N-(2-chlorobenzyl) formamide, a Novel Synthesized Antituberculosis Evaluation by Microplate Alamar Blue Assay

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***Abstract*— The multicomponent amidoalkylation reaction is done by mixing three compounds simultaneously. This method develops in the area of anti-tuberculosis. Tuberculosis (TB) is an infectious disease caused by M. tuberculosis. A novel N-(2-chlorobenzyl) formamide was synthesized by reacted with formic acid or NaBH4. The synthesized compounds were identified by structural elucidation using spectroscopic methods (1H-NMR, 13C-NMR, FTIR) and GC-MS. The antituberculosis activity test used the Microplate Alamar Blue Assay (MABA) method with a concentration series of 1000-1,954 µg / mL against M. tuberculosis bacteria strain H37Rv. The results showed that the product was successfully synthesized using a multicomponent reaction with formic acid. N-(2-chlorobenzyl)formamide has MIC value 1000 µg / mL for its evaluation on antituberculosis activity with MABA method but are less potent than INH (2 µg / mL) as drug control.**

Keywords—tuberculosis, bacteria, formamide

I. INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by the bacterium Mycobacterium tuberculosis. TB is one of the biggest health problems that requires special treatment, because this disease can cause death. This disease generally attacks the lungs (pulmonary TB), but can also attack other body parts (extrapulmonary TB). Globally, 10.0 million people were infected with TB in 2017. In Indonesia new TB cases in 2017 even became the second highest after India in the Asian region, which is as many as 1.2 million cases with an average death rate of 110 thousand soul [1].

Multidrug-resistant TB (MDR-TB) is a global problem that follows the development of TB itself. In addition to MDR-TB, also appears Extensive Drug Resistance (XDR-TB), where there was an increase in cases in 2017 to 8.5% from 6.2% in 2016. Patients with XDR-TB have a success rate of therapy of only 44% with mortality rate of 14-27% [1].

With an increase in cases of resistance to tuberculosis, research on identifying new drug targets and developing anti-TB drugs continues to be intensified so that obstacles in resistance problems can be resolved with the discovery of new anti-TB drugs that are potent and have lower side effects than anti-TB drugs. Circulating TB. Research on the discovery of new anti-TB drugs has emerged, including using multicomponent reaction methods [2][3].

Multicomponent reaction is one method of synthesis that is currently growing rapidly. Multicomponent reactions involve three or more starting materials (SM) for react to certain products which are composed of functional groups from the reacted SM. Multicomponent reactions are often known as the one pot reaction method. Until now, multicomponent reactions are still being developed using three or four component compounds (Ugi, 2001; Ramprasad et al., 2015; Weber, 2002; Ganem, 2009). The most developed multicomponent reaction is U-4CR which leads to the formation of α-acylaminocarboamides [3].

Research on the synthesis of chlorobenzyl-formamide compounds through modification of amidoalkylation using formic acid and NaBH4 as a reducing agent has been carried out and the activity of the synthesized compound against M. tuberculosis has been carried out. The basis for testing the results of the synthesis of antituberculosis compounds is the structural similarity to one of the first-line anti-tuberculosis drugs, namely isoniazid. The presence of benzene aromatic rings with Cl substitution is expected to increase the activity of compounds in inhibiting the growth of M. tuberculosis bacteria.

This research aims to synthesize chlorobenzyl-formamide compounds using multicomponent reaction method with reduced formic acid and NaBH4 activated boric acid and to test the anti-tuberculosis activity of chlorobenzyl-formamid compounds in vitro by Microplate Alamar Blue Assay (MABA) method.

II. METHOD

Chemistry: All reagents and solvent were pro analysis grade. 2-chlorobenzaldehyde (Sigma Aldrich), Formamide (Merck), formic acid 97% (Alfa Aesar), ethyl acetate (Merck), dichloromethane (Merck), chloroform (Merck), ethanol (Merck), n-hexane (Merck), toluene (Alfa Aesar), aquadest, DMSO (Merck).

Antituberculosis: M. Tuberculosis strain H37Rv, OADC (Becton Dickinson), Alamar Blue reagent (invitrogen), Tween 80, deionized water sterile, aquadest sterile and isoniazid (Sigma Aldrich).

A. Experiment

1. Synthesis target compound

a) Multicomponent Reaction using Formic Acid

A total of 10 mmol equivalent of aromatic-aldehyde compounds, 2-chlorobenzaldehyde; 150 mmol equivalent formamide, 80 mmol equivalent formic acid mixed together into the tube a reaction that contains boiling stones and is

heated using a heating mantle that has been refluxed at 150oC until the reaction is complete (Ritmaleni, Unpublished result). Reaction monitoring is done by sampling using TLC every 30 minutes. The finished reaction is marked by the spot SM which has run out / does not appear on the TLC. Then the synthesis results were added 10 mL of saturated NaHCO3 and extracted with dichloromethane (3x10 mL). The organic phase (DCM) was collected, rinsed using 10 mL saturated NaCl. The organic phase (DCM) is taken and dried with anhydrous MgSO4. Then it is filtered and the solvent is evaporated using a rotary evaporator so that the synthesized product is [4].

b) Multicomponent reactions using NaBH4 reducers

A total of 5 mmol equivalent aromaticaldehyde compounds, 2-chlorobenzaldehyde, 75 mmol equivalent formamide, and 40 mmol equivalents of NaBH4 already activated 40 mmol H3BO3 were mixed together into a round bottom flask and stirred using a magnetic bar and stirrer at room temperature until the reaction was complete. Observation when the reaction is done by taking samples (sampling) with TLC every 10 minutes. The reaction is stopped after the existence of starting material (SM) runs out or is not visible when evaluating sampling with TLC. After the reaction is complete, add 10 mL of saturated NaCl then extracted with DCM (3x10 mL). The organic phase (DCM) is collected and added anhydrous MgSO4 to dry the water footprints. Then the DCM phase is filtered and evaporated using a rotary evaporator to get the product synthesis results.

c) Purification

The purification of the compound is carried out by recrystallization using an optimized solvent system and the purity is tested using TLC with three different eluents to ensure the product compound has been formed.

A small amount of crystalline compound synthesized is dissolved in a drop of ethanol / methanol and bottled on a TLC plate using a capillary tube with a comparison of starting material compound for the synthesis using NaBH4 activated by boric acid and a mixture of compounds (crude product) for the synthesis using formic acid. Then the TLC plate is eluted in the TLC vessel which has been saturated with the eluent used. The elution limit for each TLC plate is 4 cm. When the elution process is complete, the TLC plate is visually observed under UV light of 254 nm and 366 nm, and using a KMnO4 stain solution by dipping the TLC plate into a bottle containing the stain solution then waiting for it to dry. This KMnO4 stain solution is used to identify compounds with functional groups that are sensitive to oxidation, which will appear as bright yellow patches against a light purple background. If after eluting only one spot is seen, the compound is assumed to be pure.

After that the melting point distance of the compound was tested by inserting the synthesized crystals into a special capillary tube melting point test, which was then included in the Buchi Melting Point B-540 apparatus, with an initial temperature setting of 50°C and a final temperature of 200°C using a 5°C gradient temperature. Furthermore, observed and recorded the distance of the melting point of the compound from melting to perfect melting. Melting point distance is the temperature range at which the crystals begin to melt until they melt completely.

2. Antituberculosis activity assay

All of the compounds were screened in MABA. M. Tuberculosis strain H37Rv cultures was freshly prepared in Middlebrook 7H9 broth, then adjusted to 107 CFU/mL. All the compound solved in dimethyl sulfoxide (DMSO) to obtain 4000 µg/mL stock solution. DMSO was used as negative control and Isoniazid (2 □L/mL) were used as positive control in all experiments. In this study 10 concentrations series were used; 1000; 500; 250; 125; 62.5; 31.25; 15,625; 7,813; 3,907 and 1,954 µg / mL to get Minimum Inhibitory Concentration (MIC) from MABA method.

III. RESULT

The results of the synthesis using formic acid evaluated by TLC show that synthesis using this method gives results in the form of a mixture of compounds, in accordance with the results obtained by Ivar Ugi [5] that synthesis of MCRs can produce several variations of compounds marked with the formation of many spots in the crude product.

In the results of synthesis using NaBH4 obtained a single compound which was evaluated by TLC showed the formation of just one spot after the completion of the reaction.

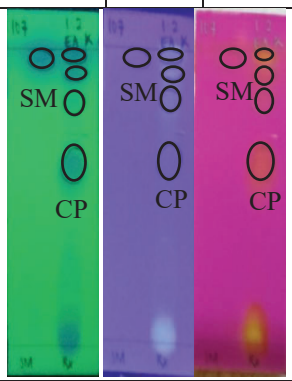
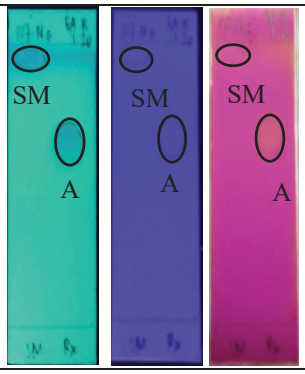
Sample of Crude Product	TLC Profile			Pro-duct	Rf
	UV254	UV366	$KMnO_4$		
LR107				K1 K2 K3 K4	0,97 0,92 0,8 0,6
Eluent: EtOAc:$CHCl_3$ (1:2)					
LR107-Na					0,65
Eluent: EtOAc:$CHCl_3$ (1:20)					

Figure 1. Crude Product of MCRs synthesis

The study of multicomponent reactions between substituted aromatic-aldehydes of chloro and formamide atoms using formic acid was based on the amidoalkylation reaction. Amidoalkylation reaction is a reaction that leads to the formation of carbon-carbon bonds with the replacement of group X in the electrophile reagent, where the X group can be halogen, –OH, -OR, –OCOR, –NHCOR, or –NR2. This

reaction can be through 2 (two) condition, namely alkaline and acid, which of the condition will be obtained imine intermediates, which are reacted with a nucleophile to form new bonds on C and N atoms which then form α-amidoalkylation products by releasing H_2O. The results of the evaluation when synthesis using formic acid provide a mixture of compounds in the synthesis product.

The basis for the use of $NaBH_4$ in the modification of the multicomponent reaction between an aromatic-aldehyde substituted by the chloro group and formamide, is the ability of the $NaBH_4$ reducing agent to reduce the imine group (C = N) to an amine group (C-N). In this research, direct reductive amination is carried out. This method allows the conversion of carbonyl functionality to amine by reacting a mixture of a carbonyl compound and an amide with a suitable reducing agent in a single reaction without intermediate isolation of imine or iminium salts formed from the results of aldehyde condensation with amides.

Products synthesized using formic acid were separated to obtain a single spot from each product using a column chromatography method with an optimum eluent. Based on the results of the separation, the 4th spot of the synthesis results showed a dominant amount so that it was thought to be a target compound from the synthesis results with formic acid reagent. Separation of single compound (LR107-K4) and single compound synthesized using boric acid activated $NaBH_4$ (LR107-Na) was recrystallized as the final stage of purification of the compound.

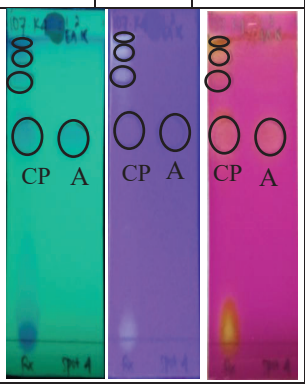
Sample of Crude Product	TLC Profile			Pro-duct	Rf
	UV254	UV366	$KMnO_4$		
LR107				K4	0,6
Eluent: EtOAc:$CHCl_3$ (1:2)					

Figure 2. Pure Product of the MCRs synthesis.

Chemistry

***N*-(2-chlorobenzyl)formamide**

Recrystallization from toluene:n-hexane : C_8H_8ClNO; M. wt 169; Yield 36,5%; m.p74,5 – 75,0°C; white needle-shaped crystal; IR (KBr) cm^{-1} 3263,56 (N-H *stretch*), 3055,24 (C-H aromatic *stretch*), 2885,51 (CH *stretch*, metilen), 1658,78 (C=O *stretch* amide), 1543,05 (N-H *bend*), 1381,03 and 1357,89 (C-N *stretch*, amide); 1249,87 (benzene substituted Cl), 1049,28 and 756,10 (C-H bend *oop* aromatic ortosubstituted). GC-MS (EI-MS, m/z) 134; 125; 107; 89; 77; 63; 51; 29; 28 (100%, *base peak*). ^{1}H-NMR (500 MHz, ppm, $CDCl_3$): δ 4,441 (2H, *d* J= 6 Hz, H3); δ 5,870 (1H, *s*, *broad peak*, H2); δ 7,213 (2H, *dd* J1= 8,5 Hz J2= 2 Hz, H5&H9); δ 7,297 (2H, *dd* J1= 8 Hz J2= 1,5 Hz, H6&H8); δ 8,252 (1H, *s*, H1). ^{13}C-NMR (125 MHz, ppm, $CDCl_3$): δ 41,686 (CH_2, C3); δ 129,341 (Caromatic, C6&C8); δ 129,130 (Caromatic, C5&C9); δ 133,747 (Caromatic, C4); δ 136,300 (Caromatic, C7); δ 161,161 (C=O, C1)

The compound then analysed with Mass Spectroscopy to find out the molecular weight of the synthesized compound to ensure it was the target compound. However, the LR107-Na (a product synthesized using $NaBH_4$ activated boric acid showed that it was not target compound. The M+ showed m/z product was 142, and the target compound should give the m/z 169. With the m/z of the Starting Material (SM) was 140, but the alcohol form of the starting material used. The addition of 2 numbers to the molecule shows that the compound formed is a form of alcohol from SM used.

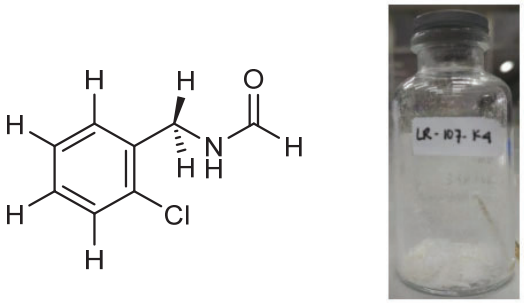
NaBH4 is a reducing agent that is inexpensive, easy to use, and environmentally friendly, so that the use of NaBH4 as a reducing agent in direct reductive amination has been done by using solvent addition or assisted with the addition of catalysts.

In this study, the starting material that used is amides, the results showed that the reaction between aromatic-aldehyde with formamide (amide group) is more difficult to occur. This can be due to the resonance in formamide which causes N atoms in amides to have weak base properties and are very weak nucleophilic compared to N atoms in amines. The low nucleophilicity of N atoms in the amides causes no attack of C carbonyl atoms in aromatic-aldehydes.

Based on the BM obtained, the product of the synthesis of the four compounds is a form of alcohol from BC used. This can happen because NaBH4 is a selective reduction of aldehyde and ketone groups, so it can quickly reduce the C = O carbonyl group of the aromatic-aldehyde BC used, before the aromatic-aldehyde SM reacts with formamide.

Meanwhile, the product synthesized by formic acid, *N*-(2-chlorobenzyl)formamide was succesfully formed. The pure white-needle-shaped crystal product was analyzed with GC-MS then the purity of 100% was obtained.

Structure **Shape**



N-(2-chlorobenzyl)formamide white-needle-shaped crystal

Figure 3. Structur and Shape of *N*-(2-chlorobenzyl) formamide

Antituberculosis assay

The two product then were evaluated their antituberculosis activity to determine the minimum inhibitory concentration through Microplate Alamar Blue Assay. This method is based on the mechanism of M. tuberculosis redox reaction that can reduce the blue

resazurin to the pink resofurin. The wells that retain the blue are the MIC value of the test compound, which indicates that M. tuberculosis bacteria have died as their metabolism cannot convert resazurin to resofurin in pink.

M. tuberculosis is a pathogen that has the highest ability to sense and adapt to the host or environment by evolving through a specific mechanism of redox system homeostasis to protect and protect itself from either oxidation or redox produced by the host or from the environment. The presence of a redox homeostasis system in M. tuberculosis causes these bacteria to transform the resazurin indicator into a resofurin with a change in pink in the wells indicating growth. Whereas in wells that remain blue indicates that there is no bacterial growth.

The results of the antituberculosis assay using MABA method on the eight synthesized compounds can be seen in the images below. The determination of MIC values was visually computed by comparing the colors of the treatment groups with those of the bacterial control group. The smallest concentration capable of preventing the change of blue to pink is set as the MIC value [6].

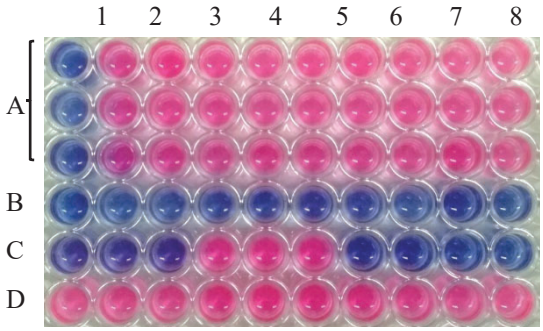


Figure 4. Results of color change after addition of Alamar Blue reagent to compound LR107-K4 (day 7)

Description: (1A) 1000.0µg / mL; (2A) 500.0µg / mL; (3A) 250.0µg / mL; (4A) 125.0 µg / mL; (5A) 62.5µg / mL; (6A) 31.2µg / mL; (7A) 15.6µg / mL; (8A) 7.8µg / mL; (9A) 3.9µg / mL; (10A) 1.9µg / mL. Number 1B-10B is a control compound (compound without bacteria). Number 1C-3C is an INH drug control of 2 µg / mL. The 4C-6C is a 1.25% DMSO solvent control. The 7C-10C number is media control. The 1D-10D number is bacterial control.

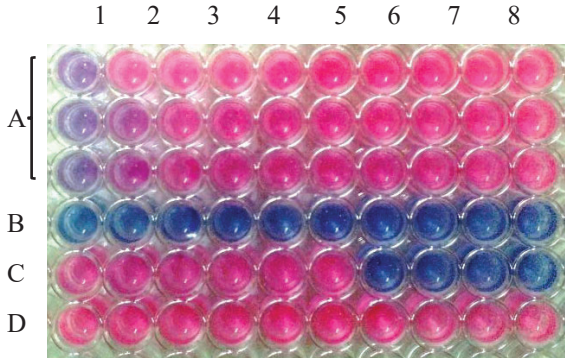


Figure 5. Results of color change after addition of Alamar Blue reagent to compound LR107-Na (day 7)

Description: (1A) 1000.0µg / mL; (2A) 500.0µg / mL; (3A) 250.0µg / mL; (4A) 125.0 µg / mL; (5A) 62.5µg / mL; (6A) 31.2µg / mL; (7A) 15.6µg / mL; (8A) 7.8µg / mL; (9A) 3.9µg / mL; (10A) 1.9µg / mL. Number 1B-10B is a control compound (compound without bacteria). Number 1C-3C is an INH 1µg / mL drug control. The 4C-6C is a 1.25% DMSO solvent control. The 7C-10C number is media control. The 1D-10D number is bacterial control.

Based on the results of the above study, it is possible to interpret the activity of each test compound of MIC value in inhibiting M. tuberculosis growth using the MABA method, which is summarized in Table 1.

Table 1. MIC value of the synthesized product

Sample	KHM (µg/mL)		
	Replicate I	Replicate II	Replicate III
LR107-K4	1000,00	1000,00	1000,00
LR107-Na	1000,00	1000,00	1000,00

Based on observational data, the MIC value for the synthesized compounds with formic acid for the compounds LR107-K4 was 1000,00 µg/mL and the synthesized compounds with $NaBH_4$ activated boric acid was also 1000,00 µg/mL.

The antituberculosis activity was affected by the presence of chloro substituent on the benzylformamide ring that enhance the inhibition activity of the molecule. Chloro substitution can significantly increase biological activity, which is associated with lipophilicity of a compound which is an important parameter in membrane permeation in biological systems. According to this study, the target compound *N*-(2-chlorobenzyl)formamide has antituberculosis activity, but is still in a high range. However, this study maybe used for a reference about MCRs synthesis as antituberculosis agent.

IV. CONCLUSION

N-(2-chlorobenzyl)formamide, a novel compounds produced by using multicomponent reaction and give moderate to excellent yields. The synthesized compound were evaluated for their in vitro antituberculosis activity using MABA method and the result indicated that the compounds have inhibitory activity of M. tuberculosis at concentrations of 1000 □g/mL. Meanwhile these compounds could not be synthesized by using the multicomponent reaction of NaBH4 method of boric acid, which was confirmed by GC-MS and did not show the molecular weight of the target compound.

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