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Research Article

EFFECT OF BENZALDEHYDE EXCESS IN THE SYNTHESIS OF LR-2 AND CYTOTOXIC ACTIVITY OF LR-2 AGAINTS HeLa CELL

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ABSTRACT

LR-2(4-phenyl-3,4-dihydro-indeno[2',1']pyramidine-2(1H)-thione; Leni Ritmaleni 2), which designed and assumed to have biologically activity as anticancer, has been successfully synthesized by using the Biginelli reaction. This research was aimed to investigate the effect of benzaldehyde excess in the synthesis of LR-2 and to evaluate the cytotoxic activity of LR-2 against HeLa cancer cell lines. The synthesis was done by reacting benzaldehyde, 2-indanone and together with thiourea at one time as said as one pot reaction synthetic methodology and the reaction was acid catalysed. The mole equivalent of benzaldehyde was in excess compare to others. The effect of benzaldehyde in excess is the higher the mole of benzaldehyde, the lower the yield of LR-2. The cytotoxicity of LR-2 was done by using MTT method and the LC_{50} was 268.15 μM.

Key words : LR-2, benzaldehyde, cytotoxic, HeLa

INTRODUCTION

Cervix cancer is most frequently case of cancer that happened to women. This type of cancer actually can be control but because of the limited access to the treatment and screening especially in developing countries. This cancer is the second large cancer case after breast cancer. Each year, there are around 400.000 new cervix cancer cases and 80% of them are in developing countries. This research was trying to evaluate the effect of the excess of benzaldehyde **1** in the synthesis of LR-2 **4** and its activity as an anticancer agent.

LR-2 (4-phenyl-3,4-dihydro-indeno[2',1'] pyrimidine-2(*1H*)-thione) **4** has been reported as one of molecule that can be synthesised through the use of Biginelli reaction (Ritmaleni *et al.,* 2011). As known, Biginelli reaction is one of synthetic methodology which is categorised in the multi component reaction (MCR) method (Russowsky *et al.,* 2004) .The main structure as the result of Biginelli reaction is the 3,4-dihydropyrimidine-2(1H)-one which has been evaluated as antimitotic (Mayer *et al.,* 1999), antihypertention (Atwal *et al.,* 1991), antibacterial and anti-inflammatory (Kappe, 1993). About the synthetic methodology of Biginelli reaction itself has been modified by some researchers like modifying in the catalyst (Kappe, 1997) and solvent type (Bose *et al.,* 2003).

LR means Leni Ritmaleni and 2 is the series number of LR compound. The LR-2 **4** synthesis was carried out by reacting among benzaldehyde **1**, 2-indanone **2** and thiourea **3** in one reaction system as catalysed by HCl and this method also has been published. The different with LR-1 is the use of urea instead of thiourea **3** as starting material (Ritmaleni and Nurcahyani, 2006). In the synthesis of LR-2 **4** the equivalent mole relative among the three is one mole for each starting material (Scheme 1). This is the same as Biginelli protocol one mole for each benzaldehyde **1**, urea and 1,3-dicarbonyl. The biological activity of LR-2 **4** also has been investigated as anticancer agent againts T47D (Ritmaleni *et al.,* 2011) and Myeloma (Susanti, 2007) cancer cell line and Vero cell line (Laksmiani, 2007)

METHODOLOGY

Materials

Benzaldehyde, thiourea, ethanol, ether, chloroform and chloride acid (concentrated) (E-Merck and *pro analysis* (p.a). 2-indanone (Sigma-Aldrich), anisaldehyde, aceton,aquadest, $MgSO_4$ silica gel 60 GF254 (E-Merck), TLC plate, 4,5-dimethyltiazol-2-il)-2,5-diphenyl tetrazolium bromide, DMSO, HeLa cell line (were obtained from the laboratorium Penelitian dan Pengujian Terpadu/ LPPT Universitas Gadjah Mada Yogyakarta), RPMI 1640 (Sigma-Aldrich), Fetal Bovine Serum (FBS) (Sigma-Aldrich), fungison (Sigma-Aldrich),penicillin-streptomycin (Sigma-Aldrich),3-(4,5-dimethyltiazol-2-il)-2,5-diphenyl tetrazolium bromide (MTT).

Instrumentation

Glassware, syringe, stirrer bar, magnetic bar, thermoneter, hot plate, pipet, uv lamp, Buchi Melting Point B-540, Shimadzu FTIR-8201 PC dan FT-IR Thermo Nicolet AVATAR 360, UV-Vis spectrophotometer (Genesys 10 dan MILTON ROY SPECTRONIC 3000), spectrophotometer 1H-NMR (1H-NMR, 60 MHz JEOLMY60), and mass spectrophotometer (GC-MS Shimadzu QP-5000), ELISA reader

Procedure

Synthesis 4-phenyl-3,4-dihydro-indeno [2', 1'] pyrimidine-2(1H)-thione (LR-2) 4

To round bottom flask 10 mL, benzaldehyde **1** (1 mmol; 106.13 mg; 101 μL), thiourea **3** (1 mmol; 76.12 mg), 2-indanone **2** (1 mmol; 132.16 mg) and ethanol (3 mL) were mixed with magnetic bar. After that, concentrated HCl (3 drops) was added and the reaction was reflux for 6 hours. This was said as reaction A. For reaction B, C and D, the same method was applied and the difference only on the amount of benzaldehyde. For reaction B, 1.5 mmol (159.19 mg; 150 μL); reaction C, 2 mmol (212.26 mg; 200 μL); reaction D, 2.5 mmol (265.33 mg; 250 μL) was added to each flask. The reaction was monitored by TLC. After 6 hours, the reaction mixture was extract with chloroform (4 x 10 mL), dried over $MgSO_4$ anhydrate, filtered and evaporated the solvent by using rotavapor yielded a crude product as a reddish solid.

A small amount of crude product of LR-2 **4** in chloroform was put as a band on the base line of the TLC preparative plate, silica gel GF 254 and eluted with $CHCl_3$: Et_2O (100 : 1). Each band was collected, incubated in $CHCl_3$ and the $CHCl_3$ was evaporated by using rotavapor to get the product. The purity test was done by checking the melting point of the LR-2 **4** by using Buchi Melting Point B540. Structure elucidation of LR-2 **4** has been published in another publication, so in this paper the elucidation only made by comparing MS data with the previous one. The structure of LR-2 **4** was confirmed as MS spectra form by using GC-MS Shimadzu QP-2010S.

The LR-2 **4** was confirmed as reddish-orange prowder, R*f* = 0,59 (Et_2O : $CHCl_3$ = 1:100) and m.p. 197.5-198.2 °C. UV-Vis, λmax = 332 nm; GC, r.t. = 16.382 menit; EI-MS, m/z 278 (100) 245, 218, 115 201 (100); IR (n cm-1) 1188.1 cm-1 (C=S) 3193,9 cm^{-1}(N-H) 1026.1 cm^{-1}(C-N) 1666.4 cm^{-1} (C=C); 1H-NMR (CDCl3, δ ppm, 60 MHz): 7,5 (H, 9x H-Ph); 4,8 (2H, 2x (-NH-); 1,8 (2H,1x (-CH_2-); 1H, 1x (-CH-)).

Cytotoxicity assay

One hundred mL of the exponentially growing cells (5 x 10^4 cells/mL) were seeded in 96-well microculture plate with a serial dilution of LR-2 **4** in a volume of 100 μL. The media without cells was used as control media and treated with the same serial dilution of LR-2 **4**, while DMSO used as control for the protein fraction. After 24 hr incubation, the number of viable cells was ascertained with MTT reaction (3-(4,5-dimethyltiazol-2-il)-2,5-diphenyl tetrazolium bromide). The absorbance was measured by ELISA reader. LC_{50} value was calculated using probit method.

Percent of dead cells was calculated as follow:

$$\% \text{ dead cells} = \frac{\text{A untreated cells} - \text{A treated cells}}{\text{A untreated cells}} \times 100\ \%$$

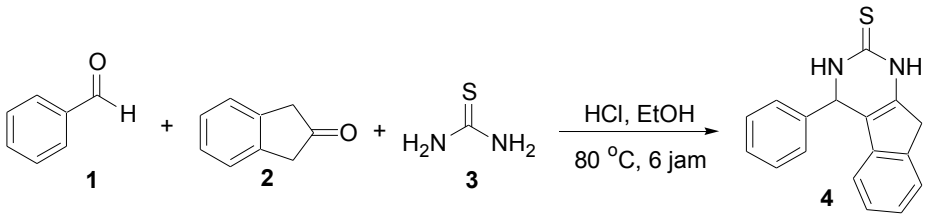

Figure 1. Synthesis of LR-2 4

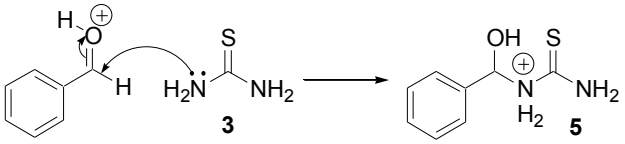

Figure 2. Reaction between benzaldehid 1 and thiourea 3 (Ritmaleni and Parmasari, 2011).

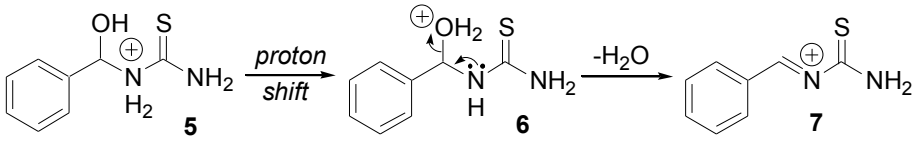

Figure 3. Water elimination to form the imminium ion 7[12]

RESULT AND DISCUSSION

The effect of the benzaldehyde excess in the synthesis of LR 2 4

To explain the effect of excess benzaldehyde in the synthesis of LR-2 **4**, first we have to know the reaction mechanism (Fig. 1). The reaction was started by formation of imminium ion **7** which is resulted from the condensation between benzaldehyde **1** and thiourea **3**. Benzaldehyde **1** is one of aldehyde that does not have alpha hydrogen. Benzaldehyde **1** in acid condition can be protonated on oxygen atom and as a result is there is a positive charge on it because oxygen uses the free electron pair to form a new bond. Afterwards, the phi bond on C-O carbonyl bond are dissociated and the electron move to oxygen to form normal oxygen, in hence the carbon atom become less electron and be more positive. This carbon atom with its positive charge acts as elctrophile and thiourea with free electron pair on sulphur as nucleophile. Imminium ion **7** is formed from the attacking of free electron pair on the nitrogen of the thiourea **3** to the carbon of the carbonyl of benzaldehyde **1**. Here, thiourea **3** acts as a nuclephile (Fig. 2).

One nitrogen on the -NH_2 will be lost as the result of proton shift to form s pecies netral and continued by the water elimination to form the imminium ion **7** (Fig. 3).

This imminium ion **7** that has been already formed will react directly with 2-indanone **2** in enol form as the nucleophile. 2-Indanone **2** is a keton which has four hydrogen alpha. This hydrogen with the absent of acid can form an enol of 2-indanone **2** and make the carbon atom on the alpha position acts as a nucleophile.

The next reaction is the attacking of free nitrogen on the thiourea **3** side of the previous species to the carbon of the 2-indanone **2** to form the hydropyrimidine ring **9**. The last step of this reaction mechanism is water elimination to form LR-2 **4**. Detail mechanism is as follow (Fig. 4):

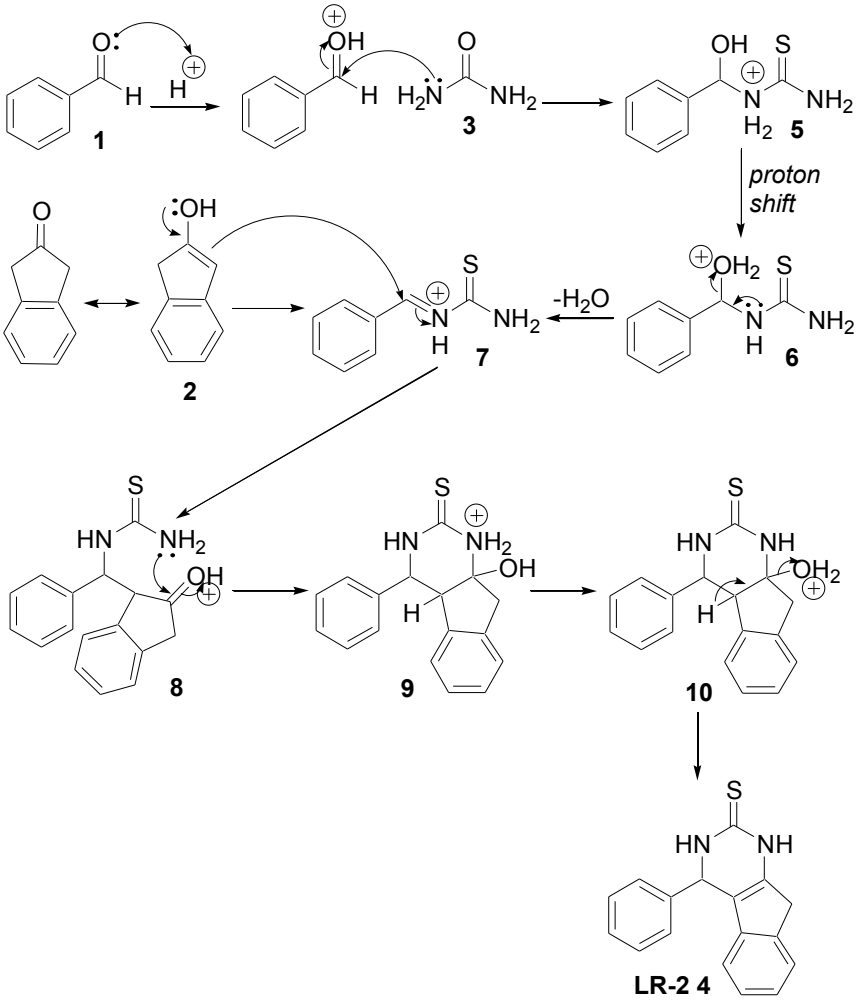

Figure 4. Reaction mechamism of LR-2 **4** (Ritmaleni, *et al., 2011*)

Optimation of the reaction to obtain a high yield can be done by variation on temperature, amount of catalyst (Ritmaleni and Parmasari, 2011), amount of thiourea **3** (Ritmaleni and Sari, 2010), type ofcatalyst (Ritmaleni and Purwitasari, 2009) and amount of 2-indanone 2 (Ritmaleni and Kusuma, 2010).

On this reaction, the research was especially investigated the effect of excess of benzaldehyde **1**. The reaction was done in four type reaction conditions as table I below.

The result showed that the increasing amount of benzaldehyde **1** was not followed by the increasing of yield of LR-2 **4**. According to Martin et. al.[16], theoretically, if one concentration of starting material is increased it means the collision frequency is also high. The possibility of each molecule to collide each other is getting higher, so the more products are also formed. The formed LR-2 **4** should more as benzaldehyde **1** are more too in the reaction system. Unfortunately, the result did not approve the theory.

One possibility that can explain this phenomena is the formation of side products. When each starting material has reacted all, there is still benzaldehyde **1** left which has possibility to attack the intermediates or even

Table I Amount of starting materials and yield

	Starting material			Yield
	2-indanone	**thiourea**	**benzaldehyde**	**LR-2 4**
1 (Flask A)	1 mmol	1 mmol	1 mmol	30 %
2 (Flask B)	1 mmol	1 mmol	1.5 mmol	27 %
3 (Flask C)	1 mmol	1 mmol	2 mmol	14 %
4 (Flask D)	1 mmol	1 mmol	2.5 mmol	8 %

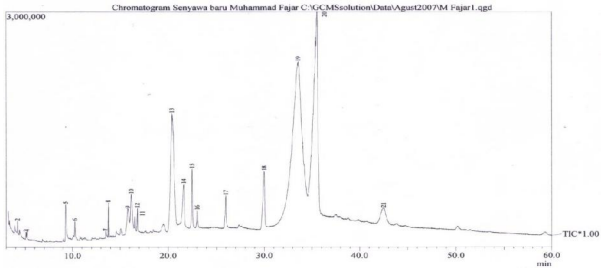

Figure 5. GC spectra of *crude product* of flask D.

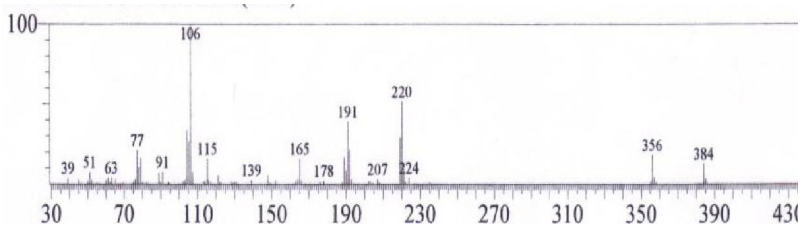

Figure 6. MS spectra of peak no. 18 on crude GC spectra of LR-2 **4.**

the product during the reaction, as seen on the reaction mechanism above, to form new side products. The GC-MS spectra was analysed to explain what kind of side products they are. Here, the GC spectra of crude product of reaction on flask D. Flask D was chosen because it gave the lowest yield so hopefully it can showed the kind of side products that are looked for. The spectra showed that there are many side products apart from LR-2 **4** (Fig. 5).

Based on the above spectra, there are 21 products formed during the reaction including LR-2 **4** (peak 20). Lets first check peak no. 18 which has molecular weight around 384 according to MS spectra (Fig. 6).

The first possibility is when benzaldehyde **1** was protonated, it always acts as electrophile and once it meets the LR-2 **4** which has free electron pair on nitrogen and it readily acts as a nucleophile and directly attack the protonated benzaldehyde **1** to form a new product **12** with MW around 384. The mechanism below explain how it forms (Fig. 8).

The second possibility is when protonated benzaldehyde **1** was attacked by nitrogen of thiourea **3** to form the imminium ion **7** like in the reaction mechanisme above, the other protonated benzaldehyde **1** was also attacked by 2-indanone **2** to form benzaldehyde-2-indanone product **14**.

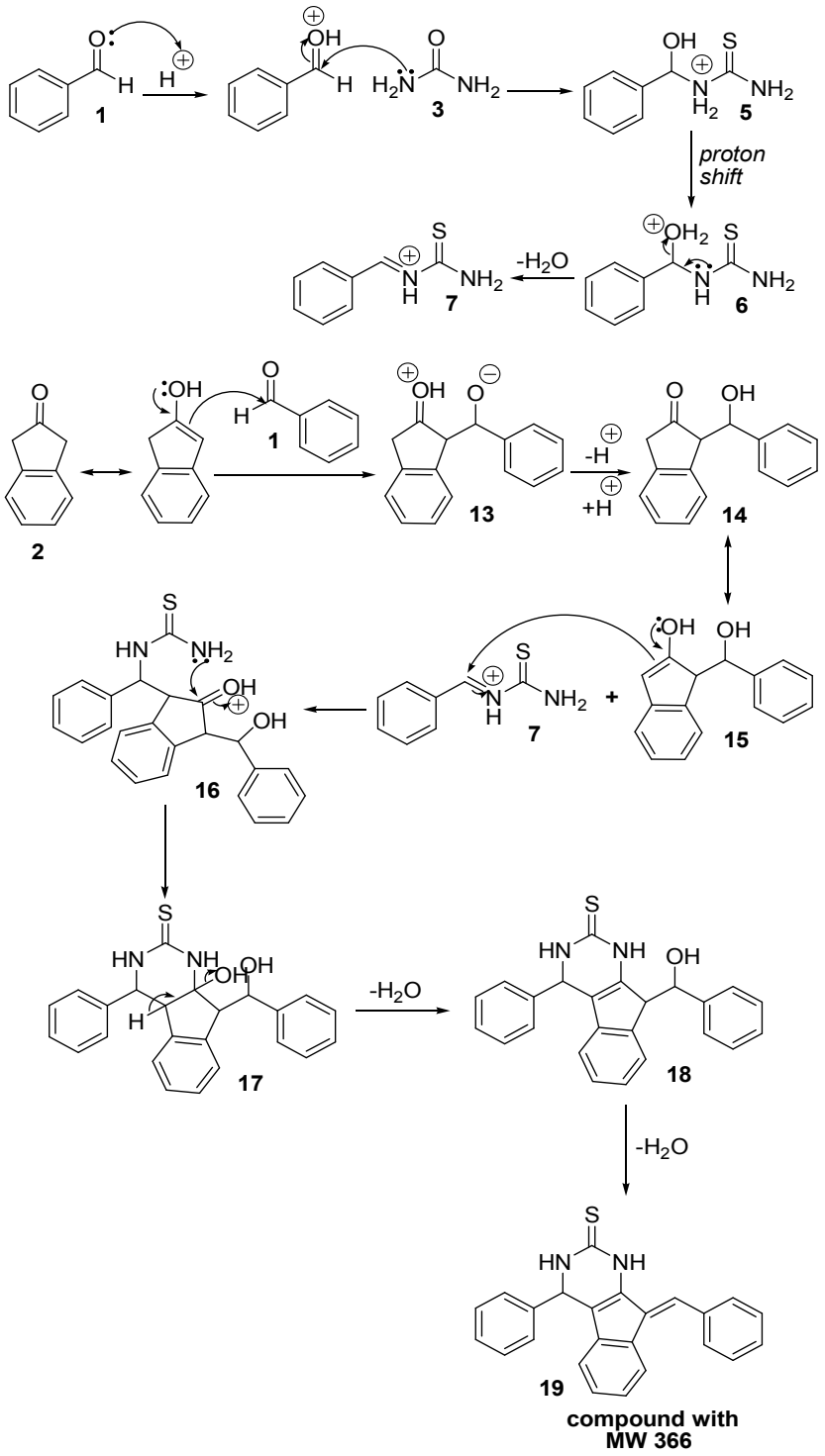

Figure 7. The formation of side product with MW 366

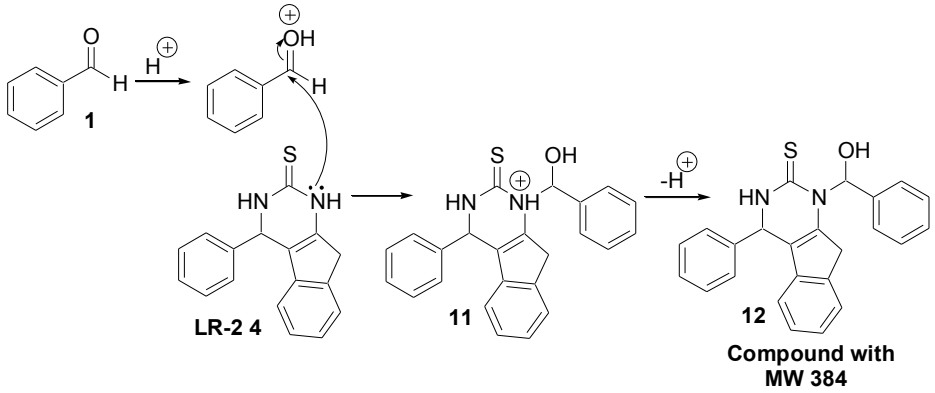

Figure 8. The formation of side product **12** with MW 384

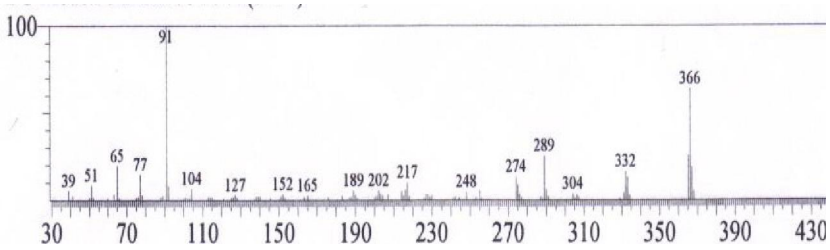

Figure 9. MS spectra of peak no. 18 on crude GC spectra of LR-2 **4.**

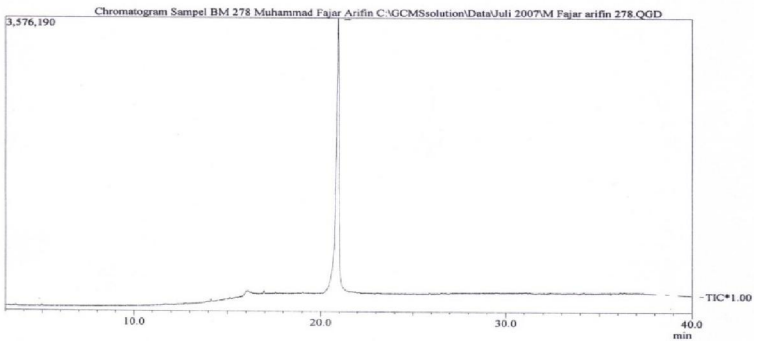

Figure 10. GC spectra of pure LR-2 **4.**

The benzaldehyde-2-indanone product **14** then reacted with imminium ion **7** to form a new product **17** as well and followed by elimination of water to produce the new molecule **19** with MW around 366. The detail mechanism is as follow (Fig. 7).

And this prediction is suitable to the MS spectra of peak no. 19 as shown below (Fig. 9).

Many other possibilities can be drawn to explain the argumentation why the higher the mole of benzaldehyde **1** in the synthesis of LR-2 **4**, the lower the yield was obtained. This result does not support the common theory about the more concentration of the starting material, the more the products will be obtained. In this case the more the mole of benzaldehyde **1**, the lower the yield of LR-2 **4**

was obtained and this has been explained that because more side products were formed in the reaction system. After doing the characterisation, LR-2 4 has the same melting point and MS spectra with previous LR-2 4.

The structure elucidation of LR-2 4 has been done by using spectroscopic methods before as published in Majalah Farmasi Indonesia. The melting point is around 199-200 °C and its purity is also high like GC spectra below (Figure 10).

Cytotoxicity activity of LR-2 on HeLa cell lines

To investigate potency of LR-2 **4** as an anticancer candidate, *in vitro* cytotoxic assay of LR-2 **4** on culture cell lines especially on Hela cell lines was carried out. The cytotoxic effects of LR-2 **4** expressed as % inhibition on HeLa cell lines were demonstrated in table II. DMSO, a solvent to solve LR-2 **4** in this research was used as a negative control.

As shown in table II, at concentration series of 101.56 - 3250 μM, LR-2 **4** was able to inhibit the growth of HeLa cell lines at a range of % inhibition between 28.84 to 92.21%. At the highest concentration (3250 μM), LR-2 **4** had the highest cytotoxic effect (92.21%). When the concentration of LR-2 **4** was reduced by serial dilution, the cytotoxic effects on Hela cell lines were also decreased. Using statistical analysis, it has been found that $r_{calculation}$ (0.839) was higher than r_{table} (0.704). Therefore, it can be concluded that there is a correlation between treatment of LR-2 **4** and % inhibition on HeLa cell lines. On the other hand, at a range of concentration of DMSO, the negative control, DMSO only caused smaller effects of % inhibition than when compared to LR-2 **4** solution. DMSO at 1.25% (v/v) only had 8.82%, while at the smallest concentration, 3.62 % inhibition on HeLa cell lines was found. It can be seen that the effects of series of DMSO on % inhibition were not too linier, even % inhibition at value 1625 μM was negative. In this regards, $r_{calculation}$ (0.528) was smaller than r_{table} (0.811). These results showed that there is no correlation between DMSO treatment and % inhibition of HeLa cell lines. In other words, DMSO treatment on HeLa cell lines at the range did not affect the growth of the cells.

The LC_{50} (a concentration at 50% letal) was calculated using probit method. The equation between log of concentration (X) and probit (Y) as shown in Table 3 was Y = 1.4151 X + 1.5636 with r = 0.9940. From this equation, the value of LC_{50} of LR-2 **4** on Hela cell lines was about 268.15 μM. Another research carried out by Ritmaleni, *et al.,* (2011) found that LC_{50} of LR-2 **4** on T47D was 159.51 μM. Maliga and Mitchison (2006) have reported that cytotoxic activity of compounds with IC_{50} between 50-200 μM are categorized as low cytotoxic activity. In another report, (Teng *et al.,* 2005) reported that a agent with anticancer activity should have LC_{50} below 50 μM (Teng *et al.,* 2005). Therefore, the LR-2 **4** has low cytotoxic activity. The IC_{50} of monastrol (the lead compound of LR-2 **4**) on *BS-C-1 green monkey kidney cells* was known as much as 5.2 ± 0.4 μM (Maliga and Mitchison, 2006).

The low cytotoxic activity of LR-2 **4** could be due to unavaibility of OH on phenil group at potition of R_4 of LR-2 **4**. The OH is important to form OH bond between the LR-2 **4** and Eg 5, a kinase that has role in cytotoxic activity mechanism. The other possibility of the low cytotoxic activity of LR-2 **4** were due to substitution of ethyl ester group in position R_5 and substitution of metal group on position R_6 by indeno. The substituents cause the LR-2 **4** is more bulky, and the effect is the interaction between LR-2 and Eg5 are inhibited (Table III).

CONCLUSION

In the synthesis of LR-2 **4**, the higher the mole of benzaldehyde **1**, the lower the yield of it was obtained. This is because the more side products was formed. The LC_{50} of LR-2 on HeLa cell lines was 268.15 μM and this stated that is not poten as an anticancer agent.

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