

SYNTHESIS AND ANTIMICROBIAL EVALUATION OF SYMMETRICAL DIQUATERNARY AMMONIUM SALTS BEARING BIS-1,3,4-OXADIAZOLE RINGS MOIETIES

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Abstract. This study describes the synthesis of some novel compounds containing bis-1,3,4-oxadiazole bearing quaternary ammonium salt moieties. The target compounds were prepared from 2-(dimethylamino)ethyl methacrylate (DMAEMA) or 2-(diethylamino) ethyl methacrylate (DEAEMA), using adipic acid as starting material. All the newly synthesized compounds showed satisfactory analytic data for the proposed structures, which were confirmed by IR and NMR (¹H and ¹³C) spectroscopy. The newly synthesized compounds were evaluated for their antibacterial activity against various gram-positive and gram-negative strains of bacteria, and the antifungal activities were tested against three phytopathogenic fungi namely, *Fusarium oxysporum*, *Fusarium commune* and *Fusarium rodelens*. Some of the tested compounds displayed promising antibacterial and antifungal activities.

Keywords: 1,3,4-oxadiazole, quaternary ammonium compounds, DMAEMA, DEAEMA, antimicrobial activities.

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Introduction

The treatment of bacterial and fungal infectious diseases remains a challenging problem because of the increasing number of microbial pathogens [1-3]. The current interest in the development of new antimicrobial agents can be partially ascribed both to the increasing emergence of bacterial resistance to antibiotic therapy and to newly emerging pathogens [4]. It reinforces the need for the development of new and potent chemical entities or an improvement in the activity of the well-known chemical compounds. Considering this statement, the synthesis of analogues can be seen as an efficient approach to optimize an active chemical structure and design new drugs [5-8], since simple structural changes can lead to better biological activities through modifications of physicochemical properties [9].

Literature survey reveals that compounds containing 1,3,4 oxadiazole nucleus have demonstrated a wide range of pharmacological activities [10,11], including: anti-inflammatory [12], antimicrobial [13], analgesic [14], antiviral [15], antiproliferative [16], antitumor [17], antihypertensive [18,19], antioxidant [20], antitubercular [21], sedative-hypnotic [22] and antibacterial and antifungal activities [23].

Quaternary ammonium salts (QAS) constitute an important class of organic compounds that are present in a large number of biologically active molecules [24]. It was reported that the quaternary ammonium compounds have antimicrobial properties [25], show adsorption on negatively charged solids, act on the cell wall and have a direct or indirect lethal effect on the cell [26]. QAS were introduced as antimicrobial agents by Domagk over eighty years ago [27].

In recent years the number of applications of QAS has been continuously increasing. They are widely used as biocides [28,29], antibiotic [30], disinfectants [31], antimalarial [32] and antimicrobial properties [33,34]. In the design of new drugs, the combination of different pharmacophores frame may lead to compounds with interesting biological profiles [35]. Also, QAS incorporating heterocycles, e.g. 1,3,4-oxadiazole, displayed various pharmacological properties [36]. Prompted by these investigations, we have synthesized compounds **5a** and **5b** containing bis-1,3,4oxadiazole fragment with the attached quaternary ammonium group and their antimicrobial activity has been evaluated.

Materials and methods

All solvents and reagents were obtained from Sigma Aldrich and BIOCHEM. The purity of compounds was routinely checked by thin layer chromatography (TLC) using silica gel F254 supplied by MERCK, and spots were observed using iodine as visualizing agent.

All melting points were determined in open capillary tubes on a BÜCHI 540 melting point apparatus and are uncorrected.

The IR spectra in the range of 4000-400 cm⁻¹ were recorded as potassium bromide discs on a Shimadzu FTIR-8300 spectrophotometer.

The ¹H and ¹³C NMR Spectra were measured in DMSO-*d*₆ on a Bruker AM 300 MHz spectrometer (University of Oran, Es-Senia), operating at 300 MHz for ¹H and 75 MHz for ¹³C nuclei, relative to the internal standard of tetramethylsilane (TMS). Chemical shift values are expressed in parts per million (δ, ppm).

Preparation of diethyl adipate 2

Diethyl adipate **2** was prepared according to the reported in the literature procedure [37]. The adipic acid (5 g, 0.034 mol) was dissolved in 200 mL of absolute ethanol and a few drops of conc. sulphuric acid was added. The mixture was heated at 80°C in an oil bath for 8 hours, the progress of the reaction being monitored by TLC. After completion of the reaction, the excess of ethanol was removed under reduced pressure and the residue was extracted with chloroform. The organic layer extract was washed with a 5% solution of sodium bicarbonate, then the solvent was evaporated and the product was collected. Yield of compound **2**-76%. Liquid, $R_f = 0.45$ ($\text{CHCl}_3/\text{CH}_3\text{OH} = 8/2$); IR (KBr, $\nu \text{ cm}^{-1}$): 1706.9 (C=O), 1070.4 (C-O-C).

Adipic acid dihydrazide 3

A mixture of diethyl adipate **2** (5 g, 0.028 mol) and an excess of hydrazine hydrate (0.05 mol) in absolute ethanol (150 mL) were refluxed for 6 hours on a water bath. The purity of the products was checked by single spot TLC, the reaction mixture was cooled. Then the solvent was evaporated under reduced pressure to give adipic acid dihydrazide **3**. Yield- 67%. Powder, m.p. 180–181°C, $R_f = 0.40$ ($\text{CHCl}_3/\text{CH}_3\text{OH} = 9/1$); IR (KBr, $\nu \text{ cm}^{-1}$): 3222.8 (NH-NH₂), 1606.6 (C=O). ¹H-NMR: 8.34 (3H, O=C-NH-NH₂), 1.99 (2H, O=C-CH₂CH₂), 1.42 (2H, O=C-CH₂CH₂). ¹³C-NMR: 178.2 (O=C-NHNH₂), 39.8 (O=C-CH₂CH₂), 26.3 (O=C-CH₂CH₂).

2,2'-butane-1,4-diylbis[5-(bromomethyl)-1,3,4-oxadiazole] (4). A mixture of adipic acid dihydrazide **3** (3g, 0.017 mol), bromoacetic acid (4.78 g, 0.034 mol) and phosphorus oxychloride (5 mL) was refluxed at 80-90°C for 6 hours. After completion of reaction, as indicated by TLC, the mixture was cooled and poured into ice water and then sodium bicarbonate solution was added. The resulting solid was collected by filtration, washed with cold water, and dried. Then the solvent was evaporated under reduced pressure to give 2,2'-butane-1,4-diylbis[5-(bromomethyl)-1,3,4-oxadiazole] **4**. Yield- 85%. Crystalline solid, m.p. 114-115°C, $R_f = 0.42$ ($\text{CHCl}_3/\text{CH}_3\text{OH} = 4/1$); IR (KBr, $\nu \text{ cm}^{-1}$): 1643.2 (C=N), 1105.1 (C-O-C), 497 (C-Br). ¹H-NMR: 8.30 (2H, O=C-CH₂-Br), 2.17 (2H, N=C-CH₂CH₂), 1.45 (2H, N=C-CH₂CH₂). ¹³C-NMR: 174.8 (N=C-CH₂CH₂), 172.1 (O=C-CH₂-Br), 39.7 (N=C-CH₂CH₂), 33.8 (N=C-CH₂CH₂), 24.4 (O=C-CH₂Br).

N,N'-[butane-1,4-diylbis(1,3,4-oxadiazole-5,2-diylmethylene)]bis[N,N-diethyl-2-(methacryloyloxy)ethan ammonium] bromide (5a). According to the procedure adopted by Guqian Lu *et al.* [38], a mixture of compound **4** (1 g, 0.003 mol) and tertiary amine DEAEMA (0.97 g, 0.005 mol) was dissolved in dry acetone (50 mL), in the presence of hydroquinone. The mixture was gently refluxed for 40 hours on water bath. After cooling, diethyl ether was added, the target diquaternary ammonium salt **5a** was collected by filtration under reduced pressure and washed several times with diethyl ether. Yield- 82%. Pasty mass, $R_f = 0.50$ ($\text{CHCl}_3/\text{CH}_3\text{OH} = 4/1$); IR (KBr, $\nu \text{ cm}^{-1}$): 1737.7 (C=O), 1625.9 (C=N), 1460 (C=C), 1026.1 (C-O-C). ¹H-NMR: 6.12-5.76 (2H, =CH₂), 4.44 (2H, N⁺CH₂CH₂), 3.73 (2H, N=C-CH₂N⁺), 3.44 (2H, N⁺CH₂CH₂), 2.55 (3H, N⁺CH₃), 1.93 (2H, N=C-CH₂CH₂), 1.49 (3H, C(CH₃)=CH₂), 1.23 (2H, N=C-CH₂CH₂). ¹³C-NMR: 174.0 (N=C-CH₂CH₂), 166.5 (C=O), 157.0 (N=C-CH₂N⁺), 136.0 (C(CH₃)=CH₂), 125.6 (=CH₂), 63.3 (N⁺CH₂CH₂), 55.7 (N=C-CH₂N⁺), 50.6 (N⁺CH₂CH₂), 47.2 (N⁺CH₃), 26.7 (N=C-CH₂CH₂), 24.4 (N=C-CH₂CH₂), 17.8 (C(CH₃)=CH₂).

N,N'-[butane-1,4-diylbis(1,3,4-oxadiazole-5,2-diylmethylene)]bis[2-(methacryloyloxy)-N,N-dimethylethan ammonium] bromide (5b). Compound **4** (1 g, 0.003 mol) was dissolved in dry acetone (40 mL) and the resulting solution was mixed with DMAEMA (0.81g, 0.005 mol), in the presence of hydroquinone. The mixture was gently refluxed for 40 hours on water bath. After completion of the reaction, the solvent was evaporated and the formed precipitate was filtered, washed with diethyl ether and dried. Yield of compound **5b**- 79%. Pasty mass, $R_f = 0.58$ ($\text{CHCl}_3/\text{CH}_3\text{OH} = 4/1$); IR (KBr, $\nu \text{ cm}^{-1}$): 1737.7 (C=O), 1647.1 (C=N), 1460 (C=C), 1022.2 (C-O-C). ¹H-NMR: 6.17-5.75 (2H, =CH₂), 4.40 (2H, N⁺CH₂CH₂), 3.71 (2H, N=C-CH₂N⁺), 3.39 (2H, N⁺CH₂CH₂), 2.78 (3H, N⁺CH₃), 2.50 (2H, N=C-CH₂CH₂), 1.90 (3H, C(CH₃)=CH₂), 1.14 (2H, N=C-CH₂CH₂). ¹³C-NMR: 174.9 (N=C-CH₂CH₂), 167.0 (C=O), 155.67 (N=C-CH₂N⁺), 136.0 (C(CH₃)=CH₂), 128.0 (=CH₂), 62.3 (N⁺CH₂CH₂), 59.0 (N=C-CH₂N⁺), 50.1 (N⁺CH₂CH₂), 43.1 (N⁺CH₃), 27.1 (N=C-CH₂CH₂), 24.3 (N=C-CH₂CH₂), 17.8 (C(CH₃)=CH₂).

Testing the biological activity**Determination of minimum inhibitory concentration (MIC)**

MIC of the synthesized compounds **3**, **4**, **5a** and **5b** was determined by using the broth microdilution method [39]. The solutions of each tested compounds were prepared in DMSO to get a concentration of 100 mg/mL. From this stock solution, serial dilutions of the compounds (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, and 0.097 mg/mL) were prepared. The MIC was recorded in each case as the minimum concentration of the compound, which inhibited the visible growth of the tested microorganism, and DMSO was used as a negative control.

Antibacterial activity

In order to evaluate the antibacterial activity eleven bacteria have been used: *Enterobacter aerogenes* (ATCC 51697), *Citrobacter freundii* (ATCC 8090), *Acetobacter aceti* (ATCC 15973), *Escherichia coli* (ATCC 25933), *Klebsiella*

pneumoniae (ATCC 70603), *Enterobacter cloacae* (ATCC 13047), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella enterica* (ATCC 13312) as gram negative bacteria and *Methicillin-resistant Staphylococcus aureus* (MRSA) (ATCC 43300), *Curtobacterium flaccumfaciens* (ATCC 53934) and *Staphylococcus wavy* as gram positive bacteria.

All microorganisms were obtained from Microbiology Laboratory, Institute of Biology, University of Dr. Moulay Tahar, Saïda. The bacterial strains were maintained on Muller-Hinton agar and the tested compounds were dissolved in DMSO to make a stock solution of 100 mg/mL and the other concentrations were prepared by dilution. A suspension of the organisms was introduced onto the surface of sterile agar plates, and then incubated at 37 °C for 18 – 24 hrs. After incubation, MIC was determined. All the tests were performed in triplicate.

Antifungal activity

Antifungal activity has been tested on three pathogenic fungi of tomato, such as *Fusarium oxysporum* MIAE 00123, *Fusarium commune* MIAE 00112 and *Fusarium rodelens* MIAE 00129 by the diffusion plate method [40].

All the fungal strains were obtained from collection of pure cultures of the laboratory of Applied Microbiology Faculty of Nature Sciences and Life, Oran University, Algeria. The test compounds were dissolved in DMSO (1 mL), mixed with potato dextrose agar PDA at concentrations of 50, 100, 150 and 200 µg/mL and poured onto sterile Petri dishes (with diameter of 9 cm). A 6 mm disc containing mycelia was transferred to the center of PDA plate. Three replications were maintained for each concentration and DMSO was used as a control solvent. Then the inoculated plates were incubated at 37°C for 4 days and the zone of inhibition was observed and measured. The relative inhibition rate of the circle mycelium compared to blank assay was calculated *via* the following equation:

$$\text{Relative inhibition rate (\%)} = [(T - C)/T] \times 100\% \quad (1)$$

where: *T* is the extended diameter of the circle mycelium during the blank assay,
C is the extended diameter of the circle mycelium during testing.

Results and discussion

Chemistry

The target compounds **5a** and **5b** were synthesized by a multiple-step procedure as shown in Figure 1. The synthetic route started from esterification of adipic acid **1** using ethanol in the presence of sulphuric acid. This ester reacted with hydrazine hydrate in ethanol to give adipic acid dihydrazide **3**. Bis-1,3,4-oxadiazole **4** was obtained by ring closure reaction upon treating acid hydrazide **3** with bromoacetic acid in phosphorus oxychloride. Finally, the products **5a** and **5b** were obtained by refluxing compound **4** with the appropriate tertiary amine (DEAEMA or DMAEMA) in acetone to give the corresponding quaternary ammonium salts.

The physicochemical data of the synthesized compounds (**2-4**, **5a** and **5b**) are presented in Table 1. The percentage yield of all the synthesized compounds was found to be in the range of 67-85%.

The purity of the synthesized compounds was checked by thin layer chromatography and melting points values. The structures of all the synthesized compounds were confirmed by IR, ¹H and ¹³C NMR spectral data.

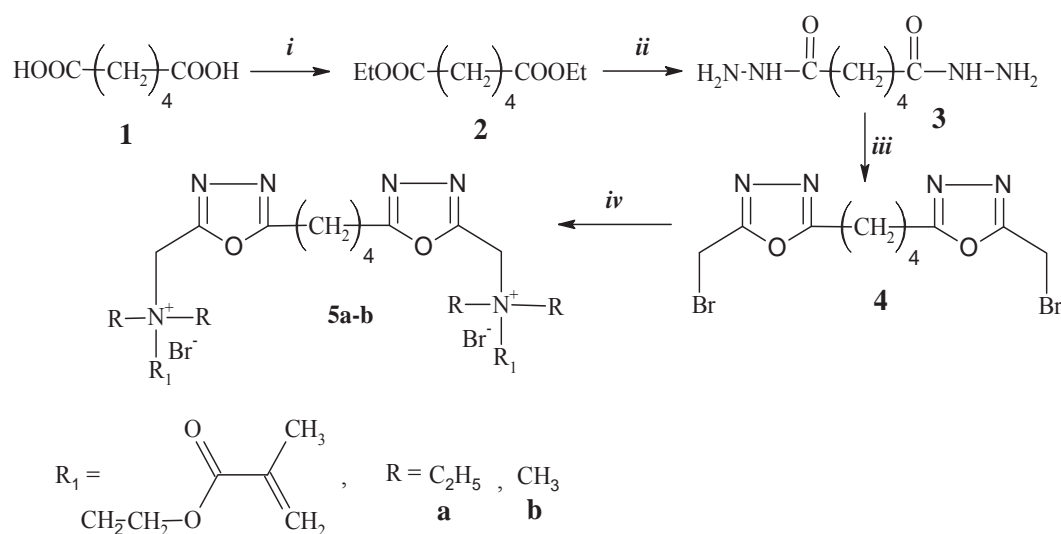


Figure 1. Synthetic route of the final compounds **5a-b**.

Reagents and conditions: (i) C₂H₅OH and conc. H₂SO₄, reflux for 8 h; (ii) NH₂NH₂·H₂O, C₂H₅OH, reflux for 5 h; (iii) POCl₃, BrCH₂COOH, reflux for 6 h; (iv) DMAEMA/DEAEMA, acetone, reflux for 40 h.

Table 1

Physical properties and analytical data of the synthesized compounds.					
Compound	Colour	Yield, %	Melting point, °C	Rf value	Solvent system CHCl ₃ /CH ₃ OH
2	Colorless	76	liquid	0.45	8/2
3	White	67	180-181	0.40	9/1
4	White	85	114-115	0.42	4/1
5a	Brown	82	semi-solide	0.50	4/1
5b	Brown	79	semi-solide	0.58	4/1

Biological activity**Antibacterial activity**

The synthesized compounds have been tested *in vitro* for their antibacterial activity against eleven bacteria, such as *Enterobacter aerogenes* ATCC 51697, *Citrobacter freundii* ATCC 8090, *Acetobacter acetii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Salmonella enterica* ATCC 13312 as gram negative bacteria and *Methicillin-resistant Staphylococcus aureus* (MRSA) ATCC 43300, *Curtobacterium flaccumfaciens* and *Staphylococcus wavy* as gram positive bacteria. The obtained results are summarized in Table 2.

Table 2

Minimal inhibitory concentration in mg/mL of synthesized compounds against tested bacterial strains by microdilution method.

Strains	Compound and values of MIC in mg/mL			
	3	4	5a	5b
<i>Enterobacter aerogenes</i> (ATCC 51697)	0.097	12.5	25	12.5
<i>Citrobacter freundii</i> (ATCC 8090)	12.5	0.39	0.19	0.097
<i>Acetobacter acetii</i> (ATCC 15973)	1.56	6.25	6.25	6.25
<i>Escherichia coli</i> (ATCC 25933)	6.25	1.56	100	50
<i>Klebsiella pneumoniae</i> (ATCC 70603)	6.25	0.78	25	25
<i>Enterobacter cloacae</i> (ATCC 13047)	3.12	12.5	12.5	12.5
<i>Pseudomonas aeruginosa</i> (ATCC 27853)	3.12	12.5	12.5	12.5
<i>Salmonella enterica</i> (ATCC 13312)	0.78	12.5	12.5	12.5
<i>Methicillin-resistant Staphylococcus aureus</i> (MRSA) (ATCC 43300)	25	0.78	0.78	0.78
<i>Curtobacterium flaccumfaciens</i> (ATCC 53934)	3.12	12.5	25	25
<i>Staphylococcus wavy</i>	1.56	12.5	12.5	25

As it is shown in Table 2, the results of evaluating the antibacterial properties suggest that all compounds have very good potential to act as antibacterial agents. The values of MIC ranged between: 0.097-100 mg/mL. Compounds **3** and **4** have shown the highest antibacterial activity against all tested microorganisms. The best activity was observed against *Citrobacter freundii* ATCC 8090 and MRSA by compound **5a** and **5b**, but relatively low antibacterial activity (MIC 50-100 µg/mL) was observed against *Escherichia coli*.

Antifungal activity

Similarly, the newly prepared compounds were screened *in vitro* for their antifungal activity against three pathogenic fungi of tomato, such as *Fusarium oxysporum* MIAE 00123, *Fusarium commune* MIAE 00112 and *Fusarium rodelens* MIAE 00129. The results of *in vitro* antifungal activities are presented in Table 3. The antifungal screening showed that the maximum inhibitory activity was found for compound **3** against all of tested fungi at concentration of 200 µg/mL, and compound **4** showed good activity against the tested fungi, compound **5a** and **5b**, showed moderate activity against *Fusarium oxysporum* and *Fusarium rodelens*. However, lower activity was observed for compound **5b** against *Fusarium commune*.

Table 3

Relative inhibition rate (%) of synthesized compounds against tested fungal strains.

Compound	Relative inhibition rate (%)											
	<i>Fusarium oxysporum</i>				<i>Fusarium commune</i>				<i>Fusarium rodelens</i>			
	Concentration in µg/mL											
	50	100	150	200	50	100	150	200	50	100	150	200
3	14.5	15.5	16.6	18.8	3.3	12.2	21.1	22.2	6.6	8.8	30	30
4	12.2	12.9	13.3	15.5	10	11.1	11.1	11.1	7.7	8.8	11.1	11.1
5a	5.5	6.6	6.6	7.7	3.3	5.5	6.6	6.6	6.6	6.6	7.7	8.8
5b	5.5	5.5	7.4	14.4	1.1	1.1	3.3	4.4	5.5	6.6	7.7	7.7

Conclusions

The present study reports on the successful synthesis and antimicrobial activity of some novel bis-1,3,4-oxadiazole compounds containing quaternary ammonium salt moiety. The 1,3,4-oxadiazole heterocycle was synthesized by ring-closing reaction, and quaternary ammonium salt was obtained by *N*-alkylation. All the synthesized compounds were structurally characterized using IR and NMR spectroscopic techniques. The newly synthesized compounds were evaluated for their antibacterial and antifungal activity. The results of biological tests indicated that most of the synthesized compounds exhibited promising results. Thus, the mixed compounds containing both 1,3,4-oxadiazole and quaternary ammonium salts moieties can serve as interesting lead molecules for further synthetic and biological evaluation.

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