SYNTHESIS AND ANTIMICROBIAL ACTIVITY EVALUATION OF ETHYL SALICYL FUMARATE AND ETHYL FURFURYL FUMARATE

SINTESIS DAN UJI KERASIAAT ANTIMIKROBA TERHADAP ETIL SALISIL FUMARAT DAN ETIL FURFURIL FUMARAT

Jumina*, iqual Tabah**, dan Abdul Karim Zulkarnain***

*Laboratorium Kimia Organik, Jurusan Kimia, Fakultas Matematika dan Ilmu Pengetahuan Alam, Universitas Gadjah Mada,
**Laboratorium Kimia Kompunst, Jurusan Kimia, Fakultas Matematika dan Ilmu Pengetahuan Alam, Universitas Gadjah Mada,
***Laboratorium Farmaseutika, Fakultas Farmasi, Universitas Gadjah Mada, Yogyakarta.

ABSTRACT

This research was conducted in order to synthesize and investigate the antimicrobial activity of ethyl salicyl fumarate and ethyl furfuryl fumarate. These two target molecules were chosen as the former is the derivative of C-9154 antibiotic containing phenolic hydroxy group, whereas the latter is an example of C-9154 antibiotic derivative bearing furan ring instead of benzeno.

Ethyl salicyl fumarate was synthesized from methyl salicylate through reduction with LiAlH4, condensation of salicyl alcohol with malic anhydride, and esterification of salicyl maleic acid with ethanol in the presence of benzenesulfonylic acid as the catalyst. These reactions gave satisfactory yields (76-92%) in all stages in-situ. Similar to this procedure, ethyl furfuryl fumarate was prepared from furfural through reduction with NaBH4, followed by condensation of the resulted furfuryl alcohol with maleic anhydride and esterification of furoyl maleic acid with ethanol in the presence of benzenesulfonylic acid. Although the reduction of furfural and the condensation of furfuryl alcohol with maleic anhydride could be performed smoothly, the esterification of furfuryl maleic acid with ethanol only gave 38% yield of ethyl furfuryl fumarate.

The results of antimicrobial activity test showed that the value of minimum inhibition concentration (MIC) of salicyl maleic acid and ethyl salicyl fumarate towards Staphylococcus aureus were 500 and 100 μg/mL, whereas towards Escherichia coli were 2000 and 4000 μg/mL, respectively. In contrast, the MIC values of furfuryl maleic acid and ethyl furfuryl fumarate towards Staphylococcus aureus and Escherichia coli were 150 and 100 μg/mL, respectively.

Keywords: synthesis, activity, C-9154 antibiotic, fumarate.

ABSTRAK

Penelitian ini dilakukan dengan tujuan melakukan sintesis dan uji khasiat antimikroba terhadap etil salisil fumaraat dan etil furfuryl fumaraat. Dua molekul target tersebut dihitung dengan pertimbangan bahwa etil salisil fumaraat adalah model suatu antibiotik C-9154 yang mengandung grup fenol, sedangkan etil furfuryl fumaraat adalah model suatu antibiotik C-9154 yang mengandung cincin furan.

Etil salisil fumaraat diisoleksi dari etil salisilat melalui reaksi dengan NaBH4, kemudian dengan asam salisilik alkohol dengan anhidrida asam, dan esterifikasi asam salisilik alkohol dengan etanol menggunakan katalis asam benzenesulfonyat. Keseluruhan tahapan reaksi tersebut membutuhkan rendemen yang
Malic acid (cit) and fumaric acid (trans) are two among the known compounds which have been used in the field of pharmacy, medicine, and agriculture since a long time ago. Specifically, the derivatives of these two isomeric compounds have been utilized for the preparation of antibiotics, antifung, antifungalism and plant growth regulators (Stecher, 1968). Just to mention some examples are the ester form of melodic acid which has been patented as an antifung (Gutten, 1993), A-10009 antibiotic discovered by Mollay (1972), fumaryl D.L-alanine antibiotic (Birkeland, 1952), and malic hydrazides which are known to be able to inhibit the growth of several horticulture plants (Schulze, 1949). In addition, combination of malic acid and chloropenicilline known as chloropenicilline malate is commonly found in some cough formulations such as Benadryl, Formula 44 and Alulis.

Another active compound which also contains fumaric fragment is C-91344 antibiotic. C-91344 (1) is a class of antibiotic isolated by Hasegawa (1975) from Streptomyces albofasciac through a fermentation process. The compound showed a broad spectral activity against Gram-positive and Gram-negative bacteria such as Escherichia coli and Streptomyces aureus with minimum inhibition concentration (MIC) values from 10-100 μg/ml and LD₅₀ in mice of 75 μg/kg. The chemical structure of C-91344 antibiotic itself basically consists of two fragments i.e. phenolic acid and fumarimidate.

Recently we have developed a new strategy for the synthesis of C-91344 antibiotic derivatives from vanillin (Jumina et al., 2000 and 2003). Within this strategy, vanillin was converted to 4-ethoxy-3-methoxybenzylalime in three stages. This benzylalime was then reacted with malic anhydride to be followed by esterification of the reacted acid with absolute ethanol at the presence of HClO₄. This method is very efficient and satisfactory yields (70-95%) were obtained in each step. The biological activity of both C-91344 derivatives obtained (2 and 3) have already been investigated in which the MIC of 2 towards Streptomyces aureus and Escherichia coli were 2500-6000 μg/ml, whereas that of 3 towards the same bacteria were 500-1000 μg/ml. Thus, conversion of the carbonylic acid group in 2 to an ester functionality in 3 has led to an increase of anti microbe activity to approximately four times.

The above method has also been extended on the case of 4-ethoxy-3-methoxybenzyl alcohol simply prepared via reduction of ethyl vanillin with NaBH₄. This type of benzy alcohol derivatives was reacted with

Majlis Ilmu Farmasi Indonesia, 13/4, 2002
malonic anhydride to be followed by treatment with absolute ethanol in the presence of H$_2$SO$_4$. Again, excellent yields (78-91%) were achieved in all steps. The acid form (4) of the derivative obtained still gave a weak antimicrobial effect (MIC 2000-2500 μg/mL) towards Staphylococcus aureus and Escherichia coli. However, a significant antimicrobial activity towards these two microbes (MIC 400-700 μg/mL) were observed on the case of the ester derivative (5).

In order to obtain a more potent C-9154 antibiotic derivative, it was of interest to synthesize C-9154 antibiotic derivatives bearing phenolic hydroxyl group and furyl moiety. Regarding the former, this should be more polar than compound 2, 3, 4, and 5 previously obtained. Whereas regarding the latter, the derivative would have a furan ring instead of benzene ring as seen in the structure of compound 2, 3, 4, and 5. These two target compounds are planned to be prepared respectively from methyl salicylate, the main constituent of oil of wintergreen, and furfural.

**METODOLOGY**

**Chemicals**

All chemicals used in this research were reagent grade from Merck. Apparatus. The equipment used in this experiment involved JEOI Model 4600 proton NMR spectrometer, Shimadzu FTIR 8200 PC spectrophotometer, and Shimadzu QP 5000 Gas Chromatograph-Mass Spectrometer.

**Reduction of methyl salicylate**

A solution of methyl salicylate (1.4 g, 9.22 mmol) in dry dioxane (15 mL) was heated at 60°C in a round bottomed flask equipped with condenser. Into this solution was then added slowly lithium aluminium hydride (1.06 g, 27.66 mmol) through the condenser. After all LAH was has been added, the mixture was heated at 95-97°C for 2 h. The mixture was allowed to cool to room temperature, then cold water (3 mL) was added slowly to destroy excess LAH. The mixture was acidified using HCl 10%, then this was extracted with ethyl acetate (3×40 mL). The combined organic layers were washed with water (70 mL), dried over anhydrous sodium sulfate and evaporated to leave sticky light yellow oil of salicylic malonic acid (3.3 g, 93%). This product was characterized by means of IR and proton NMR spectrometers.

**Condensation of salicyl alcohol with malonic anhydride**

A solution of salicyl alcohol (2.0 g, 16.13 mmol) in dioxane (15 mL) was added dropwise into a solution of malonic anhydride (1.90 g, 19.39 mmol) in dioxane (15 mL). The mixture was stirred at 70-80°C for 3.5 h. The resulting mixture was allowed to cool, then diluted with ethyl acetate (50 mL). The mixture was washed with water (2×50 mL) and the organic layer was dried over anhydrous sodium sulfate and evaporated to leave sticky light yellow oil of salicylic malonic acid (3.4 g, 92%). This product was characterized by means of IR spectrometers.

**Synthesis of ethyl salicylic formate**

A mixture of salicylic malonic acid (1.0 g, 4.50 mmol), absolute ethanol (10 mL), and benzene sulfonic acid (ca. 0.3 g) was stirred and heated at reflux for 3.5 h. The resulting mixture was allowed to cool, then the solvent was removed by means of rotary evaporator. The residue was dissolved with water (100 mL), then extracted with ethyl acetate (3×100 mL). The combined organic layers were washed with water (2×70 mL), dried over anhydrous sodium sulfate and evaporated to afford the desired ester (0.81 g, 76%) which was found as light yellow oil. This product was characterized by means of IR and proton NMR spectrometers.
Reduction of furfural

Into a solution of furfural (4.60 g, 48.14 mmol) in absolute ethanol (30 mL) was added sodium borohydride (3.64 g, 96.25 mmol). The mixture was heated at reflux for 2 hours, then allowed to cool down and evaporated to dryness. The residue was diluted with water (50 mL), then extracted with ethyl acetate (3x30 mL). The combined organic layers were washed with water (2x80 mL), dried over anhydrous sodium sulfate and evaporated to give furfuryl alcohol (2.50 g, 53 %). This product was identified by means of IR and GC-MS spectrometers.

Condensation of furfuryl alcohol with maleic anhydride

A solution of maleic anhydride (1.75 g, 17.86 mmol) in benzene (15 mL) was stirred and warmed at 50°C. Into this solution was added dropwise a solution of furfuryl alcohol (1.35 g, 17.81 mmol) in benzene (15 mL). The heating was continued for 2 hours, then the mixture was allowed to cool and diluted with ethyl acetate (70 mL). The mixture was washed with water (3x80 mL), dried over anhydrous sodium sulfate, and evaporated to afford fufurylmaleic acid (1.95 g, 72 %) as a brown oil.

Synthesis of ethyl furfurylformate

A mixture of furfuryl maleic acid (1.0 g, 4.50 mmol), absolute ethanol (10 mL), and benzene sulfonic acid (ca. 0.3 g) was stirred and heated at reflux for 3.5 h. The resulting mixture was allowed to cool, and further treatment according to procedure as described for the preparation of ethyl salicylic formate afforded ethyl furfuryl formate (0.38 g, 38 %) as a brown oil. The product was identified by means of IR spectrometer.

Determination of anti microbial activity of the derivatives synthesized

A series of solutions of each C9154 derivative were prepared and placed in tubes containing Staphylococcus aureus and Escherichia coli. The samples were incubated at 37°C, and the inhibition effect of each sample was observed.

The C9154 antibiotic derivatives obtained from the experiment were placed on petri dish containing Gram positive and negative bacteria. The samples were incubated at 37°C using agar media and the inhibition effect of each sample was observed.

RESULTS AND DISCUSSION

Synthesis of ethyl salicylic formate

As stated previously, the preparation of ethyl salicylic formate was conducted in order to generate C-9154 antibiotic derivatives possessing phenolic hydroxyl group. It was of interest to investigate whether the existence of such free hydroxyl group would enhance its antimicrobial activity or not in comparison to those of compound 2, 3, 4, and 5 previously synthesized. For this purpose, ethyl salicylic formate was prepared from methyl salicylate, the main constituent of oil of wintergreen, in 3 steps i.e. (1) reduction of methyl salicylate, (2) condensation of salicyl alcohol with maleic anhydride, and (3) esterification of salicyl maleic acid with ethanol (Scheme 1).
The reduction of methyl salicylate was conducted using LiAlH₄ in dry diethyl ether for 2 hours to give 82% yield of the desired salicylic alcohol which was found as a white waxy solid. It is interesting to note in here that a safer procedure was found when methyl salicylate was added slowly to a hot (70-80°C) suspension of LiAlH₄ in diisobutyl ketone rather than heating slowly a mixture of LiAlH₄ and methyl salicylate in diisobutyl ketone. In this latter procedure, a rather uncontrollable reaction occurred when the temperature reached at about 80°C. Such a danger was not found at all when the former procedure was applied. In addition, it is worthy to mention that the resulted salicylic alcohol is best to be stored in a fridge. It was discovered that storing of the compound at room temperature for course of days has changed it to be a waxy solid which was insoluble in most organic solvents such as dichloromethane, chloroform, ethyl acetate, diisobutyl ketone, benzene, toluene, and carbon disulfide.

The IR spectrum of the product showed the existence of two strong broad bands of OH groups at 3450 and 3163 cm⁻¹. The former is originated from CH₃OH, whereas the latter is from the phenolic OH group. The phenolic OH group appeared at a lower frequency compared to that of CH₃OH as the lone pair electrons of the oxygen atom in phenol involve in delocalization. As expected, the spectrum did not show any absorption of CO¹ stretching frequencies which normally arise at around 1700 cm⁻¹. Therefore, the IR spectrum clearly proved that the C=O group of the starting material has been reduced to CH₂OH.

The proton NMR spectrum of the resulted salicylic alcohol displayed three signals, which indicated the presence of three types of protons. A doublet multiplet appearing at 6.77 ppm (4H) is supposed to be originated from the resonance of the phenyl protons. The pattern of the peak, which is slightly irregular, may be attributed to the existence of two different substituents lying in an equal orientation in the benzene ring. Singlets appearing at 4.8 ppm (1H) and 3.7 ppm (2H) respectively correspond to the resonance of OH and methylene protons. Although the spectrum is sufficiently clean, the integration of the phenyl proton is a bit less than the expected value.

Following the above success, the resulted salicylic alcohol was reacted with malonic acid. The reaction was conducted in diisobutyl ketone at 70-80°C for 2 hours to give yellow sticky oil of salicyl malonic acid in 70% which could be viewed as the acid form of C-9154 antibiotic derivative. The IR spectrum of this product showed OH stretching frequency at 3331 cm⁻¹, carbon stretching frequency at 1778 cm⁻¹, and C=O stretching frequency at 1620-1595 cm⁻¹. The existence of only one OH absorption band rather than two as seen in the IR spectrum of salicylic alcohol indicated that a reaction has occurred toward CH₂OH. Together with the presence of a strong C=O absorption band appearing at 1778 cm⁻¹, this indicated that salicyl malonic acid has been obtained in this experiment.

It has been reported previously (Usai et al., 2000 and 2001) that ester form of C-9154 antibiotic derivatives could be prepared through esterification of the related acid using concentrated sulfuric acid as the catalyst. Referring to this report, the acid form of C-9154 antibiotic derivative obtained was esterified using absolute ethanol in the presence of concentrated sulfuric acid. The reaction was conducted at reflux for a long time (4 hours) as examination using thin layer chromatography (TLC) indicated that the reaction has not gone to completion after being heated for 2.5 hours.

Identification of the product using proton NMR spectrum led to a conclusion that the product was not the right compound. The error as the spectrum displayed only a singlet of alkoyl proton at 6.2 ppm instead of the expected two singlets even though the existence of CH₃K group is clearly indicated in the spectrum. In addition, the data indicated that the expected esterification was also followed by tautomerization which replaced the oxybutylyoxymethylene group by ethoxy group leading to the formation of M.3.0.0.0.211
diethyl malate. Such esterification is possible as α-hydroxyxymethyl group is sufficiently bulky so that replacement by smaller ethyl group would afford to a thermodynamically more stable product.

Due to the above difficulty, then the esterification of salicyl maleic acid with ethyl was performed using benzoyl peroxide which is weaker than sulfonic acid. The experiment was conducted at reflux for 3.5 hours to give yellow oil of the desired-ethyl salicyl maleate in 83 % yield. The formation of ethyl salicyl maleate as the final product rather than ethyl salicylic malonate was concluded on the basis previous findings, which showed that malonic fragment (cit) could easily be introgressed to fumaric fragment (trans) through heating at 75-100°C for 1-2 hours (Vogel, 1968). It was supposed that the heating (75°C) undertaken in the experiment that was sufficient long (3.5 hours) could simultaneously converted ethyl salicylic malonate to ethyl salicyl maleate.

Identification of the product using IR spectrum still showed the existence of OH group appearing at 3392 cm⁻¹, however, the peak is less intense and not as wide as that of salicyl maleic acid, which perhaps indicates that esterification has already occurred toward the COOH group. Another indication for the occurrence of esterification is the enhancement of CH₂ banding frequency appearing at 1257 cm⁻¹ in comparison to that of salicyl maleic acid. Thus, it could be shown in here that the esterification has occurred without cleaving the α-hydroxyxymethyl group.

The proton NMR spectrum of the resulted ethyl salicyl maleate showed a triple at 3.1 ppm (3 H) and a quartet at 4.2 ppm (2 H) indicating the existence of CH₂(CH₃) functionality. The male:CH=CH proton resonates as two singlets at 6.15 and 6.30 ppm, whereas the phenyl and methylene protons respectively appear at 6.4-7.2 ppm and 3.8 ppm. Although the spectrum did not exhibit a proper integration, the spectrum could clearly proved that the outcome of the reaction is the expected ethyl salicyl maleate.

**Synthesis of ethyl furfuryl fumarate**

As done for ethyl salicyl maleate, the preparation of ethyl furfuryl fumarate from furfural was conducted in 3 steps i.e. (1) reduction of furfural, (2) combination of furfuryl alcohol with maleic anhydride, and (3) esterification of furfuryl maleic acid (Scheme 2). This method of preparation is different from the that reported by Sunniestrah (2000) who prepared C-5-ketone derivatives through the condensation of furan-2-carboxylic maleamide.

The reduction of furfural was conducted using sodium borohydride in reducing ethanol for 2 hours. Heating at reflux (75°C) was required as most of starting materials reduced instant when the reaction was performed at room temperature. Furfuryl alcohol was obtained in 53 % yield and was found as brownish-yellow oil.

![Scheme 2](image)

The IR spectrum of the synthesized furfuryl alcohol showed the disappearance of C=O stretching frequency appearing at 1674 cm⁻¹ in the IR spectrum of furfural. Instead, the spectrum demonstrated the existence of an intense broad band of OH group appearing at 3300 cm⁻¹. These phenomena, therefore, proved that the reduction of the furfural C=O group has occurred.

Further analysis of the synthesized furfuryl alcohol was conducted using GC-MS spectrometer. The chromatogram showed that the compound was sufficiently pure. The only peak appearing in the chromatogram gave molecular ion at m/z = 98 on its mass spectrum. This m/z value is exactly the same as the
value of molecular weight of furfuryl alcohol. Other fragments appearing at m/z = 81 and m/z = 69 were supposed to be generated respectively through the fragmentation of that molecular ion by losing OH and CHO groups. Thus, there is no doubt that the reduction of furfuryl C=O group has been achieved in this experiment.

The condensation of furfuryl alcohol and malic anhydride was conducted in benzene at 50-60°C for 3 hours to give 72 % yield of furfuryl malic acid which was found as dark-brown oil. The product was identified using IR spectrum which showed the existence of OH and C=O stretching frequencies of the COOH group respectively at 3500 and 1740 cm⁻¹. In addition, the existence of malic fragment was also indicated by the presence of a medium absorption band at 1635 cm⁻¹ which corresponded to the stretching frequency of C=O bond.

As a final step, the resulted furfuryl malic acid was treated with excess ethanol in the presence of catalytic amount of benzenehexafluoride at reflux for 1.5 hours to afford the expected ethyl furfuryl fumarate in 38 % yield which was found as dark-brown oil. This relatively low yield perhaps was attributed by the instability of the furan ring especially under acidic and thermal conditions. Indeed, it was discovered that only polymeric material was obtained when the esterification was conducted using concentrated sulfuric acid as the catalyst. Likewise, polymeric material was the major product when the reaction was conducted at reflux for 3.5 hours.

Identification of the synthesized ethyl furfuryl fumarate was performed using IR spectrometer. The IR spectrum clearly showed the disappearance of OH stretching frequency appearing at 3500 cm⁻¹ in the IR spectrum of furfuryl malic acid. This proved that a reaction has occurred to the hydroxyl moiety of the COOH group. In addition, the IR spectrum exhibited a strong absorption band of C=O stretching frequency at 1724 cm⁻¹. Whereas C=O and C=O stretching frequencies respectively appeared at 2931-2981 and 1161 cm⁻¹, C=C stretching frequency appeared at 1617 cm⁻¹. Although this product has not been characterized fully, the IR spectrum obtained would provide meaningful evidence for the formation of ethyl furfuryl fumarate.

Antimicrobial activity of the synthesized C-9154 antibiotic derivatives

Antimicrobial activity evaluation towards the synthesized C-9154 antibiotic derivatives was conducted using Staphylococcus aureus and Escherichia coli respectively as the representative of Gram positive and Gram negative bacteria. Aqueous methanol was used as the control, and antimicrobial activity was determined through measurement of minimum inhibition concentration (MIC) of the compound towards the growth of the bacteria. The MIC values obtained for salicyl malic acid (4), ethyl salicyl fumarate (7), furfuryl malic acid (8), and ethyl furfuryl fumarate (9) towards Staphylococcus aureus and Escherichia coli are presented in Table 1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC towards Staphylococcus aureus (µg/mL)</th>
<th>MIC towards Escherichia coli (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicyl malic acid (4)</td>
<td>500</td>
<td>2000</td>
</tr>
<tr>
<td>Ethyl salicyl fumarate (7)</td>
<td>100</td>
<td>4000</td>
</tr>
<tr>
<td>Furfuryl malic acid (8)</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Ethyl furfuryl fumarate (9)</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

As shown in Table 1, compound 4, 6, 7, 8, and 9 are all active against Staphylococcus aureus and Escherichia coli even though compound 6 and 7 only show weak antimicrobial activity against Escherichia coli. Accordingly, it could be concluded to some extent that the synthesized C-9154 antibiotic derivatives are more effective against the Gram positive bacteria. The data also show that in general the ethyl ester forms of the C-9154 antibiotic derivatives obtained are more active than the carboxylic acid analogues. Thus, this finding is consistent with the discovery previously reported (Jumaa, 2001).

Compared to compound 2, 3, 4, and 5, which showed MIC values towards Staphylococcus aureus and Escherichia coli in the range of 400-2500 µg/mL, it can be pointed out that in general compound 4, 7, 8.

Majidah Fawzi (2002), 13(2), 2002 213
and 9 exhibit stronger antimicrobial activity. Thus, the attachment of phenolic hydroxyl group, which increases the polarity of the compound, could also enhance the antimicrobial activity. Prominent enhancement of activity compared to those of compound 2, 3, 4, and 5 is even found for compound 9 and 9 showing antimicrobial activity of 100-150 μg/mL, which are already comparable to the MIC values (25-150 μg/mL) of some common antibiotics such as penicillin, chloramphenicol, or amoxicillin (Burger, 1960 and Martin, 1992). Therefore, it can also be concluded that the existence of flavon ring instead of benzene ring in the structure of C(9)154 antibiotic derivatives could significantly enhance their antimicrobial activity. Unfortunately, due to its instability, compound 9 was rather difficult to synthesize, and the yield obtained in this experiment was only 38%.

CONCLUSIONS

Ethyl salicylate fumate (7) could be prepared in good yield from methyl salicylate through reduction followed by condensation with maleic anhydride and esterification with ethanol using benzene-sulfonamide acid as the catalyst. The similar procedure could also be applied for the synthesis of ethyl furfuryl fumate (9) even though the yield was rather unsatisfactory.

Furfuryl maleic acid (8) and ethyl furfuryl fumate (9) are sufficiently effective to inhibit the growth of *Staphylococcus aureus* and *Escherichia coli*. In contrast, salicylic maleic acid (6) is not significantly active towards *Staphylococcus aureus* and *Escherichia coli*. In the case of ethyl salicylate fumate (7), this compound is sufficiently active against *Staphylococcus aureus*, but is not sufficiently active against *Escherichia coli*.

The existence of phenolic hydroxyl group and especially flavon ring in the structure of C(9)154 antibiotic derivatives could significantly enhance their antimicrobial activity.

ACKNOWLEDGEMENT

The authors deeply thank to INDONESIA TORAY SCIENCE AND TECHNOLOGY FOUNDATION for its financial support for the implementation of most of the research activities.

REFERENCES


