Journal of Multidisciplinary Engineering Science and Technology (JMEST)
ISSN: 2458-9403
Vol. 11 Issue 2, February - 2024

Synthesis and antibacterial activity of 3,4-dichloro-5-hydroxy-2(5H)-furanone-based derivatives

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***Abstract*— Chlorine-containing 2(5H)-furanone derivatives have shown significant antitumor, antimicrobial and insecticidal activities. In this study, we have synthesized a number of functional chlorine-containing 2(5H)-furanone derivatives and evaluated their antibacterial activity. Three bacteria species (*Pseudomonas aeruginosa, Escherichia coli and Staphylococcus aureus)* were used for evaluation. The minimum inhibitory concentration was assessed on planktonic bacteria and biofilms. Results showed that the 5-position derivatization, containing two remaining chlorine atoms on 2(5H)-furanone, showed a higher antibacterial activity than those derivatized at the 4-position, with only one remaining chlorine. The derivatives with electron-donating groups attached at the 5-position demonstrated higher antibacterial activities than those with electron-withdrawing ones. The derivatives with attached hydrophilic groups such as amino, hydroxyl and carboxyl groups showed significantly reduced antibacterial activities. The results also indicate that the synthesized derivatives were more potent toward *P. aeruginosa* than *S. aureus* and *E. coli*.**

Keywords—antibacterial; 2(5H)-furanone derivatives; synthesis; evaluation; biofilm

I. INTRODUCTION

Furanones are a heterocyclic compound that contains two oxygen atoms in a five member ring. Due to their unique pharmacological and biochemical activities, these compounds are gaining increased attentions in pharmaceutical and biomedical applications [1, 2]. Furanone derivatives have exhibited numerous biological activities, including antibacterial, antifugal, antiviral, antitumor, anti-tubercular, anti-inflammatory functions, and others [3-6].

One important furanone class is 2(5H)-furanone, which is the core structure of the molecule 3,4-dichloro-5-hydroxy-2(5H)-furanone, produced during water purification and chlorination [7, 8]. This chlorine-containing 2(5H)-furanone is a highly functionalized molecule processing a pseudo hydroxyl, two chlorine atoms and a lactone-like structure. Derivatization of this molecule could make this 2(5H)-furanone more accessible to pharmaceutical and biomedical applications [6, 9]. Recent 2(5H)-furanone derivatization efforts have focused on synthesizing novel anti-infective, anti-inflammatory, and anti-cancer agents [6, 9-15]. For example, synthesized derivatives have been successfully incorporated into dental restoratives to enhance antibacterial function [16-18]. In this dental application, the derivatives were either covalently attached to polymers or in situ copolymerized with other monomers to form unleachable formulations [16-18]. Such unleachable formulations minimize or eliminate release of small toxic antibacterial agent, unlike other formulations based upon slow or extended release [19-21]. In this way, cytotoxicity of the antibacterial molecules toward oral tissues would be greatly reduced and long-term drug action would be expected. Therefore, derivatization of the compound with special functionality would broaden its usefulness.

In this report, we describe 3,4-dichloro-5-hydroxy-2(5H)-furanone derivatization at different positions and evaluate the antibacterial activity of the formed derivatives. The objective of this study was to synthesize the 2(5H)-furanone derivatives with directly or indirectly attached functional groups, evaluate the antibacterial activity of the formed derivatives, and correlate the derivatized compounds with their biocidal activity.

II. MATERIAL AND METHODS

A. Materials

2,3-Dichloromalealdehydic acid (DCMA), methanol (M), ethanol (E), mercaptoacetic acid (MAA), mercaptoethanol (ME), ethanolamine (EAm), allylamine (AAm), methylamine (MAm), sodium azide, sodium borohydride, 2-hydroxyethyl acrylate (HEA), 2-hydroxyethyl methacrylate (HEMA), acryloyl chloride (AC), benzoyl chloride (BC), glycerol dimethacrylate (GDMA), toluene, acetone, methylene chloride, tetrahydrofuran, p-toluenesulfonic acid (PTSA), triethylamine, 4-methoxyphenol, sodium chloride, sodium bicarbonate and anhydrous magnesium sulfate were used as received from Sigma-Aldrich Co. (Milwaukee, WI) without further purifications.

B. Derivative Synthesis and Characyterization

5-Position derivatives of DCMA with methanol (b) or ethanol (c). To a flask containing DCMA (0.1 mol) in methanol (0.7 mol), PTSA (2 mol %) was added.

After the mixture was refluxed at 70 ^{o}C for 72 h, the solvent was removed via a rotary evaporator. Then the crude product DM was dissolved in methylene chloride, washed with saturated sodium bicarbonate solution, and dried with anhydrous magnesium sulfate, followed by removing solvent by the rotary evaporator. The derivative with ethanol, DE, was prepared similarly. DM (b): Yellow liquid; Yield: 88%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 6.21 (1H, C<u>H</u>), 3.51 (3H, -OC<u>H</u>$_3$). DE (c): Yellow liquid; Yield: 85%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 6.28 (1H, C<u>H</u>), 3.82 (2H, C<u>H</u>$_2$), 1.26 (3H, C<u>H</u>$_3$).

5-Position derivatives of DCMA with HEA (e) or HEMA (f) or GDMA (m). To a solution containing DCMA (0.1 mol), HEMA (0.12 mol) and 4-methoxyphenol (0.1 mol %) in toluene, PTSA (2 mol %) was added. The mixture was refluxed at 100-110 ^{o}C for 3-4 h until the calculated water from a water-receiving apparatus was completely collected. Then toluene was removed via the rotary evaporator. Next, the crude product DHEA was dissolved in methylene chloride, washed with saturated sodium bicarbonate solution, brine, and distilled water, and dried with anhydrous magnesium sulfate, followed by removing solvent by the rotary evaporator. DHEMA and DGDMA were synthesized similarly. DHEA (e): Light-brown liquid; Yield: 86%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 6.32 (2H, C<u>H</u>$_2$ on C=C<u>H</u>$_2$), 6.20 (1H, C<u>H</u> on HC=C), 5.75 (1H, C<u>H</u> on DCMA), 4.25 (2H, C<u>H</u>$_2$ on HEA), 4.12 (2H, C<u>H</u>$_2$ on HEA). DHEMA (f): Yellow liquid; Yield: 89%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 6.35 (1H, C<u>H</u> on C=C<u>H</u>$_2$), 6.10 (1H, C<u>H</u> on C=C<u>H</u>$_2$), 5.71 (1H, C<u>H</u> on DCMA), 4.31 (2H, C<u>H</u>$_2$ on HEMA), 4.10 (2H, C<u>H</u>$_2$ on HEMA), 1.26 (3H, C<u>H</u>$_3$ on HEMA). DGDMA (m): Yellow liquid; Yield: 81%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 6.35 (2H, C<u>H</u> on C=C<u>H</u>$_2$), 6.10 (2H, C<u>H</u> on C=C<u>H</u>$_2$), 5.65 (1H, C<u>H</u> on MCA), 4.31 (2H, C<u>H</u>$_2$ on GDMA), 4.10 (2H, C<u>H</u>$_2$ on GDMA), 3.31 (1H, C<u>H</u> on GDMA), 1.89 (6H, C<u>H</u>$_3$ on GDMA).

5-Position derivatives of DCMA with AC (g) or BC (h). To a solution containing DCMA (0.1 mol) and 4-methoxyphenol (0.1 mol %) in toluene, AC (0.12 mol) was added. After the reaction was run at 90 ^{o}C for 3-4 h, toluene was removed via the rotary evaporator. Then the crude product DAC was dissolved in ethyl acetate, washed with saturated sodium bicarbonate solution, brine, and distilled water, and dried with anhydrous magnesium sulfate, followed by removing solvent by the rotary evaporator. DBC was synthesized similarly. DAC (g): Light-yellow liquid; Yield: 87%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 6.21 and 6.05 (2H, C<u>H</u>$_2$ on C=C<u>H</u>$_2$), 5.82 (1H, C<u>H</u> on HC=C). DBC (h): White solid; Yield: 97%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.10 (2H, C<u>H</u> on benzene), 7.90 (1H, C<u>H</u> on benzene), 7.60 (2H, C<u>H</u> on benzene), 7.35 (1H, C<u>H</u> on DCMA).

5-Position derivatives of DCMA without pseudo hydroxyl group (d). To a flask containing DCMA (0.1 mol) in methanol in an ice-bath (0 ^{o}C), sodium borohydride (0.15 mol) was added in batches. After addition, the mixture was stirred for 30 min, followed by slowly adding the pre-cooled solution of sulfuric acid (0.15 mol) in methanol and then stirring for 30-40 min. The crude product DH was then dissolved in ethyl acetate, washed with saturated sodium bicarbonate solution, brine, and distilled water, and dried with anhydrous magnesium sulfate, followed by removing solvent by the rotary evaporator. DH (d): White solid; Yield: 85%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 5.15 (2H, C<u>H</u>$_2$).

4-Position derivatives of DCMA with azido group (i) and (k). Azidonation of DCMA at 4-position was conducted between DM and sodium azide or between DH and sodium azide. For using DM as a starting compound, briefly, to a flask containing DM (0.1 mol) in acetone, sodium azide (0.2 mol) was added. After the mixture was refluxed at 70 ^{o}C for 4 h, solvent was removed via the rotary evaporator. Then the crude product DMAz was dissolved in methylene chloride, washed with brine and distilled water, and dried with anhydrous magnesium sulfate, followed by removing solvent by the rotary evaporator. The product was further purified by recrystallization from ethanol/water (1:1). In the case of DH, briefly, to a flask containing DH (0.1 mol) in tetrahydrofuran, sodium azide (0.15 mol) in water was added. After the reaction was run at room temperature for 30 min, the solvent was removed via the rotary evaporator. Then the crude product DHAz was dissolved in methylene chloride, washed with brine and distilled water, and dried with anhydrous magnesium sulfate, followed by removing solvent by the rotary evaporator. DMAz (r): Light-yellow solid; Yield: 70%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 6.40 (1H, C<u>H</u>), 3.55 (3H, -OC<u>H</u>$_3$). DHAz (k): Yellow liquid; Yield: 78%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 5.20 (2H, C<u>H</u>$_2$).

4-Position derivatives of DCMA with amino group (j) and (l). The 4-position derivative of DCMA with amino group was completed based on azide reduction between DM or DH and the reducing agent sodium borohydride. To a flask containing DMAz (0.1 mol) in tetrahydrofuran, sodium borohydride (0.15 mol) in water was added. After the reaction was run at room temperature for 5-10 min, tetrahydrofuran was removed via the rotary evaporator. Then the mixture was placed in a refrigerator overnight and crystallized. The product DMAm was filtered and dried with a freeze-dryer. DHAm was synthesized similarly. DMAm (j): White solid; Yield: 88%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 6.40 (1H, C<u>H</u>), 3.55 (3H, -OC<u>H</u>$_3$), 3.35 (2H, N<u>H</u>$_2$); DHAm (l): Yellow solid; Yield: 86%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 7.42 (2H, N<u>H</u>$_2$), 4.71 (2H, C<u>H</u>$_2$).

4-Position derivatives of DM with methylamine (n), ethanolamine (o) and allylamine (p). For methylamine derivatization, briefly, to a flask containing DM (0.1 mol), methylamine (0.15 mol) was added dropwise.

After the reaction was run for 2 h, extra methylamine was evaporated. The purified product, DMMAm was collected through column chromatography. Both allylamine and ethanolamine derivatives of DM, i.e., DMAAm and DMEAm, were synthesized similarly. DMMAm (n): Brown oil; Yield: 80%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 7.18 (1H, NH on MAm), 5.35 (1H, CH on MAm), 3.40 (3H, -OCH_3 on DCMA), 2.85 (3H, -CH_3 on MAm). DMEAm (o): Yellow oil; Yield: 75%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 7.58 (1H, NH on EAm), 5.95 (1H, OH on EA), 5.70 (1H, CH on DCMA), 3.45 (2H, CH_2 on EA), 3.35 (2H, CH_2 on EA), 3.31 (3H, -OCH_3 on DCMA); DMAAm (p): Light-brown oil; Yield: 78%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.05 (1H, NH on AAm), 5.90 (1H, CH on HC=C on AAm), 5.80 (1H, CH on C=CH_2 on AAm), 5.70 (1H, CH on DCMA), 5.15 (1H, CH on C=CH_2 on AAm), 4.00 (2H, CH_2 on AAm), 3.35 (3H, -OCH_3 on DCMA).

4-Position derivatives of DM or DCMA with mercaptoethanol (q) and (s) or mercaptoacetic acid (r) and (t). To a flask containing DM (0.1 mol) in acetone, mercaptoethanol (ME, 0.12 mol) and triethylamine (0.12 mol) in acetone were added dropwise at room temperature [15]. After the reaction was refluxed for 6 h, acetone and triethylamine were removed by the rotary evaporator. The oily residue was crystallized in distilled water, followed by filtering and freeze-drying. The crude product DMME was further purified by column chromatography [15]. DCMAME was synthesized similarly. DMMAA and DCMAMAA were also prepared similarly except for additional washing with sodium bicarbonate followed by crystallizing in distilled water. DMME (q): Colorless liquid; Yield: 81%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 6.35 (1H, CH on DCMA), 5.15 (1H, OH on ME), 3.70 (2H, CH_2 on ME), 3.45 (3H, -OCH_3 on DCMA), 3.30 (2H, CH_2 on ME). DMMAA (r): White solid; Yield: 72%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 13.25 (1H, COOH on MAA), 6.30 (1H, CH on DCMA), 4.20 (2H, CH_2 on MAA), 3.45 (3H, -OCH_3 on DCMA). DCMAME (s): White solid; Yield: 66%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.40 (1H, OH on DCMA), 6.40 (1H, CH on DCMA), 5.15 (1H, OH on ME), 3.70 (2H, CH_2 on ME), 3.30 (2H, CH_2 on ME). DCCMAMAA (t): Yellow solid; Yield: 45%. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 13.15 (1H, COOH), 8.55 (1H, OH on DCMA), 6.35 (1H, CH on DCMA), 4.10 (2H, CH_2 on MAA).

C. MIC Test for Synthesized Derivatives.

The minimal inhibitory concentration (MIC) of the synthesized antibacterial derivatives was determined following the published protocol with a slight modification [16]. In short, colonies of bacteria were prepared in 5 ml of Tryptic Soy Broth (TSB) at 37 ^{o}C for 24 h. The synthesized derivatives were dissolved in dimethylsulfoxide (2%, v/v). Two-fold serial dilutions of the synthesized derivative in dimethylsulfoxide were prepared in TSB, followed by placing in 96-well flat-bottom microtiter plates with a volume of 250 µl per well. The microtiter plate was then inoculated with bacterial strain suspension (cell concentration = 5 x 10^7 CFU/ml) and incubated at 37 ^{o}C for 24 h prior to MIC testing. The absorbance was measured at 600 nm via a microplate reader (SpectraMax M2, Molecular Devices, CA) to assess the cell growth. Three bacteria species including *P. aeruginosa, E. coli and S. aureus* were used to assess the antibacterial activity of the synthesized derivatives. Dimethylsulfoxide was used as control. Triplicate samples were used to obtain a mean value for each derivative.

D. Biofilm Evaluation.

The biofilm inhibition was evaluated using a crystal violet assay [25]. In short, bacteria suspensions were prepared in 5 ml of Tryptic Soy Broth (TSB) at 37 ^{o}C for 24 h. The synthesized derivatives were dissolved in dimethylsulfoxide (2%, v/v). Two-fold serial dilutions of the synthesized derivative in dimethylsulfoxide were prepared in TSB, followed by placing in a 96-well flat-bottom microtiter plate with 250 µl per well. The microtiter plate was then inoculated with bacterial strain suspension (cell concentration = 5 x 10^7 CFU/ml) and incubated at 37 ^{o}C for 24 h. After the plate was washed with sterile distilled water to remove planktonic cells, crystal violet (0.1% w/v) was added and the plate was incubated at room temperature for 15 min, followed by washing with sterile distilled water to remove unbound crystal violet. Finally the adhered biofilm staining with crystal violet was eluted with acetic acid (30% v/v) and the absorbance was measured at 550 nm via a microplate reader. Three bacteria species including *P. aeruginosa, E. coli* and *S. aureus* were used to assess the biofilm inhibition. Triplicate samples were used to obtain a mean value for each derivative.

E. Statistical Analysis.

One-way analysis of variance (ANOVA) with the post hoc Tukey-Kramer multiple-range test was used to determine significant differences of each measured property or activity among the derivatives in each group. A level of α = 0.05 was used for statistical significance.

III. Results and Discussion

A. Synthesis

DCMA, a chlorine-containing 2(5H)-furanone and some of its derivatives have been reported to demonstrate significant antibacterial or antimicrobial activities [6, 9-15]. However, many derivatives have been synthesized using very complicated and expensive catalyst/co-catalyst systems and some were synthesized under strict reaction conditions [10-12], which are not practical in a larger scale. In this study, we tried to use the simplest ways to derivatize DCMA at different reaction sites on the molecule and evaluate the antibacterial activities of the formed derivatives, in order to find which position and what kind of functional groups would confer the strongest

antibacterial effect (See Fig. 1 for the structures and Table 1 for derivative names and their abbreviations). By analyzing the structure of DCMA, there are three positions on DCMA which could be potentially derivatized [6, 9-15], i.e., 5, 4 and 3. According to reports [14, 15], position 5 seems to be the most reactive site, followed by 4. The position 3 was the most difficult site to be derivatized [15]. By derivatizing through the pseudo hydroxyl group at 5, we synthesized DM, DE, DHEA, DHEMA, DGDMA, DAC, DBC and DH. By derivatizing through replacing chlorine at 4, we directly synthesized DCMAMAA and DCMAME from DCMA and indirectly synthesized DMAz, DMAm, DMMAm, DMEAm, DMAAm, DMMAA and DMME from DM as well as DHAz and DHAm from DH. The synthesis of the derivatives at 5 by pseudo hydroxyl group substitution was basically the same, i.e., using PTSA as a catalyst to reflux the compound. As a consequence, the pseudo hydroxyl group was replaced by hydroxyl-containing compounds [14, 16]. DM, DE, DHEA, DHEMA and DGDMA were synthesized through this substitution. On the other hand, the pseudo hydroxyl group was also utilized to form an ester group with acid chloride, i.e., AC and BC, with the help of refluxing at 90 ^{o}C. Since the pseudo hydroxyl group at 5 is the most reactive group [13-15], an inactivation of this group would facilitate derivatization at either 4 or 3. Hence, we inactivated it by either forming DM or DH, to enhance further derivatization at position 4. DM synthesis was accomplished by refluxing with methanol in the presence of PTSA, as discussed previously. However, DH was derivatized using sodium borohydride, followed by neutralization with sulfuric acid. The compounds derivatizing from position 4 include (1) DM-based: DMAz, DMAm, DMMAm, DMEAm, DMAAm, DMMAA as well as DMME and (2) DH-based: DHAz and DHAm. DCMAMAA and DCMAME were directly derivatized at position 4 without any pseudo hydroxyl group protection or substitution, following the published protocols [15]. The majority of the derivatives in this study were obtained using simple and conventional purification processes with high yields but some were purified with column chromatography.

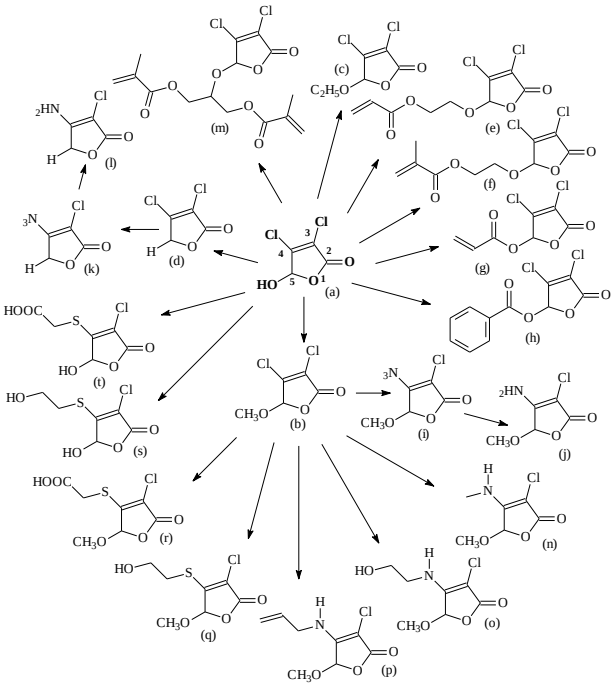


Fig. 1. SCHEMATIC CHEMICAL STRUCTURES AND ABBREVIATIONS OF THE SYNTHESIZED DERIVATIVES

TABLE I. ABBREVIATION OF THE SYNTHESIZED DERIVATIVES IN THE STUDY

Name	Derivative
DCMA	2,3-Dichloromalealdehydic acid
DM	Product of DCMA and methanol at 5-position
DE	Product of DCMA and ethanol at 5-position
DH	Product of DCMA at 5-position
DHEA	Product of DCMA and HEA at 5-position
DHEMA	Product of DCMA and HEMA at 5-position
DAC	Product of DCMA and AC at 5-position
DBC	Product of DCMA and BC at 5-position
DMAz	Product of DM and azide at 4-position
DMAm	Product of DMAz at 4-position
DHAz	Product of DH and azide at 4-position
DHAm	Product of DHAz at 4-position
DGDMA	Product of DCMA and GDMA at 5-position
DMMAm	DM with methylamine at 4-position
DMEAm	DM with ethanolamine at 4-position
DMAAm	DM with allylamine at 4-position
DMME	DM with mercaptoethanol at 4-position
DMMAA	DM with mercaptoacetic acid at 4-position
DCMAME	DCMA with mercaptoethanol at 4-position
DCMAMAA	DCMA with mercaptoaceic acid at 4-position

B. Evaluation

It is known that DCMA is a toxic compound, which is a byproduct produced during the water purification process [7,8]. Due to its toxicity, DCMA and its derivatives have demonstrated significant antimicrobial activities [6, 9-17]. The primary purpose of the present study was to functionalize DCMA in order for it to be attachable to biomaterials and/or to be potentially used for dental or other biomaterials applications. In this study, we used both minimum inhibitory concentration (MIC) and biofilm assays to evaluate the antibacterial activity of the synthesized derivatives to both planktonic bacteria cells and biofilm. Three bacteria species (*P. aeruginosa, E. coli* and *S. aureus*) were evaluated. These bacteria are some of the most concerning pathogens due to development of antibiotic resistant strains [22-23].

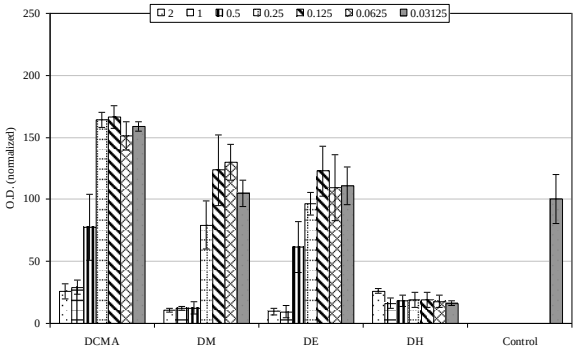
Table 2 shows the MIC values of all the synthesized derivatives to three bacteria strains. MIC values were in the decreasing order of (1) *P. aeruginosa*: DCMAMAA > DMEAm > DHAm > DMAm > DAC > DMMAA > DMAAm > DCMAME > DMME > DMMAm > DHAz > DMAz > DHEA > DBC = DGDMA = DHEMA > DE = DCMA > DM > DH. (2) *E. coli*: DMEAm > DCMAMAA > DHAm = DAC > DMAAm > DMAm > DMMAA > DMMAm > DMME > DGDMA = DHEMA > DHAz > DHEA > DCMAME > DMAz > DE > DBC > DM > DCMA > DH. (3) *S. aureus*: DMAm > DMEAm > DHAm > DAC > DCMAMAA > DMMAA > DMAAm > DMME > DMMAm > DGDMA > DCMAME > DBC = DHAz > DHEMA > DE > DMAz > DHEA = DM = DMCA > DH. Among all the three bacteria species, DH was the strongest antibacterial agent, followed by DM. From the results, three general trends in MIC appear: (1) The derivatives at the 5-position exhibited lower MIC values than the ones at the 4-position. This higher antibacterial activity is likely due to the presence of two chlorine atoms at the 3- and 4-positions) on 5-substitued molecules, compared to only one chlorine on 4-substituted molecules. (2) An electron-donating group on the 5-position exhibited lower MIC values than an electron-withdrawing one, indicating better antibacterial effects of electron-donating groups. (3) The derivatives with hydrophilic end groups, such as amino, hydroxyl and carboxyl moieties, at both the 4- and 5-positions showed much higher MIC values than those with hydrophobic groups, indicating reduced antibacterial activities. Regarding different bacteria, MIC values against *P. aeruginosa* were lower than those against *S. aureus* or *E. coli*. The values toward *E. coli* were the highest, indicating that these derivatives were more potent toward *P. aeruginosa*, followed by *S. aureus* and *E. coli*.

TABLE II. MIC VALUES OF THE COMPOUNDS SYNTHESIZED IN THE STUDY*

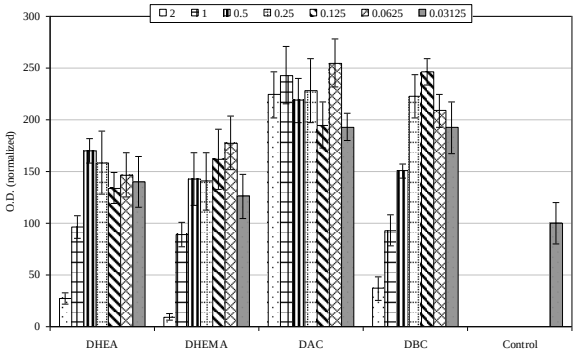
	MIC		
	P. aeruginosa	E. coli	S. aureus
DCMA	30.2 (7.8)[a]	70.8 (12)	79.2 (19)[h]
DM	15.1 (3.9)	89.2 (24)	79.2 (19)[h]
DE	30.2 (7.8)[a]	125 (25)	102.1 (27)[i]
DH	6.25 (1.6)	30.7 (7.0)	40.1 (9.4)
DHEA	95.8 (16)[b]	200 (15)	79.2 (19)[h]
DHEMA	75.0 (12)[c]	250 (50)[f]	137.5 (13)
DAC	2000 (100)	2000 (120)	1000 (110)
DBC	75.0 (12)[c]	100 (24)	158.3 (18)[j]
DMAz	112.5 (32)[b,d]	154.2 (27)[g]	90.6 (27)[i]
DMAm	2375 (750)	1500 (340)	4500 (212)
DHAz	127.5 (27)[d]	239.6 (72)[f]	156.3 (31)[j]
DHAm	3500 (901)	2000 (330)	1166.7 (76)
DGDMA	75.0 (12)[c]	250 (50)[f]	225 (15)[k]
DMMAm	156.2 (25)[d]	625 (75)	240.6 (22)[k]
DMEAm	4250 (247)[e]	>5000	1250 (130)
DMAAm	859.3 (36.9)	1562.5 (618)	447.9 (25)[l]
DMME	562 (23)	260 (109)[f]	285 (31)
DMMAA	1250 (120)	1250	458.3 (130)[l]
DCMAME	671 (31)	171.8 (36)[g]	178.1 (15)
DCMAMAA	4625 (478)[e]	3500 (141)	870.8 (120)

* MIC [μg/mL] = minimum inhibition concentration. Entries are mean values with standard deviations in parentheses and the mean values with the same superscript letter were not significantly different ($p > 0.05$).

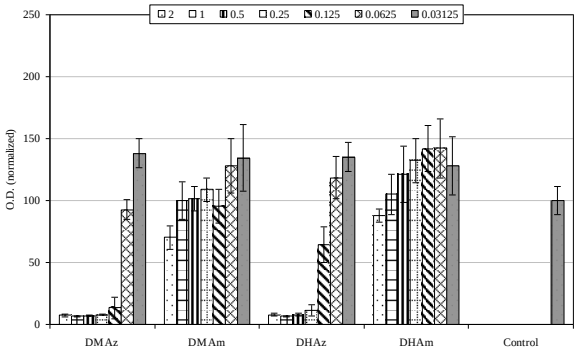
Fig. 2 shows the biofilm inhibition values of the synthesized derivatives to *P. aeruginosa*. Fig. 2a, 2b, 2c, 2d and 2e respectively show the biofilm inhibition curves of the derivatives DCMA, DM, DE and DH versus control (bacteria only) (Fig. 2a), DHEA, DHEMA, DAC and DBC (Fig. 2b), DMAz, DMAm, DHAz and DHAm (Fig. 2c), DGDMA, DMMAm, DMEAm and DMAAm (Fig. 2d), and DMME, DMMAA, DCMAME and DCMAMAA (Fig. 2e). DH showed the lowest optical density (O.D.), followed by DM, DMAz and DHAz. Minimum biofilm inhibitory concentration (MBIC) values for *E. coli* and *S. aureus* (in addition to *P. aeruginosa*) are listed in Table 3 for comparisons. The values were in the decreasing order of (1) *P. aeruginosa*: DAC > DMEAm > DCMAMAA > DMMAA > DHAm = DMAAm > DMAm > DMME > DCMAME > DHEA = DBC = DGDMA > DHEMA > DMMAm > DCMA > DE > DM > DHAz > DMAz > DH; (2) *E. coli*: DHAm > DMAm > DMEAm > DMMAA > DMME = DMCAMAA > DAC > DMAAm > DCMAME > DMMAm > DCMA > DHAz > DE > DHEMA = DGDMA > DHEA > DM > DBC > DMAz > DH; (3) *S. aureus*: DMAm > DHAm > DMEAm = DCMAMAA > DMMAA > DMME > DMAAm > DAC > DCMAME > DGDMA > DMMAm > DBC > DHAz > DHEMA > DHEA > DCMA > DE > DM > DMAz > DH. DH showed the lowest MBIC to all three bacteria species and is the strongest antibiofilm agent. The results for MBIC showed the similar trend to those for MIC, although there are some discrepancies. Again, the MBIC values were lower toward *P. aeruginosa* than *S. aureus* and *E. coli*. All the hydrophilic groups attached to either 4- or 5-position showed much higher MBIC values, and in some cases lacked any antibacterial activity.



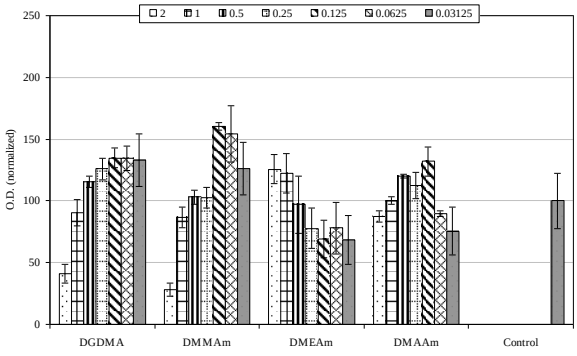
(a)



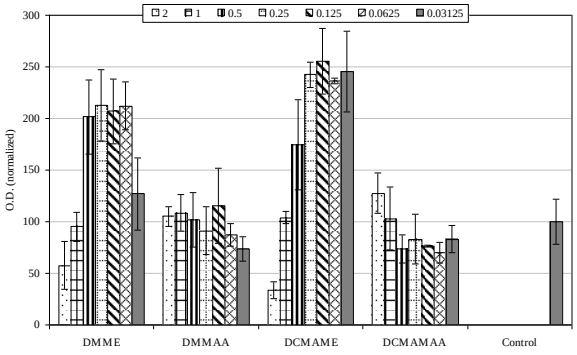
(b)



(c)



(d)



(e)

Fig. 2. Effect of biofilm inhibition concentration of the synthesized derivatives on P. aeruginosa: (a) DCMA, DM, DE and DH; (b) DHEA, DHEMA, DAC and DBC; (c) DMAz, DMAm, DHAz and DHAm; (d) DGDMA, DMMAm, DMEAm and DMAAm; and (e) DMME, DMMAA, DCMAME and DCMAMAA. Values were measured at optical density (O.D.) at 500 nm

TABLE III. MBIC VALUES OF THE COMPOUNDS SYNTHESIZED IN THE STUDY*

	P. aeruginosa		E. coli		S. aureus	
	MBIC	A	MBIC	A	MBIC	A
		77.6		24.4		39.5
DCMA	0.5	(16)	1.0	(8.8)	0.5	(13)
		83.7		39.5		80.0
DM	0.25	(19)	0.5	(9.4)^{b}	0.25	(6.5)^{e}
		96.6		67.9		82.3
DE	0.25	(8.9)	0.5	(18)^{c}	0.25	(11)^{e}
		16.3		87.4		38.9
DH	0.0312	(2.0)	0.125	(26)	0.125	(2.8)
		96.2		42.8		54.2
DHEA	1.0	(11)^{a}	0.5	(2.5)^{b}	0.5	(5.2)
		89.5		55.7		36.7
DHEMA	1.0	(12)^{a}	0.5	(14)^{c}	1.0	(8.9)
						45.5
DAC	2.0	224.2	2.0	122	2.0	(9.8)
		93.3		96.7		52.1
DBC	1.0	(11)^{a}	0.25	(7.9)	1.0	(5.7)
		92.6	0.25	67.8	0.25	43.9
DMAz	0.0625	(8.0)		(11)		(13)
		70.2	2.0	278	2.0	
DMAm	2.0	(9.6)				422.3
		64.2	0.5	88.3	1.0	43.1
DHAz	0.125	(14)		(12)		(10)
		87.9	2.0	327.8	2.0	
DHAm	2.0	(22)				277.5
		90.7		54.8		88.0
DGDMA	1.0	(11)^{a}	0.5	(5.3)^{c}	1.0	(6.9)
		86.6	1.0	57.2	1.0	73.2
DMMAm	1.0	(8.5)^{a}		(9.4)		(2.3)
DMEAm	2.0	195.4	2.0	277	2.0	251^{f}
		87.2	2.0	109.5	2.0	
DMAAm	2.0	(12)				111.2
		65.3	2.0	179.2^{d}	2.0	
DMME	2.0	(14)				119.1
DMMAA	2.0	105.1	2.0	269.3	2.0	148.2
		33.8	2.0	83.4	2.0	33.8
DCMAME	2.0	(8.5)		(14)		(8.5)
DCMAMAA	2.0	127.4	2.0	175.2^{d}	2.0	245^{f}

*MBIC [mg/mL] = minimum biofilm inhibition concentration; A = absorbance (normalized to control at 100%). Entries are mean values with standard deviations in parentheses and the mean values with the same superscript letter were not significantly different ($p > 0.05$).

As mentioned above, the primary purpose of this study was to synthesize the DCMA derivatives with directly or indirectly attachable functional groups for potential antibacterial biomaterials applications. The MIC and MBIC could be a useful indicator to show potency of the synthesized derivatives, although their antibacterial activities may be different after they are attached to biomaterials or copolymerized with other monomers. In this study, DHEA, DHEMA, DGDMA, DAC and DMAAm were really designed for polymerizing or copolymerizing with other monomers for producing antibacterial polymers, since they all contain polymerizable vinyl groups. DHEA, DHEMA, DGDMA and DAC can be used to form an in situ polymerizable formulation for dental or orthopedic applications [16-18] but DMAAm may be used to make a polymer in-house since theoretically it is not as reactive as the others [24]. Both DMAm and DHAm can be further derivatized due to the reactive amino groups. The other derivatives including DMME, DCMAME and DMEAm with attached primary hydroxyl groups, DMMAm with attached secondary amino group, and DMMAA and DCMAMAA with attached carboxyl groups, all can be further derivatized to have a variety of reactive groups for many applications [24]. Although some results showed an unfavorable conclusion or trend to those with attached hydrophilic groups, the outcome might be different after the derivatives are further attached to biomaterials. For example, amino groups will become amide or urea after reacting with carboxyl or isocyanate groups. Therefore, the future study is needed for evaluation of the derivatives after being attached to biomaterials.

IV. Conclusions

In this study we have synthesized numerous functional DCMA-based derivatives. Results showed that the 5-position derivatization with two chlorine atoms exhibited a higher antibacterial activity than those derivatizing at the 4-position, resulting in only one chlorine remaining. The derivatives with electron-donating groups attached at 5-position demonstrated higher antibacterial activities than those with electron-withdrawing ones. The derivatives with attached hydrophilic groups such as amino, hydroxyl and carboxylic groups showed significantly reduced antibacterial activities. The synthesized derivatives were more potent to *P. aeruginosa* than *S. aureus* and *E. coli*. Moreover, our results offer hope in identifying potent novel antimicrobial agents for these sometimes difficult-to-treat pathogens.

Acknowledgment

This work was partially sponsored by IUPUI BBRC Research Support Funds Grant.

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