

SYNTHESIS OF TETRAKIS-N,N,N-TRIMETHYLAMMONIUMMETHYL-C-3,4-DIMETHOXYPHENYLCALIX[4]RESORCINARENE IODIDE BASED VANILLIN AND ITS ANTIDOTE ACTIVITY FOR CHROMIUM(VI) INTOXICATION

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Received January 18, 2013; Accepted May 23, 2013

ABSTRACT

A research has been conducted to synthesize tetrakis-N,N,N-trimethylammoniummethyl-C-3,4-dimethoxyphenylcalix[4]resorcinarene iodide from vanillin and investigate its antidote capability for Cr(VI) intoxication. The synthesis was carried out in four steps i.e. (1) O-alkylation of 4-hydroxy-3-methoxybenzaldehyde, (2) HCl-catalyzed condensation of 3,4-dimethoxybenzaldehyde with resorcinol, (3) Mannich reaction of C-3,4-dimethoxyphenylcalix[4]resorcinarene with formaldehyde and dimethylamine in the presence of HCl to yield tetrakis-N,N-dimethylaminomethyl-C-3,4-dimethoxyphenylcalix[4]resorcinarene, and (4) treatment the Mannich base with methyl iodide to produce the quaternary ammonium salt of tetrakis-N,N,N-trimethylammoniummethyl-C-3,4-dimethoxyphenylcalix[4]resorcinarene iodide. According to the analysis of ¹H-NMR spectrometer, the targeted compounds tend to exist in the chair (C_{2h}) conformation. The capability of resorcinarene for Cr(VI) antidote was examined by orally injection method on mice groups. Based on LD₅₀ determination, the resorcinarene was classified as non toxic compound according to Loomis criteria. Therapeutic using quaternary ammonium-modified resorcinarene was able to reduce metal concentrations of Cr(VI) in liver, kidney, and serum in the magnitude of 78.30; 85.72, and 88.79%, respectively. The higher dose of drug administered the greater decrease in the level of heavy metal. Judging from the amount of milligrams of reduced chromium per gram of organ, kidney is the organ having highest decreasing metal concentrations.

Keywords: vanillin; tetrakis-N,N,N-trimethylammoniummethyl-C-3,4-dimethoxyphenyl-calix[4]resorcinarene iodide; LD₅₀; antidote; Cr(VI)

ABSTRAK

Telah dilakukan penelitian untuk mensintesis tetrakis-N,N,N-trimetilammoniummetil-C-3,4-dimetoksifenilkaliks[4]resorsinarena iodida dari vanilin dan menyelidiki kemampuannya sebagai antidotum keracunan Cr(VI). Sintesis dilakukan dalam empat tahap yaitu (1) O-alkilasi 4-hidroksi-3-metoksibenzaldehida, (2) kondensasi 3,4-dimetoksibenzaldehida dengan resorsinol terkatalisis HCl, (3) reaksi Mannich C-3,4-dimetoksifenilkaliks[4]resorsinarena dengan formaldehida dan dimetilamina dengan adanya HCl untuk menghasilkan tetrakis-N,N-dimetilaminometil-C-3,4-dimetoksifenilkaliks[4]resorsinarena, dan (4) reaksi basa Mannich dengan metil iodida untuk menghasilkan garam amonium kuartener tetrakis-N,N,N-trimetilammoniummetil-C-3,4-dimetoksifenilkaliks[4]resorsinarena iodida. Berdasarkan analisis ¹H-NMR, senyawa target berada dalam konformasi kursi (C_{2h}). Kemampuan resorsinarena sebagai antidotum Cr(VI) diuji dengan metode injeksi secara oral pada mencit putih. Berdasarkan penentuan LD₅₀, resorsinarena uji diklasifikasikan sebagai senyawa non toksik menurut kriteria Loomis. Pemberian resorsinarena termodifikasi ammonium kuartener mampu mengurangi konsentrasi logam Cr(VI) dalam organ hati, ginjal dan serum secara berturut-turut hingga 78,30; 85,72 dan 88,79%. Semakin tinggi dosis obat yang diberikan maka semakin besar penurunan kadar logam berat tersebut. Ditinjau dari besaran miligram kromium berkurang setiap gram organ maka ginjal merupakan organ yang paling banyak mengalami penurunan konsentrasi logam.

Kata Kunci: vanilin; tetrakis-N,N,N-trimetilammoniummetil-C-3,4-dimetoksifenilkaliks[4]-resorsinarena iodida; LD₅₀; antidotum; Cr(VI)

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INTRODUCTION

The macrocyclic molecules of resorcinarenes are cyclic tetramers easily available via one-step reaction by acid-catalyzed condensation of resorcinol and various aldehydes [1-5]. Investigation by *in vitro* method, these compounds have been studied with regard to inclusion of cations [2,6-9], anions [10-11], and organic neutral molecules [3-4,12,]. Recently, Henge-Napoli et al. [13] proved that the Calix[4]arenes containing sulphonato group is an effective antidote on reducing the heavy metal concentration *in vivo* tests on mice. The results of these experiments are consistent with the discovery of Shinkai et al. [14] who reported that the *p*-sulphonatocalix[6]arene shows much greater selectivity towards uranium than Zn^{2+} , Ca^{2+} , and Cr^{3+} . Calixarene compounds also play important role in medicine and treatment, for example are 4-sulphonatocalix[4]arene and 4-sulphonato-calix[6]arene. Both of them together with cyclodextrins increase the solubility of niclosamide in water. Thus the drug will be more quickly absorbed into the corresponding receptors in the body so it will feel the effects of drug soon [15].

Meanwhile, the presence of heavy metals in environment has been increased due to the industrial activities [16-18]. It leads to bioaccumulation of the metals in living organism, causing health problems in animals, plants, and human being [19]. In humans and animals, trivalent chromium is an essential nutrient that plays an important role in glucose, fat and protein metabolism. In contrast, products which contain a hexavalent chromium base are oxidising agents capable of inducing tissue damage leading to conditions such as dermatitis [20], lymphocyte apoptosis [21], nephrotoxicity [22-23] and hepatotoxicity [24], in addition to contributing to the potential carcinogenic, teratogenic and mutagenic risk [25].

Therefore, based on the phenomena above, we recently become interested in exploring the synthesis of calix[4]resorcinarene derivatives that has capability in binding guest of heavy metal anion of Cr(VI). In this work, we report the synthesis of quaternary ammonium modified of calix[4]resorcinarene using vanillin as starting material. Here in, we also investigate the usage of the synthesized compound as antidote for acute Cr(VI) intoxication through *in vivo* examination.

EXPERIMENTAL SECTION

Materials

Vanillin, dimethyl sulfate, sodium hydroxide, resorcinol, absolute ethanol, fuming hydrochloric acid, dimethylamine, formaldehyde, acetone, methyl iodide, carboxymethyl cellulose, and potassium dichromate. All

reagents in analytical grade were obtained from E Merck Co Inc. (Germany) and used without further purification. The specimens (mice Balb/c) were obtained from Pharmacology and Toxicology Laboratory, Medicine Faculty of Universitas Gadjah Mada, Yogyakarta.

Instrumentation

Instruments used in this study were melting point electro thermal 9100, TLC (silica gel 7730 60GF₂₅₄), Shimadzu FTIR-Prestige-21, JEOL-MY500 NMR (¹H at 500 MHz, ¹³C at 125 MHz), Bruker AC300F NMR (¹H at 300 MHz, ¹³C at 75 MHz), and Shimadzu AAS AA-6650-F.

Procedure

O-alkylation of vanillin

3,4-Dimethoxybenzaldehyde (veratraldehyde) was made from vanillin via O-alkylation with dimethylsulphate according to the previous procedure [26].

Synthesis of C-3,4-dimethoxyphenylcalix[4]resorcin arene (1)

The mixture of resorcinol (0.55 g; 5 mmol), vanillin (5 mmol), absolute ethanol (50 mL), and hydrochloric acid fuming (0.5 mL) was stirred under reflux at 80 °C for 24 h until the reactants used up at the reaction (TLC monitoring). Furthermore, the mixture was cooled and the product was filtered followed by washing with ethanol-aquadest (1:1) then dried to give the desired compound **1** as a white-orange crystal in 97.54%, 346-348 °C; FTIR (KBr) ν (cm⁻¹): 3402 (OH), 3024 (C_{sp2}-H), 1604 and 1512 (C=C aromatic), 3000-2846 (C_{sp3}-H aliphatic), 1427 (-CH methine bridge), 1373 (-CH₃); ¹H-NMR (500 MHz, DMSO-*d*₆) δ (ppm): 8.29 (8H,s,OH), 6.41-6.46 (20H,m,ArH), 5.68 (4H,s,Ar₂CHAR) (in acetone-*d*₆ the signal of this chemical shift give multiple peaks), 3.69 and 3.44 (24H,s,OCH₃); ¹³C-NMR (125 MHz, DMSO-*d*₆) δ (ppm): 152.9 (-C-OH), 152.7 (-C-OMe), 143.7, 137.0, 120.9, 120.7, 114.1, 112.7, 102.1 (Ar), 55.0 (OCH₃), 40.6 (Ar₂CHAR).

Synthesis of tetrakis-N,N-dimethyl-aminomethyl-C-3,4-dimethoxyphenylcalix[4]resorcinarene (2)

C-3,4-Dimethoxyphenylcalix[4]resorcinarene (0.5 mmol) was dissolved in 30 mL of acetone. To a stirred of this mixture was added 0.186 mL (2.5 mmol) of formaldehyde 37%, 0.316 mL (2.5 mmol) of dimethylamine, and 1 mL of HCl respectively. The mixture was stirred at room temperature for 48 h until the reactants used up at the reaction (TLC monitoring).

Solvent was removed by evaporation under reduced pressure. The residue was triturated with methanol: water (1:1). The precipitate was then filtered, washed several times with methanol, and dried to give the title compound **2** (0.912 g, 72.40%) as brown crystal; m.p. 378-380 °C; FTIR (KBr) ν (cm⁻¹): 3410 (OH), 1604 and 1512 (C=C aromatic), 3000-2785 (C_{sp3}-H aliphatic), 1465 (-CH₂-), 1373 (-CH₃); ¹H-NMR (500 MHz, DMSO-d₆) δ (ppm): 8.26 (8H,d,OH), 6.21-6.47 (16H,m,ArH), 5.64 (4H,d,Ar₂CHAR), 4.51-4.70 (8H,m,ArCH₂N-), 3.68 (24H,d,OCH₃), 3.45 (24H,s,NMe₂); ¹³C-NMR (75 MHz, DMSO-d₆) δ (ppm): 152.8 (-C-OH), 152.6 (-C-OH), 148.5 (-C-OMe), 146.5 (-C-OMe), 135.0, 130.5, 124.9, 124.5, 121.8, 113.1, 109.3, 108.7 (Ar), 54.9 (OCH₃), 47.2 (NMe₂), 46.8 (Ar-CH₂-NMe₂), 31.1 (Ar₂CHAR).

Synthesis of tetrakis-N,N,N-trimethyl-ammonium methyl-C-3,4-dimethoxyphenyl-calix[4]resorcinarene iodide (**3**)

To a stirred solution of tetrakis-N,N-dimethylaminomethyl-C-3,4-dimethoxyphenylcalix[4]resorcinarene (1.0 mmol) in 30 mL acetone was added methyl iodide (5.0 mmol) drop wise. The mixture was then stirred and refluxed for 2 h. The solvent was removed by evaporation under reduced pressure to leave a red-brick residue, washed several times with water-methanol (1:1) and dried to yield 0.912 g of product **3** (90.25%); m.p. 334-335 °C; FTIR (KBr) ν (cm⁻¹): 3484 (OH), 1600 and 1513 (C=C aromatic), 3000-2932 (C_{sp3}-H aliphatic), 1468 (-CH₂-), 1388 (-CH₃); ¹H-NMR (300 MHz, DMSO-d₆) δ (ppm): 8.59 (8H,m,OH), 6.15-6.44 (16H,m,ArH), 5.45-5.71 (4H,m,Ar₂CHAR), 4.65 (8H,d,ArCH₂N-), 3.65 (24H,s,OCH₃), 1.10-2.90 (36H,m,NMe₃); ¹³C-NMR (75 MHz, DMSO-d₆) δ (ppm): 157.4 (-C-OH), 152.5 (-C-OH), 149.8 (-C-OMe), 147.8 (-C-OMe), 134.4, 129.8, 124.6, 124.3, 121.3, 113.2, 108.8, 107.8 (Ar), 54.9 (OCH₃), 42.9, 42.3, 41.9 (NMe₃), 36.2 (Ar-CH₂-NMe₂), 31.1 (Ar₂CHAR).

Determination of oil-water partition coefficient

Oil-water partition coefficients (log P_{o/w}) was determined by solvent extraction method of the two-phase, octanol as lipid and phosphate buffer solution as the aqueous phase, according to the previous procedure [27].

Determination of acute toxicity (LD₅₀)

Acute toxicity test (LD₅₀) of compound **3** was performed using mice with relatively similar weight, both males and females each divided into 5 groups of experimental animals. Four groups of mice were given orally with resorcinarene at different doses (250, 500, 1000, and 2000 mg/kg body weight), whereas the other group was a blank sample. LD₅₀ was determined by counting the number of animal's deaths that occurred

within 24 h to 20 days after the first administration of single doses.

Antidote activity test

Five groups of mice (27±2 g) were acclimated for 1 week before experimental use. They were allowed food and water *ad libitum*. Four groups of mice (each 6 mice) were injected with a solution of K₂Cr₂O₇ orally at a dose of 0.12 mg (120 ppm) every morning for 14 days. LD₅₀ of Cr(VI) is 3.43 mg/kg body weight [28]. Six hours later, the second, third, and fourth groups of mice were consecutively injected with the resorcinarene **3** orally at different doses (25, 50, and 100 mg/kg body weight). The first group of mice was left without giving antidote as a negative control, while the fifth group was used as a blank sample. On day 29, after 2 week observation, the animals were sacrificed and the chromium content determined on samples of liver, kidney, and blood (serum), which had been digested in hydrochloric acid, using atomic adsorption spectrometry (AAS).

RESULT AND DISCUSSION

Synthesis of Quaternary Ammonium Modified Resorcinarene

In our previous work [1], reaction of resorcinol and 4-methoxybenzaldehyde in the presence of hydrochloric acid produce a cyclic tetramer of C-4-methoxyphenylcalix[4]resorcinarene. In the same way, then we introduce this method for a different starting material of aromatic aldehyde i.e. 4-hydroxy-3-methoxybenzaldehyde. The synthesis of tetra and octafunctionalized calix[4]resorcinarene **1**, **2**, and **3** compounds using vanillin as an aromatic aldehyde through several stepwise reactions as shown in Fig. 1.

The ortho positions to hydroxyl groups at the resorcin residue are the most reactive and the most possible side on resorcinarene **1** toward electrophilic substitution such as Mannich reaction. The Mannich reaction involves the nucleophilic addition of a nucleophile to an iminium ion formed by the reaction of formaldehyde with a secondary amine [29-31]. In this research, synthesis of tetrakis-N,N-dimethylamino methyl-C-3,4-dimethoxyphenylcalix[4]resorcinarene (**2**) from compound **1** according to the Mannich procedure was carried out in the solvent of acetone at room temperature by using formaldehyde and dimethylamine in the presence of HCl.

In this Mannich reaction, acid catalysts would protonize formaldehyde oxygen atom such that the carbon atom of formaldehyde is very reactive to being attacked by a lone pair of dimethylamine to form iminium ions which stabilized by resonance. The

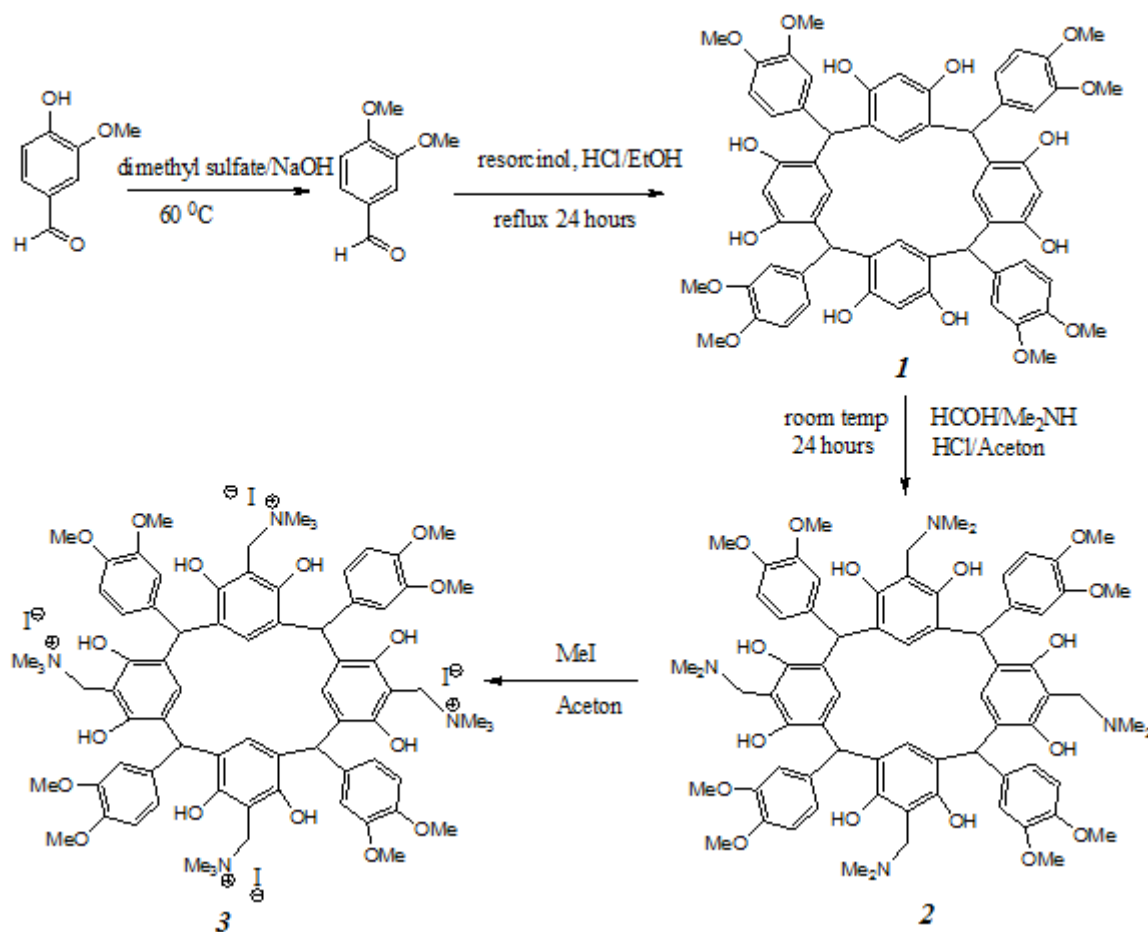


Fig 1. Overall stepwise reactions of resorcinarene 3 synthesis

existence of positive character iminium ion will push the electrophilic substitution reaction with the calix[4]resorcinarene 1. Iminium ions act as an electrophile agent. The number of iminium ion substitution on the compound 1 was proportional with the amount of iminium ion itself.

Resorcinarene 2 has 4 amine groups with certain basicity depend on the substituent. Based on these properties, amine can undergo alkylation reactions with alkyl halides via a nucleophilic substitution reaction where the amine acts as a nucleophile while the alkyl halide acting as electrophile agent. Both of the primary, secondary, and tertiary amine can react with alkyl halides through nucleophilic substitution reaction. Even the tertiary amine competes with the ammonia to react with alkyl halides with the main product of ammonium salt. In this study, 4 active groups of the tertiary amine compound 2 will be further modified to quaternary ammonium groups. The existence of positive character of quaternary ammonium group on resorcinarene compound is expected to interact well with anionic species so that the final product modified quaternary

ammonium will be effectively used as a host of heavy metal anion such as $\text{Cr}_2\text{O}_7^{2-}$.

The modifying process of the tertiary amine group into quaternary ammonium on resorcinarene was done by reacting compound 2 with MeI in acetone at room temperature for 48 h. MeI is the most powerful and reactive of primary alkyl halides when compared with other alkyl halides. The product of resorcinarene 3 was obtained as a light brown powder in 90.25% yield with melting point of 334-335 °C. The FTIR spectrum of this compound show many peaks consist of OH group (3484 cm^{-1}), C=C aromatic (1600 and 1513 cm^{-1}), $-\text{CH}_2-$ (1468 cm^{-1}), and $-\text{CH}_3$ (1388 cm^{-1}). Clear evidence of the quaternary ammonium salt was given by the $^1\text{H-NMR}$ spectrum (Fig. 2) in which the presence of additional peaks at δ 1.1-2.9 ppm with total integration consist of 36 proton correspond to the N,N,N-trimethylammonium iodide group ($-\text{N}^+(\text{Me})_3\cdot\text{I}^-$).

The non-planarity of resorcinarenes means that they can, theoretically, exist in many different of isomeric forms. However, in the most cases, only two diastereoisomers were observed, those are crown and chair conformers [32-34]. Based on the proton NMR

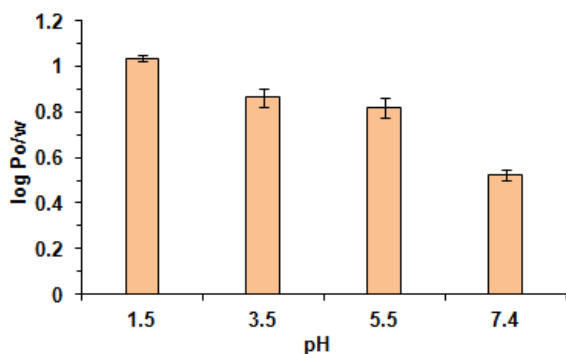


Fig 4. Oil-water partition coefficient of resorcinarene 3

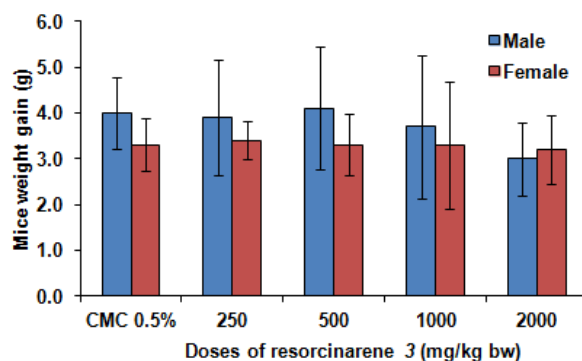


Fig 5. Effect of resorcinarene 3 on mice weight gain

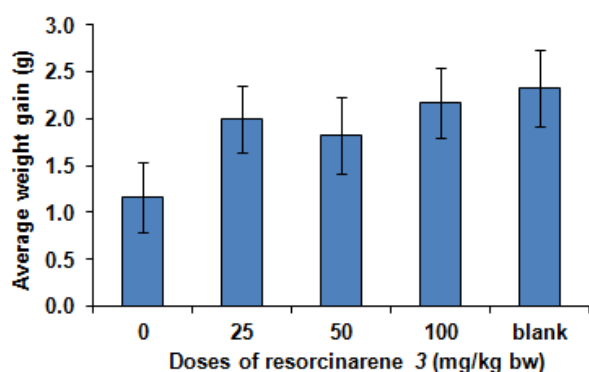


Fig 6. Influence of chromium intoxication and antidote doses on average weight gain

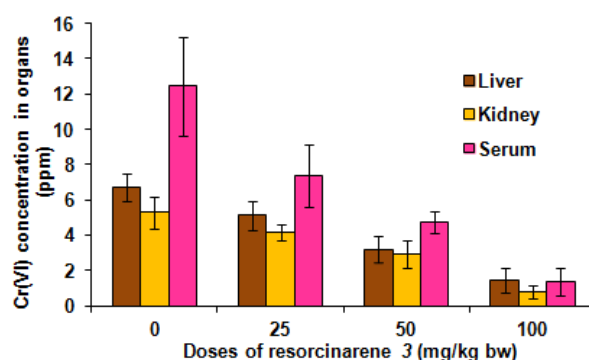


Fig 7. Effect of chromium intoxication and antidote dose on Cr(VI) concentration in organs

standard solutions at pH 1.5, 3.5, 5.5, and 7.4 (Fig. 4).

A compound which is soluble in the two solvents that do not interfere with each other then the compound will be distributed into the polar phase (water) and non-polar phase (octanol, chloroform, etc.). After equilibrium level was reached, compound in both solvents always fixed (at a constant temperature) so that the partition coefficient value can be determined. In this study, the optimum incubation time is determined when the decreasing of resorcinarene 3 concentration in n-octanol phase relatively constant, the achievement equilibrium was gained.

The result of the fat-water partition coefficient determination of 3 was presented in Fig. 4. Fat-water partition coefficient of the compound has different values at each pH experiment. Based on Fig. 4 showed the greater pH of phosphate buffer solution cause the smaller of log $P_{o/w}$. This indicate that the degree of lipophilicity of the compound decrease with increasing pH.

In general, the results of fat-water partition coefficient determination (Fig. 4) showed the value of log $P_{o/w}$ were between 0.52 to 1.03 at pH region of 1.5 to 7.4. At pH 1.54 the value of log $P_{o/w}$ of resorcinarene 3 was more than 1, which means that the value of $P > 10$. This suggests that more than 10 parts of the compound are in the organic layer and less than 1 part in water

phase. Thus lipophilicity of resorcinarene 3 is high enough so that it can penetrate through non-polar site of biological membranes. The value of log $P_{o/w}$ at pH 3.5, 5.5 and 7.4 were gradually decreased to 0.5. This gives another advantage of resorcinarene 3 to be more easily excreted or released from the body's biological system after binding the toxin target.

The Toxicity of the Drug

Determination of LD_{50} was conducted to examine the relationship of resorcinarene dose with toxic effects. This test is designed to measure the degree of toxic effects of a compound that occurs in a short time, i.e. 24 h after administration in a single dose. From the results of the study, no one of mice died after treatment. Even in the observation period of up to 3 weeks after injection of a single dose of 2000 mg/kg of the drug, there is no animal that showed clinical symptoms (toxic effects) and lead to death. Therefore, the resorcinarene 3 was classified as non toxic compound according to Loomis criteria [35].

The low degree of toxicity of the compound was also supported by data from animal weight measurements of both males and females in different doses as shown in Fig. 5. Based on the chart, there was no significant weight gain differences ($P > 0.05$)

between the control group (CMC 0.5%) and groups given orally at various drug doses. This indicates that the presence of resorcinarene **3** in the body, although it has active side of a positive character, but does not interfere with the interaction between essential metal ions (Mg, Ca, K) with physiologic ligands such as hydroxyl (-OH), carboxyl (RCOO⁻), phosphates (HPO₄⁻), carbonyl (C=O), thiol (-SH) and amine (-NH₂) groups. Thus the processes of essential metals absorption occur continuously to pose no metallic mineral deficit (anemia) and weight loss.

Antidote Activity Test

Based on observations of animal body weight (Fig. 6), control group of mice (I) with only Cr(VI) administration without any further treatment showed the lowest weight gain compared with the positive control group (blank) and groups given antidote in various doses (25, 50, and 100 mg/kg bw). This indicates that the presence of Cr(VI) in mice physically may cause interference or inhibition of the development of body weight. The mechanism of the weight gain disorder comes from the direct toxic activity of chromium in the tissue. The existence of Cr(VI) in the body will alter hormonal metabolism and reduced absorption of essential metals causing anemia and weight loss [22]. The result also showed that compound **3** affect the activity of Cr(VI) and reduce the negative impacts that are detected by normally weight gain.

Symptoms of Cr(VI) poisoning is also common on the weight disorder of vital organs of excretion involving the liver and kidneys. Based on previous studies on the toxicology of Cr(VI) stated that hexavalent chromium able to induce tissue damage. Recent studies also indicate the presence of lesions in the kidney tissue [22] after experimental animals were given food containing Cr(VI).

Therapy using resorcinarene **3** (Fig. 7) prevent and reduce metal concentrations accumulated in organs. The higher doses of drug administered the greater decrease in the levels of heavy metals. At the highest dose (100 mg/kg bw), reduction of the total chromium concentration in the liver, kidney, and serum was 78.30, 85.72 and 88.79% respectively. In the terms of the amount of milligrams (chromium) per gram of organ, the kidney was the highest on decreasing metal concentration. In general, the excretion of chromium was done mainly by the kidney and liver, as well as in smaller amounts in the bile, milk and hair. It shows that more chromium residues accumulate in the kidney than in liver. This fact corresponds to the results of investigations using radioactive ⁵¹Cr which proves that chromium deposited up to 6 times in kidney than liver and blood [22].

CONCLUSION

Tetrakis-N,N,N-trimethylammoniummethyl-C-3,4-dimethoxyphenylcalix[4]resorcinarene iodide (**3**) has been synthesized via Mannich reaction of tetrakis-N,N-dimethylaminomethyl-C-3,4-dimethoxyphenylcalix[4]resorcinarene (**2**) followed by alkylation with MeI. Resorcinarene **3** has enough solubility in fat but lower in water except in alkaline pH. It means that the resorcinarene can penetrate to the cell membrane thus it still be able to contribute on binding with chromium in the liver and kidney. Treatment using resorcinarene **3** prevent and reduce metal concentrations accumulated in organs. The higher doses of drug administered the greater decrease in the levels of heavy metals.

ACKNOWLEDGEMENT

The authors gratefully acknowledge to the Directorate General of Higher Education, Republic of Indonesia, for financial support through the research grant program on 2013/2014.

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