SYNTHESIS AND ANTIMICROBE ACTIVITY EVALUATION OF *p*-ANISYL ETHYL FUMARATE, *N*-BENZYL ALKYL FUMARAMATE, AND ETHYL *N*-PHENYL FUMARAMATE

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Abstract

This research was conducted in order to synthesize and investigate the antimicrobe activity of *p*-anisyl ethyl fumarate, *N*-benzyl alkyl fumaramate, and ethyl *N*-phenyl fumaramate. These target molecules were chosen as the former would become a para-methoxy substituted C-9154 antibiotic derivative, whereas the two latter would become examples of unsubstituted C-9154 antibiotic derivatives bearing amido-ester fumaric side chain.

p-Anisyl ethyl fumarate was synthesized from anethole through oxidation with $KMnO_4$, reduction of p-anisaldehyde with $NaBH_4$, condensation of *p*-anisyl alcohol with maleic anhydride, and esterification of *p*-anisyl maleic acid with ethanol in the presence of benzenesulfonic acid as the catalyst. These reactions gave satisfactorily yields (55-81 %) in all steps involved. In the case of *N*-benzyl alkyl fumarate, these target molecules were prepared from benzaldehyde through reaction with hydroxylamine hydrochloride, reduction of benzaldoxime with sodium in ethanol, condensation of benzylamine with maleic anhydride, and esterification of the resulted *N*-benzyl maleic acid with ethanol and methanol in the presence of concentrated sulfuric acid as the catalyst. These synthetic steps gave *N*-benzyl ethyl fumaramate and *N*-benzyl methyl fumaramate respectively in 84 % and 77 % yield. Similar to that conducted to benzylamine, ethyl *N*-phenyl fumaramate was synthesized in 84 % yield through condensation of aniline with maleic anhydride, followed by esterification with ethanol in the presence of concentrated sulfuric acid.

The results of antimicrobe activity test showed that the value of minimum inhibition concentration (MIC) of *p*-anisyl ethyl fumarate, *N*-benzyl ethyl fumaramate, *N*-benzyl methyl fumaramate, and ethyl *N*-phenyl fumaramate towards *Staphyllococcus aureus* were 15, 25, 25, and 25 μg/mL, whereas towards *Eschericia coli* were 15, 500, 25, and 25 μg/mL respectively. Thus, the data showed that the four C-9154 antibiotic derivatives obtained are sufficiently potent and possess antimicrobe activity which are comparable those of some common antibiotics such as phenicilline, kemicitine, and amoxyline.

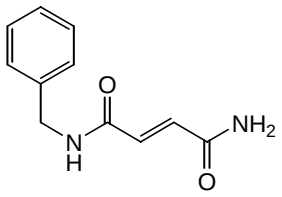
Keywords: *synthesis, activity, C-9154 antibiotic, fumarate.*

INTRODUCTION

The derivatives of maleic acid (cis) and fumaric acid (trans) are two groups 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, antifungis, antihistamins and plant growth regulators (Stecher, 1968). Just to mention some examples are the ester form of melanilic acid which has been patented as an antifungi (Ligett, 1959), A-19009 antibiotic discovered by Molloy (1972), fumaryl DL-alanin antibiotic (Birkingshaw, 1942), and maleic hydrazides which are known to be able to inhibit the growth of several horticulture plants (Schoene, 1949). In addition, combination of maleic acid and chlorpheniramine known as chlorpheniramine maleate is commonly found in some cough formulaes such as Benadryl, Formula 44 and Alerin.

Beside the active compounds as stated above, fumaric fragment also becomes part of the structure of C-9154 antibiotic. C-9154 **(1)** is a class of antibiotic isolated by Hasegawa (1975) from *Streptomyces ishigakiensis* through a fermentation process. The compound showed a broad spectral activity against Gram-positive and Gram-negative bacteria such as *Escherichia coli* and *Staphylococcus aureus* with minimum inhibition concentration (MIC) varied from 10-100 μg/mL and LD_{50} in mice of 75 mg/kg. The chemical structure of C-9154 antibiotic itself basically consists of two fragments i.e. phenylacetic acid and fumaramide.

A new strategy for the synthesis of C-9154 antibiotic derivatives from vanillin has been developed recently by our research group (Jumina et.al, 2000 and 2001). Within this strategy, vanillin was converted to 4-ethoxy-3-methoxybenzylaniline in three stages. This benzylaniline was then reacted with maleic anhydride to be followed by esterification of the related acid with absolute ethanol in the presence of H_2SO_4. This method is very efficient and satisfactory yields (70-95%) were obtained in each step. The biological activity of both C-9154 derivatives obtained **(2** and **3)** have already been investigated in which the MIC of **2** towards *Staphyllococcus aureus* and *Escherichia coli* were 2500-3000 μg/mL, whereas that of **3** towards the same bacteria were 500-1000 μg/mL. Thus, conversion of the carboxylic acid group in **2** to an ester functionality in **3** has led to an increase of anti microbe activity to approximately four times.



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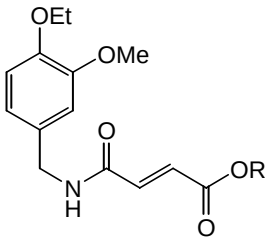
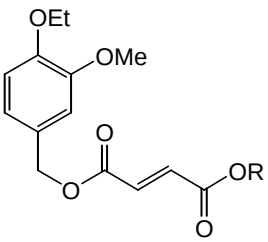


2 (R = H)
3 (R = Et)



4 (R = H)
5 (R = Et)

The above method has also been extended on the case of 4-ethoxy-3-methoxybenzyl alcohol simply prepared via reduction of ethyl vanillin with $NaBH_4$. This type of benzyl alcohol derivatives was reacted with maleic anhydride to be followed by treatment with absolute ethanol in the presence of H_2SO_4. Again, excellent yields (78-93 %) were achieved in all steps. The acid form **(4)** of the derivative obtained still gave a weak anti microbe effect (MIC 2000-2500 μg/mL) towards *Staphyllococcus aureus* and *Escherichia coli*. However, a significant anti microbe activity towards those 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 derivatives, it was of interest to synthesize *p*-anisyl ethyl fumarate **(6)**, *N*-benzyl ethyl fumaramate **(7)**, *N*-benzyl methyl fumaramate **(8)** and ethyl *N*-phenyl fumaramate **(9)**. These target molecules were chosen as all would be less bulky than compound **2**, **3**, **4**, and **5** previously obtained. In addition, the aromatic moiety of target compound **7**, **8**, and **9** would be exactly the same as that of C-9154 antibiotic. Thus, on the basis of molecular size and similarity of aromatic ring between the target molecules and C-9154 antibiotic, it was hoped that the target compound **6, 7**, **8**, and **9** would be more potent than compound **2**, **3**, **4**, and **5** previously synthesized.

METHODOLOGY

Chemicals. All chemicals used in this research were reagent grade from Merck. **Apparatus.** The equipment used in this experiment involved JEOL MY60 proton NMR spectrometer, Shimadzu FTIR 8201 PC spectrophotometer, and Shimadzu QP 5000 Gas Chromatograph-Mass Spectrometer.

Reduction of p-anisaldehyde

Sodium borohydride (0.35 g; 9.2 mmol) was added into a solution of p-anisaldehyde (0.5 g; 3.67 mmol) in absolute ethanol (4 ml). The mixture was stirred and heated at reflux for 3 hours, then the solvent was removed using a Buchii evaporator. The residue was diluted with water (15 ml), and extracted with dichloromethane (3x20 ml). The combined organic layers were washed with water (2x70 ml), dried over anhydrous sodium sulfate and evaporated to yield p-anicyl alcohol (0.30 g; 55%) as a light yellow oil. This product was identified by means of IR and GC-MS spectra.

Condensation of p-anisyl alcohol with maleic anhydride

A solution of p-anisyl alcohol (1.5 g, 10.87 mmol) in benzene (5 mL) was added dropwise into a solution of maleic anhydride (1.28 g, 13.0 mmol) in benzene (10 mL). The mixture was stirred at 90^{o}C for 2.5 h, then allowed to cool, and diluted with ethyl acetate (50 mL). The resulted solution was washed with water (3x70 mL), and the organic phase was dried over anhydrous sodium sulfate and evaporated to leave a white sticky solid (2.01 g, 78 %). This p-anisyl maleic acid was characterized by means of IR, proton NMR, and GC-MS spectrometers.

Synthesis of ethyl p-anisyl fumarate (derivative of C-9154)

A mixture of p-anisyl maleic acid (1.0 g, 4.23 mmol), absolute ethanol (10 mL), and benzene sulfonic acid (ca. 0.3 g) was stirred and heated at reflux for 3 h. The resulted mixture was allowed to cool, then the solvent was removed by means of rotary evaporator. The residue was diluted with water (40 mL), then extracted with dichloromethane (3x40 mL). The combined organic layers were washed with water (2x70 mL), dried over anhydrous sodium sulfate and evaporated to afford the desired ester (0.90 g, 81 %) which was found as a colorless oil. This product was characterized by means of IR and proton NMR spectrometers.

Synthesis of benzaldoxime

Benzaldehyde (14.0 g, 0.13 mol) was added to a stirred mixture of powdered sodium hydroxide (9.33 g, 0.23 mol) in water (27 mL). Into this mixture was then added hydroxylamine hydrochloride (10.0 g, 0.14 mol) in portions, and the mixture was stirred further at 50-60^{o}C for 2 h. The mixture was diluted with water until a clear solution was formed, then this was extracted with dichloromethane (3x50 mL). The combined organic layers were washed with water (2x80 mL), dried over anhydrous sodium sulfate and

evaporated to leave a white solid of the expected benzaldoxime (13.2 g, 84 %). This product was characterized by means of IR and GC-MS spectrometers.

Reduction of benzaldoxime to benzylamine

Benzaldoxime (4.0 g, 33.06 mmol) was dissolved in absolute ethanol (80 mL) in a round bottomed flask equipped with a reflux condenser. The mixture was stirred and heated on an oil bath at 90^{o}C. The oil bath was removed and into the hot mixture was added pieces of metallic sodium (7.60 g, 0.33 mol) in such rate which was sufficiently rapid, but without the occurrence of spoiled of the mixture through the condenser. After all sodium has been added, the mixture was heated further at reflux for 2 h. The mixture was allowed to cool down, then the solvent was removed by using a rotary evaporator. The residue was diluted with water (60 mL), then extracted with dichloromethane (3x40 mL). The combined organic layers were washed with water (2x70 mL), dried over anhydrous sodium sulfate and evaporated to leave a colorless oil of benzylamine (2.55 g, 72 %). This product was identified by means of IR and proton NMR spectrometers.

Condensation of benzylamine with maleic anhydride

A solution of benzylamine (1.16 g, 10.87 mmol) in benzene (5 mL) was added dropwise into a solution of maleic anhydride (1.28 g, 13.0 mmol) in benzene (10 mL). The mixture was stirred at 70^{o}C for 2.5 h, then allowed to cool, and diluted with ethyl acetate (50 mL). The resulted solution was washed once with 5 % HCl solution (25 mL), then followed by water (3x70 mL). The organic phase was dried over anhydrous sodium sulfate and evaporated to leave a yellow sticky solid (1.94 g, 87 %). This N-benzil maleamic acid was characterized by means of IR, proton NMR, and GC-MS spectrometers.

Synthesis of ethyl and methyl N-benzyl fumaramate (derivatives of C-9154)

A mixture of N-benzyl maleamic acid (1.5 g, 7.32 mmol), absolute ethanol (15 mL), and concentrated sulfuric acid (3 drops) was stirred and heated at reflux for 3 h. The resulted mixture was allowed to cool, then the solvent was removed by means of rotary evaporator. The residue was diluted with water (40 mL), then extracted with dichloromethane (3x40 mL). The combined organic layers were washed with water (2x70 mL), dried over anhydrous sodium sulfate and evaporated to leave the desired ethyl ester (1.45 g, 84 %) which was found as a light oil. This product was characterized by means of IR and proton NMR spectrometers.

Similar experiment was also conducted by using methanol to afford the methyl ester compound (77 %).

Condensation of aniline with maleic anhydride

Into a solution of maleic anhydride (5.0 g, 51.0 mmol) in benzene (20 mL) was added in portions aniline (4.0 g, 43.0 mmol). The resulted emulsion was then heated at reflux for 2 h. The mixture was allowed to cool down, then diluted with ethanol (120 mL) and acidified using 5 % HCl solution. The resulted suspension was filtered, and the solid obtained was dried to give N-phenylmaleamic acid (6.2 g, 76 %) which was found as a white solid.

Synthesis of ethyl N-phenyl fumaramate (derivative of C-9154)

A mixture of N-phenyl maleamic acid (1.5 g, 7.32 mmol), absolute ethanol (15 mL), and concentrated sulfuric acid (3 drops) was stirred and heated at reflux for 3 h. The resulted mixture was allowed to cool, then the solvent was removed by means of rotary evaporator. The residue was diluted with water (40 mL), then extracted with dichloromethane (3x40 mL). The combined organic layers were washed with water (2x70 mL), dried over anhydrous sodium sulfate and evaporated to leave the desired ethyl ester (1.45 g, 84 %) which was found as a light oil. This product was characterized by means of IR and proton NMR spectrometers.

Determination of anti microbe activity of the derivatives synthesized

A series of solutions of each C-9154 derivative were prepared and placed in tubes containing *Staphyllococcus aureus* and *Escherichia coli*. The samples were incubated at 37^{o}C, and the inhibition effect of each sample was observed.

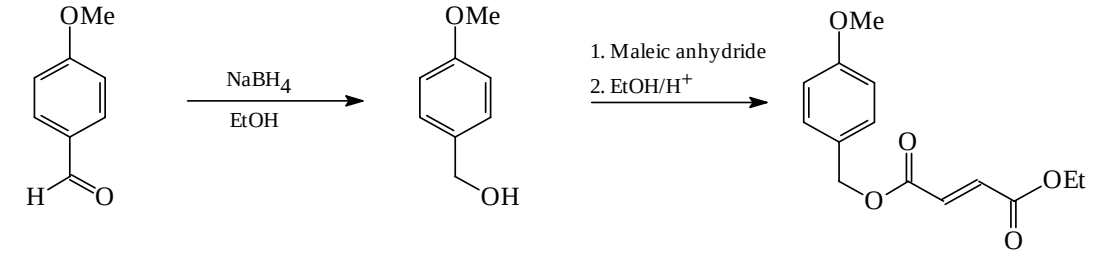
The C-9154 antibiotic derivatives obtained from the experiment were placed on petri dish containing Gram positive and negative bacteria. The samples were incubated at 37^{o}C using agar media and the inhibition effect of each sample was observed.

RESULT AND DISCUSSIONS

Synthetic approaches to the targeted C-9154 antibiotic derivatives

As mentioned previously, the experiment was designed in order to synthesize 3 types of C-9154 antibiotic derivative i.e. p-anisyl ethyl fumarate, N-benzyl alkyl fumaramate, and ethyl N-phenyl fumaramate. Whereas the first example would be a fumarate diester, the last two will be fumarate amido-esters. In the case of p-anisyl ethyl

fumarate, the whole reactions performed consisted of reduction of p-anisaldehyde with sodium borohydride, condensation of p-anisyl alcohol with maleic anhydride, and esterification of p-anisyl maleic acid with ethanol (Scheme 1).



p-anisaldehyde *p-anisyl alcohol* *p-anisyl ethyl fumarate*

Scheme 1

The reduction of p-anisaldehyde with sodium borohydride was conducted in absolute ethanol at reflux for 3 hours to give the desired *p*-anisyl alcohol in 72 % yield which was found as a light yellow oil. The reality that the reaction required a relatively long time (3 hours) under a hard condition (78^{o}C) could be correlated by the presence of the electron donating methoxy group which would decrease the reactivity of *p*-anisaldehyde carbonyl group towards nuchleophiles including hydride ions.

The IR spectrum of the resulted *p*-anisyl alcohol clearly shows the existence of a strong broad band at 3383 cm^{-1} originating from the OH stretching frequency. In addition, the disappearance of C=O absorption, which arises at 1681 cm^{-1} in the IR spectrum of the starting material, gave more evidence for the success of the reduction. Other absorption bands arising at 3100-3000 cm^{-1} (CH stretching), 1600-1500 cm^{-1} (C=C aromatic stretching), 1249 cm^{-1} (CH bending), and 1009 cm^{-1} (C-O stretching) also support the proposed structure.

Further structural assignment of the product conducted using GC-MS gave a chromatogram which indicated that the product essentially consists of a single component (t_R = 11.46 minutes, 91.5%). The mass spectrum of this major product gave a molecular ion peak at *m/z* = 138 which fits with the molecular weight of *p*-anisyl alcohol. Furthermore, fragments arising at *m/z* = 121 and *m/z* = 109 in the mass spectrum are respectively originated from the loss of OH (M-17) and CO (M-28) groups.

The resulted *p*-anisyl alcohol was then reacted with maleic anhydride in benzene at 70-80^{o}C for 2.5 hours to afford *p*-methoxybenzyl maleic acid in 86 % yield. Identification

of the product was conducted by means of IR and mass spectrometers. The IR spectrum showed stretching frequencies of OH group (3200-3700 cm^{-1}, medium), C=O group (1780 and 1732 cm^{-1}), and C=C group (1612 and 1516 cm^{-1}). The CH bending frequency of the alkyl groups appears at 1412-1463 cm^{-1} (weak), while the C-O stretching frequencies emerge as strong bands at 1175 and 1248 cm^{-1}. Identification using GC-MS indicated that the product consisted of 4 main components in which the expected anisyl maleic acid (t_R = 25.2 minutes) became the major component (60 %). The mass spectrum of this acid did not show molecular ion peak at the expected value (*m/z* 236), but it revealed a molecular ion peak at *m/z* 240. However, the fragmentation pattern of the spectrum exhibiting peaks at *m/z* 91, 121 (base peak), and 137 gave sufficient evidence for the formation of the desired *p*-anisyl maleic acid.

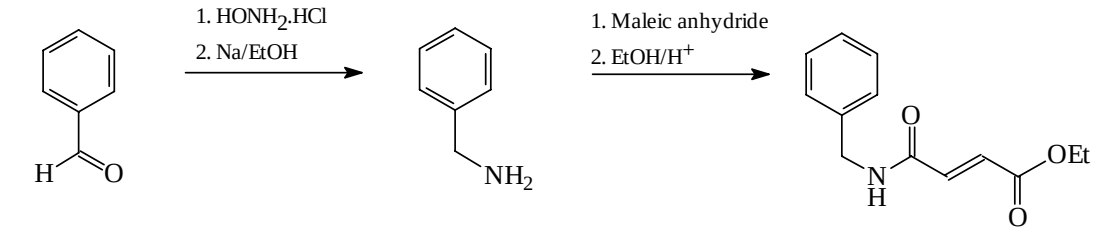
The esterification of *p*-methoxybenzyl maleic acid was first performed with absolute ethanol in the presence of concentrated sulfuric acid, which has been becoming the most common catalyst for esterification reaction. The reaction mixture was heated at reflux for 4 hours to be followed by evaporation of excess solvent and regular extraction to yield a light brown oil. Identification using proton NMR spectrum indicated that the product only gave a singlet of the alkenyl CH=CH protons appearing at 6.2 ppm instead of the expected two singlets. Although the aromatic (multiplet at 6.6-7.3 ppm), methylene (singlet at 4.35 ppm), and methoxy protons (singlet at 3.75 ppm) appear clearly in the spectrum, it also indicates the existence of two types of CH_2CH_3 groups. This last prediction is derived from the presence of two quartets (3.5 and 4.2 ppm) and overlapping triplets at 1.2 ppm. Hence, it is likely the expected esterification is also accompanied by cleavage of the *p*-methoxybenzyl substituent as seen on the case of *o*-hydroxybenzyl maleic acid.

Identification of the above product using GC-MS gave a chromatogram which showed the existence of two major constituents. The mass spectrum of peak 1 (43 %) having retention time of 6.184 minutes revealed a molecular ion peak at *m/z* 143. Based on the fragmentation pattern which gives peaks at *m/z* 99 (base peak) and 127, it is supposed that peak 1 is corresponding to diethyl maleat (M^+ = 172). In this case, the expected molecular ion peak of diethyl maleat is predicted to be not sufficiently stable and rapidly underwent fragmentation loosing CH_2CH_3 group to yield a fragment at *m/z* 143.

With regard to peak 2 (44 %, t_R = 6.192 minutes), this peak gave a mass spectrum which revealed a molecular ion peak at *m/z* 166 and base peak at *m/z* 121. Together with other peaks appearing in the spectrum, it is predicted that peak 2 is corresponding to ethyl *p*-methoxybenzyl ether (*m/z* 166). Thus, on the basis of mass and proton NMR spectra, it was concluded that the two compounds produced in the esterification of *p*-methoxybenzylmaleic acid with ethanol in the presence of concentrated H_2SO_4 were diethyl maleat and *p*-methoxybenzyl ethyl ether. The mechanism of the reaction apparently involved normal esterification of COOH side of *p*-methoxybenzylmaleic acid to be followed by CH_3CH_2OH attack of the benzylic carbon to yield *p*-methoxybenzyl ethyl ether and ethyl maleat monoacid. This last compound then underwent esterification further to form diethyl maleat.

The esterification of *p*-anisylmaleic acid with ethanol was then conducted using benzenesulfonic acid as the catalyst, which is weaker than sulfuric acid. The reaction was carried out at reflux for 3 hours to afford the desired ethyl *p*-anisylfumarate in 81 % yield which is presumably formed via isomerization of ethyl *p*-anisylmaleate. This product was identified by means of proton NMR spectrum which gives two doublets at 6.90 and 7.25 ppm (5 H) of the phenyl protons and two singlets at 6.15 and 6.25 ppm (2 H) of the fumaric CH=CH protons. The existence of CH_2 and OCH_3 protons are respectively indicated by singlets at 4.40 ppm (2 H) and 3.8 ppm (3 H). Likewise, the presence of CH_2CH_3 groups are indicated by quartet at 3.50 ppm (2 H) and triplet at 1.20 ppm (3 H). Therefore, it is clear that the result of the esterification is the desired *p*-anisyl ethyl fumarate.

With regard to N-benzyl ethyl fumaramate, these target molecules were prepared from benzaldehyde in 4 stages i.e. synthesis of benzaldoxime, reduction of benzaldoxime, condensation of benzylamine with maleic anhydride, and esterification of the resulted *N*-benzylmaleic acid with ethanol and methanol (Scheme 2).



benzaldehyde *benzylamine* *N-benzyl ethyl fumaramate*

Scheme 2

Benzaldoxime was prepared in 79 % yield through treatment of benzaldehyde with hydroxylamine hydrochloride in the presence of NaOH according to procedure as described previously (Vogel, 1968). The IR spectrum of the product showed OH absorption band at 3300 cm^{-1} (broad) and C=N stretching frequency at 1608 cm^{-1}. The resulted benzaldoxime was then reduced with metallic Na in refluxing ethanol for 3 hours to afford 68 % yield of benzylamine which was found as a colorless oil. As expected, the proton NMR of the resulted benzylamine displayed 3 signals. Singlet at 7.2 ppm (5 H) is originated from the aromatic protons, while CH_2 protons also appear as a singlet at 3.85 ppm (2 H). The resonance of NH_2 protons occurs as a singlet at 2.1 ppm. The chromatogram recorded on GC-MS for the resulted benzylamine showed that the product is sufficiently pure. The single peak observed (t_R = 6.04 ppm) gave a molecular ion peak at *m/z* 107 in its mass spectrum which fits with the molecular weight of benzylamine.

The resulted benzylamine was then reacted with maleic anhydride in benzene at 60^oC for 2 hours to afford *N*-benzylmaleamic acid in 76 % yield. This acid gave proton NMR spectrum which revealed an intense singlet at 7.3 ppm (5 H) corresponding to the aromatic protons. The maleic CH=CH protons appeared as a pair of singlets at 6.4 (1 H) and 6.5 ppm (1 H). Unusual coupling apparently occurred between CH_2 and NH protons which results to the splitting of the CH_2 signal (doublet at 4.55 ppm, 2 H). However, the resonances of the NH and OH protons only appeared as weak signals between 6.0-7.1 ppm which are slightly overlapping with those of CH=CH and aromatic protons.

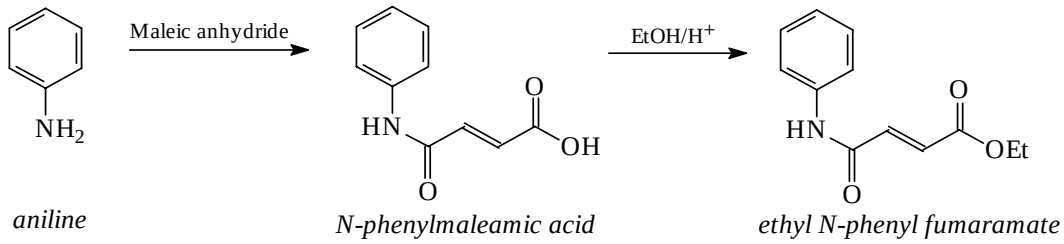
The above acid was esterified in refluxing absolute ethanol in the presence of concentrated sulfuric acid for 3 hours to yield 73 % of the desired *N*-benzyl ethyl fumaramate, which was found as a brownish yellow oil, presumably formed via isomerization at high temperature of *N*-benzyl ethyl maleamate (cis isomer). The proton NMR spectrum of *N*-benzyl ethyl fumaramate showed the existence of 7 signals. An

intense singlet at 7.3 ppm (5 H) is clearly originated from the phenyl protons, while a pair of singlets at 6.8 (1 H) and 6.2 ppm (1 H) are supposed to be originated from the CH=CH maleic protons. The methylene and NH protons are respectively appeared as singlets at 5.2 (2 H) and 4.7 ppm (1 H). The existence of CH_2CH_3 group is indicated by the appearance of a quartet at 4.2 ppm (2 H) and a triplet at 1.2 ppm (3 H) which respectively correspond to the CH_2 and CH_3 protons.

The chromatogram of *N*-benzyl ethyl fumaramate obtained from GC-MS showed that the product consisted of two components. On the basis of mass spectrum, peak 1 (20 %) having retention time of 9.401 minutes gave molecular ion peak at *m/z* 143 which is supposed to be originated from diethyl maleat. On the other hand, peak 2 (t_R = 14.992 minutes, 80 %) which revealed a molecular ion peak at *m/z* 188 is predicted to be originated from the desired *N*-benzyl ethyl fumaramate having theoretical molecular ion of 233. It is envisaged that the molecular ion of *N*-benzyl ethyl maleat is not stable enough and rapidly underwent fragmentation by loosing OEt group to yield a peak at *m/z* 188 (M-45). This GC-MS data also indicated that cleavage of the benzyl substituent of *N*-benzylmaleic acid still occurred even though only yielded 20 % of diethyl maleat. Thus, on the basis of proton NMR and GC-MS data, it was concluded that the major product fro this esterification was the targeted N-benzyl ethyl fumaramate.

In order to obtain C-9154 derivative having smaller size, the esterification of *N*-benzylmaleic acid was also conducted using methanol in the presence of sulfuric acid. The reaction was performed at reflux for 3 hours to afford 72 % of the expected *N*-benzyl methyl maleat. The proton NMR spectrum of the product showed signal of the aromatic protons as a singlet at 7.2 ppm (5 H). The CH=CH maleic protons appear as a pair of singlets at 6.4 and 6.7 ppm (2 H). The NH, CH_2, and OCH_3 protons respectively appear at 6.1 (triplet, 1 H), 4.4 (doublet, 2 H), and 3.8 ppm (singlet, 3 H). Unusual coupling between methylene and NH protons is apparently occurring in this case. The chromatogram recorded from GC-MS showed that the product is relatively pure. The mass spectrum of peak 1 which is the only peak in that chromatogram revealed a molecular ion peak at *m/z* 188 which is supposed to be originated from the expected *N*-benzyl methyl maleat by loosing OCH_3 group (M-31). As described previously, this type of fragmentation was also seen on the case of *N*-benzyl ethyl maleat.

The preparation of C-9154 antibiotic derivatives so far has been conducted towards *p*-anisyl alcohol and benzylamine. In these cases, the resulted C-9154 derivatives possess benzyl moiety whether existing as esters or amides. It was also of interest to prepare C-9154 derivatives from aniline in order to obtain C-9154 molecules having aromatic ring directly attached to the amide group. In this case the strategy consists of 2 reaction steps i.e. condensation of aniline with maleic anhydride and esterification of the resulted *N*-phenylmaleamic acid (Scheme 3).



aniline *N-phenylmaleamic acid* *ethyl N-phenyl fumaramate*

Scheme 3

The condensation of aniline with maleic anhydride was conducted in diethyl ether at room temperature for 2 hour according to the method as described by Vogel (1968) to give 92 % yield of the desired *N*-phenylmaleamic acid. The resulted *N*-phenylmaleamic acid was simply isolated through filtration. This molecule was then treated with absolute ethanol at reflux for 3 hours in the presence of concentrated sulfuric acid to afford the desired ethyl *N*-phenylmaleamate in 76 % yield.

The IR spectrum of the above ethyl *N*-phenylmaleamate revealed NH absorption band at 3307 cm^{-1}, C=O stretching frequency at 1716 cm^{-1}, and C=C stretching frequencies at 1548-1598 cm^{-1}. The proton NMR spectrum showed 5 signals indicating the presence of 5 types of protons. Broad singlet at 10.8 ppm (1 H) is originated from NH proton, whereas multiplet at 7.2-8.0 ppm (5 H) is corresponding to the aromatic protons. The appearance of a pair singlets at 6.3 and 6.4 ppm (2 H) clearly indicated the presence of CH=CH maleic protons. Similarly, the presence of a quartet at 4.5 ppm (2 H) and a triplet at 1.3 ppm (3 H) are strong evidence for the existence of CH_2CH_3 group.

The chromatogram obtained from GC-MS showed that the product consisted of two components. On the basis of mass spectrum, the major component having retention time of 18.867 minutes gave molecular ion peak at *m/z* 219 which is consistent with the

molecular weight of the expected *N*-phenyl ethyl fumaramate. Thus, on the basis of proton NMR and GC-MS data, it was strongly indicated that the outcome of the above reaction was *N*-phenyl ethyl fumaramate.

Antimicrobe activity evaluation

The experiments carried out within this research so far have led to the generation of four examples of C-9154 antibiotic derivatives i.e. *p*-anisyl ethyl fumarate **(6)**, *N*-benzyl ethyl fumaramate **(7)**, *N*-benzyl methyl fumaramate **(8)**, dan ethyl *N*-phenyl fumaramate **(9)**. Antimicrobe activity test towards these compounds was conducted using *Staphyllococcus aureus (SA)* dan *Escherichia coli (EC)* as the representative of Gram positive and Gram negative bacteria. The experiment was carried out using agar media and ethyl acetate as the solvent. The results of measurement of minimum inhibition concentration (MIC) of the above compounds towards the growth of *Staphyllococcus aureus (SA)* and *Escherichia coli (EC)* are presented in Table 1.

Tabel 1 MIC values of some C-9154 antibiotic derivatives

Compound	MIC SA (μg/mL)	MIC EC (μg/mL)
p-Anisyl ethyl fumarate **(6)**	15	15
N-benzyl ethyl fumaramate **(7)**	25	500
N-benzyl methyl fumaramate **(8)**	25	25
Ethyl *N*-phenylfumaramate **(9)**	25	25

As seen in the above table that even though N-benzyl ethyl fumarate **(7)** is sufficiently active towards *Staphyllococcus aureus* (MIC 25 μg/mL), this compound is not sufficiently active towards *Escherichia coli* (MIC 500 μg/mL). In contrast, *N*-benzyl methyl fumaramate **(8)** and ethyl *N*-phenylfumaramate **(9)** are very effective to inhibit the growth of either *Staphyllococcus aureus* (MIC 25 μg/mL) or *Escherichia coli* (MIC 25 μg/mL). A better antimicrobe activity is even found on the case of *p*-anisyl ethyl fumarate **(6)** which gives MIC value of 15 μg/mL either towards *Staphyllococcus aureus* or *Escherichia coli*. This MIC value is not only comparable to that of the authentic sample of C-9154 antibiotic, but is also comparable to the MIC values of some common antibiotics

such as phenicillin, amoxyline, kemicitine, and kloramfenicol (MIC 10-150 μg/mL; Martin, 1982 and Burger, 1960). Therefore, it is important to investigate further the biological activity, toxicity, and pharmaco-kinetic properties of the four synthesized C-9154 in order to provide knowledge for the possibility to use those C-9154 derivatives for commercial purposes.

CONCLUSIONS

p-Anisyl ethyl fumarate **(6)** could be prepared in good yield from anethole through permanganate oxidation, followed by reduction with sodium borohydride, condensation with maleic anhydride, and esterification with ethanol in the presence of benzenesulfonic acid as the catalyst.

N-Benzyl ethyl fumaramate **(7)** was prepared in satisfactorily yield (84 %) from benzaldehyde through conversion to benzaldoxime, reduction with metallic sodium in ethanol, condensation with maleic anhydride, and esterification with ethanol in the presence of concentrated sulfuric acid. When methanol was used for the esterification, the outcome was N-benzyl methyl fumaramate **(8)** in 77 % yield. Application of the last two steps towards aniline afforded N-fenil ethyl fumaramate **(9)** in 84 % yield.

p-Anisyl ethyl fumarate **(6)**, N-benzyl methyl fumaramate **(8)** and N-fenil ethyl fumaramate **(9)** all are sufficiently active to inhibit the growth of *Staphyllococcus aureus* and *Eschericia coli*. Slightly different, N-Benzyl ethyl fumaramate **(7)** is sufficiently active towards *Staphyllococcus aureus*, but is not sufficiently active towards *Eschericia coli*.

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