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Research Article

Synthesis and Bacteriostatic Activities of Bis(thiourea) Derivatives with Variable Chain Length

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A series of 1,4-bis(decoxyphenyl)carbamothioyl-terephthalamide derivatives was successfully synthesised by reaction of benzene-1,4-dicarbonyl isothiocyanate intermediates with long alkyl chain. The alkylation was performed via Williamson etherification of 4-acetamidophenol with bromoalkanes. The synthesised bis(thiourea) derivatives differed in the chain length, C_nH_{2n+1}, where $n = 10$, 12, and 14. The structures of all compounds were characterised by elemental CHN analysis, IR, ^{1}H, and ^{13}C NMR spectroscopies. Bacteriostatic activities of bis(thiourea derivatives which consisted of two folds of N-H, C=O, and C=S and long alkyl chain substituents were carried out against Gram-negative bacteria (*Escherichia coli*, ATCC 25922) via turbidimetric kinetic method. Bis(thiourea) derivatives with $n = 10$ and $n = 12$ displayed excellent activity against *E. coli* with MIC of 135 μg/mL and 145 μg/mL, respectively, while bis(thiourea) derivatives with $n = 14$ acted as cutoff point with no antibacterial properties. Similar trend was observed in binding affinity to the active site of enoyl ACP reductase (FabI), which demonstrated binding free energy of −5.3 Kcal/mol and −4.9 and −4.8 Kcal/mol, respectively.

1. Introduction

Thiourea which is also known as thiocarbamide is a white crystalline solid compound that consists of sulphur and nitrogen atoms. Thiourea moiety has become intensely synthesised due to its ability to undergo structural modifications [1]. The existing of two units of reactive primary amine groups has made thiourea a suitable precursor for a synthesis of many new compounds [2]. Thiourea derivatives are well known to display a broad spectrum of applications in pharmaceutical industry due to their biological properties such as antiparasitic [3], anticancer [4], antioxidant [5, 6], antibacterial [7–10], antifungal [11], and anti-HIV [12, 13] properties.

The synthesis and antibacterial studies of monothiourea derivative are progressing at the considerable rate while bis(thiourea) compounds are relatively less reported [14]. The presence of two or more thiourea moieties, for example, bis(thiourea), was envisaged to possess better antibacterial activity [15]. This is due to the ability of C=S and N-H groups in thiourea moieties which can be easily protonated under acidic condition and reacted with the carboxyl and phosphate groups of the bacterial surface and thus enhanced the activity [16].

Incorporation of alkyl chain as substituents in thiourea derivatives has been reported for significant biological properties [17, 18]. The presence of long alkyl chains was reported to enhance the biological activity of thiourea derivatives [19]. Alkyl chains have the ability to increase lipophilicity and promote the ability of the compound to disrupt microorganism cell wall [20–22].

In this paper, we report on the synthesis of novel 1,4-bis(decoxyphenyl)carbamothioyl-terephthalamide derivatives (**2a–c**) bearing alkyl chain of different length (C_{10}, C_{12}, and C_{14}). The compounds were demonstrated for antibacterial activities against Gram-negative bacteria (*Escherichia coli*, ATCC 25922) where the effects of different length of alkyl chains were evaluated.

2. Materials and Methods

Benzene-1,4-dioylchloride was acquired from Acros Organics. Potassium thiocyanate, 1-bromodecane, 1-bromododecane, and 1-bromotetradecane were purchased from Merck kGaa. The solvents were dried and distilled before being used under nitrogen as follows: acetone was distilled over potassium permanganate and acetonitrile was distilled over calcium hydride. All the reagents were used as received without any further purification.

Physical Measurement. Melting points were determined by using Stuart SMP3 melting point apparatus and uncorrected. The elemental CHNS analysis was provided by Universiti Teknologi MARA (UiTM) using Thermo Scientific™ FLASH 2000 CHNS/O Analyzers. Infrared (IR) spectra (ν/cm^{-1}) were recorded as KBr pellets on Perkin Elmer Thermoscientific Smart Omni Transmission Nicolet IS10 Fourier Transform Infrared Spectrometer (FTIR). ^{1}H NMR and ^{13}C NMR spectra were recorded using JEOL ECA 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) with the chemical shifts δ (ppm) reported relative to DMSO-d_6 as standards.

*2.1. Synthesis of Benzenamine Derivatives (**1a–1c**).* A mixture of 4-acetamidophenol, bromoalkane, and K_2CO_3 in acetone (20 mL) was heated at reflux constant stirring for 48 h. The reaction mixture was cooled to room temperature and taken to dryness. The solid was stirred for 1 h with 50 mL of 2% sodium hydroxide and filtered and then heated at reflux for 2 h in ethanol : concentrated hydrochloric acid (1 : 1) ratio (10 mL : 10 mL) and allowed to cool to room temperature. Water (20 mL) was added to the reaction mixture and the layers were separated. The aqueous layer was extracted with dichloromethane (20 mL). The organic layer was separated, dried over $MgSO_4$, and filtered and solvent was removed in vacuo to afford intermediate compounds **1a–1c** with yields as follows.

*4-(Decyloxy)benzenamine (**1a**).* 4-(Decyloxy)benzenamine (**1a**) was obtained from N-(4-(decyloxy)phenyl)acetamide (2.92 g, 10 mmol). (2.02 g, 81%) as milky white solid, m.p: 136-137°C ν_{max} (KBr/cm^{-1}) 3467 (NH), 2917 (CH), 1509 (Ar-C), 1169 (C-N), 1028 (C-O). δ_H (500 MHz, $CDCl_3$) 0.89 (3H, t, 1x CH_3), 1.27–1.72 (16H, m, 8x CH_2), 3.82 (2H, t, 1x CH_2), 6.71 (2H, d, J = 7.65 Hz, Ar-H), 7.33 (2H, d, J = 8.6 Hz, Ar-H), 10.10 (2H, s, 1x NH). δ_C (125 MHz, $CDCl_3$) 14.2, 22.8, 26.1, 29.3, 29.4, 29.6, 29.7, 32.0, 68.3, 115.4, 122.3, 124.7, 159.2.

*4-(Dodecyloxy)benzenamine (**1b**).* 4-(Dodecyloxy)benzenamine (**1b**) was obtained from N-(4-(dodecyloxy)phenyl)acetamide (3.20 g, 10 mmol). (1.92 g, 69%) as light brown solid, m.p: 139-140°C ν_{max} (KBr/cm^{-1}) 3467 (NH), 2916 (CH), 1509 (Ar-C), 1169 (C-N), 1028 (C-O). δ_H (500 MHz, DMSO-d_6) 0.84 (3H, t, 1x CH_3), 1.23–1.68 (20H, m, 10x CH_2), 3.94 (2H, t, 1x CH_2), 7.00 (2H, d, J = 8.6 Hz, Ar-H), 7.31 (2H, d, J = 8.6 Hz, Ar-H), 10.29 (2H, s, 1x NH). δ_C (125 MHz, DMSO-d_6) 14.0, 22.1, 25.5, 28.6, 28.7, 28.8, 29.0, 29.1, 31.3, 67.8, 115.3, 123.9, 124.5, 158.2.

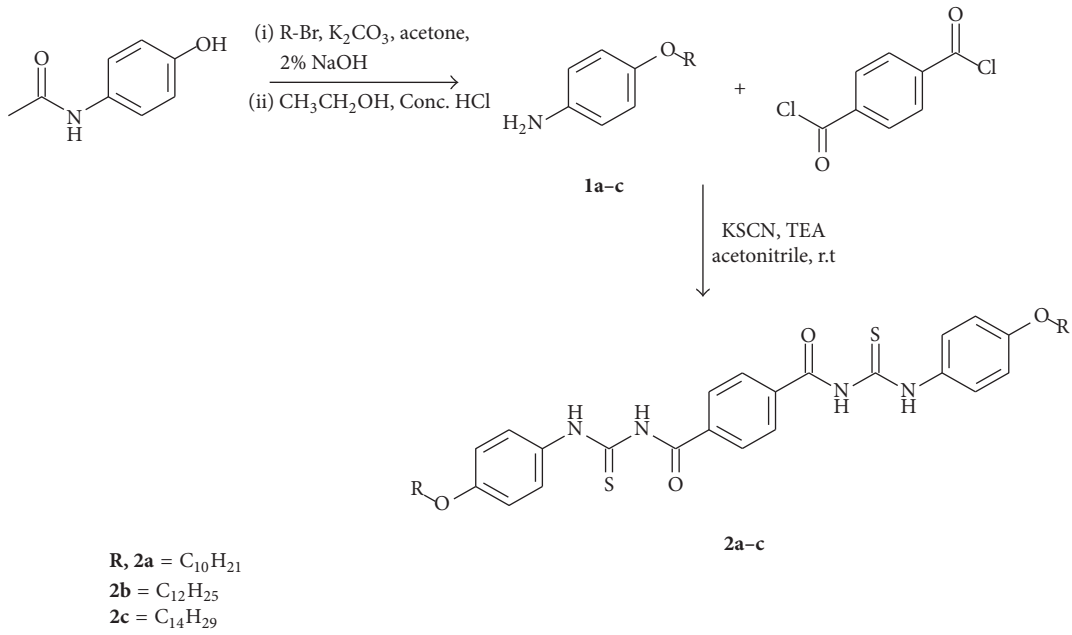
*4-(Tetradecyloxy)benzenamine (**1c**).* 4-(Tetradecyloxy)benzenamine (**1c**) was obtained from N-(4-(tetradecyloxy)phenyl) acetamide (3.50 g, 10 mmol). (2.80 g, 91%) as off-white solid, m.p: 142-143°C ν_{max} (KBr/cm^{-1}) 3467 (NH), 2915 (CH), 1510 (Ar-C), 1169 (C-N), 1039 (C-O). δ_H (500 MHz, DMSO-d_6) 0.85 (3H, t, 1x CH_3), 1.23–1.98 (24H, m, 12x CH_2), 3.94 (2H, t, 1x CH_2), 7.01 (2H, d, J = 9.55 Hz, Ar-H), 7.31 (2H, d, J = 8.6 Hz, Ar-H), 10.25 (2H, s, 1x NH). δ_C (125 MHz, DMSO-d_6) 14.0, 22.1, 25.5, 28.6, 28.7, 28.8, 29.0, 30.7, 31.3, 67.8, 115.3, 124.1, 124.3, 158.1.

*2.2. Synthesis of 1,4-bis(decoxyphenyl)carbamothioyl-terephthalamide Derivatives (**2a–2c**).* A solution of benzene-1,4-dioylchloride in dry acetone (20 mL) was added into a suspension of potassium thiocyanate in dry acetone (20 mL). The mixture was stirred at room temperature to form precipitate. The mixture was filtered and the white KCl was removed. The filtrate was used directly in next step. Triethylamine was added dropwise to benzenamine derivatives **(1a–1c)** in dry acetone (20 mL). The mixture was added to the filtrate. The reaction mixture was stirred at room temperature for 4 h and filtered. The crude solid was recrystallised in DMF : MeOH (1 : 1) mixture. The preparation of **2a–2c** with benzenamine derivatives **1a–1c** (g, mmol) and yield is shown as follows.

*N1,N4-bis[(4-Decoxyphenyl)carbamothioyl]terephthalamide (**2a**).* N1,N4-bis[(4-decoxyphenyl)carbamothioyl]tereph-thalamide (**2a**) was obtained from 4-(decyloxy)benzenamine **1a** (0.25 g, 1 mmol). (0.26 g, 69%) as bright yellow, m.p: 199-200°C; (Found: C, 67.46; H, 7.76; N, 7.49; S, 8.52. $C_{42}H_{58}N_4O_4S_2$, Requires C, 67.5; H, 7.8; N, 7.5; S, 8.6%); ν_{max} (KBr/cm^{-1}) 3393 (NH), 2922 (CH), 1668 (C=O amide), 1531 (Ar-C), 1244 (C=S), 1144 (C-N), 1116 (C-O). δ_H (500 MHz, $CDCl_3$) 0.88 (6H, t, 2x CH_3), 1.28–1.80 (32H, m, 16x CH_2), 3.97 (4H, t, 2x CH_2), 6.94 (4H, d, J = 9.15 Hz, Ar-H), 7.56 (4H, d, J = 9.2 Hz, Ar-H), 8.05 (4H, s, Ar-H), 9.14 (2H, s, 2x NH), 12.25 (2H, s, 2x NH). δ_C (125 MHz, $CDCl_3$) 14.2, 22.8, 26.1, 29.3, 29.4, 29.5, 29.7, 32.0, 68.4, 114.8, 125.9, 128.4, 158.2, 165.5, 178.2.

*N1,N4-bis[(4-Dodecoxyphenyl)carbamothioyl]terephthalamide (**2b**).* N1,N4-bis[(4-dodecoxyphenyl)carbamothioyl]terephthalamide (**2b**) was obtained from 4-(dodecyloxy)benzenamine **1b** (0.29 g, 1 mmol). (0.24 g, 60%) as light yellow solid, m.p: 202-203°C; (Found: C, 68.78; H, 8.28; N, 7.03; S, 8.03. $C_{46}H_{66}N_4O_4S_2$, Requires C, 68.8; H, 8.3; N, 7.0; S, 8.0%); ν_{max} (KBr/cm^{-1}) 3397 (NH), 2921 (CH), 1668 (C=O amide), 1532 (Ar-C), 1245 (C=S), 1145 (C-N), 1117 (C-O). δ_H (500 MHz, $CDCl_3$) 0.88 (6H, t, 2x CH_3), 1.26–1.81 (40H, m, 20x CH_2), 3.97 (4H, t, 2x CH_2), 6.95 (4H, d, J = 9.15 Hz, Ar-H), 7.56 (4H, d, J = 9.15 Hz, Ar-H), 8.06 (4H, s, Ar-H), 9.10 (2H, s, 2x NH), 12.24 (2H, s, 2x NH). δ_C (125 MHz, $CDCl_3$) 13.1, 21.5, 24.9, 28.1, 28.2, 28.4, 28.5, 30.8, 67.1, 113.4, 125.0, 127.7, 129.6, 135.1, 156.6, 166.5, 178.3.

*N1,N4-bis[(4-Tetradecoxyphenyl)carbamothioyl]terephthalamide (**2c**).* N1,N4-bis[(4-tetradecoxyphenyl)carbamothioyl]-terephthalamide (**2c**) was obtained from 4-(tetradecyloxy)-benzenamine **1c** (0.31 g, 1 mmol). (0.20 g, 46%) as yellow



Scheme 1: Synthesis of bis(thiourea) derivatives.

solid, m.p: 208–210°C; (Found: C, 69.98; H, 8.72; N, 7.36; S, 7.49. $C_{50}H_{74}N_4O_4S_2$, Requires C, 69.9; H, 8.7; N, 7.4; S, 7.5%); ν_{max} (KBr/cm^{-1}) 3397 (NH), 2918 (CH), 1669 (C=O Amide), 1531 (Ar-C), 1244 (C=S), 1148 (C-N), 1117 (C-O). δ_H (500 MHz, $CDCl_3$) 0.88 (6H, t, 2x CH_3), 1.26–1.80 (48H, m, 24x CH_2), 3.98 (4H, t, 2x CH_2), 6.95 (4H, d, J = 8.6 Hz, Ar-H), 7.56 (4H, d, J = 8.6 Hz, Ar-H), 8.06 (4H, s, Ar-H), 9.05 (2H, s, 2x NH), 12.23 (2H, s, 2x NH). δ_C (125 MHz, $CDCl_3$) 14.2, 22.8, 26.1, 29.3, 29.5, 29.7, 29.8, 32.0, 68.3, 114.8, 125.9, 128.4, 130.1, 136.2, 158.2, 165.6, 178.0.

2.3. Antibacterial Screening. The synthesised bis(thiourea) compounds **2a–2c** were screened for the antibacterial activities against Gram-negative bacteria *E. coli* and employing turbidimetric kinetic method. The bacteria were cultured on Luria-Bertani (LB) agar for 24 h at 37°C. The inoculums were prepared by allowing bacteria to grow on media containing nutrient broth at 37°C with permanent stirring at 250 rpm for 18 h. The inoculums (0.2 mL) were inoculated with 10 mL of culture medium (with increasing concentration of the compounds dissolved in propanol). The mixture was shaken at 180 rpm at 37°C. Inoculums with propanol were used as a control. Aliquots of each replicates were taken at every 1 h interval for 6 h. The transmittances (T) were recorded using a UV-Visible spectrophotometer (Optima SP-300). The antibacterial activities were determined by plotting a graph of ln Nt versus time. The ln Nt is defined as transmittance value, which represents the number colony forming units (CFU) mL^{-1} for bacteria versus time following expressions: *E. coli* ln Nt = 27.1–8.56T [23].

2.4. Molecular Docking. The docking studies on **2a–2c** were carried out using AutoDock Vina 1.1.2 program [24]. The polar hydrogens of the compounds and protein were added with AutoDock Tools 1.5.6 [25] prior to docking with AutoDock Vina program. The cubic grid box of 60 Å size (x, y, and z) with a spacing of 0.375 Å were centred on the active site of the protein. The X-ray crystal structure of the enzyme enoyl ACP reductase (FabI) of *E. coli* (PDB entry: 1C14) was retrieved from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) [26].

3. Results and Discussion

3.1. Chemistry. The synthesis of bis(thiourea) **2a–2c** involved multiple steps reactions. The reaction involved alkylation of 4-acetamidophenol with a series of bromoalkanes (C_{10}, C_{12}, and C_{14}) in the presence of K_2CO_3 followed by hydrolysis to form benzenamine derivatives (intermediate) **1a–1c**. The reaction of intermediate **1a–1c** prior to deprotonation by TEA for 3 h at room temperature gave bis(thiourea) **2a–2c** with moderate yields of 46–69%. The synthesis route of **2a–2c** is illustrated in Scheme 1.

The IR spectra showed the successful formation of **2a–2c** with the presence of frequencies at 3397–3393 cm^{-1} attributed to ν_{NH} band. The absorption attributed to aliphatic carbon chain was observed at 2922–2918 cm^{-1}, while strong absorption band at 1669–1668 cm^{-1} corresponded to $\nu_{C=O}$ amide. The aromatic ring was represented by the absorption bands at 1532–1531 cm^{-1}. The $\nu_{C=S}$ absorption bands were observed at 1245–1244 cm^{-1} which confirmed the formation of thiourea in the compounds [27]. The ν_{C-N} absorption band was observed at 1148–1144 cm^{-1} [28].

Further confirmation of the formation of **2a–2c** was supported by ^{1}H and ^{13}C NMR spectroscopies. The two singlets attributed to CSNH and CONH peak were observed at 12.25–12.23 ppm and 9.14–9.05 ppm, respectively, which indicated the formation of thiourea [7]. The peak of CSNH

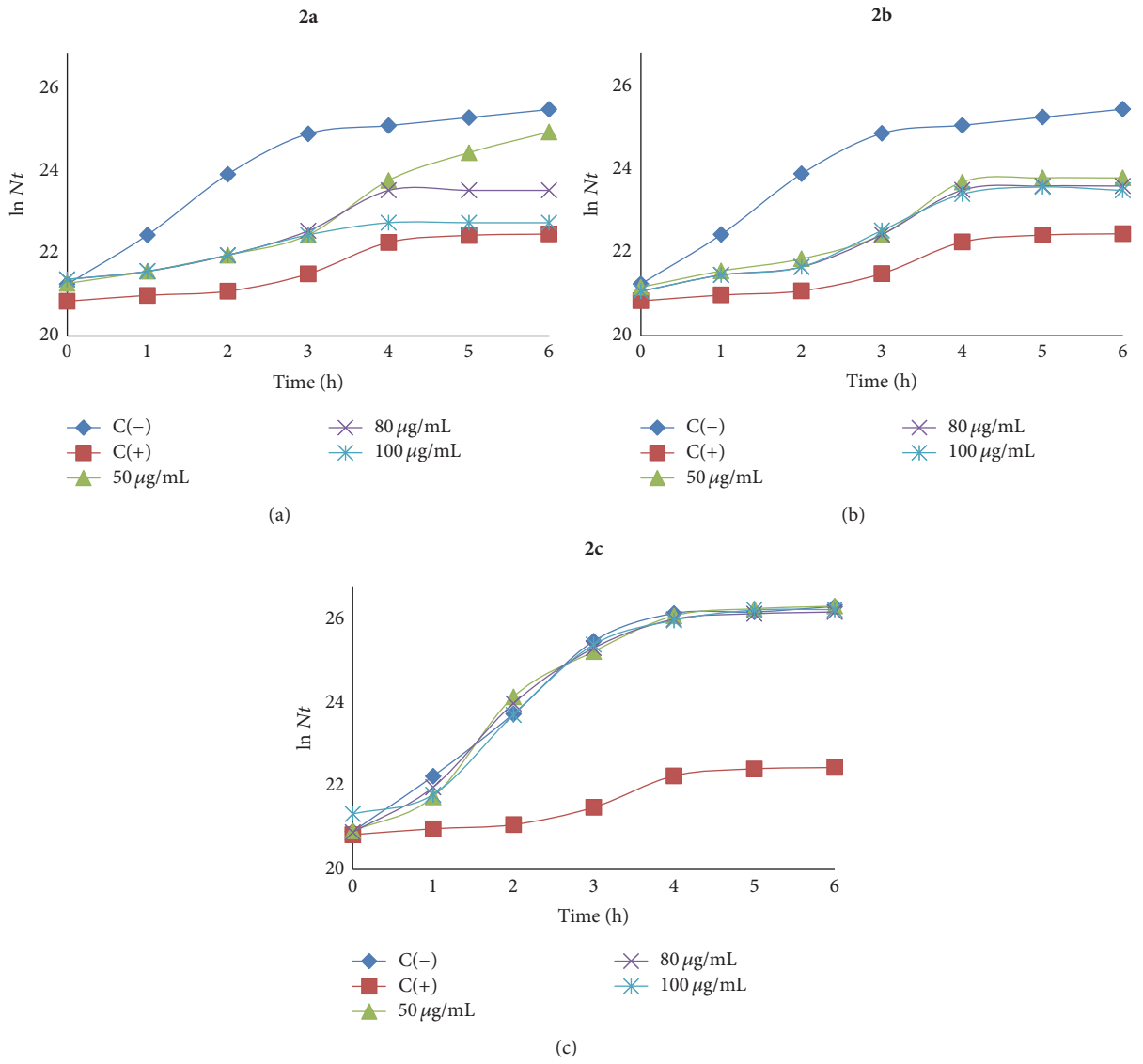


FIGURE 1: Growth of *E. coli* in media containing compounds: (a) **2a**, (b) **2b**, and (c) **2c**.

was at higher chemical shift due to deshielding effect [18]. Multiple peaks observed at 8.06–6.94 ppm were assigned to aromatic protons. The resonance as triplet at 3.97 ppm was attributed to -OCH_2 while alkyl chain (-CH_2) was represented by the peaks at 0.88–1.81 ppm.

The resonance of ^{13}C NMR at 178.3–178.2 ppm and 166.4–165.5 ppm was attributed to C=S and C=O, respectively. The multiple resonances observed at 158.2–113.4 ppm were assigned to aromatic carbons in the compound. The -OCH_2 was observed at 68.4–67.1 ppm while -CH_2 was observed as multiple resonances due to the number of carbon chains at 32.0–13.1 ppm. The -OCH_2 peak appeared at higher frequency compared to alkyl -CH_2 resonance due to downfield effect as it has been attached to higher electronegative atom (oxygen) [29].

3.2. Antibacterial Activity and Docking. The synthesised bis-(thiourea) derivatives **2a–2c** were screened for antibacterial activities using turbidimetric kinetic method. Compounds **2a–2c** were examined at the concentration of 50 μg/mL, 80 μg/mL, and 100 μg/mL against Gram-negative bacteria *E. coli* ATCC 25922. The result for an antibacterial assay for each compound is shown in Figure 1. The results were represented by graphs of ln Nt versus time (t) where ln Nt, defined as transmittance value, corresponded to the number of colony forming units (CFU) mL^{-1} for *E. coli* versus time following expression $\ln Nt = 27.1–8.56T$ [23]. Compounds **2a** and **2b** showed excellent inhibition against *E. coli* compared to **2c**.

The minimum inhibitory concentration (MIC) was also performed by extrapolating the concentration at the zero growth rate of *E. coli* ($\mu = 0$) [30]. MIC is the lowest concentration of the antimicrobial agent that inhibits the development of visible growth after overnight incubation and use to confirm the resistance of microorganisms to the antibacterial agent [31]. Propanol was used as negative control and

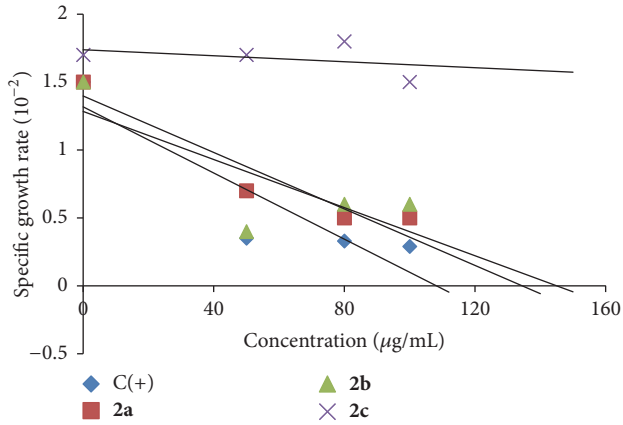


Figure 2: Minimum inhibitory concentration of **2a–2c** against *E. coli*.

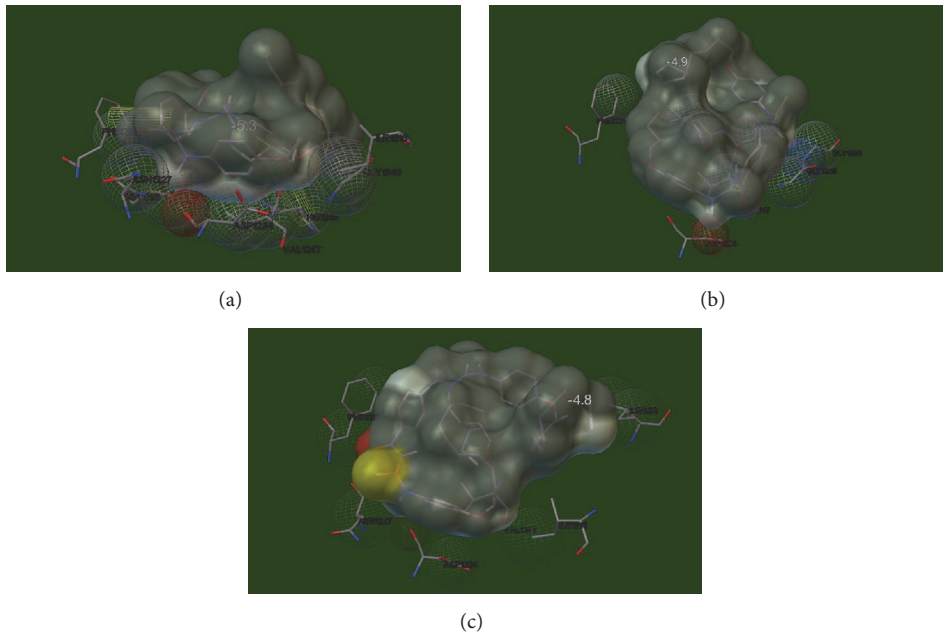


Figure 3: The molecular binding of (a) **2a**, (b) **2b**, and (c) **2c** with enzyme enoyl ACP reductase (FabI) of *E. coli*.

ampicillin as positive control. The MIC of **2a–2c** is shown in Figure 2.

The MIC data obtained showed that both **2a** and **2b** exhibited excellent antibacterial activities with MIC values of 135 μg/mL and 145 μg/mL, respectively. The MIC value of ampicillin was observed at 108 μg/mL. The MIC values less than 200 μg/mL can be suggested for pharmacological purposes [32]. It was believed that the antibacterial activities of **2a** and **2b** were due to the lipophilic character of the long alkyl chain which has the ability to disrupt microorganism cell wall [20, 21].

However, the antibacterial activities of the compounds decreased as the alkyl chains increased. Compound **2c** with C_{14} alkyl chains, for instance, showed the MIC values of >200 μg/mL. This phenomenon was due to the cutoff effect where the compound demonstrated a parabolic effect as the alkyl chain lengths increased from 14 to 16 [20]. In comparison to **2a** and **2b**, **2c** which has the longest carbon chain could have imitated the molecule in the lipid bilayer of organism and afforded less disruption in the bacteria membrane [33].

For the further understanding of the interactions between Gram-negative bacteria *E. coli* and bis(thiourea) derivatives **2a–2c** as potential antibacterial agents, **2a–2c** were interacted via docking to the active site of the enzyme enoyl ACP reductase (FabI) of *E. coli* (PDB entry: 1C14) using AutoDock Vina 1.1.2 program [24–26]. The binding interaction for **2a–2c** is shown in Figure 3.

The binding affinity of **2a–2c** was evaluated based on binding free energies (ΔG_b, Kcal/mol) [34]. Based on the binding model depicted in Figure 3, **2a** possessed the highest binding affinity with a binding free energy of −5.3 Kcal/mol followed by **2b** and **2c** with −4.9 and −4.8 Kcal/mol, respectively. Compound **2a** is strongly bound to enzyme enoyl ACP

reductase (FabI) of *E. coli* through π-π bond interactions (yellow colour cylindrical wireframe) with hydrophobic pockets of Phe 1231 and His 1246. The hydrophobic interaction between phenyl rings has increased the lipophilicity of the compound [32]. Other basic residues such as Asn 1227, Ser 1228, Asp 1224, Val 1247, Gly 1249, and Ile 1216 (Figure 3) were observed in close proximity to compound **2a,** which suggested that a strong electrostatic interaction was also involved in the binding process [35]. On the other hand, the increase of carbon chain in **2b** and **2c** was accountable for lesser binding affinity [36].

4. Conclusions

Novels bis(thiourea) derivatives **2a–2c** were successfully synthesised and evaluated for antibacterial activities against *E. coli*. The MIC data obtained showed that **2a** and **2b** with $n = 10$ and $n = 12$ exhibited excellent antibacterial activities with MIC values of 135 μg/mL and 145 μg/mL, respectively. The increase of carbon chain has decreased the antibacterial activities. Further studies on the correlation of the molecular structure and binding affinity via molecular docking interaction study revealed the binding interaction of **2a–2c** with enzyme enoyl ACP reductase (FabI) of *E. coli*, in a similar trend to that of the biological activities. Based on the molecular docking and antibacterial assay study, it can be suggested that **2a** is a potential antibacterial agent for pharmaceutical applications.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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