Asn 97	Lys 32, Tyr 36, Ser 63, Ile 64, Asn 97, Val 106

Based on the molecular docking results, it seems that ISO-1 (the native ligand) has a strong interaction with MIF, as indicated by the Moldock score of -127.57 Kcal/mol. This is due to the presence of 3 hydrogen bonds and 3 steric interactions, which occur on the same amino acids, namely Pro 1, Lys 32, and Asn 97. It is interesting to note that the hydrogen bond between the phenolic -OH group of ISO-1 and the Asn 97 amino acid is particularly important, as it acts as both a hydrogen donor and acceptor and has a distance of 2.6 Å. In addition, Asn 97 also provides the most significant steric interaction compared to the other amino acids, Pro 1 and Lys 32.

Compound 4 was found to have a lower Moldock score compared to the native ligand, ISO-1. It is thus predicted to have a stronger potential for inhibiting MIF. The presence of the nitrobenzene group (-NO_2) in compound 4 allows for the formation of two hydrogen bonds with the amino acid Asn 97, which is predicted to have a significant impact. Additionally, compound 4 has two hydrogen bonds that are not present in ISO-1, with the amino acids Ser 63 and Ile 64, as well as four steric interactions with the amino acids Tyr 36, Ser 63, Ile 64, and Val 106, which are also not present in ISO-1.

Compounds 1 and 2 have higher moldock scores than ISO-1 since the halobenzene groups (dichlorobenzene) are neither hydrogen donor nor acceptor substituents, so they could not form hydrogen bond interactions with Asn 97. Compound 3, on the other hand, has a cyano group (-CN) that is still a hydrogen acceptor but weaker than the nitrobenzene group, so compound 3 only has hydrogen bond interactions with Asn 96 amino acid with a bond distance of 2.9 Å, which is not as good as the -OH group on ISO-4 and the -NO_2 group on compound 4. These are shown in Table 1 and Fig. 4.

Compounds resulted from synthesis

N-Benzoyl-N'-phenylthiourea derivatives have been successfully synthesized with varying yields. The reaction mechanism involves a nucleophilic attack from the free electron pair of the (-NH_2) group on the phenylthiourea compound to the carbonyl (C=O) group of the benzoyl chloride derivatives compound (see Fig. 5 and Supplementary data). The presence of an electron-donating group (-Cl) on the benzene ring of benzoyl chloride increases the electron density on the benzene ring. The benzene ring, which is δ- and

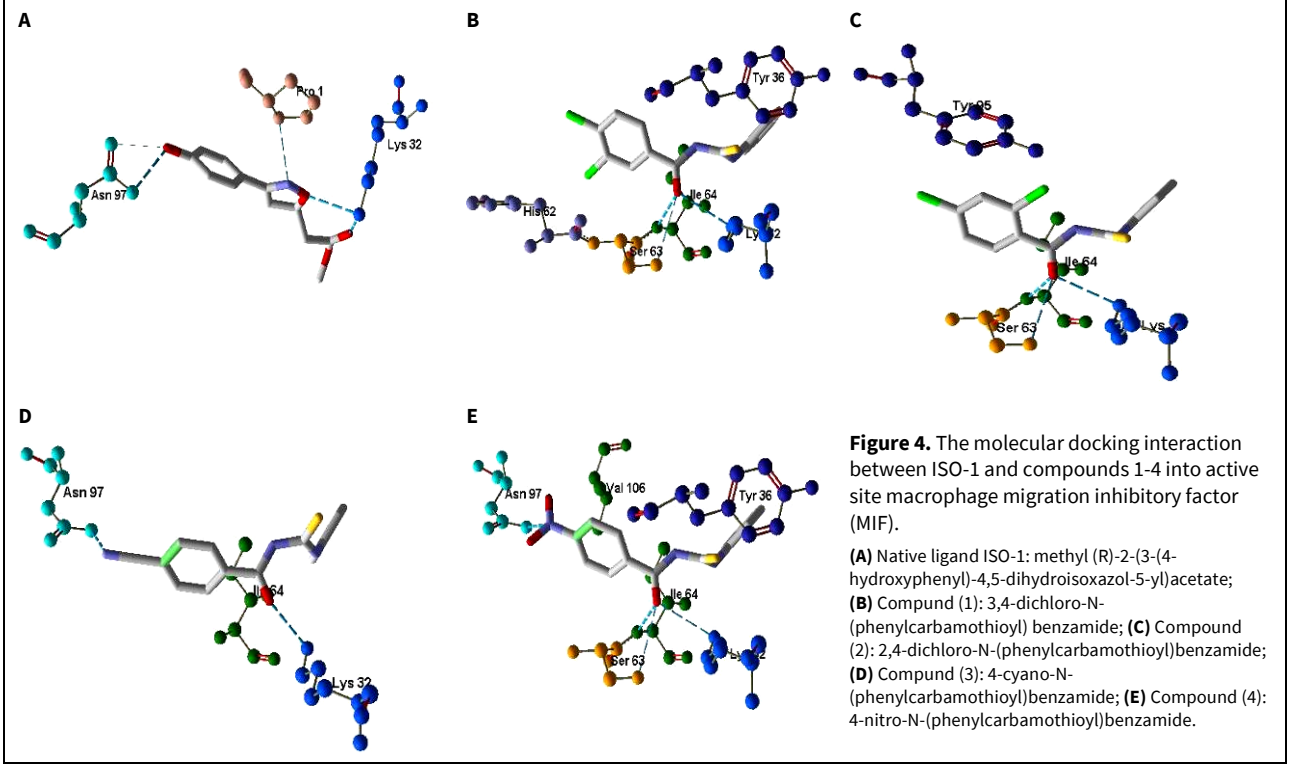


Figure 4. The molecular docking interaction between ISO-1 and compounds 1-4 into active site macrophage migration inhibitory factor (MIF).

(A) Native ligand ISO-1: methyl (R)-2-(3-(4-hydroxyphenyl)-4,5-dihydroisoxazol-5-yl)acetate; **(B)** Compound (1): 3,4-dichloro-N-(phenylcarbamothioyl) benzamide; **(C)** Compound (2): 2,4-dichloro-N-(phenylcarbamothioyl)benzamide; **(D)** Compound (3): 4-cyano-N-(phenylcarbamothioyl)benzamide; **(E)** Compound (4): 4-nitro-N-(phenylcarbamothioyl)benzamide.

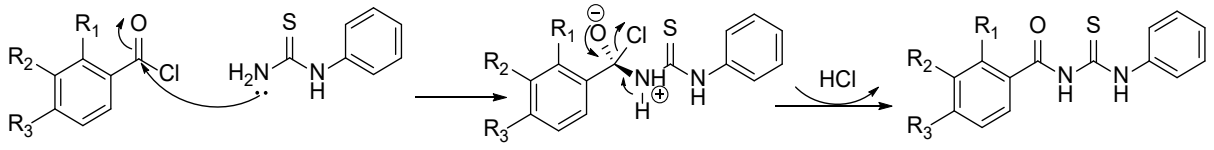


Figure 5. The mechanism synthesis of N-benzoyl-N'-phenylthiourea derivatives.

close to the δ+ carbon atom of the carbonyl group, exhibits attractive forces, making it more stable. This effect causes compounds 1 and 2 to be synthesized more easily with good yields (>70%). However, compounds 3 and 4 showed electron-withdrawing groups (-CN) and (-NO_2), respectively, which decrease the electron density on the benzene ring. The benzene ring, which is more δ+ and close to the δ+ carbon atom of the carbonyl group, exhibits repulsive forces, making it less stable (Putra et al., 2017). This effect causes compounds 3 and 4 to be more difficult to synthesize, resulting in lower yields (<25%).

Compounds 1-4 have been successfully synthesized, and their chemical structures have been confirmed using FTIR, NMR, and MS data, which are presented below (see also Supplementary data).

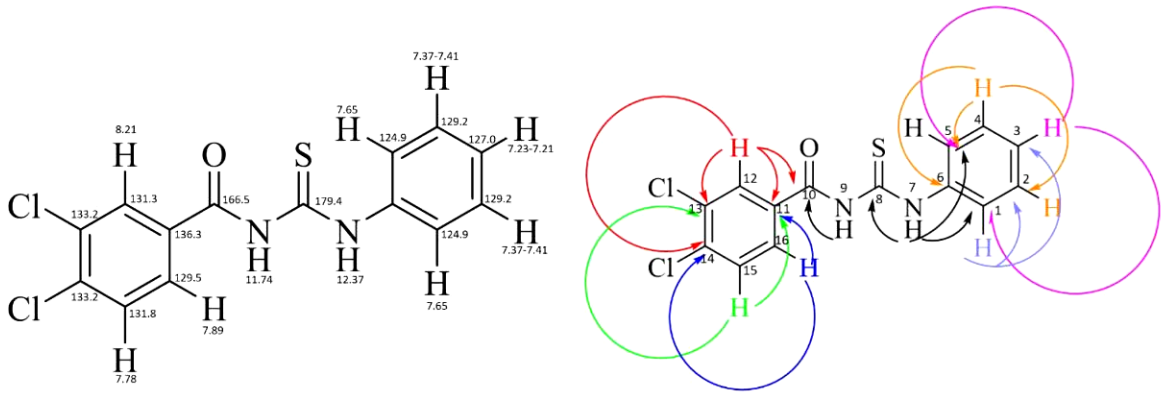
Compound (1): 3,4-dichloro-N-(phenylcarbamothioyl)benzamide

Obtained (74%), mp 140-141°C. FT-IR (KBr disk) cm^{-1}: 3160 (-NH), 1685 (-C=O, amide), 15371 (C=S), 1623 and 1470 (C=C aromatic), 779 (C-Cl), ^{1}H-NMR (400 MHz, CDCl3): δ 12.23 (s, 1H), δ 11.98 (1H, S); δ 7.64-7.66 (2H, m); 7.64-7.66 (1H, m); δ 7.52 (1H, dd, J=1.96Hz; 8.3Hz); δ 7.40(2 H, t, J=7.6 Hz); δ 7.25 (1H, t, J=7.6 Hz); δ 7.24 (1 H,d, J=1.96). ^{13}C-NMR (125 MHz, CDCl3): 179.0 (C=S); 167.3 (C=O); 138.3; 136.4; 133.8; 133.2; 131.8; 131.1; 129.3 (2 C); 127.1; 127.0; 124.9 (2 C). COSY, HMBC, and HSQC data are presented in Table 2. ESI oa-TOF MS: m/z calculated $[M-H]^+$: 322.9813, found: 322.9816.

Compound (2): 2,4-dichloro-N-(phenylcarbamothioyl)benzamide

Obtained (67%), mp 118–119°C. FT-IR (KBr disk) cm^{-1}: 3168 (-NH), 1686 (-C=O, amide), 1537 (C=S), 1633 and 1474 (C=C aromatic), 770 (C-Cl), ^{1}H-NMR (400 MHz, $CDCl_3$): δ 12.23 (s, 1H), δ 11.98 (1H, S); δ 7.64-7.66 (2H, m); 7.64-7.66 (1H, m); δ 7.52 (1H, dd, J=1.96Hz; 8.3Hz); δ 7.40 (2H, t, J = 7.6 Hz); δ 7.25 (1H, t, J=7.6 Hz); δ 7.24 (1H,d, J=1.96). ^{13}C-NMR (100 MHz, $CDCl_3$): 179.0 (C=S); 167.3 (C=O); 138.3; 136.4; 133.8; 133.2; 131.8; 131.1; 129.3 (2 C); 127.1; 127.0; 124.9 (2 C). COSY, HMBC, and HSQC data are presented in Table 3. ESI oa-TOF MS: m/z calculated $[M-H]^+$: 322.9813, found: 322.9816.

Table 2. Result of chemical structure elucidation compound (1): 3,4-dichloro-N-(phenylcarbamothioyl)benzamide.



No.	HSQC		COSY	HMBC
	HNMR	CNMR		
1 & 5	7.65 (2 H, d, J = 7.84)	124.9 (2 C)	H2; H4	C2; C3; C4
2 & 4	7.37-7.41 (2 H, m)	129.2 (2 C)	H1; H5; H3	C2; C5; C1; C4
3	7.21-7.23 (1 H, m)	127.0	H2; H4	C1; C5
6	-	138.5		
7	12.27 (1 H, s)	-		C1; C5; C8
8	179.4	-		
9	11.74 (1 H, s)	-		C10
10	-	166.5		
11	-	136.3		
12	8.21 (H, d, J = 1.92 Hz)	131.3		C10; C11; C13; C14
13	-	133.2		
14	-	133.2		
15	7.78 (1 H,d, J = 8.48)	131.8	H16	C11; C13
16	7.89 (1 H, dd, J = 1.92; 8.48 Hz)	129.5	H15	C11; C14

Compound (3): 4-cyano-N-(phenylcarbamothioyl)benzamide

Obtained (24%), mp 118–119°C. FT-IR (KBr disk) cm^{-1}: 3168 (-NH), 1686 (-C=O, amide), 1537 (C=S), 1633 and 1474 (C=C aromatic), 2240 (CN), ^{1}H-NMR (400 MHz, $CDCl_3$): δ 12.39 (s, 1H), δ 11.81 (1H, S); δ 8.05 (2H, d, J= 8.28 Hz); 7.96 (2H, d, J=8.32 Hz); δ 7.65 (2 H, d, J=7.84 Hz); δ 7.39(2 H, t, J=7.84 Hz); δ 7.24 (1H, t, J=7.56 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$): 179.4 (C=S); 167.5 (C=O); 138.4; 137.0; 132.8 (2C); 130.0(2C); 129.2 (2C); 127.0; 124.9 (2 C); 118.6; 115.5.

Compound (4): 4-nitro-N-(phenylcarbamothioyl)benzamide

Obtained (23%), mp 118–119°C. FT-IR (KBr disk) cm^{-1}: 3160 (-NH), 1681 (-C=O, amide), 1534 (C=S), 1620 and 1465 (C=C aromatic), 1356 (NO_2), ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.29-8.26 (m, 4H), δ 8.31-8.10 (5H, m). ^{13}C-NMR (100 MHz, $CDCl_3$): 165.3 (C=S); 164.7 (C=O); 159.7 (2C); 146.2; 143.2; 135.7; 135.9 (2C); 131.1 (2C); 131.06 ; 124.3 (2 C).

Compounds 1 and 2 are isomers, so to distinguish them precisely, we added 2D NMR data, namely COSY, HSQC, and HMBC, which can be seen in Tables 2 and 3.

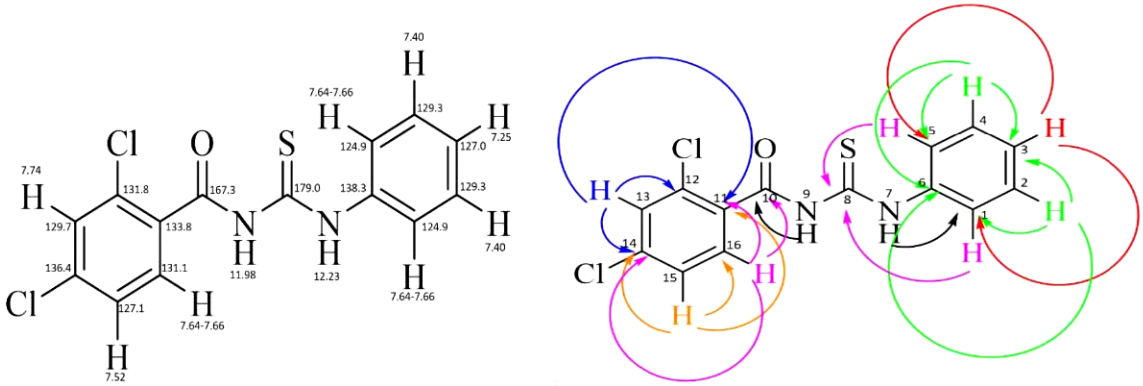
MIF activity test results

SDS-PAGE results on the proteins that have been modified showed that in fractions 3, 4, and 5, there were proteins about 10-15 kDa in size (Fig. 6). Further testing of tautomerase activity showed that the fraction had a clear tautomerase activity. This indicated that the eluted protein was an MIF protein. In addition, there were no other bands in the SDS-PAGE results indicating that the process of purification of MIF proteins was going well.

MIF target protein activity assay

The compounds tested in the MIF assay were compounds 1 and 2. Compounds 3 and 4 were not tested because the yield of the synthetic products was still less than 25%, indicating the need for optimization to produce a good product. Compounds 1 and 2 showed only a 10.1% and 6.2% (Fig. 7) decrease in tautomerase activity of the MIF target protein, respectively. The inhibitor control used was the heavy metal Cu^{2+}, as it can cause protein degradation, leading to the absence of MIF protein formation (Xio et al., 2020).

Table 3. Result of chemical structure elucidation compound (2): 2,4-dichloro-N-(phenylcarbamothioyl)benzamide.



No.	HSQC		COSY	HMBC
	HNMR	CNMR		
1 & 5	7.64-7.66 (2 H, m)	124.9 (2 C)	H2; H4	C3
2 & 4	7.40(2 H, t, J = 7.6 Hz)	129.3 (2 C)	H1; H5; H3	C2; C5; C1; C4
3	7.25 (1 H, t, J = 7.6 Hz)	127.0	H2; H4	C1; C5
6	-	138.3		
7	12.23 (1 H, s)	-		C1
8	-	179.0		
9	11.98 (1 H, s)	-		C10
10	-	167.3		
11	-	133.8		
12	-	131.8		
13	7.24 (1 H,d, J = 1.96 Hz)	133.2		C11; C12; C14
14	-	136.4		
15	7.52 (1 H, dd, J = 1.96 Hz; 8.3 Hz)	127.1	H16	C11; C14; C16
16	7.64-7.66 (1 H, m)	131.1	H15	C11; C10

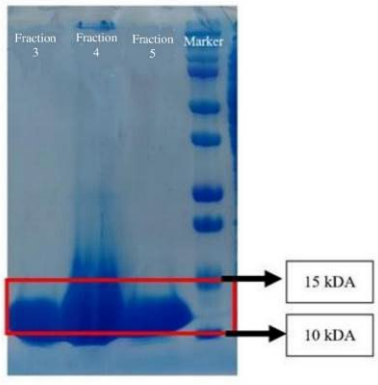


Figure 6. SDS-PAGE of purified MIF, indicating a band at around 13 kDa, which corresponds to the size of the protein.

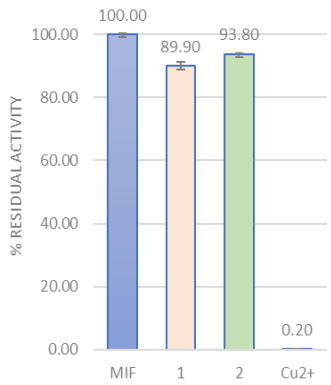


Figure 7. Percentage of MIF inhibition.

Normalization % residual activity with triplicate (n = 3): MIF (positive control) = 100 ± 0.2%; Compound 1 = 89.9 ± 0.14%; Compound 2 = 93.8 ± 0.03%; Cu^{2+} (inhibitor control) = 0.2 ± 0.01%.

CONCLUSION

The prediction of the activity of N-benzoyl-N'-phenylthiourea derivative compounds against their inhibitory activity on macrophage (MIF) coded PDB: 1LJT is better than that of the comparison compound (ISO-1), as indicated by the docking score. These compounds can be synthesized from phenylthiourea starting materials, which react with several benzoylchloride derivatives. The compounds resulting from the synthesis underwent structural analysis and identification using FT-IR, UV-Vis, ^{1}H-NMR, ^{13}C-NMR, and MS, confirming their correspondence to the expected structures. However, compounds 1 and 2, despite their structural accuracy, exhibited low inhibitory activity on macrophage (MIF) and acted as fewer effective inhibitors of the MIF target protein's tautomerase activity compared to the positive control inhibitors. Nevertheless, the study highlights the potential of N-benzoyl-N'-phenylthiourea derivatives and suggests further exploration in drug development research.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

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Kesuma D, Putra GS, Yuniarta TA.(2022a). Synthesis and cytotoxic activity of N-(2,4-dichloro)benzoyl-N'- phenylthiourea against human breast cancer cell line. Thai J Pharm Sci 46(2): 173–176.

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AUTHOR CONTRIBUTION:

Contribution	Kesuma D	Putra GS	Yuniarta TA	Suhud F	Sumartha IGA	Boengas S	Sulistyowati MI	Kok T
Concepts or ideas	x	x						
Design	x		x	x		x		x
Definition of intellectual content	x							
Literature search	x	x	x	x	x	x	x	x
Experimental studies		x	x		x			x
Data acquisition	x		x	x	x	x		x
Data analysis	x	x	x				x	x
Statistical analysis	x	x	x			x	x	x
Manuscript preparation		x	x				x	
Manuscript editing		x	x				x	
Manuscript review	x	x	x	x	x	x	x	x

Citation Format: Kesuma D, Putra GS, Yuniarta TA, Suhud F, Sumartha IGA, Boengas S, Sulistyowati MI, Kok T (2023) Synthesis and *in vitro* activity tests of N-benzoyl-N'-phenylthiourea derivatives as macrophage migration inhibitory factor. J Pharm Pharmacogn Res 11(5): 902–925. https://doi.org/10.56499/jppres23.1657_11.5.902

Supplementary data

Compound 1: 3,4-dichloro-N-(phenylcarbamothioyl)benzamide

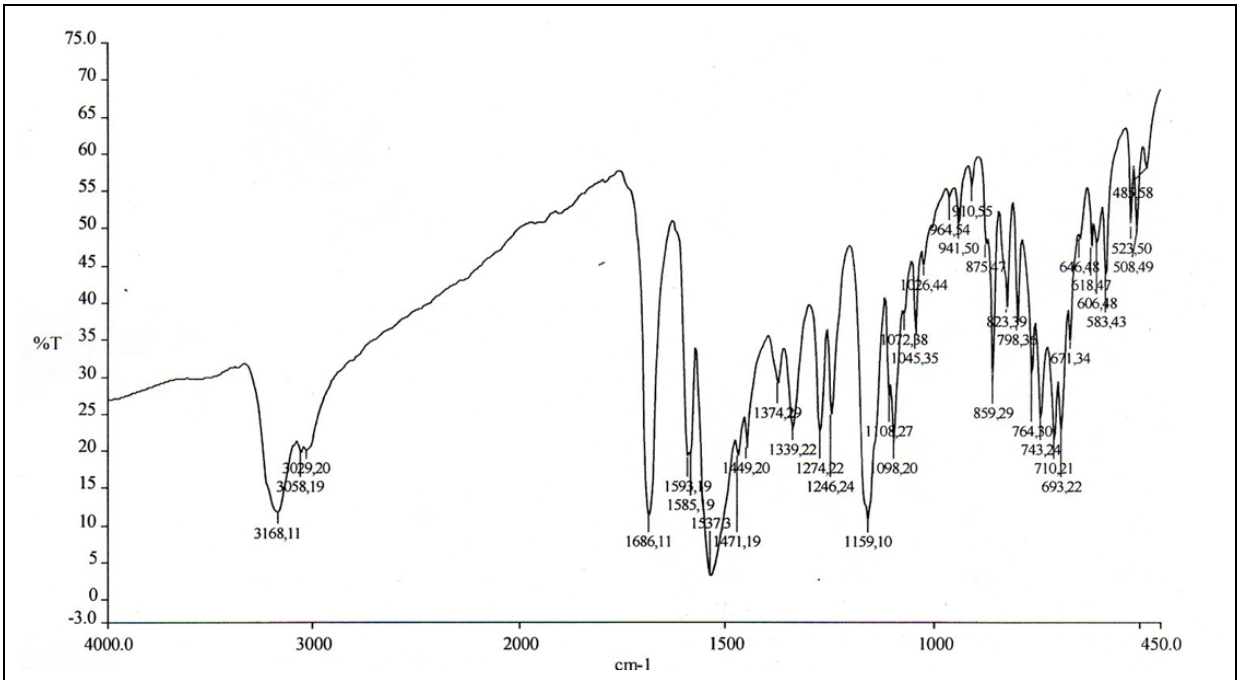


Figure 1S. FTIR of 3,4-dichloro-N-(phenylcarbamothioyl)benzamide.

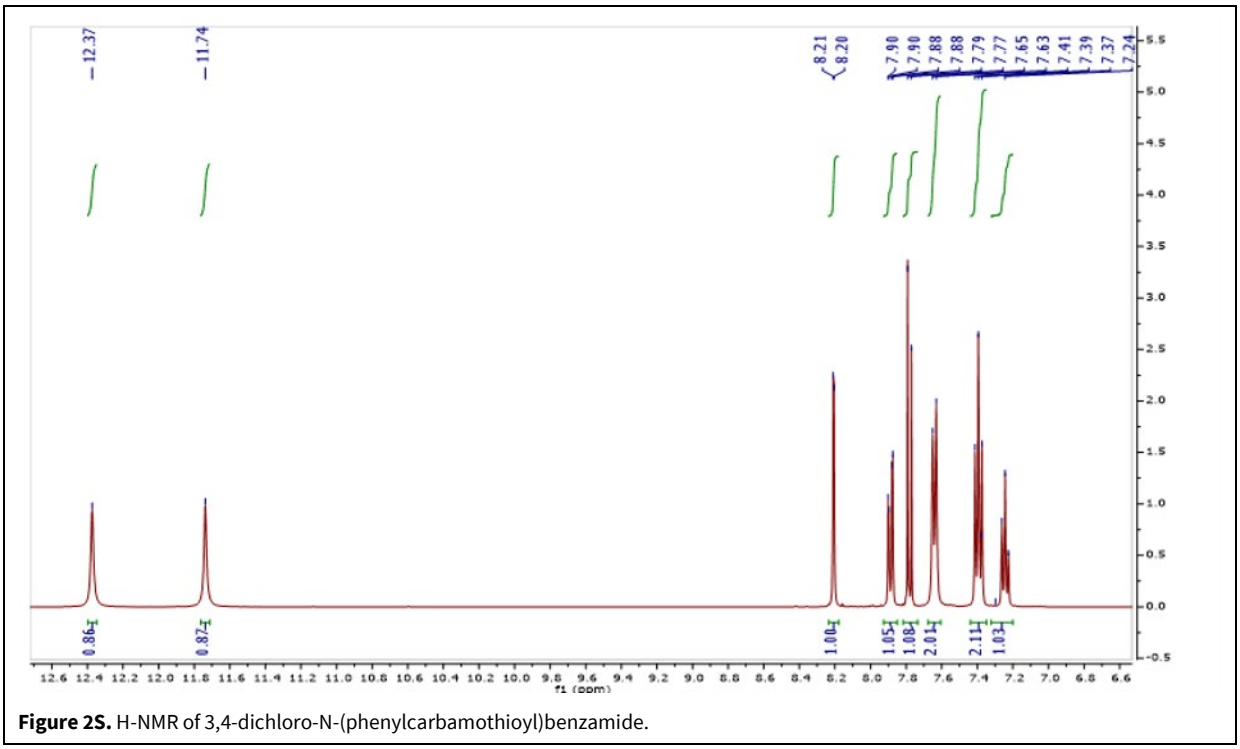


Figure 2S. H-NMR of 3,4-dichloro-N-(phenylcarbamothioyl)benzamide.

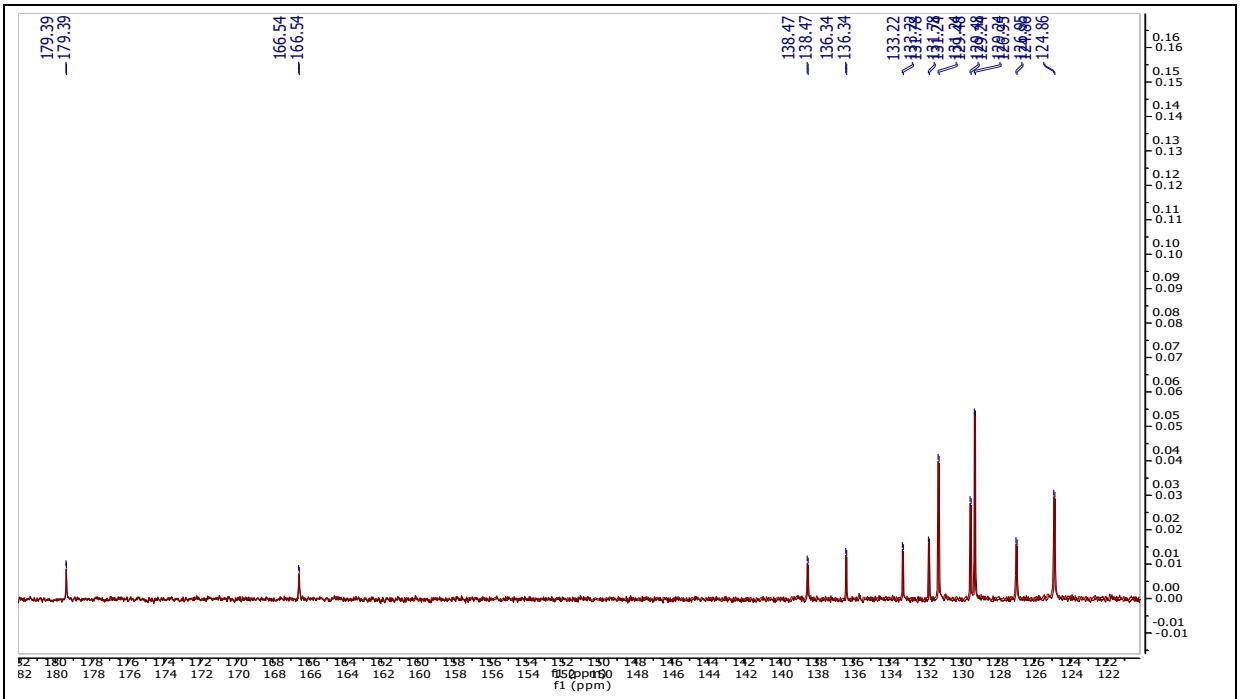


Figure 3S. C-NMR of 3,4-dichloro-N-(phenylcarbamothioyl)benzamide.

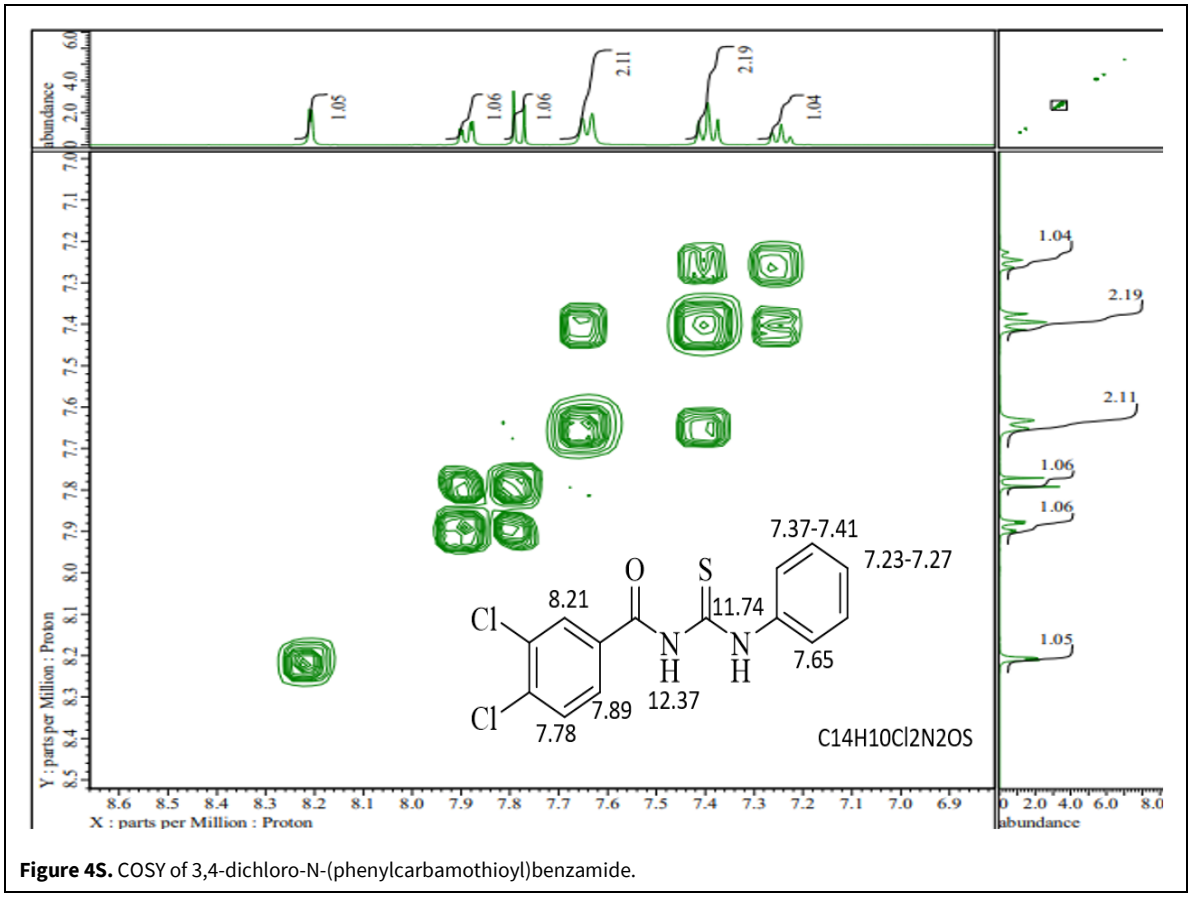


Figure 4S. COSY of 3,4-dichloro-N-(phenylcarbamothioyl)benzamide.

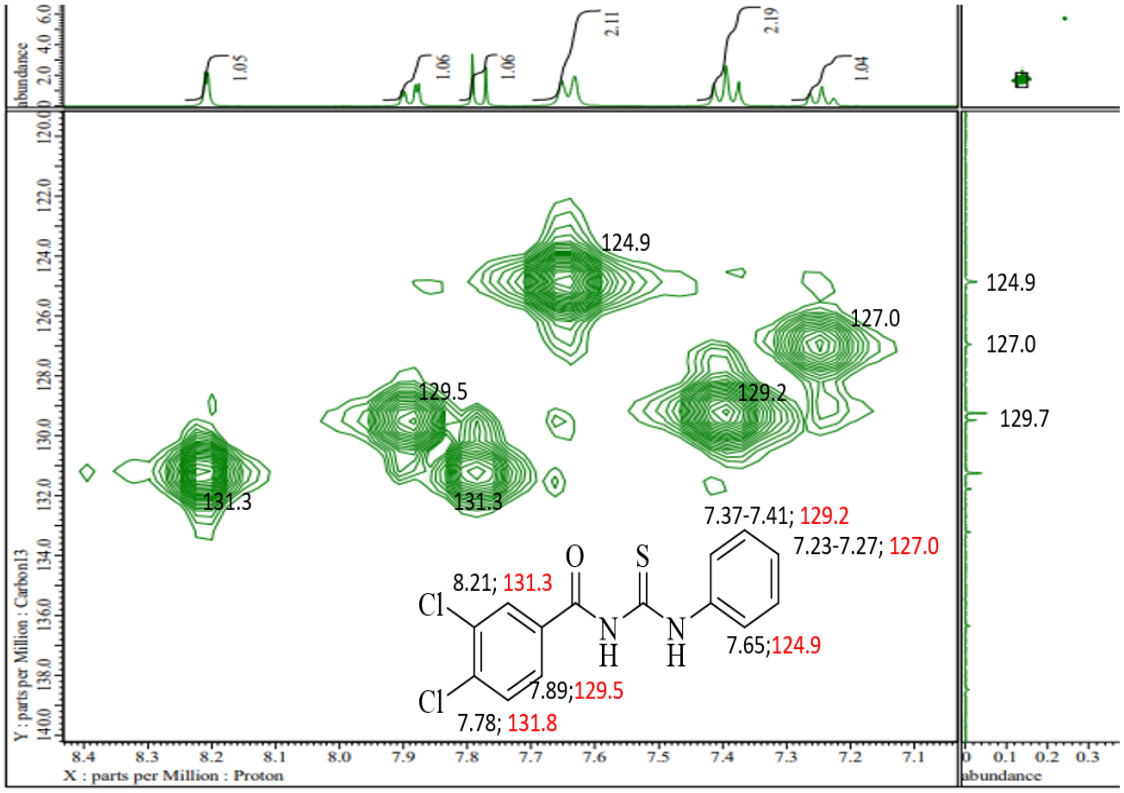


Figure 5S. HSQC of 3,4-dichloro-N-(phenylcarbamothioyl)benzamide.

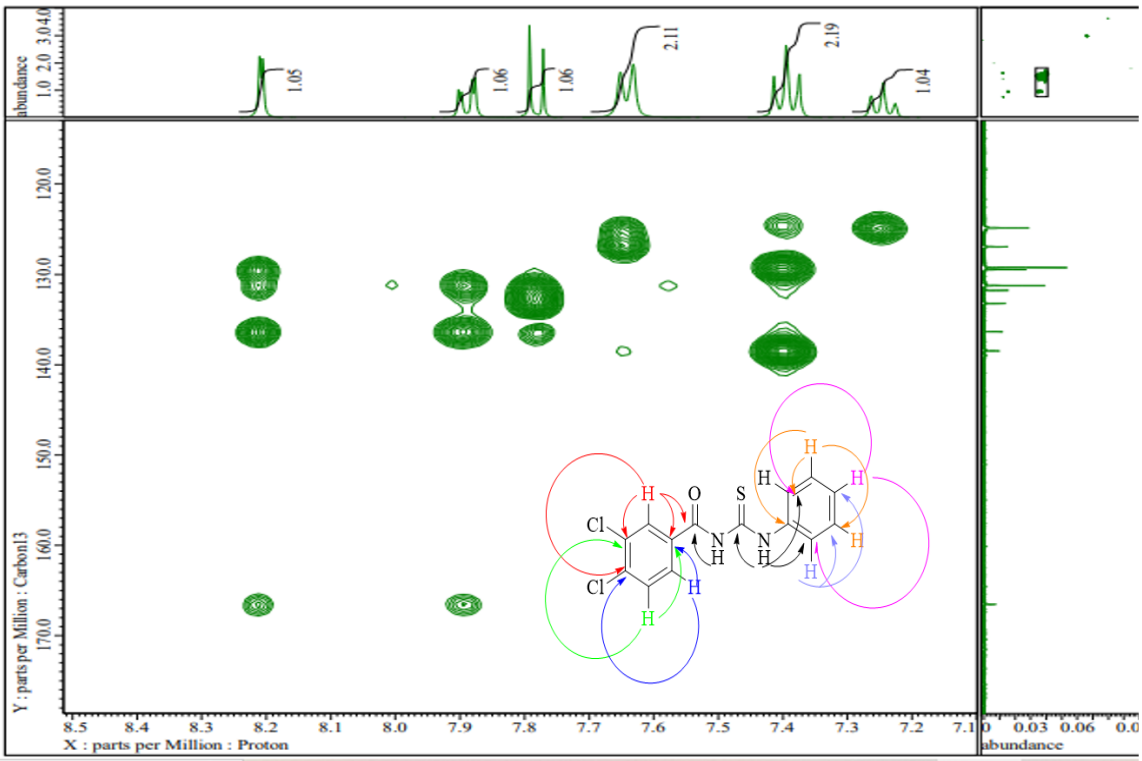


Figure 6S. HMBC 1 of 3,4-dichloro-N-(phenylcarbamothioyl)benzamide.

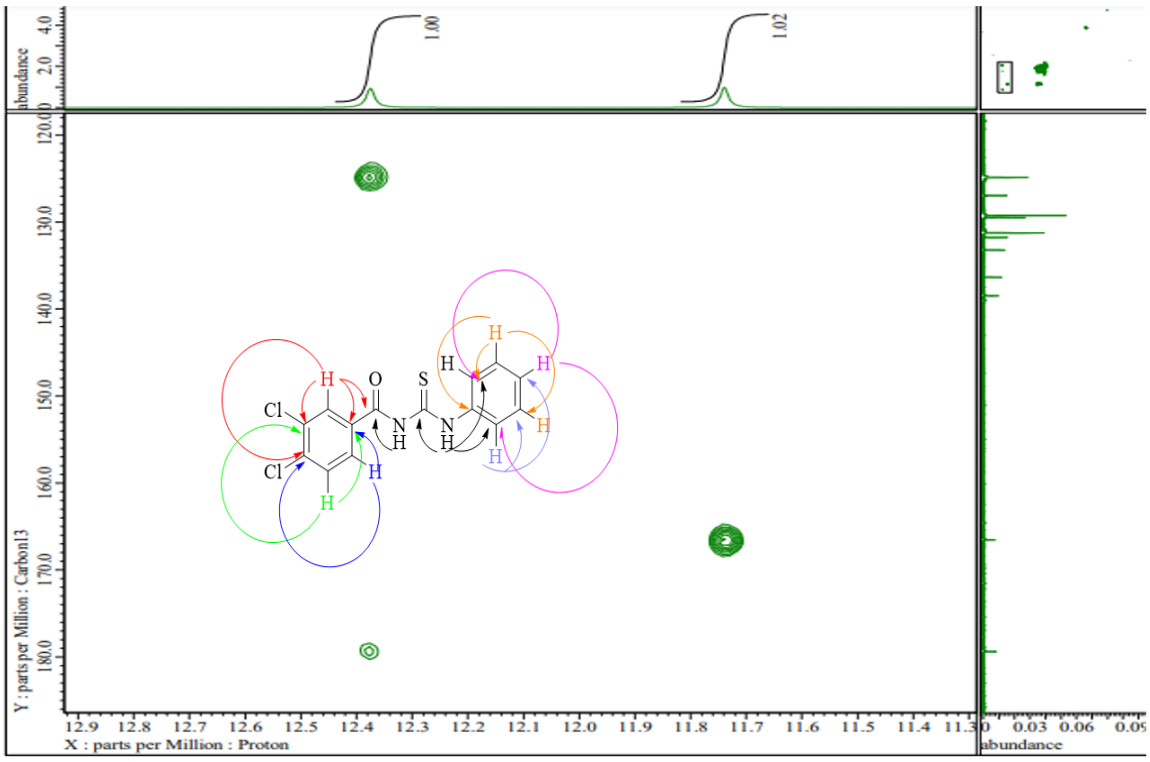


Figure 7S. HMBC 2 of 3,4-dichloro-N-(phenylcarbamothioyl)benzamide.

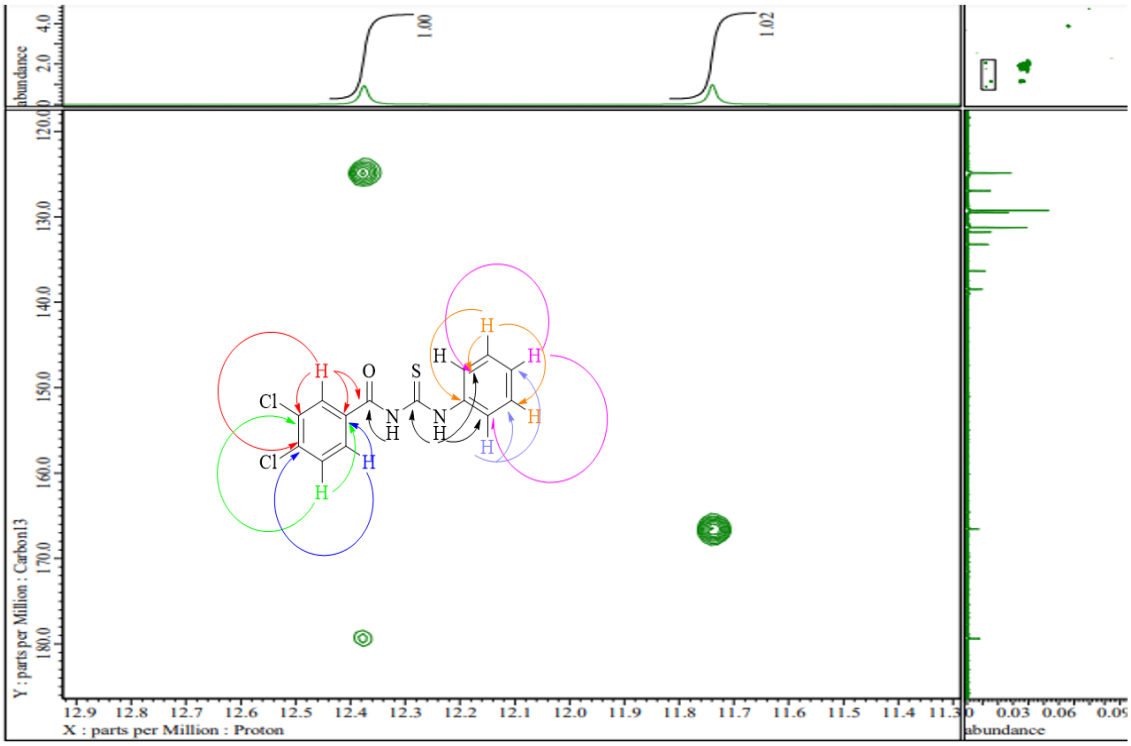


Figure 8S. HMBC 2 of 3,4-dichloro-N-(phenylcarbamothioyl)benzamide.

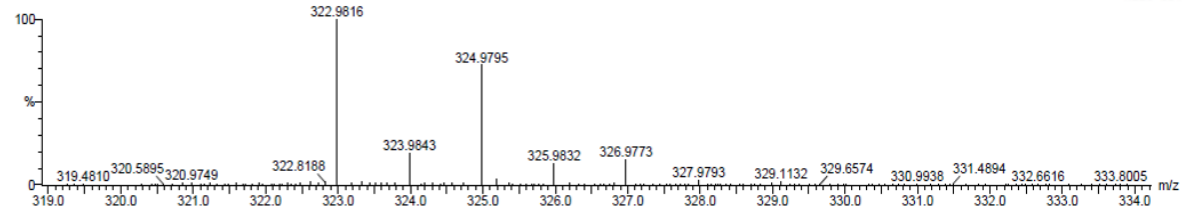
Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 5

Monoisotopic Mass, Even Electron Ions
6540 formula(e) evaluated with 25 results within limits (up to 5 best isotopic matches for each mass)
Elements Used:
C: 0-1000 H: 0-1000 N: 0-500 O: 0-500 S: 0-100 Cl: 0-8

standard
Unair_Dini_seny4-neg 12 (0.374) Cm (12:13)

TOF MS ES-
1.22e+004



Minimum:				-1.5			
Maximum:		30.0	5.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
322.9816	322.9813	0.3	0.9	10.5	299.7	0.0	C14 H9 N2 O S Cl2
	322.9824	-0.8	-2.5	12.5	312.7	12.9	C7 H N12 Cl2
	322.9823	-0.7	-2.2	4.5	313.6	13.9	C12 H16 S4 Cl
	322.9811	0.5	1.5	7.5	314.0	14.3	C6 H5 N8 O4 Cl2
	322.9815	0.1	0.3	5.5	314.3	14.6	C11 H12 O5 S2 Cl

Figure 9S. MS of 3,4-dichloro-N-(phenylcarbamothioyl)benzamide.

Compound 2: 2,4-dichloro-N-(phenylcarbamothioyl)benzamide

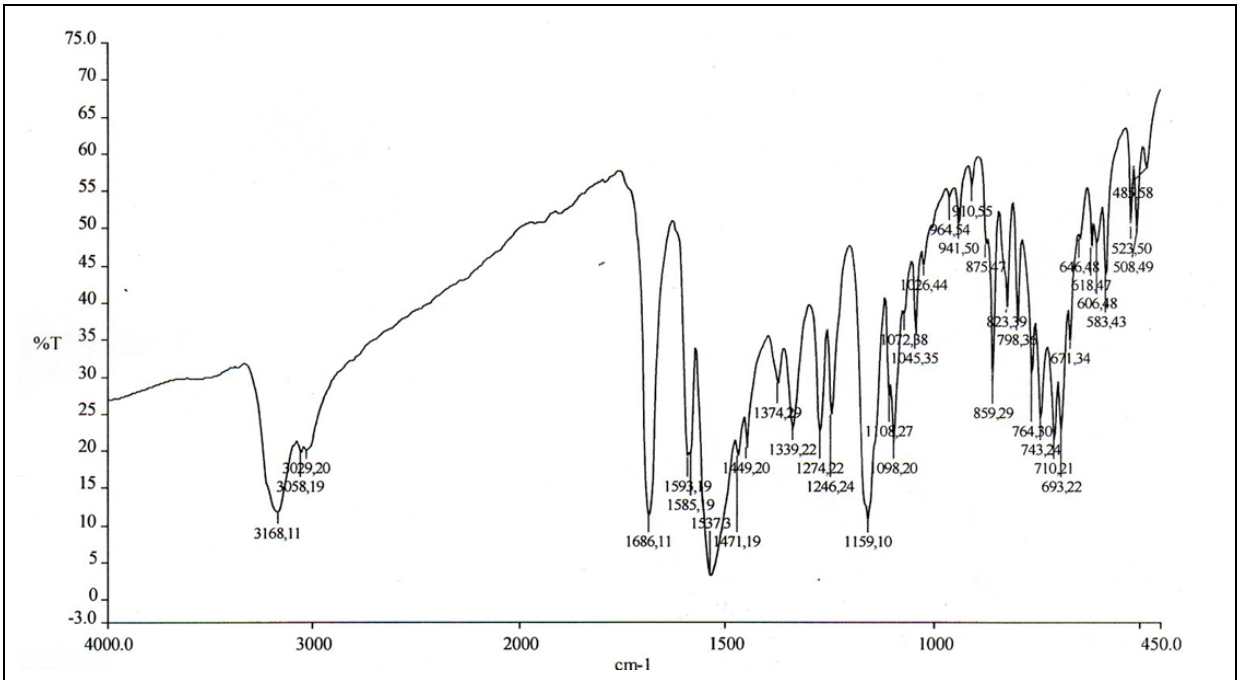


Figure 10S. FTIR of 2,4-dichloro-N-(phenylcarbamothioyl)benzamide.

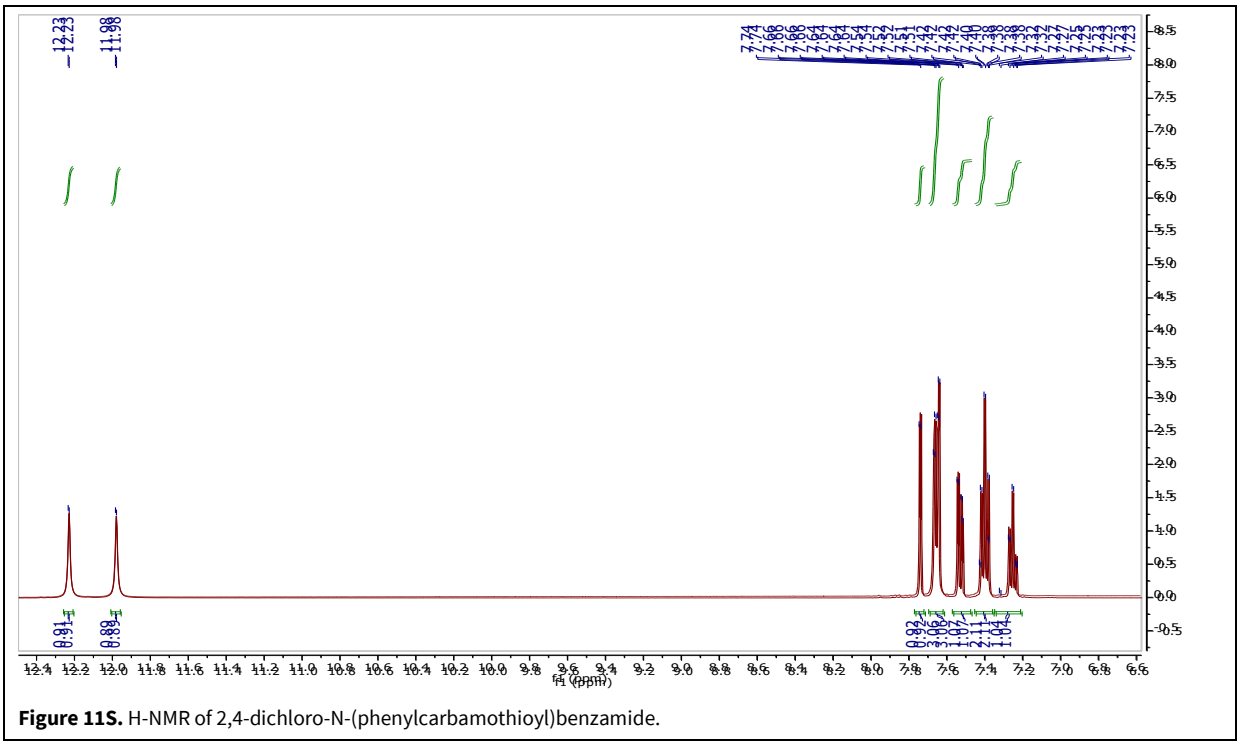


Figure 11S. H-NMR of 2,4-dichloro-N-(phenylcarbamothioyl)benzamide.

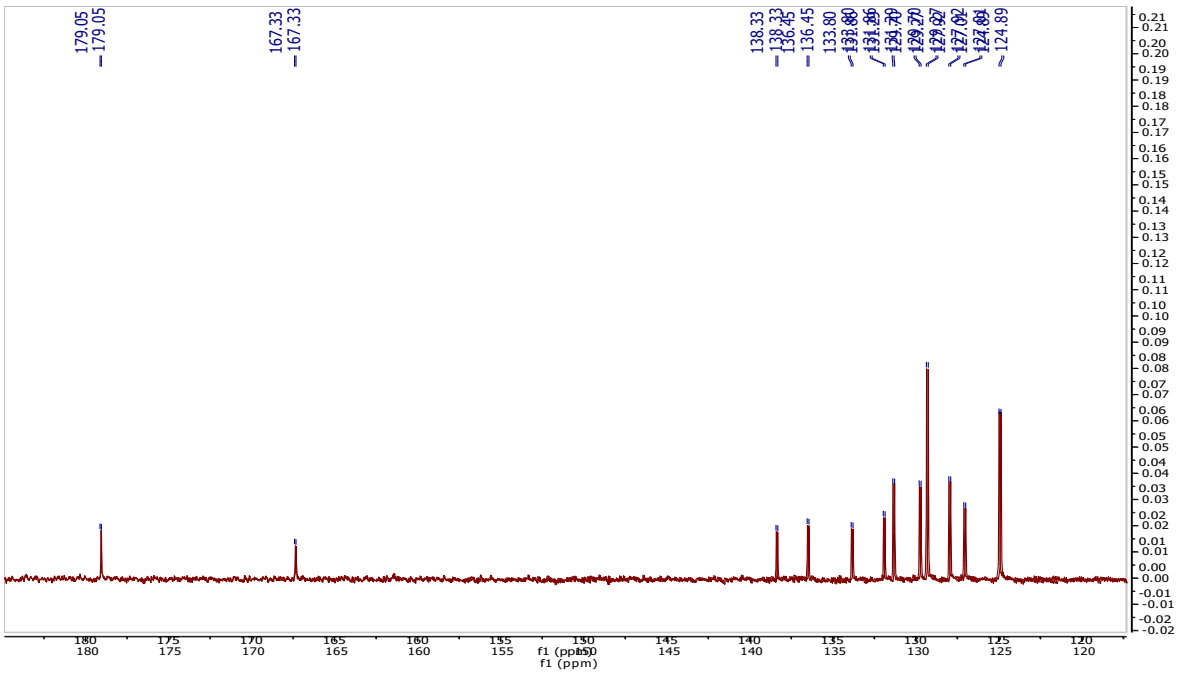


Figure 12S. C-NMR of 2,4-dichloro-N-(phenylcarbamothioyl)benzamide.

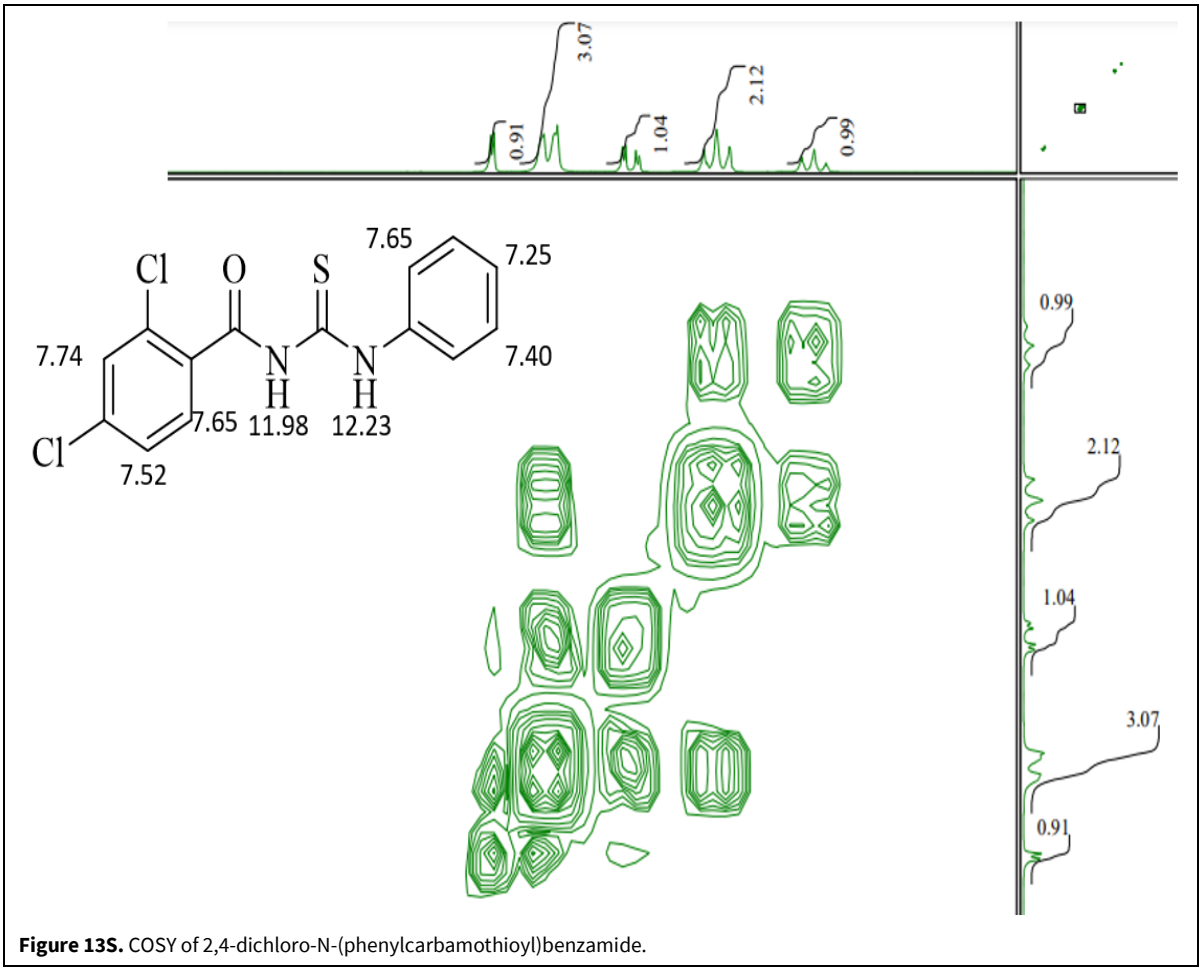


Figure 13S. COSY of 2,4-dichloro-N-(phenylcarbamothioyl)benzamide.

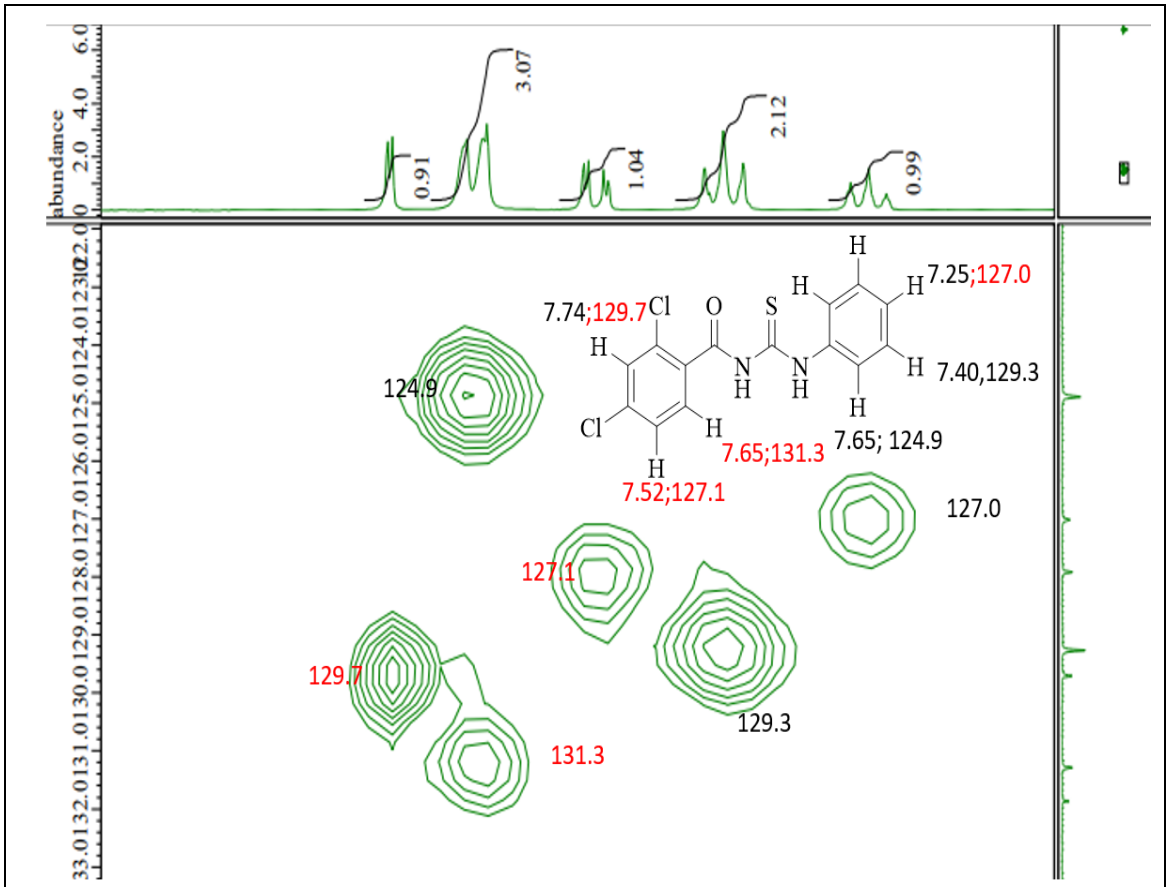


Figure 14S. HSQC of 2,4-dichloro-N-(phenylcarbamothioyl)benzamide.

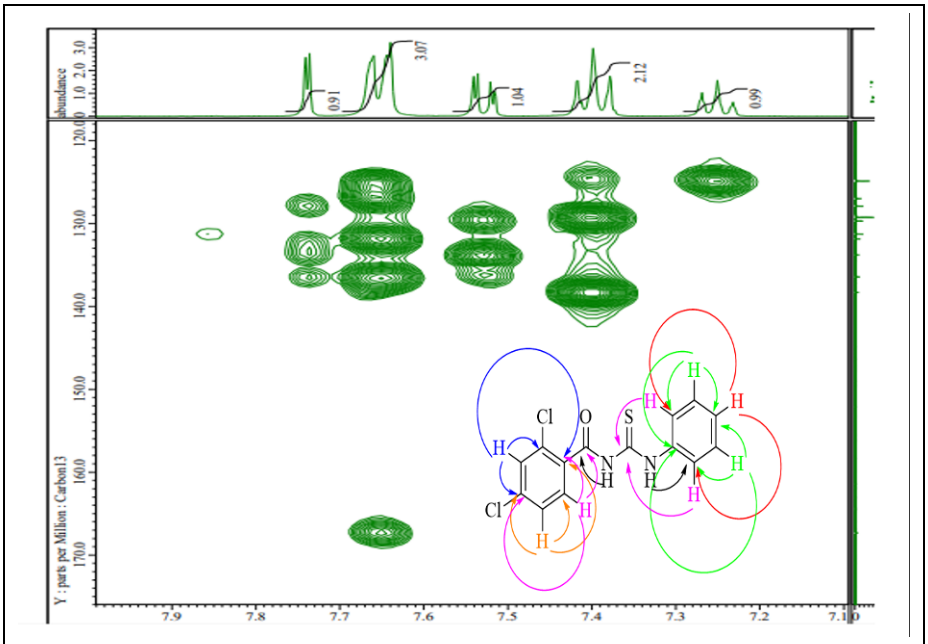


Figure 15S. HMBC 1 of 2,4-dichloro-N-(phenylcarbamothioyl)benzamide.

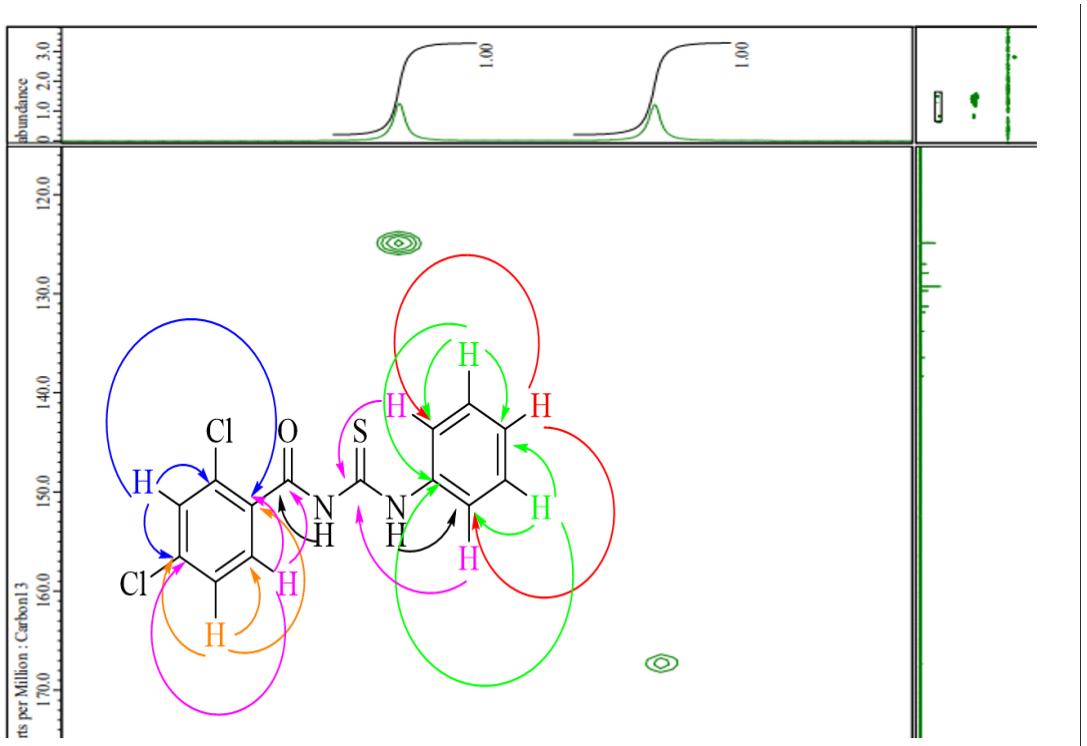


Figure 16S. HMBC 2 of 2,4-dichloro-N-(phenylcarbamothioyl)benzamide.

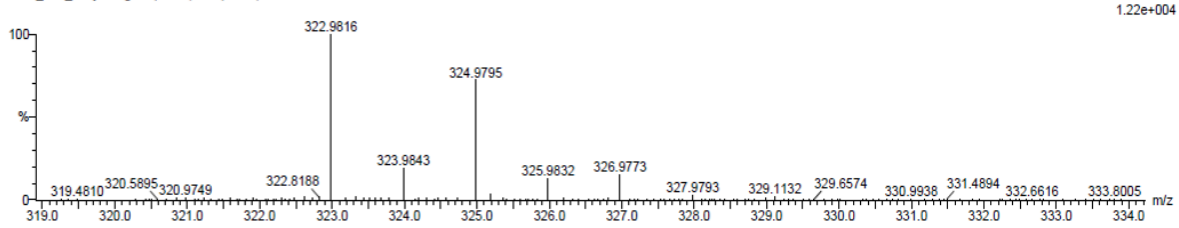
Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 5

Monoisotopic Mass, Even Electron Ions
6540 formula(e) evaluated with 25 results within limits (up to 5 best isotopic matches for each mass)
Elements Used:
C: 0-1000 H: 0-1000 N: 0-500 O: 0-500 S: 0-100 Cl: 0-8

standard
Unair_Dini_seny4-neg 12 (0.374) Cm (12:13)

TOF MS ES-
1.22e+004



Minimum:				-1.5			
Maximum:		30.0	5.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
322.9816	322.9813	0.3	0.9	10.5	299.7	0.0	C14 H9 N2 O S Cl2
	322.9824	-0.8	-2.5	12.5	312.7	12.9	C7 H N12 Cl2
	322.9823	-0.7	-2.2	4.5	313.6	13.9	C12 H16 S4 Cl
	322.9811	0.5	1.5	7.5	314.0	14.3	C6 H5 N8 O4 Cl2
	322.9815	0.1	0.3	5.5	314.3	14.6	C11 H12 O5 S2 Cl

Figure 17S. MS of 2,4-dichloro-N-(phenylcarbamothioyl)benzamide.

Compound 3: 4-cyano-N-(phenylcarbamothioyl)benzamide

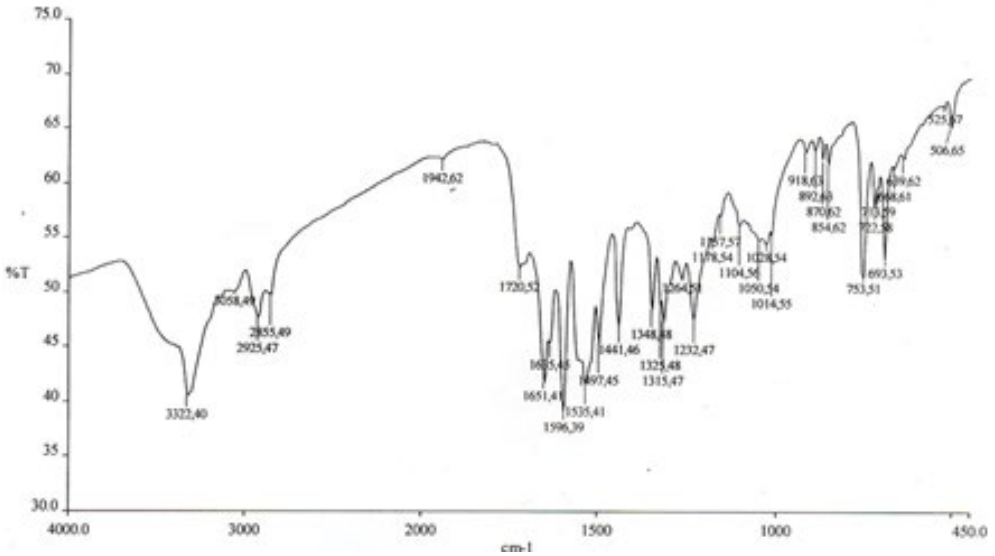


Figure 18S. FTIR of 4-cyano-N-(phenylcarbamothioyl)benzamide.

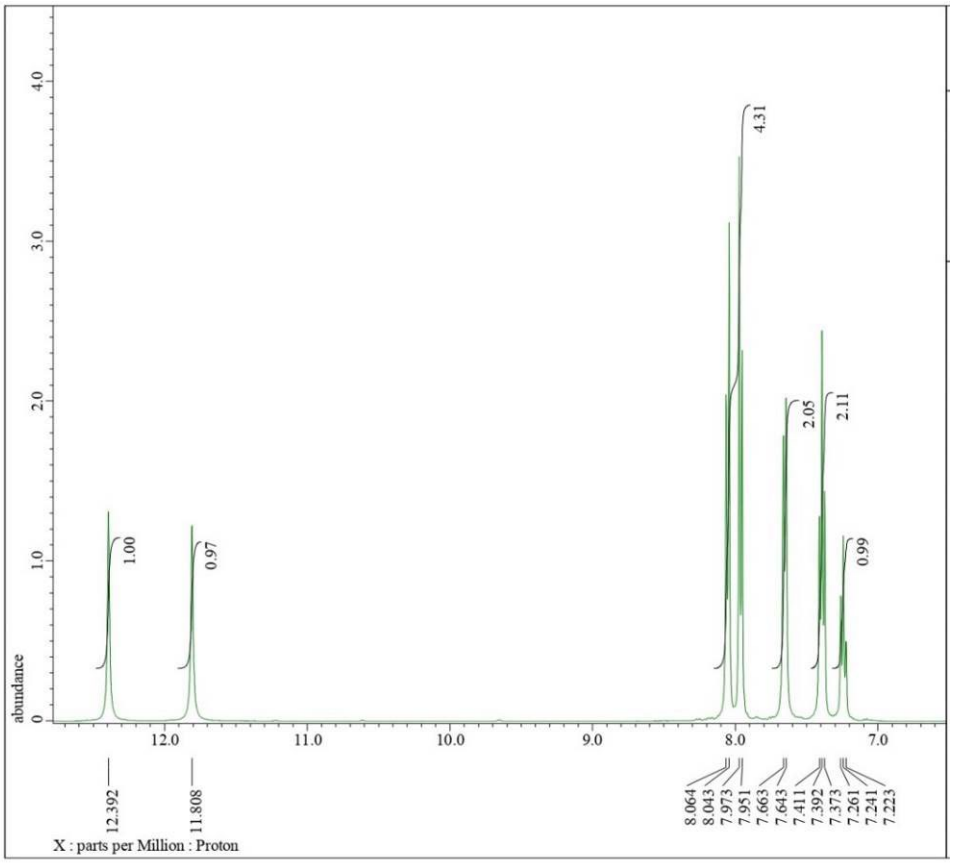


Figure 19S. H-NMR of 4-cyano-N-(phenylcarbamothioyl)benzamide.

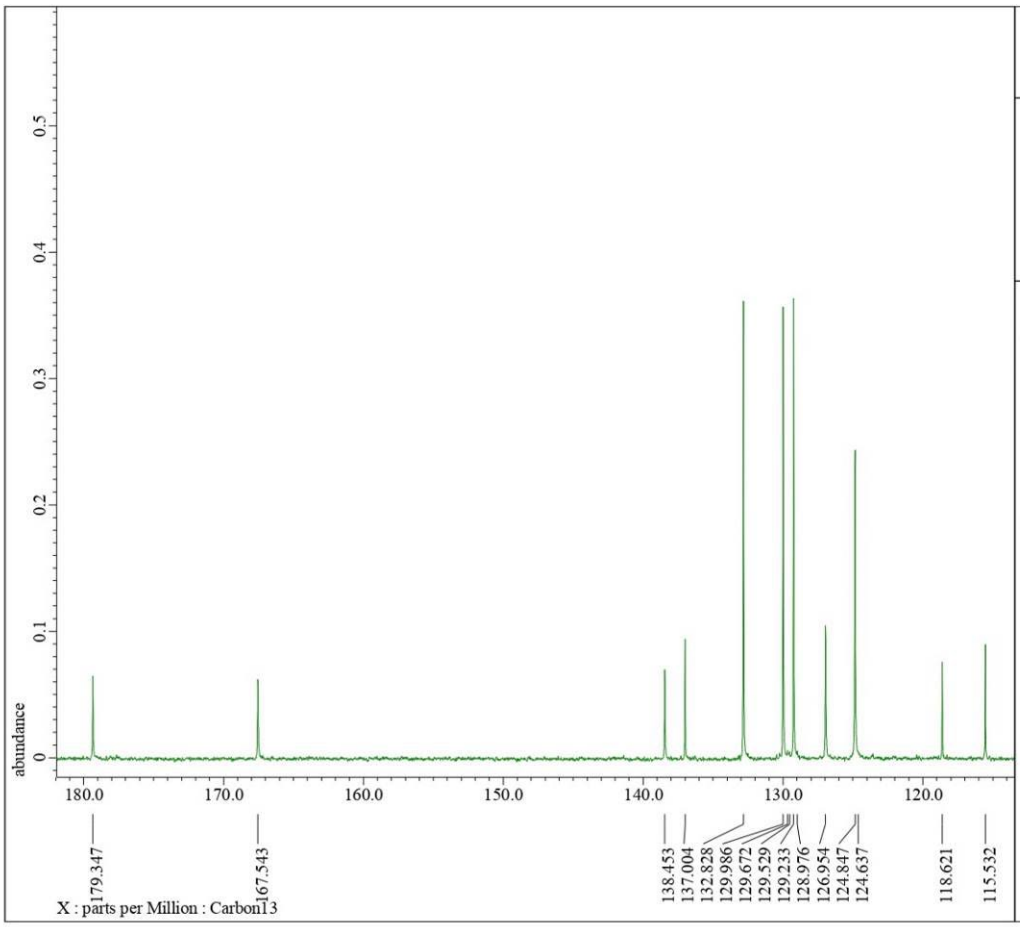


Figure 20S. C-NMR of 4-cyano-N-(phenylcarbamothioyl)benzamide.

Compoud 4: 4-nitro-N-(phenylcarbamothioyl)benzamide

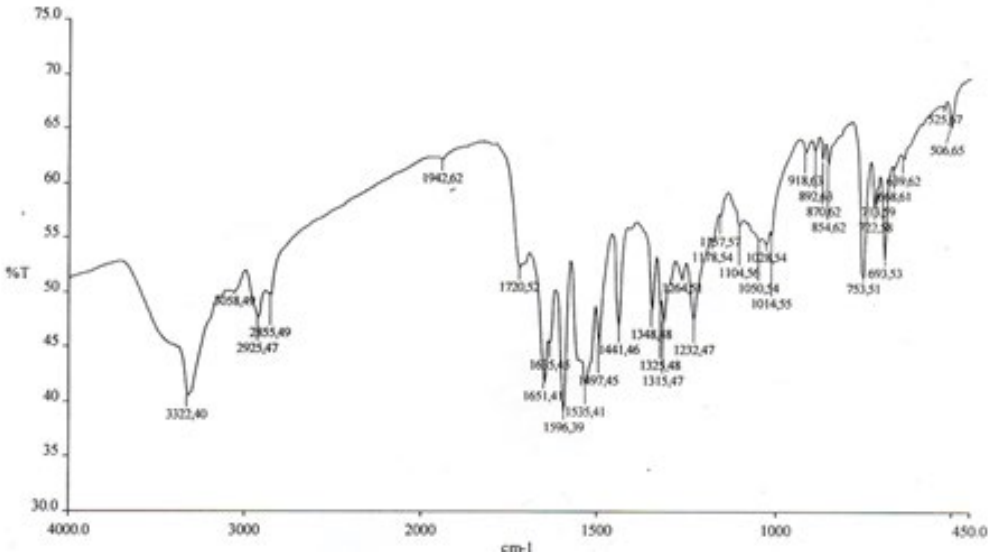


Figure 21S. FTIR of 4-nitro-N-(phenylcarbamothioyl)benzamide.

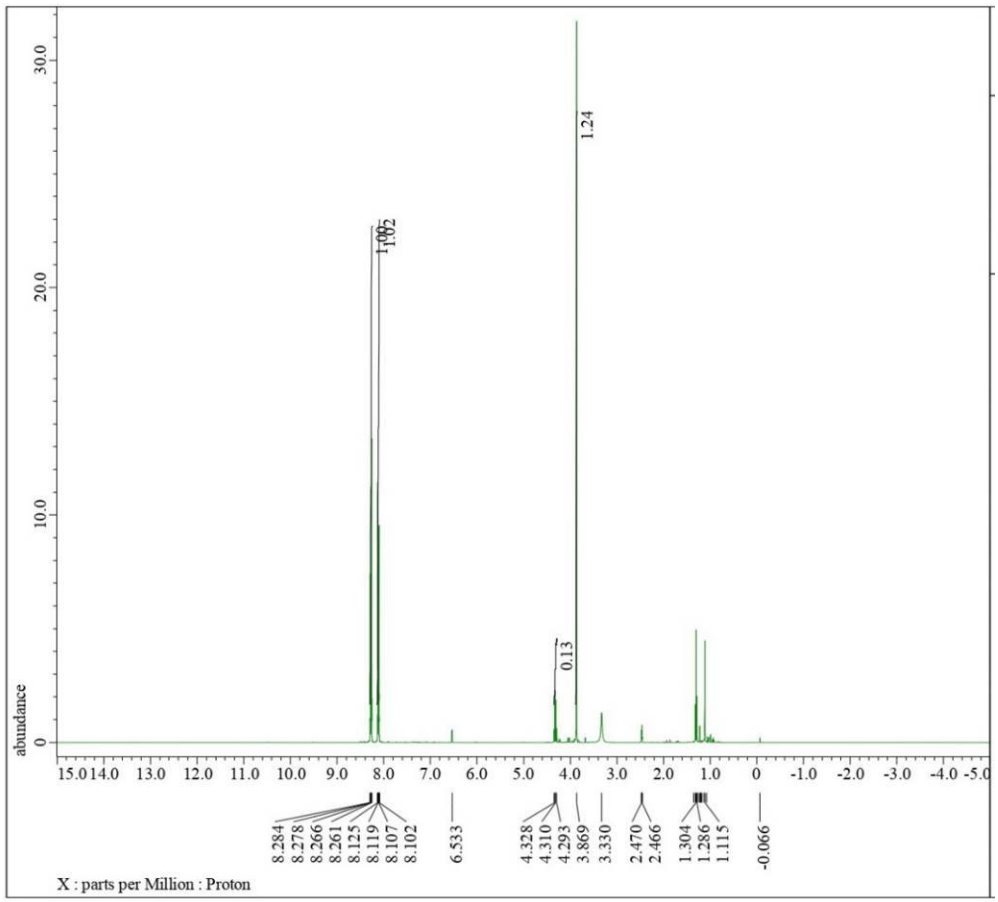


Figure 22S. H-NMR of 4-nitro-N-(phenylcarbamothioyl)benzamide.

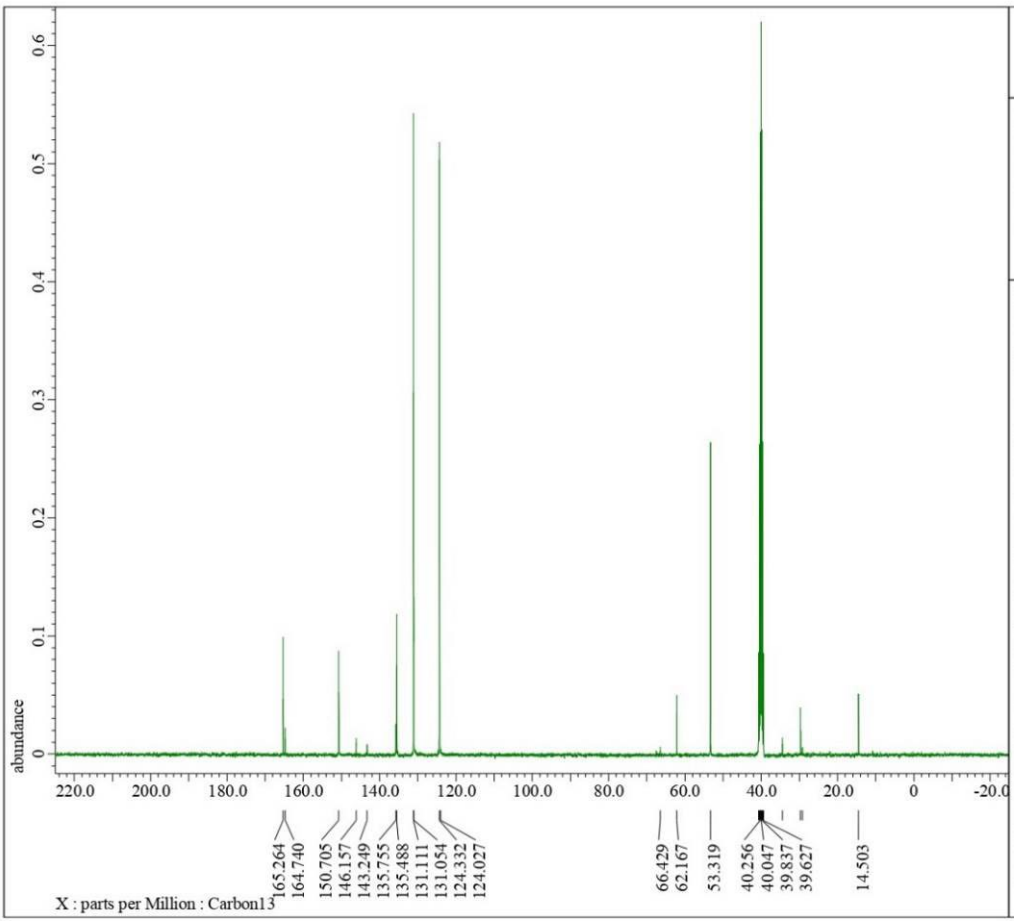


Figure 23S. C-NMR of 4-nitro-N-(phenylcarbamothioyl)benzamide.

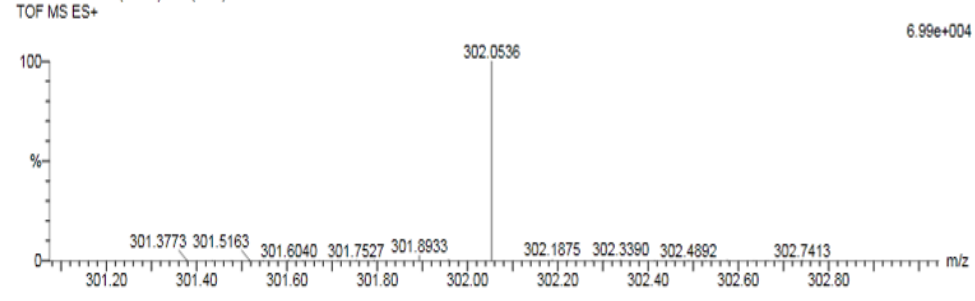


Figure 24S. MS of 4-nitro-N-(phenylcarbamothioyl)benzamide.

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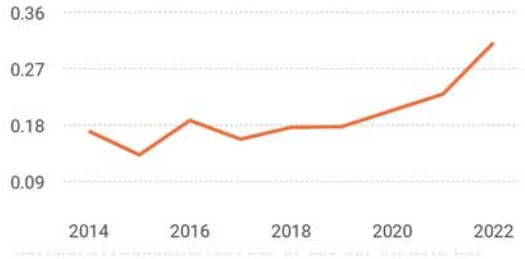

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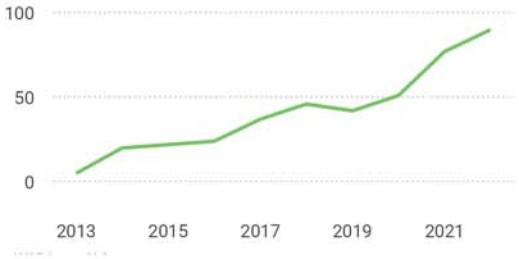

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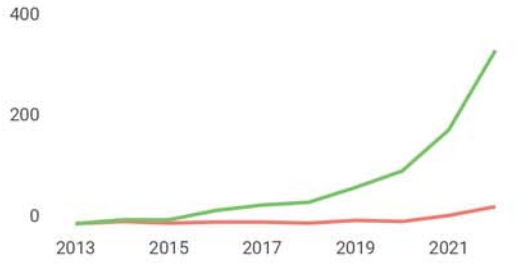

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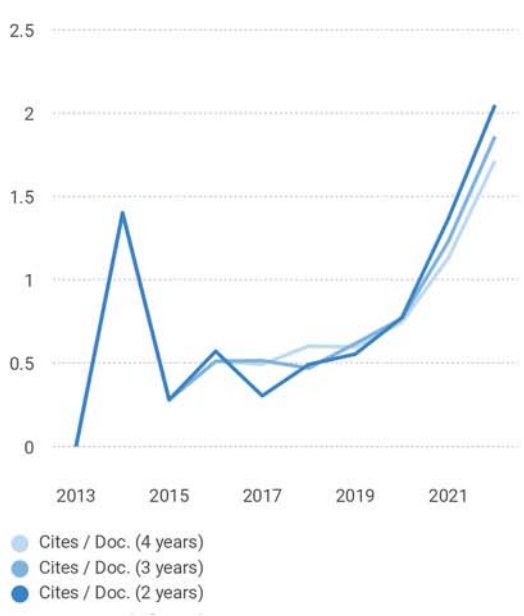

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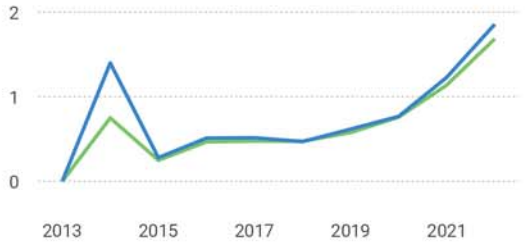

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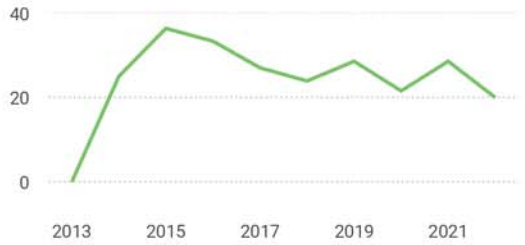

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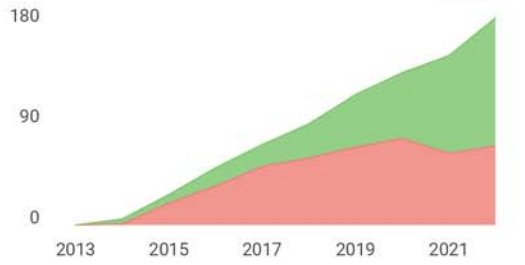

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