Evaluation of Low Molecular Weight Bis-Urea Derivatives as Antimicrobial Agents

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ABSTRACT

Antibiotic resistance against common microbes is an ongoing concern worldwide. This warrants continuous studies that aim to discover new compounds with antimicrobial properties. In this study, sixteen low molecular weight bis-urea derivatives were screened for their in vitro antimicrobial properties using agar well diffusion method. The structure of the bis-urea compounds is comprised of cyclic and aromatic linkers and a variety of symmetric end groups such as aliphatic chains and heteroaromatic groups. Significant antimicrobial activity against strains of *Escherichia coli, Staphylococcus aureus*, and *Klebsiella pneumoniae* was observed for compounds with long aliphatic chains compared to those with benzyl and heteroaromatic end groups. Further studies on the minimum inhibitory concentration and cytotoxicity can aid in the development of these compounds as antimicrobial agents as well as for other possible biomedical and environmental applications.

Keywords: antimicrobial activity, bis-urea derivatives, agar well diffusion, antimicrobial agents

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INTRODUCTION

Misuse and abuse of antibiotics have been prevalent in the Philippines for a long time due to misconceptions about their proper use and the availability of the drugs in local stores (Barber et al. 2017). This attitude towards antibiotics, among other factors, contributes to the emergence and spread of antibiotic-resistant strains, making this a global health concern (Nys et al. 2004). Common bacteria such as *Escherichia coli (E. coli)* are already resistant to antibiotic drugs such as cefazolin, gentamicin, ciprofloxacin, and older drugs including ampicillin, oxytetracycline, trimethoprim, and chloramphenicol (Nys et al. 2004). In other studies, *Staphylococcus aureus (S. aureus)* was also found to exhibit resistance against chloramphenicol, tetracycline, and ciprofloxacin (Juayang et al. 2014) while drugs such as ceftazidime and avibactam have been ineffective against *Klebsiella pneumoniae* (*K. pneumoniae)* strains (Chou et al. 2016). The growing antibiotic resistance is a serious threat to modern society, so there has been a necessity to discover new antimicrobial agents that would be effective against strains yet to be discovered (Moellering 2011; Kumar B et al. 2016).

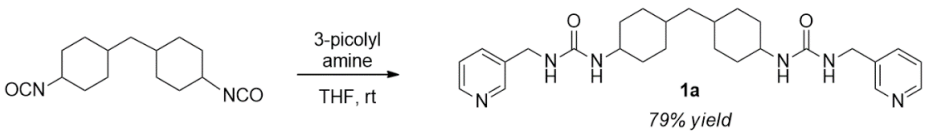
Some of the more diverse antibiotic agents discovered to aid this problem are low molecular weight compounds, which include peptides (Baquero et al. 1978; Marshall and Arenas 2003), steroids (Moore et al. 1993), and other compounds isolated from natural sources (Höltzel et al. 2000). Synthetic low molecular weight compounds have also been reported as antimicrobial agents (Rufián-Henares and Morales 2007; Keche et al. 2012; Poonia et al. 2020). Specific examples of these synthetic compounds include quinoline derivatives which contain urea groups (Keche et al. 2012) and bis (urea-1,2,3-triazole) compounds (Poonia et al. 2020). Urea moieties have been incorporated into molecules used as a scaffold for bioactive molecules with both organic and medicinal applications (Gündüz et al. 2020). Specifically, urea derivatives have been known to possess both antimicrobial and antiviral properties (Sun et al. 2006; Abdel-Rahman and Morsy 2007; Džimbeg et al. 2008). Although more specific mechanisms explaining the antimicrobial properties of urea derivatives are yet to be studied, there are few studies that have been reported. For instance, Gündüz et al. (2020) observed that 1,3-disubstituted urea derivatives support the quorum sensing inhibition of *Pseudomonas aeruginosa* which decreased the biofilm formation of the bacteria. In another study by Lal et al. (2020), urea-1,2,3-triazole-amide hybrid compound with a good antimicrobial activity was observed to have significant docking interactions with the active site of *S. aureus* DNA gyrase complexed with DNA.

In this study, sixteen (16) readily synthesized low molecular weight bis-urea compounds were screened for antimicrobial activity against three organisms, *E. coli, K. pneumoniae,* and *S. aureus* using agar well diffusion method. The linkers used for the bis-urea compounds are cyclic and aromatic moieties, whereas the end groups are either aliphatic chains, benzyl, or picolyl (pyridyl-containing) groups. Pyridine derivatives are well-studied for their anti-hypertensive, anti-neoplastic, and anti-inflammatory properties (Lednicer and Mitscher 1977) and have also been previously reported to exhibit antimicrobial properties (Patel et al. 2010; Kumar S et al. 2016). Moreover, the incorporation of long aliphatic chain lengths enhances the antimicrobial properties as observed by Akedo et al. (1977), Violette et al. (2006), and Yong et al. (2007).

MATERIALS AND METHODS

Synthesis of bis-urea compounds

A series of bis-urea compounds were synthesized following the representative scheme shown below (Genio and Paderes 2021). Following the method used by Rutgeerts et al. (2019), various aromatic and cyclic diisocyanates and amines containing aliphatic, aromatic, and heteroaromatic groups were used to synthesize the bis-urea compounds. The diisocyanates and amines were stirred in anhydrous tetrahydrofuran (THF) at room temperature for an hour. The reaction mixture was kept under a nitrogen atmosphere and anhydrous THF was used because of the sensitivity to moisture of the starting reagents. Products were precipitated out, filtered, washed with diethyl ether, and dried under a vacuum.



Scheme 1. Representative procedure for the synthesis of bis-urea compound **1a**.

The detailed method and characterization of the compounds used in this study were previously reported by Genio and Paderes (2021). ^{1}H NMR, ^{13}C NMR, mass spectrometry, and FTIR spectroscopy were among the methods performed to confirm the synthesis of the compounds.

Agar Well Diffusion Method

Screening of the antimicrobial properties was performed by the Microbial Research and Services Laboratory (MRSL) of the Natural Sciences Research Institute (NSRI), University of the Philippines Diliman using the agar well diffusion method. Samples were all prepared in 98% ethanol using a 5 µg/mL concentration. Ethanol, which is the solvent used for the sample solutions, was used as the negative control. For the positive controls, commercially available antibiotics cloxacillin (CLOX) and cefalexin (CEF) were used. Both compounds were prepared in ethanol at 5 µg/mL concentration to compare their antimicrobial activities. The organisms used were *Escherichia coli* UPCC 1195, *Klebsiella pneumoniae* UPCC 1360, and *Staphylococcus aureus* UPCC 1143.

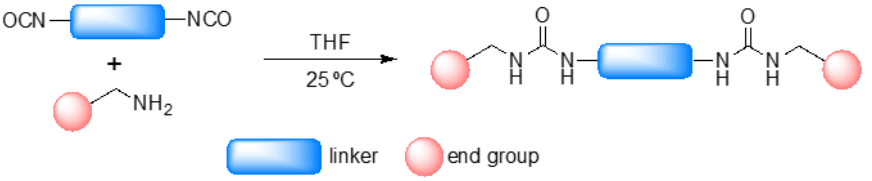
Using peptone water as suspending medium, suspensions of the test organisms were prepared from 24 h old culture of microbes. These microbial suspensions were introduced and swabbed onto the surface of about 3 mm thick, pre-poured nutrient agar plates. The swab was distributed evenly twice over the agar surface. Portions of 200 µL samples were placed on the three equidistant wells of 10 mm diameter made on the agar plate. The agar plates were left for 24 h at 35 °C and clearing zones were measured and used to calculate the antimicrobial index (AI) following the formula:

$$AI = \frac{\text{Diameter of clearing zone} - \text{Diameter of well}}{\text{Diameter of well}} \quad (1)$$

Formula for calculating antimicrobial index (AI)

RESULTS AND DISCUSSION

As shown in Scheme 2, the synthesis of low molecular weight bis-urea compounds was performed through condensation of various diisocyanates and amines. The reaction produced white powdery precipitates at 49-97% yields and were easily filtered off from the mixture (Genio and Paderes 2021). The solid products were washed several times with organic solvents such as diethyl ether to remove any unreacted or excess starting materials and ensure the purity of the bis-urea compounds. Spectra obtained from the characterization of these compounds confirmed the formation of the urea groups and showed only peaks that correspond to the predicted product, implying high purity. More specifically, the absence of the diisocyanate peaks and the appearance of carbonyl peaks were noted in the IR spectra. Proton peaks at a chemical shift of around 4-6 ppm were also observed in the ^{1}H NMR spectra, indicating the successful formation of urea groups.



Scheme 2. General scheme for the synthesis of bis-urea compounds.

The synthesized bis-urea compounds, shown in Figure 1, were tested for antimicrobial activity against gram-positive bacteria, *S. aureus,* and gram-negative bacteria, *E. coli* and *K. pneumoniae*. Examples of the resulting wells from the assay using compound **6b** are shown in Figure 2. Three wells in the agar plate were observed to have clearing areas where the microbial organisms did not grow. These areas were measured to calculate the antimicrobial index per compound against specific organisms.

The results of the clearing zones in millimeters and the calculated antimicrobial indices of all sixteen compounds against each of the three organisms are summarized in Table 1. For comparison, results for the antimicrobial activity of the commercial antibiotics, cloxacillin and cefalexin, prepared at 5 µg/ml using ethanol were included in Table 1.

The results show that all compounds, except **1d** and **1e,** exhibited zones of inhibition when tested against *E. coli*. However, only the antimicrobial indices of **2b**, **4b,** and **6b** are significantly larger than those with the other derivatives. Among the three compounds, **2b** was observed to have the largest AI value, which is at 2.1. For the bacteria *K. pneumoniae*, most compounds displayed a zone of inhibition except for compounds **1a** and **1d**. Similarly, good activity was observed for compounds **2b**, **4b,** and **6b,** with **2b** and **4b** showing significantly larger AI (2.6 and 2.2, respectively). Moreover, compounds **2b**, **4b**, **5b,** and **6b** also displayed notable AI values against *S. aureus*.

Generally, compounds containing long aliphatic end groups (**2b**, **4b**, **5b,** and **6b**) demonstrated potential antimicrobial activity against the three organisms. The antimicrobial activities seemed to be dependent both on the linker and the alkyl end groups. For instance, bis-urea compounds with dicyclohexyl group as the linker (compounds **1**) did not exhibit good activity against all the organisms tested. Compounds with cyclohexyl group (**2b**) and aromatic linkers (**4b**, **5b,** and **6b**) on the other hand, displayed significant antimicrobial activity.

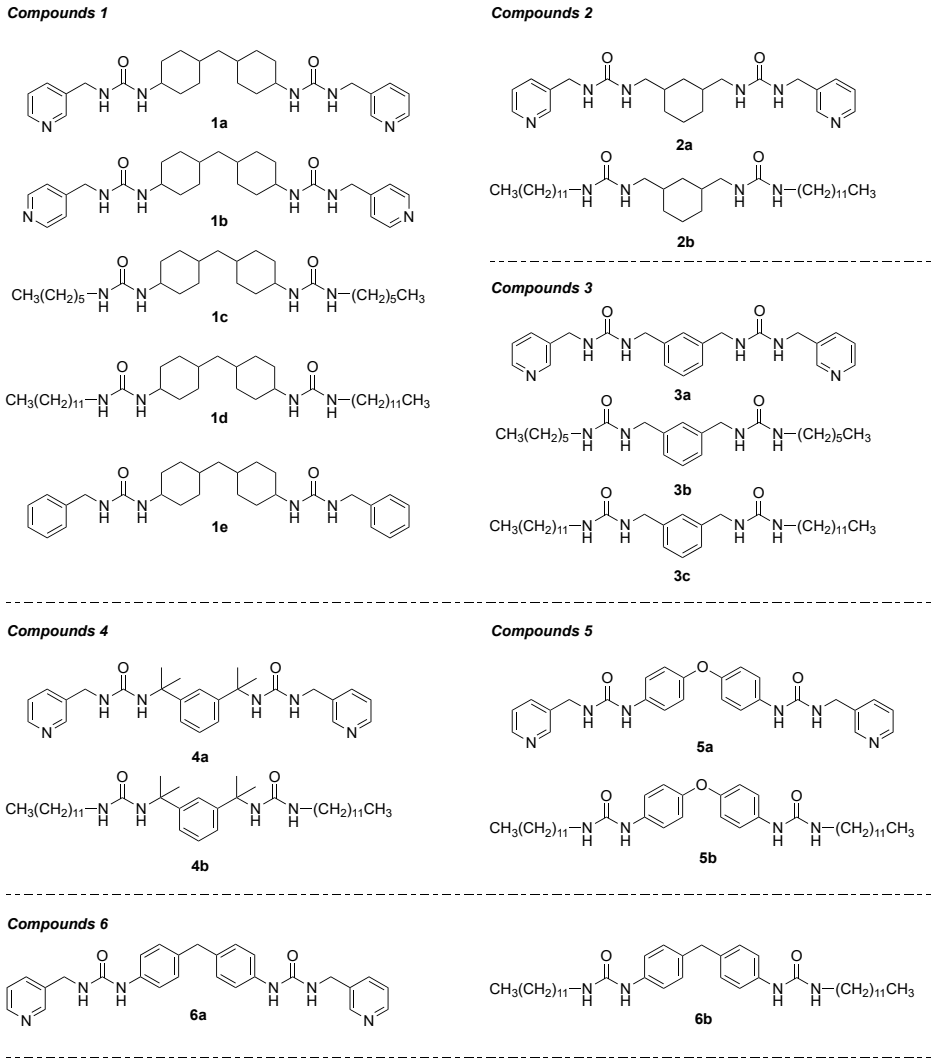


Figure 1. Structures of synthesized bis-urea compounds.

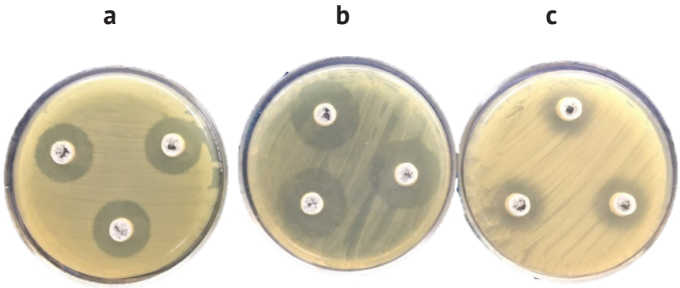


Figure 2. Images of the clearing zones of compound **6b** in agar well diffusion antimicrobial assay against a) *E. coli*, b*) K. pneumoniae,* and c) *S. aureus.*

Table 1. Results of antimicrobial activity assay

Sample[a]	*E. coli*		*K. pneumoniae*		*S. aureus*	
	Clearing zone, mm[b]	AI	Clearing zone, mm[b]	AI	Clearing zone, mm[b]	AI
1a	11.0 ± 0.0	0.1	-	0	12.0 ± 0.0	0.2
1b	11.0 ± 0.0	0.1	11.0 ± 0.0	0.1	12.0 ± 0.0	0.2
1c	11.0 ± 0.0	0.1	11.0 ± 0.0	0.1	12.0 ± 0.0	0.2
1d	-	0	-	0	11.0 ± 0.0	0.1
1e	-	0	12.0 ± 0.0	0.2	12.0 ± 0.0	0.2
2a	11.7 ± 0.6	0.2	12.7 ± 0.6	0.3	12.7 ± 0.6	0.3
2b	30.7 ± 0.6	2.1	36.0 ± 0.0	2.6	25.0 ± 0.0	1.5
3a	13.3 ± 0.6	0.3	14.0 ± 0.0	0.4	12.0 ± 0.0	0.2
3b	12.3 ± 0.6	0.2	14.0 ± 0.0	0.4	12.7 ± 0.6	0.3
3c	11.0 ± 0.0	0.1	12.0 ± 0.0	0.2	12.0 ± 0.0	0.2
4a	12.0 ± 0.0	0.2	12.0 ± 0.0	0.2	12.0 ± 0.0	0.2
4b	21.0 ± 0.0	1.1	32.3 ± 0.6	2.2	20.0 ± 0.0	1
5a	12.3 ± 0.6	0.2	12.7 ± 0.6	0.3	12.0 ± 0.0	0.2
5b	15.0 ± 0.0	0.5	11.0 ± 0.0	0.1	25.3 ± 0.6	1.5
6a	12.0 ± 0.0	0.2	13.0 ± 0.0	0.3	12.7 ± 0.6	0.3
6b	24.3 ± 0.6	1.4	29.3 ± 0.6	1.9	16.3 ± 0.6	0.6
CLOX[c]	13.3 ± 1.2	0.3	11.3 ± 0.6	0.1	25.7 ± 1.5	1.6
CEF[d]	16.3 ± 1.2	0.6	12.3 ± 1.2	0.2	12.7 ± 1.2	0.3
EtOH[e]	12.0 ± 0.0	0.2	13.0 ± 0.0	0.3	12.0 ± 0.0	0.2

[a]Samples were prepared at 5 µg/mL in ethanol; [b]Clearing zone is the average of three trials; [c]CLOX = Cloxacillin (positive control) at 5 µg/mL in ethanol; [d]CEF = Cefalexin (positive control) at 5 µg/mL in ethanol; [e]EtOH = ethanol; (-) No clearing zone was observed.

The observed difference in the antimicrobial activity between compounds with the heteroaromatic end group (**2a, 4a, 5a,** and **6a**) versus compounds with the aliphatic end groups (**2b**, **4b**, **5b,** and **6b**) may be due to increased hydrophobicity brought by the long alkyl chain lengths (Li et al. 2013). The hydrophobic nature of compounds that were previously analyzed from membrane binding and efficacy can be a factor affecting the compounds' antimicrobial activity (Kuroda et al. 2008).

Some of the antimicrobial indices obtained in this study are either comparable to or higher than the observed values for the antibiotics cloxacillin and cefalexin. Compounds **2b, 4b,** and **6b** have significantly higher AI values than both CLOX and CEF against the gram-negative bacteria, *E. coli* and *K. pneumoniae*. On the other hand, only **2b** and **5b** have comparable AI values with CLOX against the gram-

positive bacteria, *S. aureus*. These results imply the potential of these compounds as antimicrobial agents with properties that could be readily fine-tuned through structural modifications. However, further studies may be required to explore this potential antimicrobial property.

CONCLUSIONS

Sixteen bis-urea compounds were evaluated for their antimicrobial activity against *E. coli*, *K. pneumoniae*, and *S. aureus*. Bis-urea compounds with long aliphatic side chains (**2b**, **4b**, **5b,** and **6b**) showed significant activity against the tested organisms. Compound **2b** was found to be effective against all bacteria with AI values of 2.1 (*E. coli*), 2.6 for (*K. pneumoniae*), and 1.5 (*S. aureus*). Compounds **4b** and **5b** were both selective with **4b** being the most effective against *K. pneumoniae* and **5b** against *S. aureus.* Compound **6b** on the other hand, showed activity with both *E. coli* and *K. pneumoniae*. Further analysis of the potential of these bis-urea compounds as antimicrobial agents through antimicrobial assays against different organisms, cytotoxicity tests, and minimum inhibitory concentration assays can be conducted to explore more applications of these compounds as drugs or compatible ingredients to formulations. Although water is more commonly used as solvent for antimicrobial activity assays since it does not usually affect the antimicrobial activity of the compounds, the synthesized compounds were found to be insoluble in this solvent. Thus, incorporating more polar end groups and linkers can also be explored in future research.

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