SYNTHESIS AND HEME POLYMERIZATION INHIBITORY ACTIVITY (HPIA) ASSAY OF ANTIPLASMODIUM OF (1)-*N*-(3,4-DIMETHOXYBENZYL)-1,10-PHENANTHROLINIUM BROMIDE FROM VANILLIN

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ABSTRACT

The synthesis of (1)-N-(3,4-dimethoxy-benzyl)-1,10-phenanthrolinium bromide had been conducted from vanillin. Heme polymerization inhibitory activity assay of the synthesized antiplasmodium has also been carried out. The first step of reaction was methylation of vanillin using dimethylsulfate and NaOH. The mixture was refluxed for 2 h to yield veratraldehyde in the form of light yellow solid (79% yield). Methylation product was reduced using sodium borohydride ($NaBH_4$) with grinding method and yielded veratryl alcohol in the form of yellow liquid (98% yield). Veratryl alcohol was brominated using PBr_3 to yield yellowish black liquid (85% yield). The final step was benzylation of 1,10-phenanthroline monohydrate with the synthesized veratryl bromide under reflux condition in acetone for 14 h to afford (1)-N-(3,4-dimethoxy-benzyl)-1,10-phenanthrolinium bromide (84%) as yellow solid with melting point of 166-177 °C. The structures of products were characterized by FT-IR, GC-MS and ^{1}H-NMR spectrometers. The results of heme polymerization inhibitory activity assay of (1)-N-(3,4-dimethoxybenzyl)-1,10-phenanthrolinium bromide showed that it had IC_{50} HPIA of 3.63 mM, while chloroquine had IC_{50} of 4.37 mM. These results indicated that (1)-N-(3,4-dimethoxybenzyl)-1,10-phenanthrolinium bromide was more potential antiplasmodium than chloroquine.

Keywords: *vanillin; (1)-N-(3,4-dimethoxybenzyl)-1,10-phenanthrolinium bromide; heme polymerization; antiplasmodium*

ABSTRAK

Telah dilakukan sintesis senyawa (1)-N-(3,4-dimetoksibenzil)-1,10-fenantrolinium bromida dari vanilin. Selanjutnya, dilakukan pula uji aktivitasnya pada penghambatan polimerisasi hem sebagai uji awal anti plasmodium. Sintesis anti plasmodium dari turunan 1,10-fenantrolin dilakukan dalam beberapa tahap. Pada tahap pertama, vanilin dimetilasi dengan dimetil sulfat dan NaOH yang direfluks selama 2 jam sehingga dihasilkan senyawa veratraldehida berupa padatan putih-kuning dengan persen hasil sebesar 79%. Senyawa veratraldehida selanjutnya direduksi menggunakan $NaBH_4$ dengan cara digerus dalam mortar dan pestle dan diperoleh senyawa veratril alkohol berupa cairan kuning kental dengan persen hasil 98%. Senyawa veratril alkohol kemudian dibrominasi dengan reagen PBr_3 dan diperoleh senyawa veratril bromida berupa cairan kuning kehitaman dengan persen hasil 85%. Reaksi tahap akhir adalah benzilasi senyawa 1,10-fenantrolin monohidrat menggunakan produk brominasi. Reaksi berlangsung pada suhu refluks mengunakan pelarut aseton selama 14 jam menghasilkan senyawa (1)-N-(3,4-dimetoksibenzil)-1,10-fenantrolinium bromida berupa padatan kuning yang mempunyai persen hasil dan titik leleh berturut-turut sebesar 84% dan 166-177 °C. Struktur produk hasil reaksi dibuktikan dengan spektrometer IR, GC-MS dan ^{1}H-NMR. Hasil uji aktivitas penghambatan polimerisasi hem terhadap senyawa (1)-N-(3,4-dimetoksibenzil)-1,10-fenantrolinium bromida menunjukkan bahwa senyawa tersebut memiliki aktivitas penghambatan polimerisasi hem dengan nilai IC_{50} 3,63 mM sementara klorokuin memiliki nilai IC_{50} 4,37 mM. Hasil tersebut mengindikasikan bahwa (1)-N-(3,4-dimetoksibenzil)-1,10-fenantrolinium bromida memiliki potensi sebagai anti plasmodium yang lebih baik daripada klorokuin.

Kata Kunci: *vanilin; (1)-N-(3,4-dimetoksibenzil)-1,10-fenantrolinium bromida; polimerisasi hem; anti plasmodium*

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INTRODUCTION

Malaria continues to be main health problem and deadly parasitic disease in the world. During 100 years, the world has not given clear contribution to the curing of the disease. In addition, World Health Organization (WHO) [1] reported that 41% of world population (2.3 billion people) was threatened, 300-500 million were infected, 1.5-2.7 million were died by malaria.

In recent years, progress has been made in malaria control as a result of insecticide-treated bed nets and effective treatment. But the development of resistance to insecticides and medicines as well as the poor quality of the health systems in many affected countries pose threats to these achievements [1]. Malaria could be treated by oral medication. However, it constantly changes, especially through the development of parasite (such as *P. falciparum*), which is resistance to standard anti malaria drugs, such as chloroquin. Therefore, the discovery and development of new effective anti malaria drugs are urgently required to solve the problems.

As the effort to overcome the malaria cases, our research group has conducted several malaria-related-researches. The group has synthesized and studied the anti plasmodium activity of 1,10-phenanthroline derivatives. Hadanu et al. [2], [3] and Yapi et al. [4] had synthesized 8 new antiplasmodium of *N*-alkyl- and *N*-benzyl-1,10-phenanthroline and found that *N*-benzyl-1,10-phenathrolinium iodide displayed very high activity against *P. Falciparum* strain of FCR3 [5]. The presence of nitrogen atom, softer counter anion and the benzylic group on phenanthroline skeleton was predicted to be responsible to the activity.

By considering the previous results, the another 1,10-phenanthroline derivative was designed and synthesized from vanillin (which could be derived from clove oil) as the main raw material. In addition, development of the synthesis method by using *green* method would be also evaluated. Then, the heme polymerization inhibitory activity assay of the synthesized compound would be also carried out

EXPERIMENTAL SECTION

Materials

The materials employed were vanillin, sodium hydroxide (NaOH), dimethyl sulphate (DMS), sodium borohydride ($NaBH_4$), sodium bicarbonate ($NaHCO_3$), phosphorus tribromide (PBr_3), 1,10-phenanthroline monohydrate, sodium sulphate (Na_2SO_4) anhydrous, hematin, acetic acid glacial, chloroquine diphosphate, dimethyl sulfoxide (DMSO), chloroform, dichloromethane and acetone. All chemicals, except PBr_3 (was synthesized from red phosphorus and bromine based on method in [6] by previous researcher [7]) were purchased from Merck with high grade and used without any further purification.

Instrumentation

Melting point was determined using Electrothermal 9100 melting-point apparatus and was not corrected. Infra red spectra were obtained using Shimadzu-Prestige 21 spectrometer. ^{1}H-NMR spectra were recorded at 60 MHz with a JEOL JNM-MY and at 500 MHz JEOL JNM-ECA spectrometer using TMS as an internal reference. Chromatogram and mass spectra were measured on a Shimadzu QP-2010 GCMS spectrometer.

Procedure

Synthesis of antiplasmodium of 1,10-phenanthroline derivative

Synthesis of veratraldehyde (2). This procedure was adopted from literature [8]. Vanillin **(1)** (33 mmol) and 12 mL hot water were added into a 250 mL of three-necked flask until it was completely dissolved. As much as 12 mL NaOH 5 M was heated until 100 °C and added to the mixture. The DMS (4.6 mL) was added dropwise into the mixture and after 45 min, 1.3 mL DMS was added into the mixture. The mixture were heated for 10 min and 3 mL NaOH 5M was added dropwise. Alternately addition of NaOH solution and DMS was more than twice, so a total of DMS were added was 8.4 mL (88 mmol). Last addition was 4 mL of NaOH solution. Heating was stopped after 15 min of the last addition of DMS. The mixture was cooled rapidly to 25 °C while stirring and was then extracted with 3 x 10 mL dichloromethane. The organic layer was combined, dried with Na_2SO_4 anhydrous and evaporated with rotary evaporator to afford **(2)** as white-yellow solid (79%). M.p.: 40.6–43.8 °C (standard: 40-43 °C). FTIR (KBr) υ_{max}: 2839-2762 (C_{sp}^3-H), 3078, 1589 and 1512 (ArH), 1681 (C=O), 1342 (CH_3), 1273 (OCH_3) cm^{-1}. 1H-NMR (60 MHz, $CDCl_3$) δ: 9.9 (1H, s, CHO), 7.3-7.7 (2H, m, ArH), 6.8-7.2 (1H, m, ArH), 4.0 (6H, s, OCH_3). GC-MS (purity 100%). MS relative intensity (m/z): 166 [M^+, basepeak], 151, 137, 123, 95, 77, 65, 51.

Synthesis of veratryl Alcohol (3). This reduction was carried out according to the method as previously described [9]. A mixture of **(2)** (10 mmol) and $NaBH_4$ (10 mmol) was ground with an agate mortar and a pestle for 10 min. The mixture was quenched with a saturated aqueous solution of $NaHCO_3$. The mixture was then extracted with 2 x 10 mL of dichloromethane. The organic layer was dried with Na_2SO_4 anhydrous to

be followed with evaporation of the solvent to give **(3)** as yellow liquid (98%). FTIR (neat) υ_{max}: 3402 (OH), 2931-2839 (C_{sp}^3-H), 3078, 1597 and 1512 (ArH), 1458 (CH_2), 1265 (OCH_3) cm^{-1}. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 6.5-7.1 (3H, m, ArH), 4.5 (2H, s, CH_2), 3.9 (6H, s, OCH_3), 3.2 (1H, s, OH). GC-MS (purity 98.99%). MS relative intensity (m/z): 168 [M^+, basepeak], 151, 137, 121.

Synthesis of veratryl bromide (4). This procedure was adopted from literature [7]. Compound **(3)** (10 mmol) and 30 mL chloroform were poured into 100 mL three-necked flask equipped with condenser and funnel dropper and then was stirred with magnetic stirrer. The mixture was cooled until -5 °C. PBr_3 (10 mmol) in 15 mL cooled chloroform were added dropwise, the mixture was then stirred for 30 min in ice bath, to be followed at room temperature for 1 h. The reaction was continued with reflux in 60 °C for 3 h. Ice water (15 mL) was added. The mixture was then extracted with 2 x 15 mL chloroform. The organic layer was combined, dried with Na_2SO_4 anhydrous and evaporated with rotary evaporator to get **(4)** as yellowish black liquid (85%). FTIR (neat) υ_{max}: 2931-2839 (C_{sp}^3-H), 3008, 1597 and 1512 (ArH), 1458 (CH_2), 1265 (OCH_3), 1334 (C-Br) cm^{-1}. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 7.3 (1H, s, ArH), 6.8-7 (2H, m, ArH), 4.4 (2H, s, CH_2), 3.9 (6H, s, OCH_3). GC-MS (purity 89%). MS relative intensity (m/z): 231 [M^+], 151 (basepeak), 139, 108, 77.

Synthesis of N-(3,4-dimethoxybenzyl)-1,10-phenan throlinium bromide (5). This reaction was performed according to the method as previously reported [4]. Compound **(4)** (1 mmol) and 1,10-phenanthroline monohydrate (1 mmol), and 15 mL acetone were added to a 100 mL three-necked flask. The mixture was refluxed for 12 h and cooled to room temperature. The precipitate was then washed with acetone to yield **(5)** as yellow solid (84%). m.p. 166-177 C. FTIR (KBr) υ_{max}: 2993-2839 (C_{sp}^3-H), 3047, 1597 and 1512 (ArH), 1456 (CH_2), 1257 (OCH_3) cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 9.74-9.76 (1H, d, *J*=5.85 Hz, H8), 9.48-9.49 (1H, d, *J*= 8.45 Hz, H3), 9.31-9.32 (1H, dd, *J*=1.95 Hz, H7), 8.77-8.79 (1H, dd, *J*=1.3 Hz, H2), 8.49-8.52 (1H, dd, *J*= 5.85 Hz, H4), 8.43 (2H, s, H5 and H6 of ArH), 8.03-8.05 (1H, dd, *J*=3.85 Hz, H9), 6.64-6.67 (1H, dd, *J*=1.95 Hz, H11), 6.78-6.80 (1H, d, *J*=8.4 Hz, H12), 7.176-7.178 (1H, d, *J*=1.3 Hz, H10), 7.26 (2H, s, H1a and H1b), 3.69 (3H, s, OCH_3), 3.64 (3H, s, OCH_3).

Heme polymerization inhibitory activity assay of 1,10-phenanthroline derivative

Effects of antimalarial compounds *in vitro* tests was conducted, i.e. heme polymerization inhibitory activity test method. This test was conducted by Basilico et al. [10] which modified the dose of hematin solution and the sample used. A total of 100 mL solution of 1 mM hematin in 0.2 M NaOH was put into the microtube, then added 50 mL of test material with various dose levels, ie 20, 10, 5, 2.5, and 1.25 mg/mL. Replication was conducted for 3 times for each dose. To initiate the polymerization reaction hem, 50 mL glacial acetic acid solution (pH 2.6) was added in the microtube which already contains hematin solution and sample, then were incubated at 37 °C for 24 h. Positive control used was chloroquine diphosphate, whereas the negative control was aquadest.

After incubation, the microtube was centrifuged at 8000 rpm for 10 min. Supernatant was removed and the precipitate was washed 3 times with 200 mL DMSO. Each microtube was washed by centrifugation speed 8000 rpm for 10 min. The precipitate obtained was added with 200 mL 0.1 M NaOH. Each 100 mL of the solution obtained were put in microplate 96 wells and OD values was read by Elisa reader at a wavelength of 405 nm.

Heme polymerization inhibitory activity values expressed in IC_{50}, i.e. levels that could inhibit heme polymerization by 50% compared to the negative control. Standard curve was constructed by making a series of concentrations of hematin (which was dissolved in 0.2 M NaOH). A total of 100 mL of each concentration was added to the wells and 96 wells microcultures OD value by Elisa reader at a wavelength of 405 nm. Heme polymerization inhibitory IC_{50} value was calculated using probit analysis.

RESULT AND DISCUSSION

Synthesis of Antiplasmodium of 1,10-Phenan-throline Derivative

According to the previous studies (either experimental and theoretical studies) [2-5] related with the potential of 1,10-phenanthroline skeleton as the antimalaria, it was predicted that the activity was correlated with the counter anion (I^- or Br^-), the presence and the charge of benzylic group as well as the nitrogen atom in the phenanthroline. The benzylic group might be introduced via bimolecular substitution reaction between 1,10-phenanthroline and benzyl halide, at which the halide might be varied. The later could be prepared from benzaldehyde.

The synthesis of antiplasmodium of 1,10-phenantroline was commenced from the renewable starting material of vanillin, which might be either isolated from vanillin plant of derived from eugenol isolated from clove leave oil [8]. Vanillin **(1)** was initially methylated using DMS in the presence of base to yield veratraldehyde **(2)** (Fig. 1). The methyl group introduced to vanillin might be also used as the protecting group in further steps.

Table 1. The IC_{50} values of (1)-*N*-(3,4-dimethoxybenzyl)-1.10-phenanthrolinium bromide, negative and positive controls based on heme polymerization inhibitory activity assay

Sample	Concentration (mg/mL)	Average dose of hemozoin (mM)	Average Inhibition Percentage	IC_{50} (mM)
N-(3,4-dimethoxybenzyl)-1,10-phenanthrolinium bromide	5	15.60 ± 1.93	90.37 ± 1.19	3.63
	2.5	26.07 ± 3.74	83.91 ± 2.31	
	1.25	97.67 ± 12.40	39.71 ± 7.65	
	0.63	145.13 ± 4.82	10.41 ± 2.97	
	0.31	173.27 ± 3.91	- 6.95 ± 2.41	
Chloroquine	5	12.67 ± 4.69	92.18 ± 2.90	4.37
	1.25	99.13 ± 17.01	38.81 ± 10.50	
	0.63	129.87 ± 17.46	19.83 ± 10.78	
	0.31	142.53 ± 16.36	12.02 ± 10.10	
Aquadest		162.00 ± 2.65	0.00 ± 0.00	

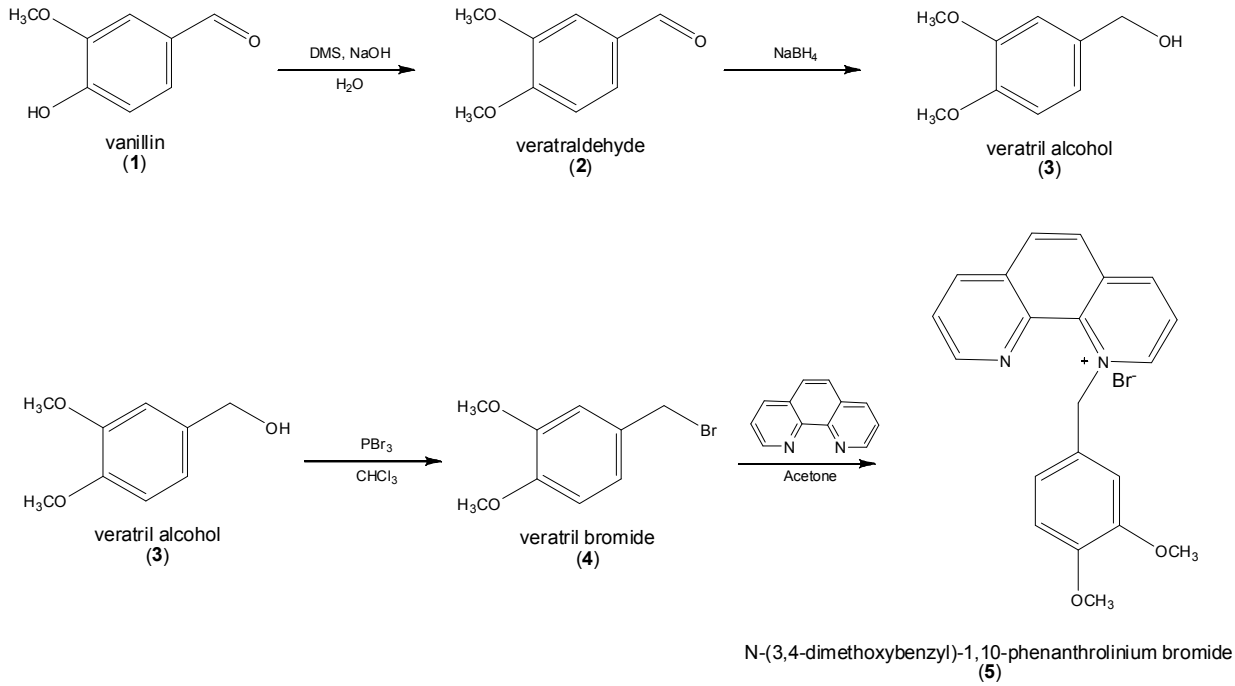

Fig 1. Synthesis of antimalaria of 1,10-phenanthroline derivative

The second step was solvent-free-reduction of **(2)** using $NaBH_4$. The reaction was conducted by grinding all of the reactant together. This method was proved to be more effective and efficient than the conventional one (which used the alcoholic solvent at high temperature for long reaction time). The obtained product was veratryl alcohol **(3)** (Fig. 1).

The hydroxyl group in benzylic alcohol is known as a bad leaving group. Therefore, it should be derived into benzyl halide in order to ease the bimolecular substitution with 1,10-phenanthroline. This reaction was important as the counter anion of the antiplasmodium was introduced in this part. In this case, veratryl alcohol (**3)** was reacted with PBr_3 to give veratryl bromide **(4)** (Fig. 1).

The last step was the bimolecular substitution between veratryl bromide **(4)** and 1,10-phenanthroline to give *N*-(3,4-dimethoxybenzyl)-1,10-phenanthrolinium bromide. The reaction was bimolecular nucleophilic substitution, at which the nitrogen of phenanthroline substituted the bromide to give the phenanthroline salt with bromide as the counter ion.

Structural determination of (1)-*N*-(3,4-dimethoxybenzyl)-1,10-phenanthrolinium bromide was made based FTIR and ^{1}H-NMR studies. The FTIR spectrum showed peak in 3425 cm^{-1} indicated the product of reaction has hydrogen bonding, probably from the free nitrogen atom from the unit of phenanthroline. In the ^{1}H-NMR spectrum, the product showed 14 proton species, which was the same with

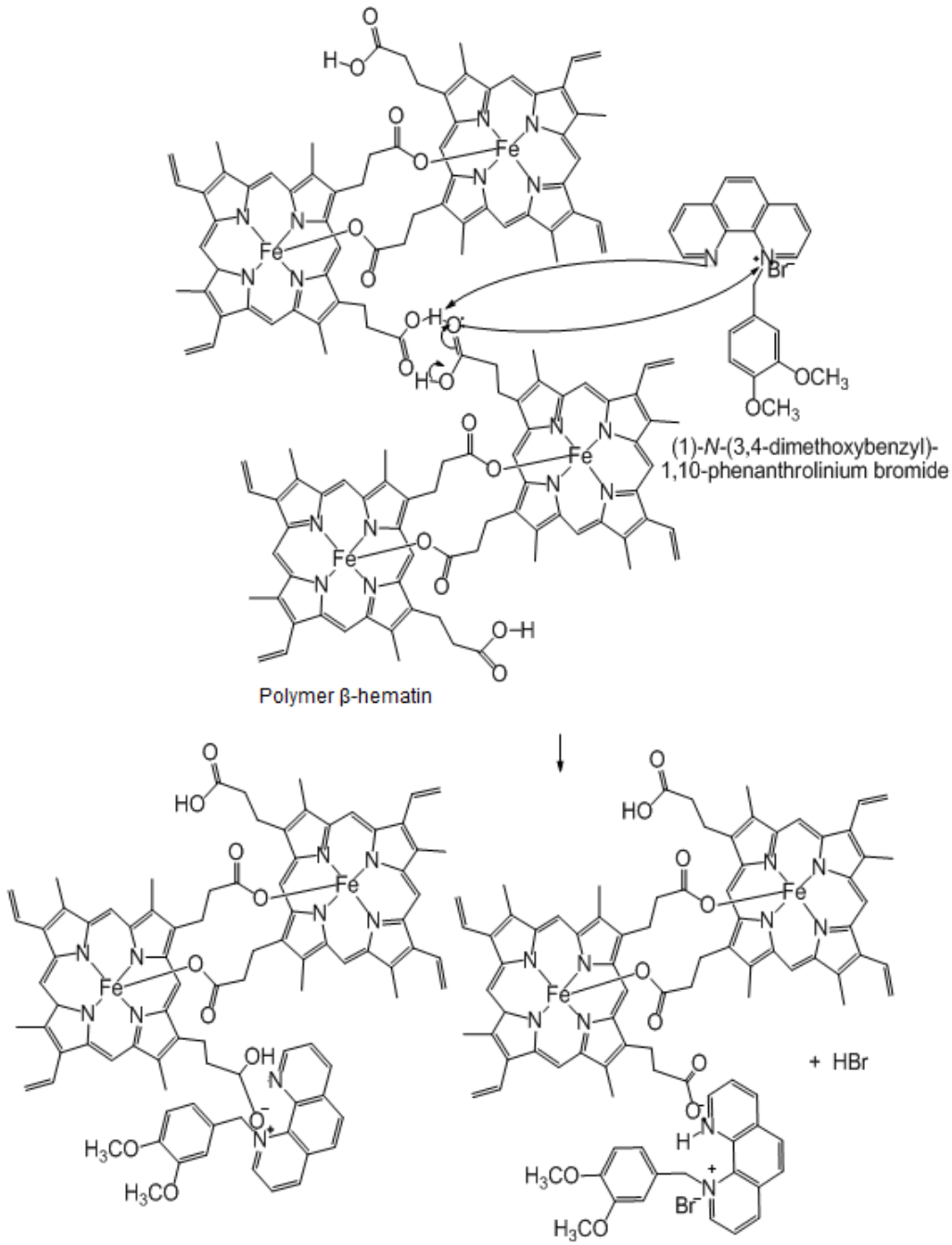

Fig 2. The mechanism of inhibition (1)-*N*-(3,4-dimethoxybenzyl)-1,10-phenanthrolinium bromide against β-hematin polymer

number of proton in the target compound. One evidence that showed that the product has formed was the presence of singlet peak at δ 7.26 ppm coming from methylene (-CH_2-) bridge which connect benzyl group and 1,10-phenanthroline group. Based on infrared and ^{1}H-NMR spectra, the product of *N*-benzylation reaction was *N*-(3,4-dimethoxybenzyl)-1,10-phenanthrolinium bromide **(5)**.

Heme Polymerization Inhibitory Activity Assay of 1,10-Phenanthroline Derivative

For its survival, *P. Falciparum* in erythrocytes will break hemoglobin into globin and free heme in the digestive vacuole. Globin will be decomposed into amino acids as raw materials for the *Plasmodium* protein synthesis. Free heme, i.e. feroprotoporfirin IX, will be oxidized to feriprotoporfirin IX in the acidic

digestive vacuole, and was toxic to host cells and *Plasmodium*. Free heme is highly toxic because it can form radical oxygen species that can cause death for *Plasmodium* [11].

By *Plasmodium*, the free heme is converted to the inert dimer form, namely hemozoin (malaria pigment). A polymer which is identical to hemozoin, i.e. β-hematin, can be formed *in vitro* from hematin under acidic conditions. With FTIR spectrophotometer, shows that the bond between the iron-carboxylate ion of the two molecules have the same hemozoin heme in the analogues, namely β-hematin [12]. β-Hematin crystal can be further measured by Elisa reader absorption at a wavelength of 405 nm. Amount of β-hematin crystal formed is inversely proportional to the activity of antimalarial agents inhibiting the heme polymerization [10].

Under acidic condition, hematin will polymerize into β-hematin crystals. Test compounds that could inhibit hematin polymerization will reduce β-hematin crystals which formed. The IC_{50} values of the compound, negative and positive controls were listed in Table 1.

The results showed that the compound N-(3,4-dimethoxybenzyl)-1.10-phenanthrolinium bromide and positive control chloroquine has IC_{50} values of 3.63 and 4.37 mM, respectively. According Baelsman et al. [13], a compound could be considered to have heme polymerization inhibitory activity if it has heme polymerization inhibitory IC_{50} values smaller than the limit of chloroquine diphosphate, (37.5 mM or 12 mg/mL). Thus, (1)-*N*-(3,4-dimethoxybenzyl)-1,10-phenanthrolinium bromide displayed heme polymerization inhibitory activity.

The activity of the 1,10-phenanthroline derivative was described in Fig. 2. β-Hematin formation will be proceeded by the formation of amorphous precipitate of hem, and followed by a slow conversion into crystalline β-hematin. (1)-*N*-(3,4-dimethoxybenzyl)-1,10-phenanthrolinium bromide has two nitrogen. The positively-charged nitrogen would interact with the electronegative oxygen at ferriprotoporfirin IX, while the other nitrogen (base) will react with the acidic site at ferriprotoporfirin IX (carboxylic acid group). Therefore, the polymerization process might be prevented.

CONCLUSION

The compound of (1)-*N*-(3,4-dimethoxybenzyl)-1,10-phenanthrolinium bromide was prepared from vanillin via methylation, reduction, bromination and substitution. Results of heme polymerization inhibitory activity showed that the IC_{50} values of the sample and chloroquine 3.63 and 4.37 mM, respectively.

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