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Antibacterial Activities of 3-Substituted Coumarin-Scaffolds Synthesized under Microwave Irradiation

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Abstract: Global infections, such as diarrhea and acute respiratory disease, have urged the researchers in this area to discover coumarin-scaffold antibacterial agents, most widely used therapeutic drugs worldwide. This research aims to synthesize 3-substituted coumarins using microwave-assisted and evaluate their antibacterial activities against *Escherichia coli* and *Staphylococcus aureus*. In this study, salicylaldehyde with numerous activated methylenes has been used to synthesize 3-substituted coumarins following Knoevenagel condensation assisted by microwave irradiation. The highest yield of 3-acetylcoumarin was achieved using ethyl acetoacetate at the molar ratio of 1.2:1 (mol/mol) and 30 mol% of diethylamine using 100W microwave irradiation for 60 s under neat condition (43.65$\pm$0.50%). Moreover, ethyl coumarin-3-carboxylate (16.33%) was successfully synthesized using the optimum condition of 3-acetylcoumarin from diethyl malonate as activated methylene with a longer reaction time (900 s). However, the trial to afford 3-cyanocoumarin was failed from ethyl cyanoacetate and malononitrile as activated methylenes under the optimum condition of 3-acetylcoumarin. The inhibition zones of 3-acetylcoumarin and ethyl coumarin-3-carboxylate on disc diffusion assay towards *E. coli* at concentrations higher than 250 ppm showed weak activity, ranging from 1.9-3.6 mm. In contrast, both compounds showed no inhibition zone against *S. aureus* at all tested concentrations. This finding reveals that 3-substituted coumarins can be synthesized by low wattage microwave irradiation and solvent-free with moderate yield with ethyl acetoacetate is the most reactive methylene. Furthermore, this work proposes some corrections to assign proton and carbon chemical shifts in 3-substituted coumarins by analyzing HSQC and HMBC experiments in NMR measurement.

Keywords: antibacterial, coumarin, Knoevenagel condensation, microwave irradiation.

微波輻照合成的3-取代香豆素-支架的抗菌活性

摘要：全球感染，如腹瀉和急性呼吸道疾病，已促使該領域的研究人員發現香豆素支架抗菌劑，這是全球使用最廣泛的治療藥物。本研究旨在利用微波輔助合成 3-取代香豆素，並評估其對大腸桿菌和金黃色葡萄球菌的抗菌活性。在這項研究中，水楊醛與許多活化的亞甲基已被用於在微波輻射輔助下的克諾文納格爾縮合後合成 3-取代的香豆素。3-乙酰香豆素的最高產率是使用乙酰乙酸乙酯以 1.2:1 (痣/痣) 的摩爾比和 30痣% 的二乙胺在純淨條件下使用 100 W 微波照射 60 s (43.65+0.50%) 實現的。此外，以丙二酸二乙酯為活化亞甲基3-乙酰香豆素的最佳條件成功合成了香豆素-3-羧酸乙酯（16.33%），反應時間較長（900 s）。然而，在3-

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乙酰香豆素的最佳條件下，以氰基乙酸乙酯和丙二腈為活化亞甲基，提供3-氰基香豆素的試驗以失敗告終。3-乙酰香豆素和香豆素-3-甲酸乙酯對大腸桿菌的圓盤擴散試驗在濃度高於250百萬分之幾時顯示出較弱的活性，範圍為1.9-3.6毫米。相反，兩種化合物在所有測試濃度下均未顯示出針對金黃色葡萄球菌的抑菌圈。這一發現表明，3-取代香豆素可以通過低功率微波輻射合成，並且無溶劑且產率適中，其中乙酰乙酸乙酯是最具反應性的亞甲基。此外，這項工作通過分析核磁共振測量中的異核單量子相干和異核多鍵相關實驗，提出了一些修正，以指定 3-取代香豆素中的質子和碳化學位移。

关键词：抗菌，香豆素，克諾文納格爾冷凝，微波照射。

1. Introduction

The lack of environmental awareness has a direct impact on life and health. Acute respiratory disease and diarrhea are two main infections caused by an unclean environment [1]. Both ailments are caused by the most well-known bacteria, such as *Escherichia coli* and *Staphylococcus aureus* [2]. However, their activities can be inhibited with the addition of antibacterial. An antibacterial is an agent that stops the growth of bacteria or kills them [3]. In recent years, antibacterial needs to be turned into a developing issue due to the rapid rate of bacterial illnesses and its resistance to traditional antibiotics. Therefore, identifying and developing effective antibacterial with novel action mechanisms becomes urgent for infectious disease research programs [4, 5].

Coumarin moiety will be an outstanding candidate among the *O*-heterocyclics for antibacterial agents since Chlorobiocin and Novobiocin, which have a coumarin nucleus, are established as antibacterial worldwide [5, 6]. Coumarins, known as benzo-2-pyrone derivatives, are commonly used for a broad view of pharmaceutical and medicinal purposes. They also possess interesting therapeutic activities such as antimicrobial, antifungal, antiviral (including anti-HIV), analgesic, antimalarial, antileukemic, antidiabetic, antioxidant, anticancer, anti-Alzheimer's, anti-asthmatic, anti-tuberculosis, anti-platelet, anti-proliferative, anticonvulsant, anti-inflammatory, anti-hypertensive, and vasorelaxants, as well as COX, tyrosinase, DNA gyrase, and acetylcholinesterase inhibitors [7-12]. Further, their biological activities play an important role in new drugs therapy development [13]. For this reason, several methods and techniques have been developed to synthesize coumarins, such as Knoevenagel, Pechmann, Claisen, Perkin, Baylis-Hillman, Reformatsky, Wittig, Vilsmeier-Haack, and Suzuki cross-coupling by using conventional heating, microwave irradiation, or ultrasound-assisted [14, 15].

Coumarins substituted at C–3, on the other hand, are an important class of organic compounds that are commonly used in pharmaceuticals as intermediates in the synthesis of bioactive compounds [12, 16]. Therefore, the current study was concerned with the microwave-assisted synthesis of 3-substituted coumarins via Knoevenagel condensation. Knoevenagel reaction was chosen due to its simplicity and utilized readily available aldehydes or ketones and activated methylenes in acid or base catalysts. At the same time, microwave irradiation was selected since this condition is more convenient and effective than the thermal condition in organic reactions [17-19]. In addition, the antibacterial activities of the synthesized 3-substituted coumarins were also evaluated following the disc-diffusion method.

2. Material and Methods

2.1. Chemicals and Instrumentations

The chemicals used for synthesis were salicylaldehyde (1), ethyl acetoacetate (2a), diethyl malonate (2b), ethyl cyanoacetate (**2c**), malononitrile (2d), diethylamine, acetonitrile, ethanol, *n*-hexane, ethyl acetate, acetone, and chloroform. All chemicals were purchased from Merck without any purification prior to use. Microwave irradiation synthesis was conducted in a domestic Samsung ME-731K/XSE microwave oven with a general laboratory apparatus and infrared thermometer GM320. Reactions were evaluated using thin-layer chromatography (TLC) on precoated 0.25 mm thickness of Si 60 GF_{254} plates (Merck), and TLC spots were visualized under ultraviolet light UVP UV GL 55 (λ 254 and 366 nm). The melting point was measured using Electrothermal Melting Point Apparatus 9100. At the same time, structural elucidation was established by using FTIR

Shimadzu IR Prestige 2 in KBr pellet (Merck), UV-Vis Shimadzu UV2450 in methanol spectroscopic grade (Merck), 500 MHz NMR Bruker Avance operating at 500 MHz (^{1}H) and 125 MHz (^{13}C) in acetone-d_6 and $CDCl_3$ (Merck), and LC-MS with LC system Waters ACQUITY UPLC® H-Class System and MS Xevo G2-S QTof in MeOH spectroscopic grade (Merck) with the addition of formic acid (Merck). LC-MS parameters were adapted from previous research [20]. For the disc-diffusion assay, nutrient agar, nutrient broth, and DMSO from Merck and ciprofloxacin HCl monohydrate® from PT. Bernofarm Pharmaceutical Company was used. In addition, the bacteria culture of *Escherichia coli* and *Staphylococcus aureus* were obtained from Biochemistry Laboratory, Department of Chemistry, Universitas Indonesia.

2.2. Microwave-Assisted Synthesis of 3-Substituted Coumarins

The Knoevenagel condensation in basic condition using 1 and 2a was selected as a model reaction for optimizing the reaction parameters. A mixture of 1 and 2a was mixed at various molar ratios (1.1:1, 1.2:1, and 1.3:1, mol/mol), and several diethylamines as a catalyst (0, 10, 20, 30, and 40 mol% depended on 1) were added. This mixture then was irradiated under 100W microwave for several reaction times (50, 60, 70, 80, and 90 s) without any solvent or with various solvents (ethanol and acetonitrile). The reaction was worked up by cooling to room temperature washing with cold ethanol, and the product was isolated by filtration. The product was then recrystallized with hot ethanol and dried in the air. Further, the dried product was weighted, identified using TLC with *n*-hexane: EtOAc 8:2 (v/v), measured its melting point, and followed by structural elucidation using FTIR, UV-Vis, NMR, and LC-MS. Some experiments were conducted in two trials. The optimum conditions of model reaction were then applied in preparing other 3-substituted coumarins using various activated methylene, i.e., 2b-d.

2.2.1. 3-Acetyl Coumarin (3a)

3a: a pale-yellow solid; yield (%): 43.65 ± 0.50; R_f 0.66 (*n*-hexane:EtOAc 8:2, v/v); MP (°C): 119.1-121.0; FTIR (KBr pellet) ν_{max} (cm^{-1}): 3069.2 (C–H *sp*^{2}), 2953.8 (C–H *sp*^{3}), 1757.5 (–C=O lactone), 1688.5 (–C=O acetyl), 1630.8-1592.3 (C=C), 1473.1-1330.8 (C–H), and 1230.8-1142.3 (C–O); UV-Vis (methanol) λ_{max} (nm): 210.0 (lactone), 293.5 (benzoyl), and 340.0 (cinnamoyl); ^{1}H-NMR (500 MHz, acetone-d_6) δ (ppm): 8.55 (1H, *s*, H–4), 7.92 (1H, *dd*, *J* = 7.5, 1.5 Hz, H–5), 7.76 (1H, *td*, *J* = 7.5, 1.5 Hz, H–7), 7.43 (1H, *td*, *J* = 7.5, 1.5 Hz, H–6), 7.40 (1H, *dd*, *J* = 7.5, 1.5 Hz, H–8), and 2.62 (3H, *s*, $–CH_3$ of 3-acetyl); ^{13}C-NMR (125 MHz, acetone-d_6) δ (ppm): 195.5 (–C=O of 3-acetyl), 159.5 (C–2), 156.2 (C–8a), 147.5 (C–4), 135.1 (C–7), 131.5 (C–5), 125.9 (C–3), 125.7 (C–6), 119.4 (C–4a), 117.1 (C–8), and 30.3 ($–CH_3$ of 3-acetyl); and LC-MS (MeOH-HCOOH): LC rt (min) 8.31, MS (25-60 eV, m/z): 189.0550 $[M+H]^+$, corresponds to chemical formulae of $C_{11}H_9O_3$.

2.2.2. Ethyl Coumarin-3-Carboxylate (3b)

3b: a white solid; yield (%): 16.39; R_f 0.25 (*n*-hexane:EtOAc 8:2, v/v); MP (°C): 92.5-94.0; FTIR (KBr pellet) ν_{max} (cm^{-1}): 3068.8 (C–H *sp*^{2}), 2974.6-2916.7 (C–H *sp*^{3}), 1771.7 (–C=O lactone/ester), 1615.9-1558.0 (C=C), 1474.6-1293.5 (C–H), and 1228.3-1123.2 (C–O); UV-Vis (methanol) λ_{max} (nm): 212.0 (lactone), 293.5 (benzoyl), and 337.0 (cinnamoyl); ^{1}H-NMR (500 MHz, acetone-d_6) δ (ppm): 8.64 (1H, *s*, H–4), 7.87 (1H, *dd*, *J* = 7.5, 1.5 Hz, H–5), 7.74 (1H, *td*, *J* =7.5, 1.5 Hz, H–7), 7.41 (1H, *td*, *J* = 7.5, 1.5 Hz, H–6), 7.38 (1H, *dd*, *J* = 7.5, 1.5 Hz, H–8), 4.33 (2H, *q*, *J* = 7.0 Hz, $–CH_2$ of *O*-ethyl), and 1.35 (3H, *t*, *J* = 7.0 Hz, $–CH_3$ of methyl); ^{13}C-NMR (125 MHz, acetone-d_6) δ (ppm): 163.6 (–C=O of carboxyl), 156.6 (C–2), 156.0 (C–8a), 148.8 (C–4), 135.0 (C–7), 130.9 (C–5), 125.6 (C–6), 119.7 (C–3), 119.0 (C–4a), 117.1 (C–8), 62.0 ($–CH_2$ of *O*-ethyl), and 14.5 ($–CH_3$ of methyl); and LC-MS (MeOH-HCOOH): LC rt (min) 9.06, MS (25-60 eV, m/z): 219.0659 $[M+H]^+$, corresponds to chemical formulae of $C_{12}H_{11}O_4$.

2.3. Disc Diffusion Protocol on Antibacterial Screening

Antibacterial activities of isolated products were performed using the disc diffusion method by measuring the diameter of a clear zone formed in mm according to the established procedure with some modifications [21]. A 200 µL of *E. coli* and *S. aureus* was spread into a nutrient agar in a Petri dish. A 3 mm paper disc was dipped into numerous concentrations of tested compounds (62.5-1000 ppm) and then was placed above the agar. The Petri dish was incubated in a 37°C for 24 h. The inhibition zone in mm was then observed and measured. Ciprofloxacin was used as a positive control at 1000 ppm and DMSO as a negative control. Duplicates were maintained, and the experiment was repeated twice.

3. Results and Discussion

In this study, 3-substituted coumarins from the various activated methylenes have been successfully synthesized via Knoevenagel condensation under microwave irradiation. The Knoevenagel reaction is one of the most useful C=C bond formation processes involving the condensation between benzaldehydes and activated methylenes in the presence of an amine. Furthermore, a 2-hydroxy substituent in the aromatic aldehydes is required to generate the coumarin skeleton [22]. Two different mechanisms have been proposed for this reaction. The first proposal includes forming an imine or iminium salt from the amine with benzaldehyde, which is then followed by a reaction with the enolate from the activated methylene, elimination of the amine, and intramolecular ring-closure. In the second option, the carbanion from the amine's deprotonation of the activated methylene attacks the carbonyl group in aromatic aldehyde to obtain the intermediate. The intermediate then undergoes proton transfer, ring-closure via acyl substitution, and dehydration [23].

3.1. Synthesis of 3-Substituted Coumarins Using Microwave Irradiation

For the model reaction in Fig. 1, the reaction parameters were optimized under microwave conditions, as recapped in Table 1. The optimal condition for the corresponding reaction was established by reacting 1.2:1 (mol/mol) of 1 and 2a with 30 mol% Et_2NH for 60 s under solvent-free condition and 100W microwave irradiation, as shown in Table 1 entry 2. The variety of precursors' molar ratios (1.1:1 and 1.3:1 mol/mol of 1 and 2a) had a considerable impact on yields (Table 1, entries 1 and 3). Furthermore, a decrease of irradiation time to 50 s and an increase up to 80 s did not result in a substantial yield loss (Table 1, entries 4-6). However, raising the reaction time to 90 s reduced the yield further (Table 1, entry 7). At a longer reaction time, the formation of by-products such as chromones and self-condensation of 2a and isomerization and cleavage of 3a could result in a lower yield [19, 24].

In the selected reaction, the effect of solvent on reaction yield was also optimized. Ethanol exemplified polar protic solvent, whereas acetonitrile was chosen to represent polar aprotic solvent. The addition of ethanol did not significantly affect the yield, but the addition of acetonitrile dropped the yield dropped drastically (Table 1, entries 8 and 9). The reduced yield in the presence of solvent is due to the competition between reactants and solvents at the catalyst's active site, resulting in a distinct interaction between the solvent and the catalyst [25]. Moreover, the catalytic activity of Et_2NH was then investigated by modifying the catalyst quantity. Increasing Et_2NH amount to 40 mol% could not improve the yield with a half reduction in yield (Table 1, entry 10). Besides, the use of lower catalyst amounts up to 10 mol% afforded a significant reduction in yields (Table 1, entries 11 and 12) due to the selectivity reduction in the catalyst's active site [25]. Further, no yield from the uncatalyzed process was found (Table 1, entry 13), indicating that this reaction requires a base catalyst, as mentioned earlier [23].

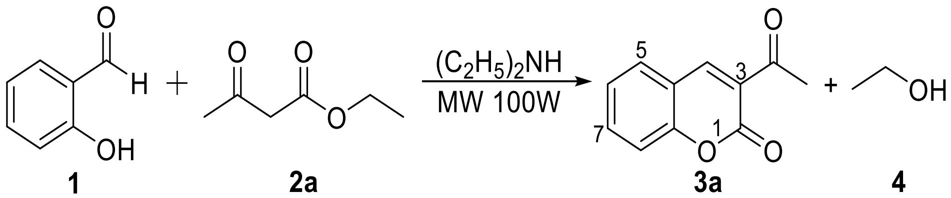

Fig. 1 Reaction scheme of 3-acetyl coumarin synthesis

Table 1 Optimization reaction conditions of 3-acetyl coumarin synthesis

Entry	1:2a molar ratio (mol/mol)	$(C_2H_5)_2NH$ (mol%)	Time (s)	Solvent	Isolated yields[a] (%)
1	1.1:1	30%	60	-	29.99[b]
2	1.2:1	30%	60	-	43.65 ± 0.50
3	1.3:1	30%	60	-	27.82[b]
4	1.2:1	30%	50	-	41.4[b]
5	1.2:1	30%	70	-	42.83 ± 0.95
6	1.2:1	30%	80	-	41.74 ± 0.93
7	1.2:1	30%	90	-	27.82 ± 0.88
8	1.2:1	30%	60	EtOH	43.33 ± 0.04
9	1.2:1	30%	60	MeCN	8.57[b]
10	1.2:1	40%	60	-	22.07[b]
11	1.2:1	20%	80	-	1.03[b]
12	1.2:1	10%	80	-	trace[b]
13	1.2:1	0%	80	-	-

Notes: [a] reaction condition: MW 100W; [b] single entry

Numerous reactions using the activated methylene series were also carried out to study the scope of the reaction using the optimum model conditions (Fig. 2). A study on the activated methylenes' reactivity was observed under similar conditions except for the irradiation time, as summarized in Table 2. The 1 and 2a produced the highest yield (Table 2, entry 1). It was discovered that the yield of the equivalent reaction with 2b was lower even with the longer reaction time (Table 2, entry 2). Furthermore, the reaction between 1 and 2c or 2d gave the desired product in a mixture (Table 2, entries 3 and 4). These results demonstrated that 2a was the most reactive among the methylenes tested. The reactivity of activated methylenes can be explained by referring to their acidities. The more acid the activated methylene, the easier it will be for amine to deprotonate it. Afterward, the activated methylene will form an active nucleophilic to attack the carbonyl group during the Knoevenagel reaction [26].

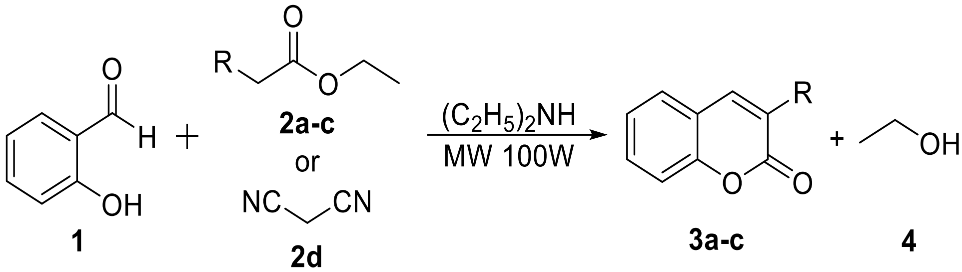

Fig. 2 Reaction scheme of 3-substituted coumarins synthesis

Table 2 Synthesis of 3-substituted coumarins

Entry	Activated methylene	Reaction Product	Time (s)	Isolated yields[a] (%)
1	2a, R = $COCH_3$	3a, R = $COCH_3$	60	43.65 ± 0.50
2	2b, R = $COOC_2H_5$	3b, R = $COOC_2H_5$	900	16.39[b]
3	2c, R = CN	3c, R = CN	390	-[c]
4	2d	3c, R = CN	60	-[c]

Notes: [a] reaction condition: MW 100W, solvent free, **1**:**2** derivatives molar ratio 1.2:1 (mol/mol), 30 mol% of $(C_2H_5)_2NH$; [b] single entry; [c] product in a mixture

Compounds 3a and 3b were obtained as pale-yellow and white solid, respectively. In the same eluent for TLC (*n*-hexane: EtOAc 8:2, v/v), 3b (R_f 0.25) was found to be more polar than 3a (R_f 0.66) due to the presence of ester group as a substituent at C–3 in coumarin scaffold [27]. In addition, the melting points of 3a and 3b were observed at 119.1-121.0 and 92.5-94.0°C, respectively, similar to the previous research [28]. The melting point of 3b was lower than 3a since the positive charge on the carbon of the carbonyl group in ester was comparatively less than ketone due to its resonance. As a result, the dipole-dipole interaction in ester will be weaker, resulting in a lower melting point [29].

The characteristic peaks of coumarin moiety were exhibited in the FTIR spectra of 3a and 3b, which belong to the C–H sp^2 stretching, –C=O lactone, C=C, C–H bending, and C–O [7, 8, 16, 30]. In addition, a signal for C–H sp^3 in both compounds also indicated an alkyl group as a substituent on the coumarin skeleton. Only 3a showed an extra peak for –C=O acetyl as the additional substituent. Furthermore, the UV-Vis spectra of both compounds showed the maximum wavelength which corresponding to carbonyl lactone, benzoyl, and cinnamoyl chromophores, confirming the typical peaks of coumarins [9, 14, and 28].

NMR characterization of 3a and 3b in 1D (^{1}H and ^{13}C) and 2D (HSQC and HMBC) was recorded to support FTIR and UV-Vis analysis. Briefly, ^{1}H-NMR spectra of both compounds revealed four proton signals of ortho-disubstituted benzene with coupling patterns of two doublets of doublets (*dd*) and two triplets of doublets (*td*) as well as one proton signal of trisubstituted alkene that appeared as singlet (*s*), indicating the α-benzopyrone scaffold with substituted in C–3. Besides, 3a also showed one proton signal of acetyl, whereas 3b appeared two proton signals of ethyl of ester. Moreover, ^{13}C-NMR spectra of both compounds displayed one carbonyl lactone, six-carbon of a benzene ring including one oxy-aril carbon, and two-carbon of alkene, belonging to the backbone of coumarin. In addition, 3a also exhibited one carbonyl ketone and one carbon of alkane composing acetyl substituent, while 3b presented one carbonyl ester with two-carbon sp^3 indicating ethyl carboxylate derivative. The ^{1}H- and ^{13}C-NMR spectral data were in good accordance with earlier findings [15, 28]. However, in this paper, we proposed some corrections on assigning proton and carbon chemical shifts in coumarin moiety by analyzing HSQC and HMBC experiments (Fig. 3) which previous researchers had not performed. Further, mass spectra of 3a and 3b revealed $[M+H]^+$ values of 189.0550 and 219.0659 m/z, respectively, confirming to chemical formulae of $C_{11}H_9O_3$ and $C_{12}H_{11}O_4$, respectively.

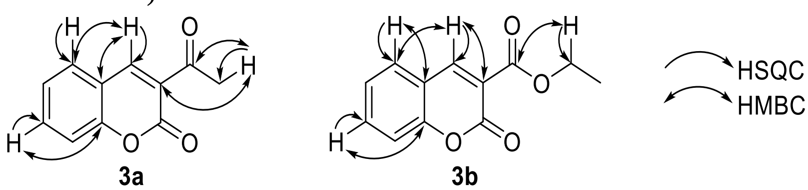

Fig. 3 Key HSQC and HMBC of compounds 3a and 3b

3.2. Antibacterial Activities of Isolated 3-Substituted Coumarins

Antibacterial screening of 3a and 3b towards *E. coli* (gram-negative) and *S. aureus* (gram-positive) followed the disc diffusion method. According to Table 3, both compounds were sensitive to *E. coli* at concentrations higher than 250 ppm rather than *S. aureus*, which were not shown any inhibition zone at all tested concentrations. However, based on classification by Davis and Stout [21], both compounds showed weak activity against *E. coli* with the inhibition zone ranging from 1.9-3.6 mm.

Table 3 Antibacterial activities of compounds 3a and 3b towards *E. coli* and *S. aureus*

	Inhibition zone (mm) in various concentration (ppm)[a]									
	E. coli					*S. aureus*				
	62.5	125	250	500	1000	62.5	125	250	500	1000
3a	0	0	1.9	2.8	3.4	0	0	0	0	0
3b	0	0	0	3.2	3.6	0	0	0	0	0

Notes: [a] Inhibition zone of ciprofloxacin as positive control: 24 mm (1000 ppm) and DMSO as negative control: 0 mm

4. Conclusion

The effect of various activated methylenes for 3-substituted coumarins synthesis was successfully investigated under microwave irradiation. Ethyl acetoacetate was discovered to be the most reactive methylenes among others. This study also proposed some corrections on assigning proton and carbon chemical shifts in 3-substituted coumarins by analyzing HSQC and HMBC experiments in NMR measurement. Moreover, microwave-assisted is a useful and straightforward approach for achieving a reasonable yield of coumarins via Knoevenagel condensation under neat and mild conditions (low wattage) in a short reaction time with solvent-free and easier product work-up. Besides, using the disc diffusion method, the antibacterial activity of 3-acetyl coumarin and ethyl coumarin-3-carboxylate demonstrated that both synthesized compounds had activity against gram-negative rather than gram-positive bacteria. This research had limitations on assaying in one representative bacteria on each gram-classified. Therefore, it is suggested to examine the antibacterial activities with other bacteria. Further structural modification of 3-substituted coumarins also needs to be carried out to improve the antibacterial activity.

Acknowledgments

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