Antituberculosis Activity Test of *N*-Methylbenzyl-*p*-coumaramide (MBC) Against *M. tuberculosis* H37Rv

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Abstract: An antituberculosis activity test of N-p-Methylbenzyl-p-coumaramide (MBC) against M. tuberculosis H37Rv has been carried out. The purpose of this study was to determine the antituberculosis activity of MBC against M. tuberculosis H37Rv. The study was conducted using agar diffusion method. The test solution was prepared by dissolving MBC in 20 mL Ogawa medium to a final concentration of 0.25; 0.50; 1; and 2 mg/L. PDA media that had been inoculated with M. tuberculosis H37Rv (seeded agar) were poured over the base layer on the petridish surface. Paper disks that have been immersed in the test solution were put symmetrically on the seeded agar. Furthermore, the seeds were incubated at 37 ºC for 48 hours. Then the diameter of the inhibition zone was measured to the accuracy of 0.5 mm with a ruler. The results showed that MBC has biological activity as an antituberculosis. MBC can inhibit the growth of M. tuberculosis H37Rv at a concentration of 0.25; 0.50; 1; and 2 mg/L with a diameter of inhibitory zones respectively 8.9; 13.7; 18.5; and 21.3 mm. This showed that the inhibition of MBC on the growth of M. tuberculosis H37Rv increased with increasing concentration of MBC used.

Keywords: tuberkulosis (TB), antituberkulosis, N-p-Metilbenzil-p-kumaramida (MBC), M. tuberculosis H37Rv

INTRODUCTION

Tuberculosis (TB) is one of the deadliest diseases in the world caused by *Mycobacterium tuberculosis*. TB can be cured by administering appropriate antituberculosis. The most commonly used antituberculosis drugs are isoniazid, rifampicin, pyrazinamide, streptomycin, and ethambutol. These five types of drugs are referred to as first-line drugs (Blasko et al., 2010). The problem of TB is exacerbated by the presence of *M. tuberculosis* strains that are resistant to two or more antituberculosis known as Multi Drug Resistance (MDR). The presence of *M. tuberculosis* resistance to antituberculosis has encouraged the use of other more toxic alternative drugs such as ethionamide, aminosalicylic acid, cycloserine, capreomycin, ciprofloxacin, and nofloxacin. These six types of drugs are referred to as second-line drugs. Provision of a more toxic drug is of course more dangerous because the drug can have greater side effects on its users. The TB problem is further compounded by the discovery of *M. tuberculosis* which is also resistant to a second-line drug called Extreme Drug Resistance (XDR) (Retnoningrum et al., 2004). Therefore, research on TB is very active, especially in the field of development and discovery of new drugs with maximum inhibition and toxicity that is safe for the body.

There are five commonly used antituberculosis drugs namely isoniazid (INH), rifampicin, pyrazinamide, streptomycin, and ethambutol (Figure 1). For active TB, these five antituberculosis drugs are often used together. Combination therapy is used to prevent the development of drug resistance and shorten the duration of treatment. The resistance of *M. tuberculosis* to these five antituberculosis drugs develops rapidly if used without other antibiotics, with an estimated rate of resistance in the laboratory between 10^{-7} to 10^{-10} per TB bacteria per generation. As for latent TB, the five antituberculosis drugs are often used alone. These five antituberculosis drugs can also be used to treat non-tuberculosis mycobacterial infections, such as *M. avium, M. kansasii, M. bovis, M. leprae and M. xenopi* (Wikipedia, 2019). The five compounds below can be used as a reference in synthesizing new compounds that have the potential as antituberculosis drugs.

a. Isoniazid (INH)   b. Pyrazinamide   c. Ethambutol

d. Rifampicin   e. Streptomycin

Figure 1. Structure of five first-line antituberculosis drugs (Wikipedia, 2019)

(Dali & Dali, 2017) has successfully synthesized the *N-p*-methylbenzyl-*p*-kumaramide (MBC) compound from *p*-coumaric acid and *p*-methylbenzylamine using an orthoboric acid catalyst (Figure 2).

H₃BO₃
reflux, 25°C, 24 h

p-Coumaric acid   *p*-Methylbenzylamine
(Starting Material)

N-p-Methylbenzyl-*p*-coumaramide   Water
(MBC)
(Target Molecule)

Figure 2. Synthesis of the reaction of the compound *N-p*-methylbenzyl-*p*-coumaramide from *p*-coumaric acid and *p*-methylbenzylamine (Dali & Dali, 2017)

MBC compounds are included in amide derived compounds which have hydroxyl functional groups. The MBC compound has a structural similarity to the five antituberculosis compounds above because the five compounds also belong to a group of amine and amide derivatives which have hydroxyl functional groups. Therefore, MBC compounds are potentially used as antituberculosis drugs.

RESEARCH METHODS
Materials and Tools

MBC is synthesized by referring to the method reported by Dali (2017). *p*-coumaric acid p.a., *p*-methylbenzylamine p.a., H_3BO_3 p.a., *N,N*-dimethylformamide (DMF) p.a., diethylether p.a., Na_2SO_4 anhydrous p.a., chloroform p.a., acetone p.a., aquabidest (Onelab Waterone), TLC plate, filter paper (paper disk), sodium chloride, glycerol, eggs, Potato Dextrose Agar (PDA), isoniazid (INH), Loewenstein Jensen medium, Middlebrook 7H9 medium, and *M. tuberculosis* H37Rv culture obtained from the American Type Culture Collection (Rockville, Md.). Chemicals are ordered from Merck and/or Sigma Aldrich.

Three-neck rounded bottom flask, condenser, analytical balance (Ohaus), Celsius thermometer, magnetic stirrer, heating mantle, desiccator, refrigerator, sterile cupboard, oven, evaporator, incubator, autoclave, vortex, Buchner funnel, effendorf tube , petridishes, and glassware.

Procedures
Synthesis of N-p-methylbenzyl-p-coumaramide (MBC) from p-Coumaric Acid

A total of 0.5 g (3 mmol) of *p*-coumaric acid was put into a 100 mL three-neck rounded bottom flask equipped with a condenser. A total of 0.037 g (0.6 mmol) orthoboric acid and 30 mL DMF were added to the three-neck rounded bottom flasks until it dissolved. Then 0.849 g (3 mmol) of *p*-methylbenzylamine are added to the mixture. Then the mixture is stirred and refluxed for 24 hours at room temperature. Every 2 hours the mixture was TLC tested. Reflux is stopped after the reaction product is formed, then the solution is cooled to room temperature. 10 mL cold aquabidest was added to the solution, then the solution was extracted with 3 x 30 mL diethylether. The organic phase is dried with anhydrous Na_2SO_4, then the water free organic phase is evaporated until yellow solids are formed. Next the yellow solid is recrystallized with chloroform and acetone to form a white solid (Dali & Dali, 2017).

Manufacture of PDA Media

PDA media are made by suspending 17.55 g of PDA in 450 mL of aquabidest (39 g in 1000 mL of water). Then the PDA media is heated using a hot plate until it boils and dissolves completely. After that, the PDA media is allowed to stand and sterilized in an autoclave at 121 ºC and 1 atm pressure for 15 minutes (Firdaus et al., 2011).

Manufacture of Ogawa Medium

Every 3.75 g of Lowenstein Jensen medium was dissolved in 600 mL aquabidest and 12 mL glycerol. The medium was sterilized in an autoclave for 15 minutes at 121ºC, then the medium was cooled to 50ºC on a hotplate. A total of 5 eggs which have been homogenized were added to the medium. Then the medium is stirred with a magnetic stirrer until it is well mixed (Andrews, 2001).

Preparation of M. tuberculosis H37Rv Inoculum

M. tuberculosis H37Rv culture was grown on Middlebrook 7H9 liquid media with Oleic Acid Bovine Serum Albumin Dextrose Catalase (OADC) complex at 37℃ and strongly agitated once a day. For inoculums, *M. tuberculosis* H37Rv suspension was made in NaCl 0.85 on turbidity of Standard No. 1 McFarland (OD 0.257 at 600 nm) containing about 3×10^8 CFU/mL (Andrews, 2001).

Preparation of Medium Containing MBC

A total of 20 mL of Ogawa medium was put into 4 erlenmeyers. MBC was added to erlenmeyer 1 to a final concentration of 0.25 mg/L. MBC was added to erlenmeyer 2 to a final concentration of 0.50 mg/L. MBC was added to erlenmeyer 3 to a final concentration of 1 mg/L. MBC was added to erlenmeyer 4 to a final concentration of 2 mg/L. The four mediums are homogeneously sterile with a magnetic stirrer. Each medium was piped 5 mL sterically and put in 4 test tubes. The negative control used was 5 mL of Ogawa medium without the addition of *M. tuberculosis* H37R and MBC cultures. While the positive control used was a mixture of 3 mL Ogawa medium, 1 mL INH, and 1 mL culture of *M. tuberculosis* H37R. Then the medium was tilted and inspected in an incubator at 85℃ for 48 hours (Rattan et al., 1998).

Antituberculosis Activity Test

PDA media as a base layer are poured into petridish aseptically. A total of 20 mL of PDA media was put into an effendorf tube. Then 10 μL of *M. tuberculosis* H37Rv inoculum was added to the effendorf tube. Furthermore, the mixture is homogeneously sterile with a magnetic stirrer. A total of 5 mL of PDA media that had been inoculated with *M. tuberculosis* H37Rv (seeded agar) was poured on top of the base layer in a circular motion until seeded agar so that it was docked on the petridish surface. Then seeded agar to stand for a few minutes until solidified. Paper disks that have been immersed in the test solution (0.25; 0.50; 1; 2) mg/L are placed symmetrically with a distance of at least 20 mm from the petridish edge. Test solutions, negative controls, and positive controls of 20 μL were dripped with a sterile pipette on the paper disk. The tip of the pipette is used to press the paper disk on the seeded agar. Next petridish closed tightly. After that, the seeded agar are incubated at 37 °C for 48 hours or until the growth of the colony can be observed with the eye. The diameter of the inhibition zone is measured to the nearest 0.5 mm with a line or colony counter. The results are positive if there is no growth of the colony in the media. On the other hand, the results would be negative if there was a growth in the media colony (Dali & Dali, 2004).

RESULTS AND DISCUSSION
Synthesis Results

The MBC compounds obtained were in the form of white solid (yield 75.83%), melting point 156-158℃, and TLC (SiO_2, *n*-hexane : chloroform = 5 : 3 v/v, Rf = 0.37) (Dali & Dali, 2017).

Antituberculosis Activity Test Results

The results of measurement of inhibition zone diameters from MBC are presented in Table 1. From Table 1 it appears that the higher the concentration of MBC used, the wider the diameter of the inhibition zone obtained. This shows that the inhibitory power of MBC on the growth of *M. tuberculosis* H37Rv increases with increasing concentration of MBC used.

Table 1. Results of measurement of inhibition zone diameters from MBC

Treatment	MBC Concentration (mg/L)	Inhibitory Zone Diameter (mm)		Response to Growth Obstacles
		Experiment	Reference*	
Ogawa Medium + MBC	0,25	8,9	6-10	Weak
	0,50	13,7	11-15	Medium
	1	18,5	16-20	Strong
	2	21,3	≥ 21	Very Strong
Positive Control (Ogawa Medium + INH + *M. tuberculosis* culture)	-	17,5	16-20	Strong
Negative Control (Ogawa Medium)	-	0	≤ 5	None

*Source: Rastina et al., 2015.

The mechanism of action of MBC as an antituberculosis is that the MBC interacts with the transmembrane protein (porin) on the outer membrane of the *M. tuberculosis* H37Rv cell wall to form a strong hydrogen bond between the carbonyl group (C=O) of MBC with the NH group of porin and/or the NH group of MBC with the C=O group of porin, resulting in damage to porin (Figure 3). As a result, *M. tuberculosis* H37Rv cells will lack nutrients, so the growth of *M. tuberculosis* H37Rv can be inhibited or die.
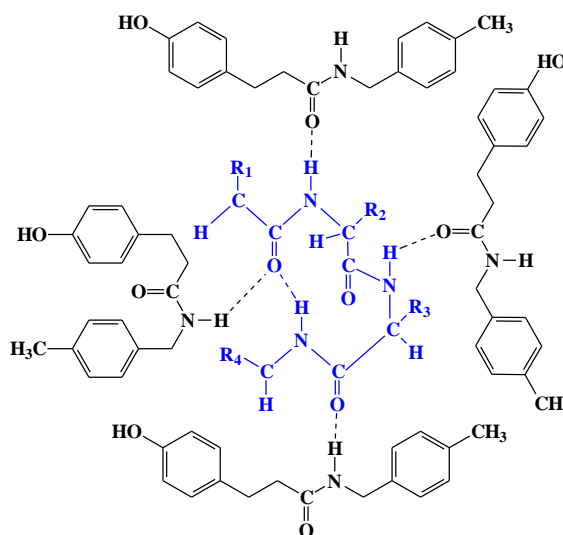
Figure 3. Interaction of hydrogen bonds between C=O and NH groups of MBC with NH and C=O groups of transmembrane protein (porin)

The cell wall of *M. tuberculosis* H37Rv is composed of bag-shaped a single molecule that surrounds the cell membrane and is called peptidaglycan. The peptidaglycan repeating unit is tetrapeptide (Figure 4). Tetrapeptides are peptide molecules composed of four amino acid residues that have one free carboxylic group and one free amino group. In the presence

of these two groups, tetrapeptides can form cross-link with heteropolysaccharide-peptide chains parallel to the oligopeptide bridge (Page, et al., 1989).
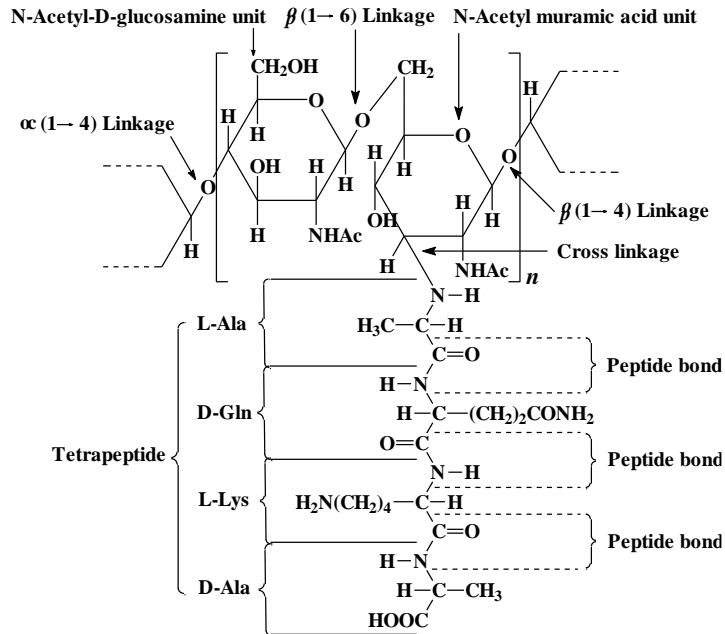

Figure 4. Peptidaglycan repeating unit of *M. tuberculosis* H37Rv cell wall (Page, et al., 1989)

The mechanism of action of MBC as an antituberculosis is by inhibiting the formation of peptidaglycan in the *M. tuberculosis* H37Rv cell wall (Figure 5), so that the *M. tuberculosis* H37Rv cell wall layer is not formed intact and causes the death of the *M. tuberculosis* H37Rv cells.
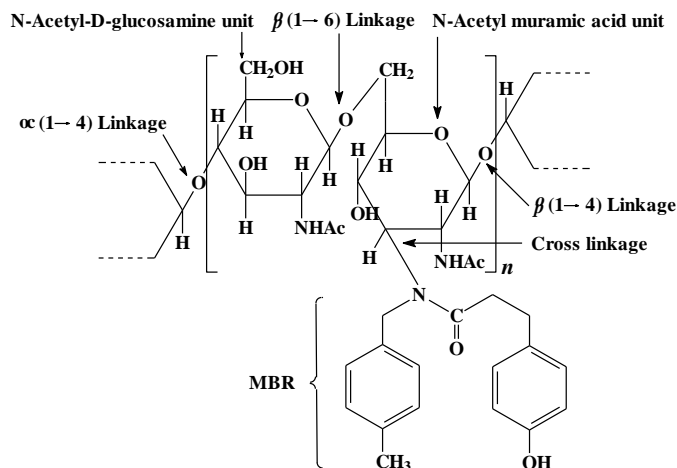
Figure 5. Inhibition of the formation of peptidaglycan in *M. tuberculosis* H37Rv cell walls by forming cross-link of heteropolysaccharide-MBC chains

The use of INH as a positive control because INH is one of the first-line antituberculosis drugs. The mechanism of action of INH as an antituberculosis is by inhibiting the formation of *M. tuberculosis* H37Rv cell walls (Figure 6). In the first stage,

INH is activated by the enzyme catalase-peroxidase of bacterium in *M. tuberculosis* H37Rv called KatG [35]. The second stage, KatG catalyzes the formation of isonicotinic acyl radicals which spontaneously pair with NAD$^+$ to form nicotinoyl-NAD$^+$ complexes. In the third stage, the nicotinoyl-NAD$^+$ complex is closely bound to the enzyme enoyl-[acyl-carrier-protein] reductase [NADH] or InhA, which prevents the enoyl-ACP reductase [NADH] substrate reacting with the enzyme fatty acid synthase-I (FAS-I). This process inhibits the synthesis of mycolic acid, which is a required component of the *M. tuberculosis* H37Rv cell wall.
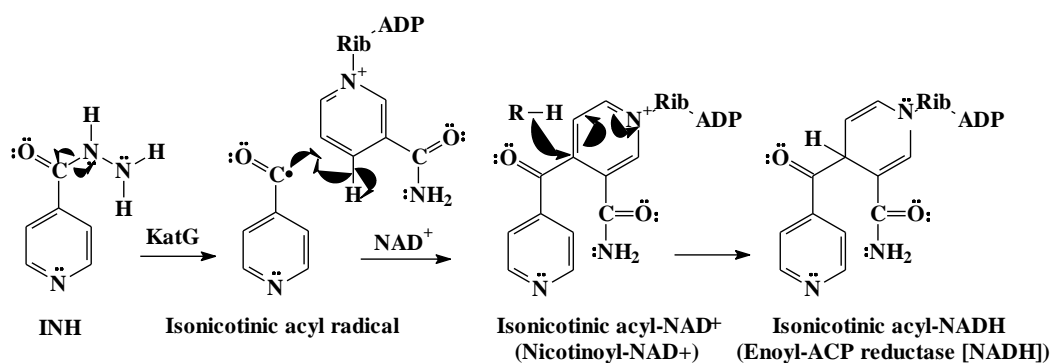

Figure 6. Mechanism of action of INH as antituberculosis (Wikipedia, 2019)

CONCLUSIONS

MBC compounds have biological activity as antituberculosis. MBC can inhibit the growth of *M. tuberculosis* H37Rv at a concentration of 0.25; 0.50; 1; and 2 mg/L with a diameter of inhibitory zones respectively 8.9; 13.7; 18.5; and 21.3 mm. This shows that the inhibition power of MBC on the growth of *M. tuberculosis* H37Rv increases with increasing concentration of MBC used.

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