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In silico and *in vitro* assay of Hexagamavunon-6 analogs, Dibenzilyden-N-Methyl-4-piperidone as antibacterial agents

Tesia Aisyah Rahmania[1], Ritmaleni Ritmaleni[1*], Erna Prawita Setyowati[2]

[1]Departement Of Pharmaceutical Chemistry, Gadjah Mada University, Sekip Utara, Yogyakarta 55281, Indonesia.
[2]Departement Of Pharmaceutical Biology, Gadjah Mada University, Sekip Utara, Yogyakarta 55281, Indonesia.

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ABSTRACT

A new series of analogs Hexagamavunon-6 (HGV-6) was prepared and screened for antibacterial activity against *Streptococcus mutans* (ATCC 25175), *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633), *Psedomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae,* and *Enterococcus faecalis* (ATCC 29212) using disk diffusion method. The antibacterial results showed that 3,5-Bis-(2',4'-dichlorobenzilyden)-N-methyl-4-piperidone (**1c**) compound display significant inhibition and broad-spectrum antibacterial activity. This is in accordance with the *in silico* evaluation showing that compound **1c** has a lower docking score than both compounds **1a** and **1b**. None of the tested compounds were as active as the reference standard drug Amoxicillin.

INTRODUCTION

Bacteria are microorganisms consisting of Gram-positive and Gram-negative. Gram-positive bacteria are composed of a peptidoglycan wall containing a specific surface polysaccharide and having polar properties, whereas Gram-negative bacteria have three layers of cell, namely, outer membrane, cell wall, and inner plasma membrane so that antibacterial compounds will be more difficult to inhibit Gram-negative bacterial activity (Glauert and Thornley, 1969). Some bacteria can cause serious infections such as endocarditis (Holland *et al*., 2016), meningitis (Wenger *et al*., 1990), pneumonia (Dasaraju and Liu, 1996), and sepsis (Minasyan, 2019).

Chlorine, the most common disinfectant, is moderately oxidative and reacts with various components of bacterial cells (Kim *et al*., 2008) so that the presence of Cl halogen atoms can play a role in inactivating various microorganisms such as *Escherichia coli, Enterococcus faecalis, Psedomonas aeruginosa, Bacillus subtilis,* and *S. aureus* but remain safe for humans because chlorine has lower toxin levels compared to other halogen compounds such as fluorine and bromine (McDonnell and Russell, 1999). In addition to the presence of Cl atoms, heterocyclic rings play a role in medicinal chemistry as therapeutic agents including acting as antitumor (Chen *et al*., 2014; El-Sawy *et al*., 2013), antifungal (Cao *et al*., 2014; Russell and Soiket, 2015), antiviral (Salem *et al*., 2013; Stamatiou *et al*., 2003), anti-inflammatory (El-Sawy *et al*., 2014), and broad spectrum of antibacterial drugs (El-Sawy *et al*., 2013; Mustafa, 2018).

Hexagamavunon-6 (HGV-6) is a curcumin analog which was synthesized by the Faculty of Pharmacy, Gadjah Mada University, and has been known to have active antibacterial activity on Gram-positive bacteria with 1,000 µg/ml inhibitory concentrations (Sardjiman, 2000). The activity of HGV-6 predicted because the structure has four chloro and two hydroxy groups in which plays a role in denaturing proteins in the bacterial cell wall.

The objective of this research was to synthesis analogs curcumin carried out by reacting 2-chlorobenzaldehyde,

*Corresponding Author
Ritmaleni Ritmaleni, Departement Of Pharmaceutical Chemistry, Gadjah Mada University, Sekip Utara, Yogyakarta 55281, Indonesia.
E-mail: ritmaleni @ ugm.ac.id

4-chlorobenzaldehyde, and 2,4-dichlorobenzaldehyde with N-methyl-4-piperidone which is a heterocyclic compound with a nitrogen atom that still has one pair of free electrons. All of the synthesized compounds tested for antibacterial activity against Gram-positive and Gram-negative bacteria using the Agar diffusion method. Each compound has a chloro atom in an aromatic group and N atom in a heterocyclic group of N-methyl-4-piperidone.

To assist in identifying molecular targets from the active side of the compound structure, the chemical docking method is used (Meng *et al*., 2011). Structural models of proteins in bacteria will be inhibited by compounds obtained through Protein Data Banks (PDB).

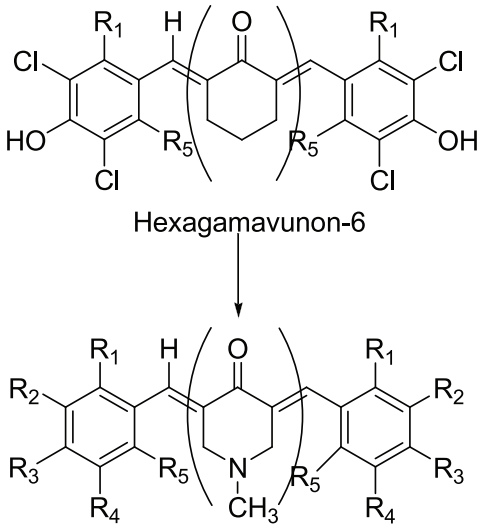
MATERIALS AND METHODS

Materials

All starting materials, reagents, and solvents were of reagent grade for analysis. Synthesis was controlled using UV lamp 254 and 366 nm (Desaga Heidelberg) with Thin Layer Chromatography (TLC) silica gel F254 (Merck). Melting point was recorded by Buchi melting point B540, Elucidation 0structure of the compound was recorded on Fourier-transform infrared spectroscopy (FTIR) Perkin Elmer and beam splitter KBr/Gemid-infrared, Direct Inlet-Mass Spectrometry (DI-MS) (QP2010S SHIMADZU), ^{1}H-NMR (JOEL), and ^{13}C-NMR (JOEL).

General procedure for synthesis

N-methyl-4-piperidone (1 mmol) was stirred with sodium hydroxide (0,1 gram) as a catalyst. Then aromatic aldehydes (2 mmol) were added as the starting material, and ethanol (2 ml) as the solvent was added dropwise until it was dissolved. The stirring reaction is carried out until the starting material is not left over, controlled by using TLC. The reaction is stopped when the TLC plate has formed a single product spot and then the mixture was washed with aquadest and dried at 45°C for 24 hours (Gregory *et al*., 2013).



Scheme 1. Basic frameworks.

In vitro antimicrobial activity assay

The antibacterial activities were tested using the agar diffusion method. Bacterial culture (*Streptococcus mutans, E. coli* ATCC 25922*, B. subtilis* ATCC 6633*, P. aeruginosa* ATCC 27853, *Klebsiella Pneumoniae,* and *E. faecalis* ATCC 29212) was prepared in Brain Heart Infusion broth and adjusted with Optical Density at a wavelenght of 600 nm to 10^{8} cfu/ml (CLSI, 2012). Mueller Hintor Agar was poured on a different sterile petri dish and allowed to solidify. Then, the surface of the agar medium was inoculated with a sterile swab with the bacterial strain. All test samples were prepared in Dimethyl sulfoxide (DMSO) (2 mg/ml) and 10 µl of each concentration added in a paper disk. DMSO was used as negative control, and amoxicilin (31.25 µg/ml) was used as positive control in all experiments. Finally, all petri dishes were incubated for 24 hours at 37°C. The results of antibacterial activity were evaluated as diameter inhibiton. It was determined by the inhibition zone diameter on test plates against the compound and compared with control antibiotic inhibition zone (Balouiri *et al*., 2016).

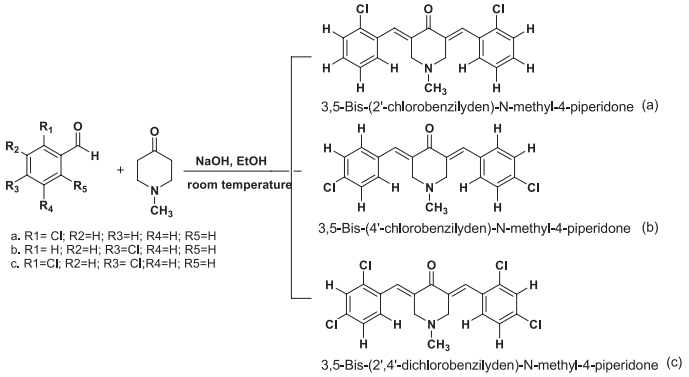
Molecular modeling studies

Preparation of ligand structure

All of the compounds (**1a-c**), HGV-6 and Amoxicillin, the reference molecules of the docking simulations, were built in the two-dimensional model in the form of Molecular Operating Environment (MOE) database. The determination of conformation was then performed on the database. Furthermore, its minimal energy was calculated (Wijianto *et al*., 2019).

Docking simulations

MOE was used to evaluate the interaction between the compound and target protein of curcumin analogs so that it can inhibit the bacterial activity. The target protein was obtained from the PDB (ID code: 5OJ0). 5OJ0 is a protein for the bacterium *Streptococcus pneumoniae*. The bacterium is a Gram-positive bacterium whose bacterial cell wall structure can be used as a reference to see interactions in the cell wall structure of other Gram-positive bacteria. Protein contains a reference molecule that is Cefixime as the native ligand and group of Penicillin-Binding Protein 2X (PBP2X). All of the docking conformations were analyzed, and the best value with the precise pose was selected for further interaction study.



Scheme 2. Reagent and conditions of synthesis (Gregory *et al*., 2013).

RESULTS AND DISCUSSION

Synthesis

*Synthesis of 3,5-Bis-(2'-chlorobenzilyden)-N-methyl-4-piperidone **(a)***

Yellow crystal, yield = 72%, m.p. = 145°C

IR (KBr, cm^{-1}) 3425,58 (N-CH_3 streatching); 3062,96 (=C-H streatchcing aromatic); 2939,52 (C-H streatching aliphatic); 1674,21 (C=O streatching aromatic); 1627,92 (C=C streatching aliphatic); 1597,06 (C=C streatching aromatic); 756,10 (subtitued ortho).

^{1}HNMR δ 2,347 (3H, *s*,N-CH_3); 3,588 (4H, *s,* CH_2); 7,207 (2H, *m,* C-H aromatic); 7,267 (4H, *m,* C-H aromatic); 7,415 (2H, *m,* C-H aromatic); 7,979 (2H, *s,* C-H alkene)

^{13}CNMR δ 45,756 (R_2-N-**C**H_3); 56,881 (R_2-N-**C**H_2-C-R); 126,625 (C-aromatic); 130,148 (C-aromatic); 130,215 (C-aromatic); 130,541(C-aromatic); 133,814 (C-aromatic); 134,217 (Cα unsaturated); 134,448 (Cβ unsaturated); 135,369 (C-Cl); 186,358 (C=O)

DI-MS (EI, 70 eV) 100% area with [M+1] 357

*Synthesis of 3,5-Bis-(4'-chlorobenzilyden)-N-methyl-4-piperidone **(b)***

Pale yellow crystal, yield = 44%, m.p. = 170°C

IR (KBr, cm^{-1}) 3448,72 (N-CH_3 streatching); 3032,10 (=C-H streatching aromatic); 2939,52 (C-H streatching aliphatic); 1674,21 (C=O streatching carbonil); 1627,92 (C=C streatching aliphatic); 1581,63 (C=C streatching aromatic); 825,53 (subtitued para).

^{1}HNMR δ 2,444 (3H, *s*,N-CH_3); 3,701 (4H, *d,* CH_2); 7,292 (4H, *m,* C-H aromatic); 7,367 (4H, *m,* C-H aromatic); 7,723 (2H, *s,* C-H alkene).

^{13}CNMR δ 45,121 (R_2-N-**C**H_3); 57,178 (R_2-N-**C**H_2-C-R); 129,092 (C-aromatic); 131,799 (C-aromatic); 133,584 (C-aromatic); 133,786 (Cα unsaturated); 135,340 (Cβ unsaturated); 135,398 (C-Cl); 186,713 (C=O).

DI-MS (EI, 70 eV) 100% area with [M+1] 357

*3,5-Bis-(2',4'-dichlorobenzilyden)-N-methyl-4-piperidone (**c**)*

Yellow crystal, yield = 60%, m.p = 151°C

IR (KBr, cm^{-1}) 3425,58 (N-CH_3 streatching); 3070,66 (=C-H streatching aromatic); 2939,52 (C-H streatching aliphatic); 1689,64 (C=O streatching carbonil); 1612,49 (C=C streatching aliphatic); 1581,63 (C=C streatching aromatic).

^{1}HNMR δ 2,356 (3H, *s*,N-CH_3); 3,548 (4H, *s,* CH_2); 7,136 (2H, *d,* C-H aromatic); 7,153 (2H, *d,* C-H aromatic); 7,456 (4H, *d,* C-H aromatic); 7,889 (2H, *s,* C-H alkene).

^{13}CNMR δ 45,813 (R_2-N-**C**H_3); 56,814 (R_2-N-**C**H_2-C-R); 127,095 (C-aromatic); 130,119 (C-aromatic); 133,584 (C-aromatic); 131,194 (C-aromatic); 132,259 (C-aromatic); 133,142 (Cα unsaturated); 134,755 (Cβ unsaturated); 135,571 (C-Cl); 136,166 (C-Cl); 185,945 (C=O).

DI-MS (EI, 70 eV) 100% area with [M+1] 427

Antibacterial activity assay

Synthesized compounds were screened for *in vitro* activity by determining the inhibitory diameter against three Gram-positive and three Gram-negative bacteria and compared with the standard antibiotic, amoxicillin, by the Agar diffusion method. The inhibitory diameter of microbes is seen from the presence of clear areas formed around the disk paper which can then be measured using a ruler in millimeters (mm). DMSO solvent is used to dissolve the synthesized compound with a concentration of 100% and also used as a negative control. The positive control used is the antibiotic amoxicillin for the antibacterial test which is a class of β-lactam antibiotics (Akhavan and Vijhani, 2019). The amoxicillin concentration used is 31.25 µg/ml. After incubation at 37°C for 24 hours, the zone of inhibition of growth around each disk was measured and zone diameters were interpreted. The experiment was performed in triplets, and the results of the antibacterial activity test can be seen in Table 1, which is the result of observations of the inhibition diameter of bacterias using synthesized compounds by analog curcumin **1a-c.**

Most of the synthesized compounds have inhibition on the Gram-positive and Gram-negative bacteria at a concentration of 1,000 µg/ml except *K. pneumonia* in compound **1b**. Compound **1c** was found to have significant inhibition in both Gram-positive and Gram-negative bacteria up to a concentration of 125 µg/ml. Compounds **1a** and **1b** display moderate antimicrobial activity in Gram-positive bacteria and weak antimicrobial activity in Gram-negative bacteria. The result of antibacterial activity shows that the compound **1c** has four chloro substitution on a benzene ring carrying electron-withdrawing groups which have three free electron pairs so that it can form more hydrogen bonds with amino acids in bacterial cells compared to compounds **1a-b** which only have two chlorine atoms. The inhibition of *S. mutans*, *B. subtilis,* and *E. faecalis* bacteria, which are a class of Gram-positive bacteria, has a greater average inhibition diameter compared to *E. coli*, *P. aeruginosa,* and *K. pneumoniae* bacteria which are a Gram-negative bacterium. Gram-positive bacteria are composed of peptidoglycan wall containing a specific surface polysaccharide and have polar properties. Whereas Gram-negative bacterias have three layers of cell wrapping, namely, outer membrane, cell wall, and inner plasma membrane so that antibacterial compounds will be more difficult to inhibit Gram-negative bacteria activity (Silhavy *et al*., 2010).

To further investigate the molecular inhibitory activity of compounds in bacteria, an *in silico* study was conducted using docking. Docking results showed S values in Table 2, whereas the S value is the Gibbs free energy to measure the strength of the bond between the ligand with the site of action on the target protein. The smaller the S value, the stronger the bond formed with the ligand. Compound **1c** has the S value that closest to the positive control, amoxicillin.

Figure 1 shows the docking interaction of amoxicillin with the two amino acids Asn on bacterial cell wall through hydrogen bonds that are formed in the lone pair of electrons from the nitrogen atom in β-lactam structure and in the NH_2 group. Figure 2 shows the docking interaction of compound **1c** with one Asn amino acid in bacteria cell wall through nitrogen atoms in heterocyclic groups, N-methyl-4-piperidone. So that compound **1c** has the best activity compared to other synthesis compounds but has not been able to be better than Amoxicillin.

Table 1. *In vitro* antibacterial activity of the compound tested by Agar diffusion method as the diameter (mm) of the inhibition zone.

Compound	Concentration (µg/mL)	Gram-positive			Gram-negative		
		S. mutans	*E. faecalis*	*B. subtilis*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*
1a	1000	9	10	9	7	8	9
	500	8	-	7	-	8	-
	250	-	-	-	-	7	-
	125	-	-	-	-	-	-
	62.5	-	-	-	-	-	-
	31.25	-	-	-	-	-	-
1b	1000	8	8	7	8	8	-
	500	7	7	-	-	-	-
	250	-	6	-	-	-	-
	125	-	-	-	-	-	-
	62.5	-	-	-	-	-	-
	31.25	-	-	-	-	-	-
1c	1000	10	15	9	11	9	9
	500	9	8	8	8	8	7
	250	7	7	7	7	7	6
	125	7	6	6	7	7	-
	62.5	-	-	-	-	-	-
	31.25	-	-	-	-	-	-
DMSO		-	-	-	-	-	-
Amoxicillin	31.25	9	7	7	7	7	10

Note: Diameters of paper disc 5 mm.
= There is no clear area around paper disc

Table 2. S value of docking results from each test compound

Compound	S Value
1a	-10,7041
1b	-9,9642
1c	-12,1780
Amoxicillin	-13,0276

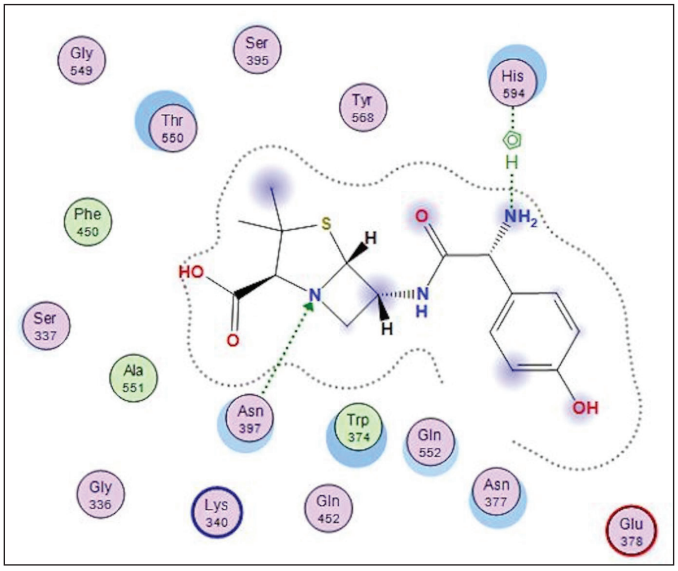


Figure 1. Docking interaction 5Oj0 with Amoxicillin.

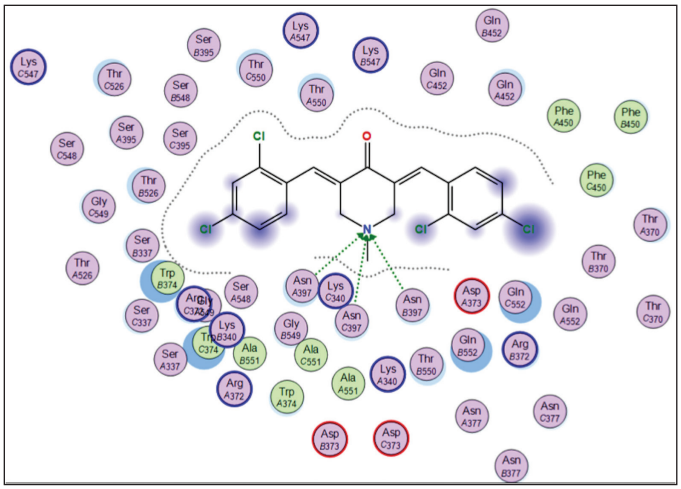


Figure 2. Docking interaction 5Oj0 with 1c compound.

CONCLUSION

All of the compounds can be synthesized using condensation reaction and produce moderate to excellent yields. The synthesized compound was evaluated for their *in vitro* antibacterial activity and indicated that all of the compounds have inhibitory activity in Gram-positive and Gram-negative bacteria at concentrations of 1,000 µg/ml except *K. pneumonia* in compound **1b**. The 3,5-Bis-(2',4'-dichlorobenzilyden)-N-methyl-4-piperidone (**1c**) compound has the most significant inhibition activity which

is Gram-positive bacteria up to a concentration of 62.5 µg/ml and Gram-negative bacteria to a concentration of 125 µg/m. Compound **1c** has the potential to be used as a drug with antibacterial activities through interaction and PBP2X inhibition confirm by docking studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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