Microwave-Assisted Claisen-Schmidt Condensation Reaction of Ethyl *p*-methoxycinnamate to Synthesize *p*-Methoxystyryl Ketone Derivatives and Evaluate Anti-inflammatory Activity of Synthetic Products

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Abstract. The rhizome of *Kaempferia galanga* has been known to contain ethyl *p*-methoxycinnamate (EPMC) (**1**) as a main component, which has anti-inflammatory properties. Previously we have reported the structural modification of EPMC (**1**) to form another ester, nitrostyrene, and cinnammamide derivatives and then studied their structure-activity relationships as anti-inflammatory agent. In continuing our research, in this paper, we report the microwave-assisted Claisen-Schmidt condensation of EPMC (**1**) to synthesize a series of *p*-methoxystyryl ketones followed by a study of their anti-inflammatory activity. The reaction begins with microwave-assisted cleavage oxidation of hydrolyzed product of EPMC, *p*-methoxycinnamic acid (**2**) with $Ca(NO_3)_2$ for the synthesis of *p*-methoxybenzaldehyde (**3**). Furthermore, **3** was reacted with acetophenone, ethyl methyl ketone and acetone via microwave-assisted Claisen-Schmidt condensation for the synthesis of (*E*)-1-(4-methoxyphenyl)-3-phenylprop-2-en-1-one (**5a**) (*E*)-1-(4-methoxyphenyl)pent-1-en-3-one (**5b**) and (*E*)-4-(4-methoxyphenyl)but-3-en-2-one (**5c**), respectively. The reaction products were characterized using spectroscopic techniques and were then tested for anti-inflammatory activity using *in vitro* anti-denaturation of protein assay. It was found that converting EPMC (**1**) to **5a** and **5b** reduced anti-inflammatory activity, while **5c** retained anti-denaturation activity with an IC_{50} of 72.8 µg/ml.

Keywords: *Claisen-Schmidt condensation, (E)-1-(4-methoxyphenyl)-3-phenylprop-2-en-1-one, (*E*)-1-(4-methoxyphenyl)pent-1-en-3-one, (*E*)-4-(4-methoxyphenyl)but-3-en-2-one*

1 Introduction

Ethyl *p*-methoxycinnamate is a major volatile component of the rhizome of *Kaempferia galanga* and has been shown to possess anti-inflammatory

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properties [1,2]. This compound has also been reported to have other pharmacological effects, such as antimicrobial, anti-cancer, larvicidal, anti-tuberculosis, nematicidal, mosquito repellent, angiogenesis inhibition, anticarcinogenic, and anti-inflammatory activity [2]. Previously, we have reported the conversion of EPMC (**1)** to another ester, cinnamamide, and nitrostyrene derivatives followed by a study of the structure-activity relationship of these compounds to their anti-inflammatory activity [1,3,4]. It has been proposed that ethyl ester is part of EPMC (**1**), which causes anti-inflammatory activity, and that decreasing and increasing the number of carbon atoms in the ester group results in decreased activity [3]. The conversion of the structure to nitrostyrenes did not give a significantly different effect, while conversion to hydroxyl cinnnamamide derivatives tended to increase the anti-inflammatory activity [1]. The fact that the ester part of EPMC (**1**) is a functional group that plays an important role in its pharmacological action leads to a concern about the stability of this compound. It has been noticed that some ester drugs are unstable in blood, plasma and serum samples due to their ability to be hydrolyzed both *in vitro* and *in vivo*. The instability of drugs can cause a substantial underestimation of the actual drug concentration [5-7].

Curcumin is a promising natural pharmaceutically active compound that is extracted mainly from *Curcuma longa* L. [8,9]. Many curcumin activities are related to their ability to suppress acute and chronic inflammation [10]. Half of the curcumin structure is a styryl ketone moiety, a skeleton that possesses an aromatic and α,β-unsaturated ketone group. The styryl ketone derivatives themselves have been found to have various pharmacological effects, such as neuroprotective [11], antitumor [12], anti-inflammatory [13], antioxidant [14] and cytotoxic [15] activity. The conjugated system of the styryl ketones is expected to be one of the parts of the structure that is responsible for its biological activities [16]. Another part of styryl ketone compounds that also contributes to its biological activity is the halogenated phenyl part [13]. Various methods of synthesizing styryl ketones have been described in the literature, where Claisen-Schmidt condensation is one of the simplest methods to synthesize this compound [16].

As part of our research, which focuses on the study of the structure-activity relationship of EPMC as anti-inflammatory agent, in the current study we attempted to convert EPMC (**1**) to *p*-methoxystyryl ketone derivatives. The conversion was carried out through microwave-assisted Claisen-Schmidt condensation. The structure of the synthetic compounds was characterized by using spectroscopic data and comparison with previously reported data. *In vitro* anti-denaturation assay was used to further test the pharmacological activity of the synthetic products.

2 Material and Methods

2.1 Materials and Instrumentation

EPMC (**1**) was obtained from the extraction and purification of the rhizome of *K. galanga* [1]. The following chemicals used were purchased from Merck: acetone, acetophenone, ethyl acetate, ethyl methyl ketone, HCl, glacial acetic acid, H_2SO_4, NaCl, NaOH, Na_2SO_4, silica gel, TLC plate 60 F250. Bovine serum albumin and Na diclofenac were purchased from Sigma Aldrich. The melting point was measured using a Stuart SMP10 apparatus (without correction). The synthetic product was analyzed using an Agilent Technology GC/MS-MSD 7890A/5975C system [1,3]. The ^{1}H-NMR was measured on a Jeol-500 MHz instrument. An unmodified oven microwave (Samsung) was used for the synthesis reaction.

2.2 Hydrolysis of 1

EPMC (**1**) was hydrolyzed as described previously to give **2** as colorless crystals [1], m.p. 175 °C (lit. 169 °C).

2.3 Conversion of 2 to 3

2.0 g *p*-Methoxycinnamic acid (**2**) and 5.0 g $Ca(NO_3)_2$ (Merck) were dissolved in 5 mL acetic acid glacial and irradiated for 2 minutes using an microwave oven at 300 W. Cold distilled water was added to the reaction product, which was then extracted with *n*-hexane to yield 0.95 g of yellow oil of (**3**) (45.7% yield) [18]. The ^{1}H-NMR data was in agreement with previously reported data [19].

2.4 Claisen-Schmith Condensation

2.4.1 Synthesis of 5a

In an Erlenmeyer flask, 0.22 mg of *p*-methoxybenzaldehyde **(3**) (1.6 mmol), 5 mL of NaOH, and 195.8 µl of acetophenone (1.6 mmol) were mixed and irradiated for 7 minutes in a microwave oven at 600 W. During the reaction, the solution was taken from the oven every 30 minutes and put in an ice bath. When the color of the solution turned orange, the reaction was stopped, followed by extraction of the product with ethyl acetate and purification with chromatography to obtain 138 mg of **5a** (62.2% yield), m.p. 71-72 °C, $C_{16}H_{14}O_2$, GCMS: 238[M]$^+$, 207, 161, 133, 108, 77 and 51. The ^{1}H-NMR spectra followed those previously reported and are tabulated in Table 2 [20].

2.4.2 Synthesis of 5b

In an Erlenmeyer flask, 214 mg of *p*-methoxybenzaldehyde (**3**) (1.6 mmol) and 8 ml of NaOH 5% were mixed, then 140 µl of ethyl methyl ketone (**4b**) (1.6 mmol) was added under continuous irradiation using a microwave oven at 600 W for 3 minutes. During the reaction, the mixture was removed from the oven every 30 seconds, followed by cooling in an ice bath. After the reaction was completed, the solution was poured into ice-cold water, neutralized with cold HCl and then extracted with ethyl acetate. Further purification of ethyl acetate extract using the chromatographic method gave 28 mg of **5b** (13.5% yield), m.p. 49-51°C, $C_{12}H_{14}O_2$, GCMS: 190 $[M]^+$, 161 (base peak), 145, 133, 118, 103, 89, 76, 63. The ^{1}H-NMR data is tabulated in Table 2.

2.4.3 Synthesis of 5c

In a test tube with cap, 60 mg of *p*-methoxybenzaldehyde (**2**) (0.44 mmol), 1.1 mL of NaOH 10% (0.44 mol), and 33.3 µL of acetone (**4a**) were combined and placed in a microwave oven and then irradiated for 20 minutes at 600 W. During irradiation, the mixture was removed from the oven every 10 seconds followed by cooling in an ice bath. After the reaction was completed, the product was poured into ice-cold water, neutralized with cold HCl and extracted with a mixture of ethyl acetate and *n*-hexane. Purification of ethyl acetate extract gave 40.4 mg of **5c** (67.3% yield), m.p. 73-74 °C, $C_{11}H_{12}O_2$, GCMS: 176 $[M]^+$, 161 (base peak), 145, 118, 103, 89, 77, 63, 44. The ^{1}H-NMR spectra were consistent with those previously reported and are tabulated in Table 2 [21].

2.5 Anti-denaturation Assay

Samples in concentrations of 0.1, 1, 10, 100 ppm were prepared and evaluated for anti-denaturation by using heated bovine serum albumin assay following our previous report [1,3,22]. Na diclofenac was used as standard.

3 Results and Discussion

Claisen-Schmidt condensation is a reaction that occurs when an aromatic carbonyl compound that does not have α-hydrogen reacts with aldehydes or ketones that have α-hydrogen. α,β-Unsaturated ketones such as chalcones, flavanones, and styryl ketones are typically synthesized using this reaction [16,23]. Recently, effective Claisen-Schmidt condensation reactions have been conducted through a microwave-assisted reaction between ketones and aromatic substituted aldehyde to synthesize α,β-unsaturated ketone compounds [16,24].

In this study, the conversion of EPMC (**1**) to α,β-unsaturated ketone, *p*-methoxystyryl ketones was carried out in two stages. The reaction begins with

the conversion of EPMC (**1**) to *p*-methoxybenzaldehyde (**3**), followed by microwave-assisted Claisen-Schmidt condensation of **3** to *p*-methoxystyrylketones. At first, the conversion of EPMC (**1**) to *p*-methoxybenzaldehyde (**3**) was considered an undesirable product of the cold nitration reaction that was developed from the previous work of Bose *et al.* [25]. Instead of an electrophilic substitution reaction, the reaction between EPMC (**1**) and $Ca(NO_3)_2$ causes cleavage oxidation of the vinyl of EPMC (**1**) to give *p*-methoxybenzaldehyde (**3**). This reaction produces a mixture that needs to be further purified using a chromatographic method. As an alternative way of producing the reaction product as the major product, the hydrolyzed product of EPMC (**1**), *p*-methoxycinnamic acid (**2**) was reacted with $Ca(NO_3)_2$. This reaction succeeded in producing the major product of *p*-methoxybenzaldehyde (**3**) with a yield of 45.7% without using chromatography. The spectroscopic data of **3** followed previously reported data [19].

In adopting and modifying the previous work of Shakil *et al.* [24], the microwave-assisted Claisen-Schmidt condensation of EPMC (**1**) was started by conducting a reaction between **3** and acetophenone (**4a**) in a test tube with a cap. Variations of microwave-oven power and reaction time were used. The solution was removed from the oven and cooled in an ice bath every 30 seconds during the reaction. When the solution's color turned orange, the reaction was complete, and the solution was cooled in cold water before being neutralized with cold HCl. The reaction product was then extracted with an organic solvent and monitored using TLC. Reactions at 300 and 450 W for each 10, 15, 25 minutes did not give the targeted product. The synthesis product was formed as the major product when the mixture was irradiated at 600 W for 25 minutes, as seen on the TLC plate. Furthermore, we tried to use this condition to irradiate the mixture that was placed in an Erlenmeyer flask and irradiated at 600 W. Monitoring of the TLC showed a single spot of **5a** within 7 minutes of irradiation. Further purification of the product reaction obtained **5a** at a yield of 62.2%. The optimization of the Claisen-Schmidt condensation reaction is given in Table 1. The spectroscopic data of **5a** was in agreement with previously reported data [20].

Other ketones were optimized by putting **3** and **4b** in an Erlenmeyer flask and irradiating at 600 W for 3 and 5 minutes. The mixture was removed from the microwave oven and cooled in an ice bath every 30 seconds during the 3 minutes reaction to produce **5b** at a yield of 13.3%. Extension of the reaction time to 5 minutes gave a mixture of the reaction product. To the best of our knowledge no spectroscopic data of this compound have been previously reported. According to the MS data of GCMS, the molecular weight of **5b** is 190, which corresponds to the molecular formula of $C_{12}H_{14}O_2$. A prominent peak appears at m/z 161, indicating the loss of C_2H_5 from the structure, where a

peak at m/z 161 usually suggests the presence of *p*-methoxylcinnamic moiety in the structure [26]. The ^{1}H-NMR spectrum exhibited the presence of four integrated protons at δ 6.91 (2H, *d*, J = 9 Hz, 2CH-Ar), 7.50 (2H, d, J = 9, 2CH-Ar), two trans-coupled peaks at 6.63 (1H, *d*, J = 16 Hz, =CH) and 7.52 (1H, d, J = 16 Hz, =CH), three integrated protons at δ 3.86 (3H, s, OCH_3), indicating the presence of *p*-methoxy cinnamic acid moiety [26]. The peaks at 1.16 (3H, t, CH_3) and 2.66 (2H. d, CH_2) suggest the presence of C_2H_5. The H-NMR data of **5b** is tabulated in Table 2. Hence, it is suggested that this compound is an (*E*)-1-(4-methoxyphenyl)pent-1-en-3-one.

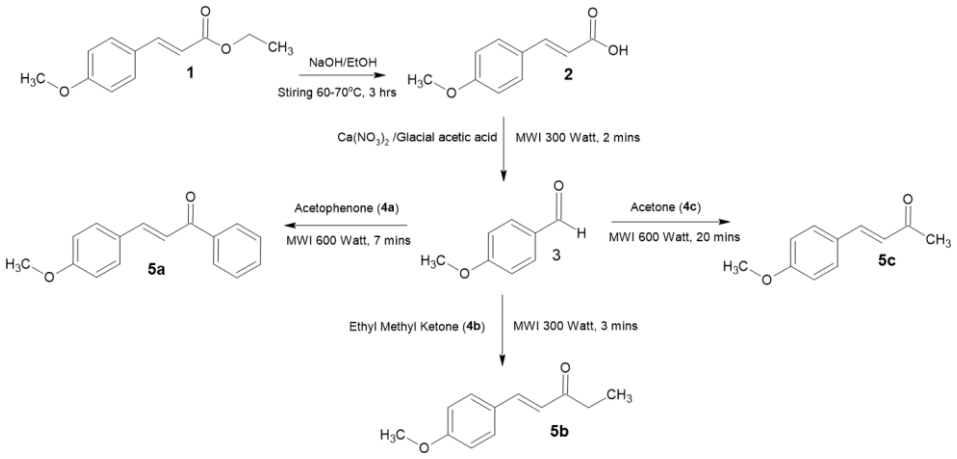
Table 1 Optimization of Claisen-Schmidt condensation for synthesis of *p*-methoxystyryl ketone derivatives.

Reagents	Molar Ratio (3:4)	Power (W)	Time (minutes)	Product (yield%)
Acetophenone (**4a**)	1:1	300 (In test tube)	10	-
			15	-
			25	-
		450 (In test tube)	10	-
			15	-
			25	**5a** as minor
		600 (in a test tube)	10	-
			15	-
			25	**5a** as major
Ethyl methyl ketone (**4b**)	1:1	600 (in Erlenmeyer)	7	**5a** (62.2%)
		600 W (in Erlenmeyer)	3	**5b** (13.3%)
			5	-
Acetone (**4c**)	1:1	300 (in a test tube)	5	-
			10	-
			20	-
		450 (in a test tube)	5	-
			10	-
			20	-
		600 (In test tube)	5	-
			10	-
			20	**5c** (67.3%)

(-): The product reaction was not formed.

In optimization reaction of **3** and **4c,** the mixture of **3** and **4c** was placed in a test tube with the cap and irradiated at 300, 450, and 600 W in 5, 10, 20 minutes. TLC analysis of the reaction results revealed that when the mixture was irradiated at 600 W for 20 minutes, the formation of **5c** (67.3% yield) occurred. During the reaction, the mixture was removed from the microwave every 10 seconds and cooled in the ice bath. The spectroscopic data of **5c** was consistent

with the previously reported data [21]. The scheme of conversion of EPMC (**1**) to a series of *p*-methoxystyryl ketones is shown in Figure 1.



Note: MWI: Microwave irradiation

Figure 1 Scheme of synthesis of styryl ketones.

Table 2 ^{1}H-NMR data of **5a**, **5b**, and **5c** in $CDCl_3$.

5a **δ (ppm), *J* (Hz)**	**5b** **δ (ppm), *J* (Hz)**	**5c** **δ (ppm), *J* (Hz)**
3.85 (3H, s; OCH_3)	1.16 (3H, t; CH_3)	2.36 (3H, s; CH3)
6.94 (2H, d, *J* = 9; 2CH-Ar)	2.66 (2H. d; CH_2)	3,85 (3H ,s; OCH3)
7.42 (1H, d, *J* = 16; =CH)	3.86 (3H, s; OCH_3),	6,61 (1H, *d*, *J* = 16; =CH)
7.50 (2H, t, *J* = 7*;* 2CH-Ar)	6.63 (1H, d, *J* = 16; =CH)	6,92 (2H, *d*, *J* = 9; 2CH-Ar)
7.56 (1H, tt, J_1 = 7, J_2 = 2; CH-Ar)	6.91 (2H, d, *J* = 9; 2CH-Ar)	7,48 (1H, d, *J* = 16, =CH)
7.60 (2H, d, *J* = 9; 2CH-Ar)	7.50 (2H, d, *J* = 9; 2CH-Ar)	7.50 (2H, d, *J* = 9, 2CH-Ar)
7.78 (1H, d, *J* = 16; =CH)	7.52 (1H, d, *J* = 16; =CH).	
8.0 (2H, d, *J* = 7; 2CH-Ar)		

Protein anti-denaturation assay was used to assess the anti-inflammatory activity of the synthetic products. Protein denaturation has been shown to increase the production of advanced glycation end products (AGEs), which are chemical mediators of inflammation, especially in rheumatoid arthritis. Therefore, compounds that have protein anti-denaturation activity are considered to have anti-inflammatory properties [27-29]. The result of the bioassay indicated that **5c** showed anti-denaturation activity with an IC_{50} value of 72.48 µg/ml. Meanwhile, **5a** and **5b** were considered not to have anti-inflammatory properties (Table 3). This study suggests that **5c** still had anti-inflammatory properties and that along with the increase of lipophilicity, the activity gradually decreased. This result is in line with our previous research, which reported that the increasing lipophilicity of the ester of EPMC (**1**) caused

a decrease in anti-denaturation activity [3]. On the other hand, the conversion of EPMC (**1**) to hydroxyl cinnammamide derivative, which is more hydrophilic than EPMC (**1**), caused increased anti-denaturation activity [1]. EPMC (**1**) showed anti-denaturation activity with an IC_{50} value of 53.3 µg/ml. Structurally, drugs that contain a ketone functional group are known to be more stable to hydrolysis than those containing an ester group [7]. Therefore, conversion of the ester EPMC (**1**) to *p*-methoxystyryl ketones provides more stable derivatives.

Table 3 Result of anti-denaturation assay.

Compound	IC_{50} Values (µg/ml)
1	53.3±4.3
3	na*
5a	na*
5b	>100
5c	72.8±4,7
Na Diclofenac	5.04±1.1

*Did not inhibit heat BSA denaturation; IC_{50} values are expressed as mean ± SD (n = 3)

4 Conclusion

Microwave-assisted conversion of EPMC (**1**) to several *p*-methoxystyryl ketones was successfully conducted in 2 steps. First, EPMC (**1**) was converted to *p*-methoxybenzaldehyde (**3**), followed by the reaction of compound **3** with various ketones. The reaction of **3** with acetophenone (**4a**), ethyl methyl ketone (**4b**) and acetone (**4c**) succeeded in producing of (*E*)-1-(4-methoxyphenyl)-3-phenylprop-2-en-1-one **(5a**) (*E*)-1-(4-methoxyphenyl)pent-1-en-3-one (**5b**), and (*E*)-4-(4-methoxyphenyl)but-3-en-2-one (**5c**), respectively. The bioassay result indicated that **5c** still had anti-inflammatory activity, with an IC_{50} value of 72.8 µg/ml. Meanwhile, both **5a** and **5b** were considered not to have anti-inflammatory activity. The structure-activity study suggested that the increasing lipophilicity of *p*-methoxystyryl derivatives will decrease the anti-inflammatory activity.

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