



Synthesis of stigmasteryl oleate, palmitate and stearate applying ethyl chloroformate as activator

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ABSTRACT

Ethyl chloroformate as activator was investigated for esterification of long chain carboxylic acids (oleic, palmitate, and stearate) with stigmasterol at mild temperatures. Optimal conditions for ethyl chloroformate activity were found under anhydrous and basic conditions at 4°C to room temperature. Esterification products were isolated and purified using preparative radial chromatography and characterized by GC-MS and ¹H-NMR spectroscopy. The GC-MS analysis results showed a fragment of (m/z) = 676.8, 650.5, and 679.8, which are correlated with the molecular weights of stigmasteryl oleate, stigmasteryl palmitate, and stigmasteryl stearate, respectively. Another confirmation was done by ¹H-NMR, showing a change of chemical shift of 3.5 ppm given by the stigmasterol's third carbon atom (C-OH) was deshielded to 4.6 ppm as it turned into (C-OR). GC-MS and ¹H-NMR spectra indicated that stigmasteryl oleate, stigmasteryl palmitate, and stigmasteryl stearate have been successfully synthesized, suggesting that ethyl chloroformate is a worthy alternative activator for stigmasterylester synthesis.

Keywords: ethyl chloroformate, oleic acid, palmitic acid, stearic acid, stigmasterylesters synthesis

INTRODUCTION

Stigmasterols are a part of phytosterols, which are getting attention for its ability to reduce cholesterol levels [1,2,3,4]. Phytosterols could be obtained from fresh vegetables or fruits or from processed foods such as juice, jam, margarine, and yoghurt enriched with phytosterolester [4]. However, due to its low fat solubility, it is necessary to perform chemical modification of this phytosterol [5]. Ester formation with fatty acid is a suitable method to increase fat solubility of phytosterol.

The common problem frequently encounters in an esterification reaction is reversible reaction which resulted in a non-stoichiometric reaction, and hence low yield of product. Many ways can be done to avoid this problem, such as excess addition of reactants and/or continuous separation of product during synthesis process, so that reaction equilibrium is always moving towards the product(s). In general, the esterification reaction occurs in the presence of acidic or basic catalyst and the selection of appropriate catalysts is important to note [6]. Esterification of a carboxylic acid with an alcohol occurs slowly, due to low electrophilic properties of carboxylic acid. Therefore, it is necessary to activate a carboxylic acid prior an esterification reaction. One of the activation method of carboxylic acid is by anhydride formation using ethyl chloroformate. Chloroformates have been used in analytical chemistry for the treatment of amino and hydroxy groups only [7]. To our knowledge, the application of ethyl chloroformate as activator of long chain carboxylic acids prior to esterification with stigmasterol have not been reported yet. In this paper, it will be shown that ethyl chloroformate was applied to activate three fatty acids, i.e. oleic acid, palmitic acid, and stearic acid, prior to esterification with stigmasterol. Since stigmasterylesters of fatty acids are either not commercially available or are very expensive, these esters must be synthesized as standards to characterize the same

compound derived from plants. Esterification products were isolated and purified applying preparative radial chromatography and characterized by IR, GC-MS, and ¹H-NMR spectroscopy.

EXPERIMENTAL SECTION

1. Materials

Stigmasterol, oleic acid, palmitate acid, and stearic acid were purchased from Sigma. All other chemicals were purchased from Merck and were used as received, unless otherwise stated [8].

2. Methods

2.1. Esterification of oleic acid with stigmasterol (Esterification #1)

In a reaction flask cooled in an ice bath at a constant temperature of 4°C, 1 mmol (297 mg) oleic acid was dissolved in 10 mL anhydrous methylenchloride. 1 mmol ethyl chloroformate (127.4 mg) dissolved in 5 mL anhydrous methylenchloride and 2 mmol (209 mg) triethylamine dissolved in 5 ml anhydrous methylenchloride were subsequently added to the solution. The mixture was then stirred for 30 minutes. 1 mmol (435.8 mg) of stigmasterol was dissolved in 15 mL of anhydrous methylenchloride. The solution was then added into the solution of activated oleic acid at room temperature and stirred for 3 hours [9,10,11]. All experiments were carried out in duplicates. The esterification reaction was monitored by thin layer chromatography (TLC).

2.2. Esterification of palmitic acid with stigmasterol (Esterification #2)

In a reaction flask cooled in an ice bath at a constant temperature of 4°C, 2 mmol (585.9 mg) palmitic acid was dissolved in 20 mL anhydrous methylenchloride. 2 mmol (247.8 mg) ethyl chloroformate dissolved in 10 mL anhydrous methylenchloride and 2 mmol (230.7 mg) triethylamine dissolved in 10 ml anhydrous methylenchloride were subsequently added to the solution. The mixture was then stirred for 30 minutes. 1 mmol (435.8 mg) of stigmasterol was dissolved in 15 mL of anhydrous methylenchloride. The solution was then added into the solution of activated oleic acid at room temperature and stirred for 3 hours [9,10,11]. All experiments were carried out in duplicates. The esterification reaction was monitored by TLC.

2.3. Esterification of stearic acid with stigmasterol (Esterification #3)

In a reaction flask cooled in an ice bath at a constant temperature of 4°C, 2 mmol (649.8 mg) stearic acid was dissolved in 20 mL anhydrous methylenchloride. 2 mmol (247.8 mg) ethyl chloroformate dissolved in 10 mL anhydrous methylenchloride and 2 mmol (230.7 mg) triethylamine dissolved in 10 ml anhydrous methylenchloride were subsequently added to the solution. The mixture was then stirred for 30 minutes. 1 mmol (435.8 mg) of stigmasterol was dissolved in 15 mL of anhydrous methylenchloride. The solution was then added into the solution of activated oleic acid at room temperature and stirred for 3 hours [9,10,11]. All experiments were carried out in duplicates. The esterification reaction was monitored by TLC.

3. Identification

3.1. Thin layer chromatography (TLC)

A small quantity of sample was spotted on TLC plate using aluminium precoated with silica gel GF₂₅₄. Then the TLC plate was developed using a mixture of ethylacetate / n-hexane (1:9,v/v). After being developed, the plate was observed under UV light (254 nm), sprayed with a 10% H₂SO₄ solution in methanol, and finally heated at 120°C for 3minutes.

4. Product isolation

The reaction mixture was evaporated, yielding powder containing esterification product. 300 mg powder was elucidated using a mixture of ethylacetate : n-hexane (0.25:9) as developing solvent and 1-mm-thick precoated silica gel GF₂₅₄ plate as stationary phase with 2 mL/min flow rate by radial chromatography, followed by solvent evaporation. Stigmasteryl ester yields were then determined by comparing obtained yields to their theoretical values. The isolates were characterized by infrared, GC-MS, and ¹H-NMR spectroscopy.

5. Product Characterization

5.1. Infrared (IR) spectroscopy

The IR spectrum was recorded on a Jasco 4200 fourier-transformed infrared spectrometer (FTIR). Prior to measurement, the sample was crushed along with dry KBr in a mortar and then compressed into a transparent disc using a hydraulic press.

5.2. Gas chromatography mass spectroscopy (GC-MS)

Mass spectrum was recorded at high resolution on a Varian 320MS GC instrument equipped with an Rtx-5MS column and helium gas as the mobile phase. Anhydrous methylene chloride was used as solvent. Preparation of

sample solution was done by ultra-sonic treatment. The operational conditions for the instrument were as follows: mobile phase flow rate of 1 mL/min, column temperature of 100°C for 2 minutes that was risen up to 320 °C at the rising speed of 10 °C/minute and kept constant at 320°C for 25 minutes. Ionization mode was electron ionization (IE) with electron energy of 70eV [13]. The volume of sample loaded was 1 µL. The data were given in m/z values.

5.3. Proton nuclear magnetic resonance (¹H-NMR) spectroscopy

¹H-NMR spectra were recorded on a JEOL, ECA 500, 500 MHz MR spectrometer. CDCl₃ was used as solvent and tetramethylsilane (TMS) was used as internal standard (7.26 ppm for ¹H).

RESULTS AND DISCUSSION

In performing esterification reaction of oleic, palmitic, and stearic as long chain carboxylic acids with stigmasterol, there are three important factors to be noted. First, ethyl chloroformate plays an important role as activating agent. In this case, it will increase the electrophilic properties of carbonyl C atom of these carboxylic acids by means of anhydrides formation of these acids. Therefore, the carbonyl C atom can now undergo electrophilic attack on a nucleophile, in this case the hydroxyl group of stigmasterol. Second, triethylamine which is as an *auxiliary base* will help to deprotonate the hydroxyl group and hence increase its nucleophilic properties which in turn also facilitate the esterification reaction. The protonated triethylamine will then form an ion pair with chloride anion released from ethylchloroformate. This ion pair is water soluble and hence can be easily extracted from organic phase with water. Third, the reaction should be carried out under anhydrous condition. The presence of water will inhibit the formation of ester, in this case water, that also has nucleophilic properties and hence will compete with the hydroxyl group of stigmasterol. The reaction of water with the anhydride will facilitate the formation of free carboxylic acid instead of ester as the product. Fig.1. illustrates the esterification reaction with ethyl chloroformate as activator.

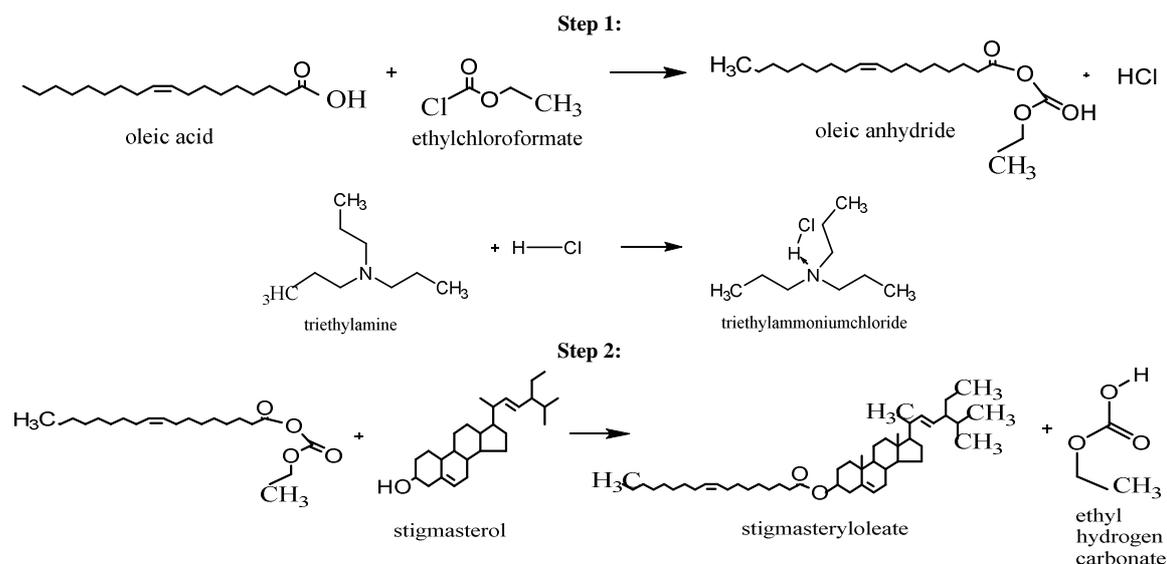


Fig. 1. Formation of stigmasteryloleate

TLC profile showed that esterification #1, #2, and #3 products with stigmasterol yielded a new product having higher R_f values (0.8, 0.9, and 0.86, respectively) compared to stigmasterol itself (0.16) (Fig. 2). Esterification #1 product is less polar than stigmasterol, as expected, since the esterification of stigmasterol using oleic acid was postulated as a possible way to increase stigmasterol's solubility in lipid.

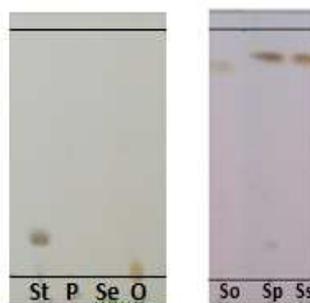


Fig. 2. TLC profile of St: stigmasterol; P: palmitic acid; Se: stearic acid; O: oleic acid, So: purified esterification #1 product; Sp: purified esterification #2 product; Ss: purified esterification #3 product

Following solvent evaporation, 900, 700, and 600 mg of each esterification #1, #2, and #3 products were obtained as white and rather sticky powder. After preparative radial chromatography purification, quantity of esterification #1, #2, and #3 products were 30mg, 35mg, and 20 mg which were equivalent to the yields of 4.2%, 21.5%, and 11.8% of theoretical values, respectively. The yield of esterification products were relatively low, might be caused by the structure of carboxylic acids and stigmasterol. The structure of carboxylic acid that has a long chain carbon ($C > 12$) and the structure of stigmasterol that has two double bonds create a larger, more bulky hydrophobic group giving steric hindrance, which also may affect solubility properties. In addition, the alkyl chain, due to its double bond, was more rigid. Consequently, free rotation of alkyl chain was inhibited [14]. Thus the rate of ester formation will decline and the yield thereof was reduced. Further characterizations of the products were done using IR, GC-MS, and $^1\text{H-NMR}$ spectroscopy. IR, GC-MS, and $^1\text{H-NMR}$ spectra data of the free carboxylic acids as starting materials were compared to those of esterification products. The results were summarized in Tab. 1, 2, and 3.

Tab. 1. Spectroscopic data of esterification #1

Spectroscopic techniques	Data		
	Oleic acid	Stigmasterol	Esterification #1 product
IR (cm^{-1})	2927.41 (OH stretch), 1712.48 (C=O stretch)	3756.65 (-OH stretch), 3343.96 (-CH=CH-stretch), 1666.2 (C=C), 1457.92 (CH_2), 1056.8 (cycloalkane moieties)	1743.33 (C=O stretch), 1083.8 (oleic C[=O]-O stretch)
GC-MS (m/z)	29.0, 54.9, 69.0, 83.0, 222.2, 264.3, 282.3	55.0, 159.0, 173.1, 255.2, 351.4, 412.4	54.9, 255.2, 394.4, 676.8, 677.7, 679.2
$^1\text{H-NMR}$ (CDCl_3)	δ 2.327 (t, 2H, H-2), 5.338 (t, 1H, H-9), 5.335 (t, 1H, H-10), 0.873 (t, 3H, H-18)	δ 3.5175 (m, 1H, H-3), 5.346 (m, 1H, H-6), 1.008 (s, 3H, H-28), 0.696 (s, 3H, H-29), 5.1505 (m, 1H, H-20), 5.027 (m, 1H, H-21), 0.809 (d, 3H, H-26), 0.843 (d, 3H, H-27)	Parts of stigmasterol: δ 4.609 (m, 1H, H-3), 5.369 (m, 1H, H-6), 0.695 (s, 3H, H-28), 1.020 (s, 3H, H-29), 5.153 (t, 1H, H-20), 5.014 (s, 3H, H-21), 0.8095 (d, 3H, H-26); Parts of oleic acid: δ 2.263 (t, 2H, H-3)

Tab. 2. Spectroscopic data of esterification #2

Spectroscopic techniques	Data	
	Palmitic acid	Esterification #2 product
IR (cm^{-1})	2919.7-2850.27 (OH stretch), 1700.91 (C=O stretch)	1739.48 (C=O stretch), 1172.51 (palmitic C[=O]-O stretch)
GC-MS (m/z)	42.9, 59.9, 129.0, 157.0, 213.2, 256.2	27.9, 54.9, 393.7, 394.7, 650.6, 651.6
$^1\text{H-NMR}$ (CDCl_3)	δ 2.344 (t, 2H, H-2), 0.879 (t, 3H, H-16)	Parts of stigmasterol: δ 4.611 (m, 1H, H-3), 5.3695 (m, 1H, H-6), 0.697 (s, 3H, H-29), 1.022 (s, 3H, H-28), 5.155 (t, 1H, H-20), 5.017 (s, 3H, H-21), 0.697 (d, 3H, H-26) Parts of palmitic acid: δ 2.263 (t, 2H, H-3), 0.840 (t, 2H, H-3)

Tab. 3. Spectroscopic data of esterification #3

Spectroscopic techniques	Data	
	Stearic acid	Esterification #3 product
IR (cm^{-1})	2919.7-2854.13 (OH stretch), 1704.76 (C=O stretch)	1727.91 (C=O stretch), 1122.37 (stearic C[=O]-O stretch)
GC-MS (m/z)	29.0, 73.0, 129.0, 185.1, 241.2, 284.3	59.9, 129.0, 395.1, 679.8, 680.8, 682.5
$^1\text{H-NMR}$ (CDCl_3)	δ 2.345 (t, 2H, H-2), 0.880 (t, 3H, H-18)	Parts of stigmasterol: δ 4.610 (m, 1H, H-3), 5.3685 (m, 1H, H-6), 0.696 (s, 3H, H-29), 5.154 (t, 1H, H-20), 5.016 (s, 3H, H-21), 1.021 (t, 3H, H-24), Parts of stearic acid: δ 0.880 (t, 3H, H-18)

It is clear that the GC-MS analysis results of esterification #1, #2, and #3 products show fragments of (m/z) = 676.8, 650.5 and 679.8, which are correlated with the molecular weights of stigmasteryl oleate (Fig.3), stigmasteryl palmitate (Fig.4), and stigmasteryl stearate (Fig.5) respectively.

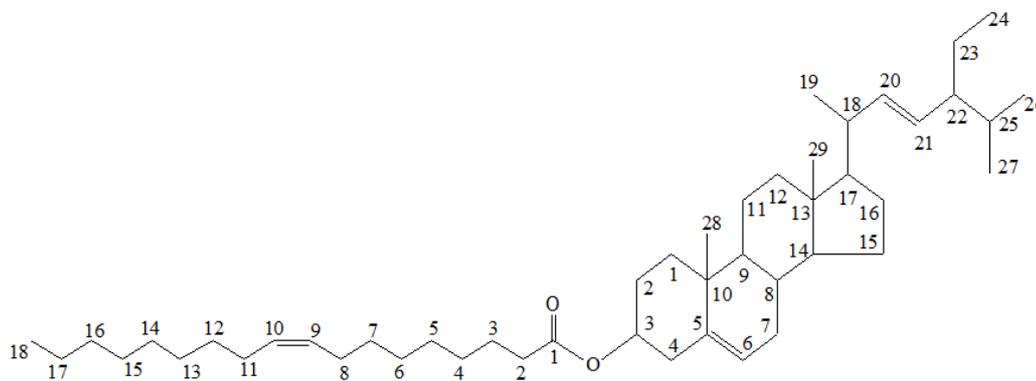


Fig.3. Chemical structure of stigmasteryl oleate

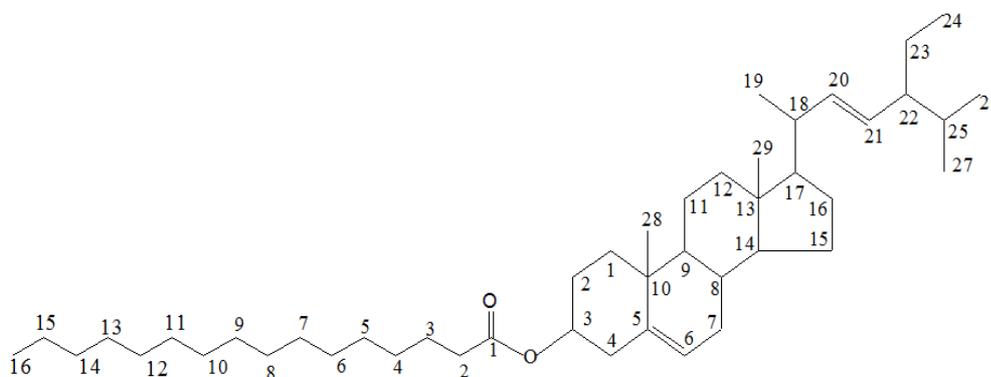


Fig.4. Chemical structure of stigmasteryl palmitate

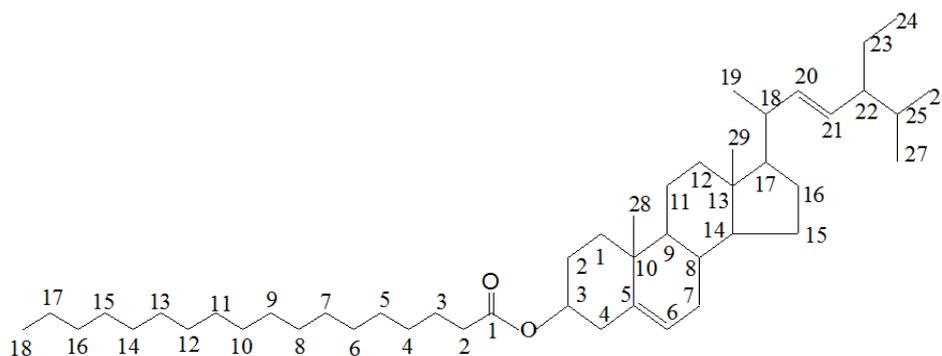
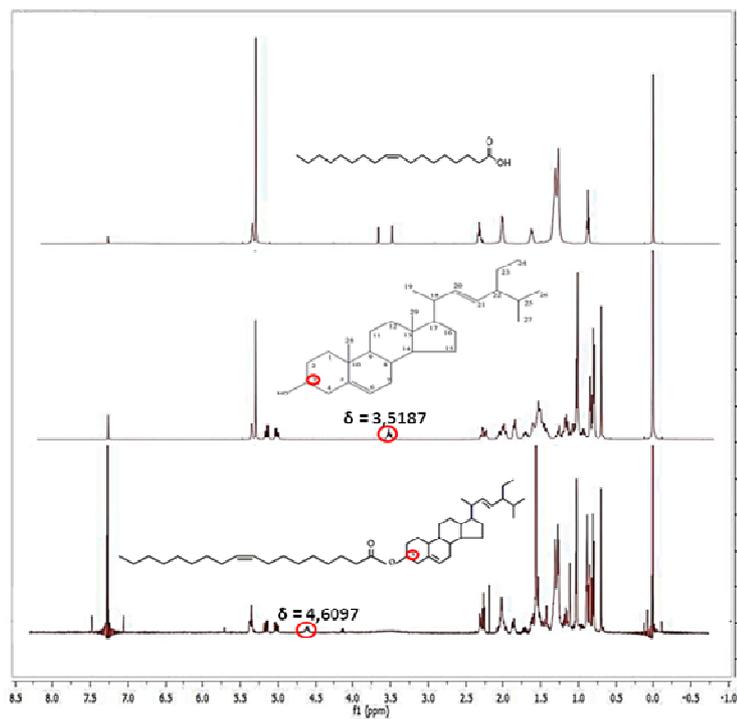
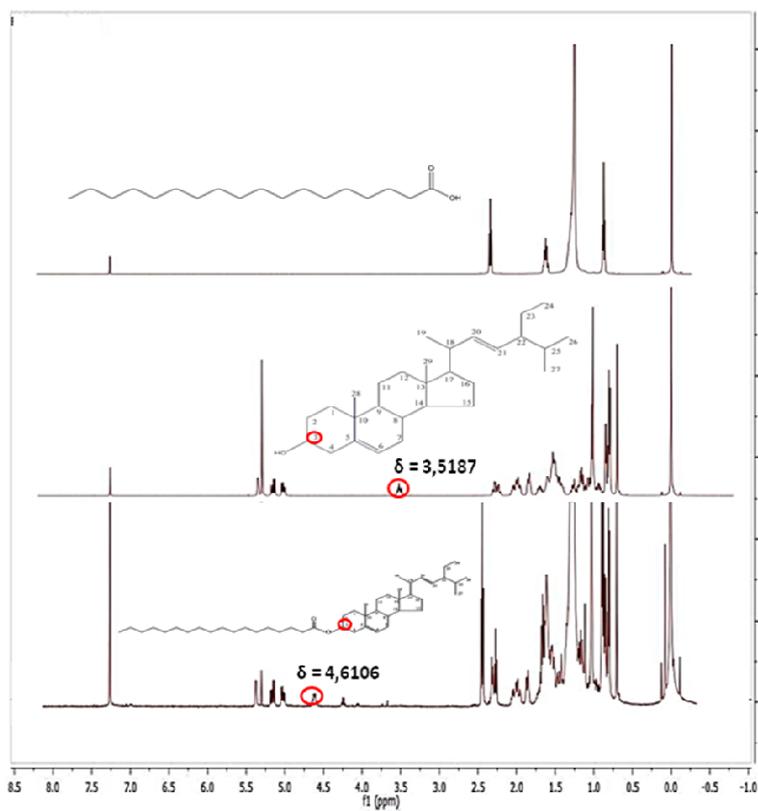


Fig.5. Chemical structure of stigmasteryl stearate

Another confirmation was done by $^1\text{H-NMR}$, showing a change of chemical shift (δ) caused by the alteration of chemical environment as the third carbon atom of stigmasterol (C-OH) was esterified. δ of 3.5187 ppm given by (C-OH) was deshielded to 4.6097 ppm, 4.6116 ppm, and 4.6106 ppm in esterification #1, #2, and #3 products, respectively, as it turned into (C-OR), because the carbonyl of carboxylic acids were an electron withdrawing group that would withdraw the electron surrounding it. The $^1\text{H-NMR}$ spectra hence supported the proof of ester group formation of oleic, palmitic, and stearic acids with stigmasterol. Fig. 6, 7 and 8 display $^1\text{H-NMR}$ spectrums of the stigmasterylesters each.

Fig. 6. ¹H-NMR spectrums of stigmasteryl oleateFig. 7. ¹H-NMR spectrums of stigmasteryl palmitate

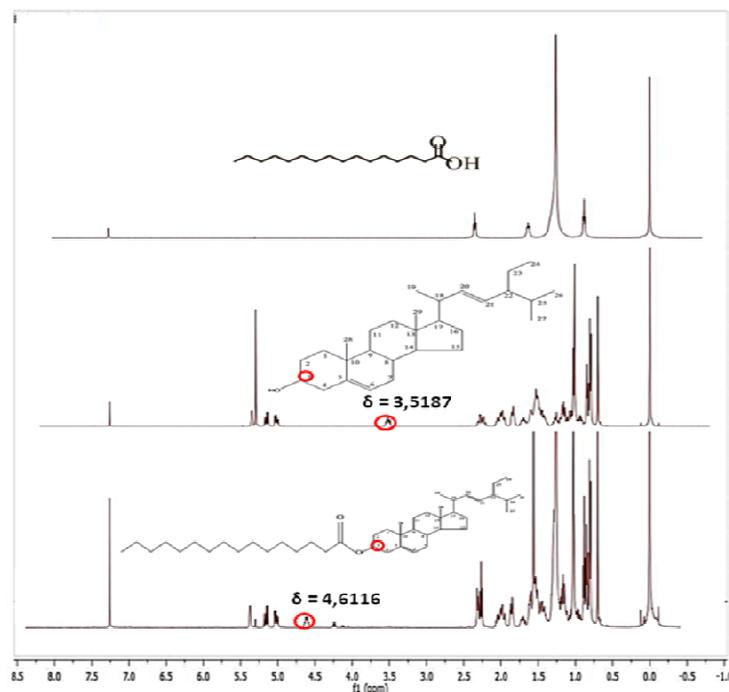


Fig. 8. ¹H-NMR spectra of stigmasteryl stearate

The melting points of these compounds were 40.8 – 42.0°C, 41.0 – 42.0°C and 56.3 - 56.6°C, respectively.

CONCLUSION

We have shown that ethyl chloroformate could activate oleic acid, palmitic acid, and stearic acid prior to esterification with stigmasterol. For future production of stigmasterylestere from long chain carboxylic acid with stigmasterol, both economical and environmental concerns should be taken into account. Thus, further studies on yield improvement through optimization condition of esterification reaction is worthy to be undertaken.

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