

Synthesis of Ethyl Octahydrospiro (indene-2,3'-pyrrolizidine) Derivative and Assessment of Its Antibacterial Activity against *Escherichia coli* and *Staphylococcus aureus*

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Article Info	ABSTRACT
<p>Article type: Original Article</p> <p>Article History: Received: 2024/07/11 Revised: 2024/10/05 Accepted: 2024/12/19 Published Online: 2024/12/30</p> <p>✉ Correspondence to: Mehri Kouhkan</p> <p>Email: mehrikouhkan@gmail.com</p>	<p>Objective: The emergence of resistance in bacteria, and the existence of various types of infection and contamination in the hospital and community is the basis for research to find new antibacterial compounds.</p> <p>Materials: Ethyl Octahydrospiro[indene-2,3'-pyrrolizidine]-1,3-diones-pyrrolizidine]-2'-carboxylate (5) was synthesized during one-pot reaction of ninhydrin, proline and ethyl acrylate in ethanol solvent. The antibacterial effect of the synthesized derivative on the mentioned bacteria was investigated by agar plate culture and broth micro dilution methods and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for the synthesized derivative.</p> <p>Results: The analysis of the results showed that the MIC and MBC values of the newly synthesized compound against <i>Staphylococcus aureus</i> (<i>S. aureus</i>) bacteria were 1.56 and 3.12 µg/mL, respectively. In addition, the MIC and MBC values of the synthetic compound against <i>Escherichia coli</i> (<i>E. coli</i>) were 0.39 µg/mL and 0.78 µg/mL, respectively.</p> <p>Conclusion: The antimicrobial test results revealed that the synthesized compound at an equivalent concentration exhibited a higher inhibitory impact on the Gram-negative <i>E. coli</i> bacteria than Gram-positive <i>S. aureus</i>. The compound causes the rapid bactericidal effect on Gram-negative bacteria by destroying or disrupting the function of the outer membrane. This combination could be effective in treating infections caused by Gram-negative bacteria.</p> <p>Keywords: Chiral azomethylene ylide, 1,3 dipolar cyclization, indane, <i>Escherichia coli</i>, <i>Staphylococcus aureus</i></p>
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Introduction

Antibiotic resistance is a significant global health challenge that jeopardizes the effectiveness of current antibiotics. The emergence and spread of bacteria resistant to multiple antibiotics highlights the urgent need for new antibacterial agents that can overcome these resistance mechanisms. Estimates suggest that over 2 million infections caused by resistant strains occur globally each year, resulting in approximately 30,000 deaths in the United

States alone [1]. *Escherichia coli*, a common agent of urinary infections, exhibits significant resistance to various antibiotics like fluoroquinolones and cephalosporins. Also referred to as *E. coli*, this bacterium is a gram-negative bacillus belonging to the enterobacteriaceae family, commonly found in the intestines of warm-blooded animals. While most *E. coli* strains are harmless, certain serotypes like O157:H7 can lead to food poisoning and diarrhoea. Clinical manifestations of this infection include frequent urination, dysuria, haematuria, and pyuria. Notably, this bacterium possesses three enzymes with closely located genes, regulated by a single section [4-6].

Staphylococcus aureus (*S. aureus*) is a significant gram-positive, facultative anaerobic cocci and holds the utmost importance within the staphylococcus genus from a medical perspective. *S. aureus*, specifically methicillin-resistant *S. aureus* (MRSA), is accountable for numerous antibiotic-resistant infections. MRSA, a strain of golden *staphylococcus*, is impervious to betalactam antibiotics like Penicillin, Nafcillin, and Oxacillin, as well as Cephalosporins, rendering these antibiotics ineffective against infections associated with this strain. MRSA is notably more prevalent in hospitals, patient care facilities, individuals with open wounds, patients with prosthetic devices, and those with compromised immune systems. [2-5]

Hong et al. studied the one-step synthesis of spirooxindole-pyrrolidine derivatives and evaluated their antimicrobial and acetylcholinesterase inhibitory activities. Antimicrobial activities and acetylcholinesterase inhibitory

activity of the synthesized spiro compounds were also evaluated, where spiro (indoline-3,'3-pyrrolidine) 4d showed the strongest antimicrobial activity against several human pathogens [6].

In another study, the synthesis and examination of the antimicrobial properties of two derivatives of pyrrolidine-2,5-dione, connected to a dibenzobarrelen backbone at positions 3 and 4, were conducted by Fanjou and colleagues. A new diazo derivative of a pyrrolidine-2,5-dione connected to a dibenzobarrelen backbone at positions 3 and 4 was synthesized using the coupling method of the previously reported N-arylsuccinimide precursor with aryl diazonium ion aniline. The first derivative (MIC 128-32 µg/mL) and the second derivative (MIC 256-16 µg/mL) along with the initial precursor (MIC 128-64 µg/mL) exhibited significant antimicrobial activity against selected bacterial and fungal species compared to the standards nystatin (MIC 2-0.5 µg/mL) and ciprofloxacin (MIC 10.5-0.5 µg/mL) [7]. Isidine and indenic alkaloids in urine have important biological and pharmaceutical as well as antibacterial, antifungal and anticancer properties. More than seven thousand such compounds have been verified. Spiro cyclic oxindoles are valuable kinetic reaction intermediates which form main units of many pharmaceuticals and alkaloids [8]. Due to a variety of biological antibacterial, antimicrobial, antifungal, antiviral, and local anesthetic properties, these compounds have attracted the attention of several chemists [9]. Therefore, various synthetic pathways have been devised for their synthesis [10, 11]. Bipolar cycloaddition reaction is

an efficient method for the synthesis of 5-membered heterocycles and Spiro heterocycles such as pyrrolidines, piperazines, pyrrolizidines which are commonly present in natural products and biologically active compounds. Despite several methods for their synthesis, production of new Spiro heterocycles is still very popular and great effort has been devoted to their synthesis in several research works [12-14].

The new compound Ethyl Octahydrospiro[indene-2,3'-pyrrolizidine]-1,3-diones-pyrrolizidine]-2'carboxylate (5) which is produced via a one pot and spatioselective reaction as a single diastromer with high purity. Heterocycles containing several rings with one spiro center and four chiral centers are produced. Presence of indene and pyrrolizidine cyclic systems intensify the possibility of pharmaceutical properties in these compounds [15]. This compound was synthesized as a single diastromer with high purity and this paves the way for the investigation of their microbiological properties [16].

Application of new synthetic compounds and evaluation of their efficiency are research hot topics and deserve to be extensively investigated. The aim of this research was to investigate and evaluate the new synthetic Ethyl Octahydrospiro[indene-2,3'-pyrrolizidine]-1,3-diones-pyrrolizidine]-2'carboxylate (5) on *S. aureus* and *E. coli* bacteria.

Methods and Materials

Synthesis of new compounds (5)

In the beginning of the preparation of Ethyl Octahydrospiro[indene-2,3'-pyrrolizidine]-1,3-diones-pyrrolizidine]-2'carboxylate (5) was synthesized as follows: in a 50cc round bottom vessel, a mixture of ninhydrin (0.178g, 1mM), proline (0.115g, 1mM) and methyl metacrylate (0.086g, 1mM) in pure ethanol was prepared and put in microwave for 2 minutes. The reaction took place by emitting CO₂ gas. Reaction process and its completion was explored with chromatography technique, after the completion of the reaction, the solvent was discarded under vacuum and dark yellow crystals were extracted from the mixture [fig.1].

Preparation of microbial suspension

To do so, 24-h pure bacterial cultivation was applied. About 3 or 4 bacterial colonies were taken and dissolved in sterile Phosphate buffered saline (PBS) in a tube and for the homogeneity of the suspension, vortex was performed. Then, using a spectrophotometer, initial bacterial concentration in the suspension was set at to be 1.5×10⁸ CFU/mL. Then, final bacterial concentration of 5×10⁵ CFU/mL was prepared from the initial suspension for broth micro dilution tests. Optical absorption of 0.08-0.13 in the suspension at wavelength 625 nm indicated bacterial concentration of 1.5×10⁸ CFU/mL in the suspension.

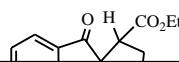
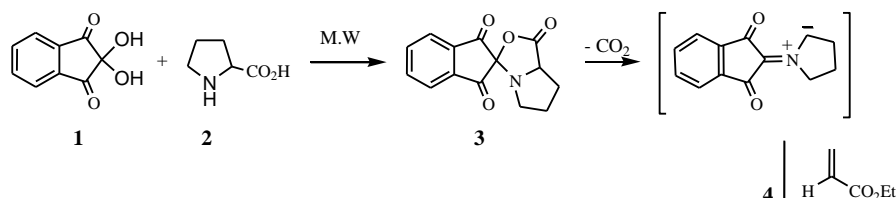


Fig. 1. synthesis of the new compound Ethyl Octahydro spiro[indene-2,3'-pyrrolizidine]-1,3-diones-pyrrolizidine]-2'carboxylate (5)

Preparation of Moller-Hinton broth

Using the information provided in on the container of the cultivation medium, the amount of required powder was calculated and weighted using a digital scale and dissolved in the required amount of water. Then, the mixture was heated on flame and autoclaved after being divided into test tubes. After autoclave, the tubes were cooled and stored in fridge for later use.

Preparation of Moller-Hinton agar (MHA) medium:

Using the information provided in on the container of the cultivation medium, the amount of required powder was calculated and weighted using a digital scale and dissolved in the required amount of water in a clean round bottom vessel. Then, the mixture was heated on flame and the mixture vessel was put in autoclave for sterilization. After autoclave, the tubes were cooled (in room temperature to maximum temperature of 50°C) and placed in disposable sterilized plates (this was done beside the flame under hood). After setting, plates were stored in fridge for later use.

Investigation of antimicrobial activities Minimum inhibitory concentration (MIC)

MIC is the minimum concentration of an antimicrobial substance that can inhibit microbial growth in laboratory conditions. The test followed the broth microdilution method and adhered to CLSI protocols [17]. Serial dilutions of Ethyl Octahydro spiro[indene-2,3'-pyrrolizidine]-1,3-diones-pyrrolizidine]-2'carboxylate (5) ranging from 0.19 to 25 µg and control antibiotics ranging from 0.03 to 200 µg were prepared in 100 µl of Moller-Hinton broth (MHB) in a 96-well microplate. Each well received 100 µL of a prefabricated bacterial suspension with 5×10^5 CFU/mL and was then incubated at 37°C for 24 hours. The MIC of the newly synthesized compound and antibiotics was determined using microplate spectrophotometry (Epoch-BioTek Co., Winooski, VT, USA) by measuring light absorption at a wavelength of 625 nm. Sterile, bacteria-free Mueller-Hinton broth (MHB) served as the negative control, while MHB with bacteria was used as the positive control. Vencomycin and gentamicin antibiotics were employed for *S. aureus* and *E. coli*, respectively, as controls for comparison with the test results.

Minimum bactericidal concentration (MBC)

MBC is defined as the minimum concentration of an antibacterial compound which can kill 99.99% of the bacteria. MBC of newly synthesized compound (5) and antibiotics were determined based on CLSI protocol. To perform these tests, 10 µl of each cells prepared based on MIC approach were cultivated in MHA medium and incubated at 37°C for 24 h. then, the colonies were counted after 18-24 h.

Figure 2. Mass spectrum of compound (5)

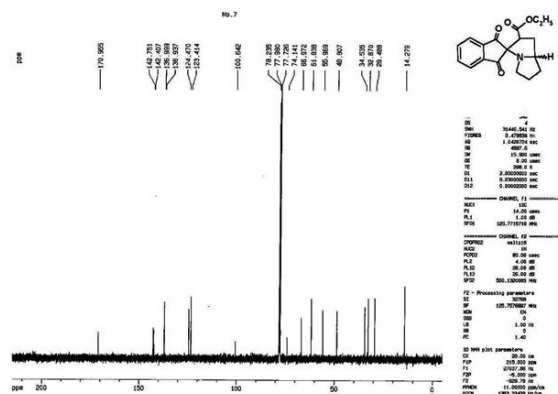


Figure 3. ¹HNMR (CDCl₃) spectrum of compound (5)

Results

In this research, Ethyl Octahydro spiro[indene-2,3'-

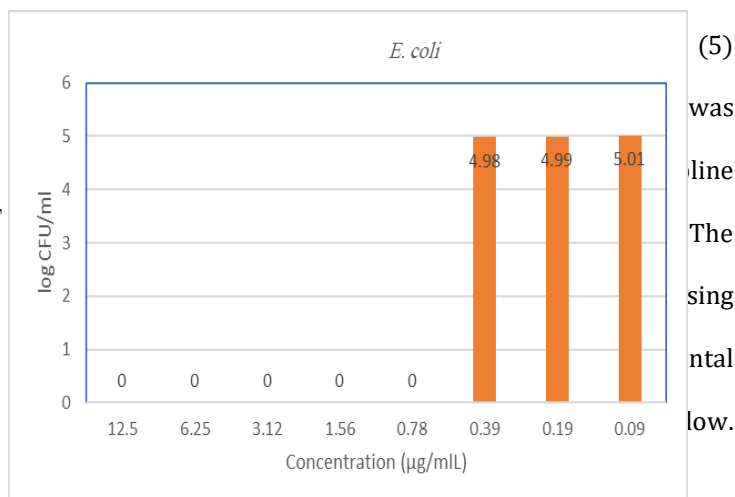


Figure 4. Antimicrobial effect of compound (5) on *E. coli* bacteria

Ethyl Octahydro spiro[indene-2,3'-pyrrolizidine]-1,3-ones-pyrrolizidine]-2'carboxylate (5): Dark-yellow ism (EtOH), 81–83% yield, m.p. 111–113 °C. 1H NMR (CDCl₃, 500 MHz) δ 0.77 (3H, t, J = 7.1 Hz, CH₃ (de1)), 0.87 (3H, t, J = 7.1, CH₃ (de2)), 1.57-1.76 (1H, m, 7'-CH), 1.78-1.89 (1H, m, 7'-CH), 1.93-2.01 (2H, m, 6'-CH₂), 2.34-2.46

(2H, m, 1'-CH₂), 2.51-2.55 (1H, m, 5'-H), 2.65-2.69 (1H, m, 5'-H), 3.73-3.87 (3H, m, OCH₂, 2'-H), 3.93-4.10 (1H, m, 7a'α-H), 7.84-7.88 (2H, m, ArH), 7.91-7.95 (1H, m, ArH), 8.01-8.04 (1H, m, ArH), (de ratio: 95:5). ¹³C NMR (CDCl₃, 125 MHz) δ 14.28 (CH₃), 29.49, 32.87, 34.54, 48.81, 55.97, 61.84 (OCH₂), 66.97 (Cspiro), 74.14 (CH-N), 123.41, 124.47, 136.98 (4CH, aromatic), 142.41, 142.75 (2Cipso, aromatic), 170.95, 202.86, 203.92 (3C=O). IR (ν_{max}/cm⁻¹, KBr) 1700, 1743 (C=O). MS (m/e, %) 313 (M⁺, 88), 240 (M⁺-CO₂Et, 45), 212 (240-C₂H₄, 46), 184 (212-C₂H₄, 46). Anal. calcd. for C₁₈H₁₉N₄O₄ (313.348): C, 68.99; H, 6.11; N, 4.47; O, 20.42%. Found: C, 69.00; H, 6.12; N, 4.47; O, 20.43%.

MIC of compound (5) against standard strain of *E. coli* bacterium were found to be 0.39 µg/mL. MIC of compound (5) against *S. aureus* were 1.56 µg/mL, respectively (Table 2).

MBC of compound (5) against standard strain of *E. coli* and *S. aureus* bacterium were found to be 0.78 and 3.12 µg/mL.

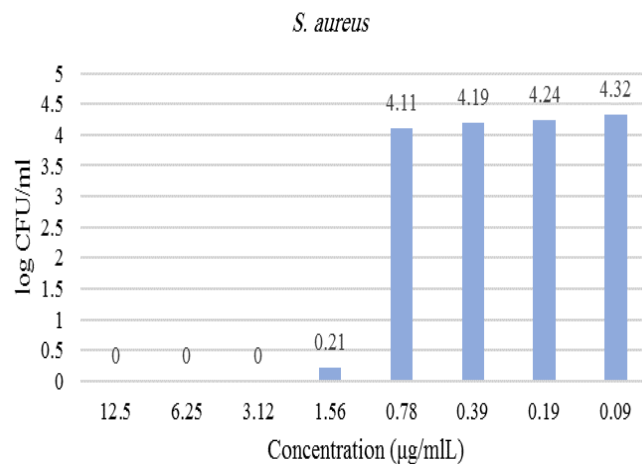


Figure 5. Antimicrobial effect of compound (5) on *S. aureus* bacteria

Table 1. Comparison of the minimum lethal and inhibitory concentrations of the new synthetic substance (5) with control antibiotics

	The new synthesized compound (5)		Gentamycine		Vancomicine	
	MIC	MBC	MIC	MBC	MIC	MBC
<i>E. coli</i>	0.39(µg/µL)	0.78(µg/µL)	3.2(µg/µL)	6.4(µg/µL)	-	-

<i>S. aureus</i>	1.56($\mu\text{g}/\mu\text{L}$)	3.12($\mu\text{g}/\mu\text{L}$)	-	-	0.2($\mu\text{g}/\mu\text{L}$)	0.2($\mu\text{g}/\mu\text{L}$)
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Discussion

The analysis of the ^1H NMR spectrum is an indispensable tool in elucidating the configuration and spatial arrangement of chemical products. Within this framework, the region between 3.76 and 3.86 ppm reveals a notable resonance linked to the 2-H proton, which is characterized by a doublet appearance. The coupling constants observed 6.6 Hz and 12.6 Hz provide insight into the interactions that the 2-H proton has with its neighboring protons, designated as α_1 and β_1 , respectively.

The resultant splitting pattern serves not only to clarify the interactions between these protons but also supports the selectivity of the reaction under investigation. The manifestation of the doublet indicates that the 2-H proton's environment is influenced by both adjacent protons, thereby affirming a distinct stereochemical configuration. This level of detail is crucial, as it aligns with and substantiates theoretical models positing the regioselectivity of the reaction.

Notably, for several Gram-positive and Gram-negative bacteria evaluated, Substance (5) demonstrates lower MBC values relative to the control antibiotics. This indicates its potential viability as an effective alternative or adjunctive treatment, especially in light of increasing antibiotic

resistance observed in clinical pathogens. Moreover, the synergistic properties of Substance (5), when combined with existing antibiotics, warrant further investigation to enhance therapeutic outcomes.

The comparative analysis presented underscores the promising antimicrobial properties of the new synthetic substance (5). While it exhibits significant potential as an alternative therapeutic agent, further studies are imperative to fully elucidate its mechanism of action, safety profile, and applicability in clinical settings. The continued exploration of such novel compounds is vital in addressing the growing challenge of antibiotic resistance and improving patient care outcomes.

The MIC of new synthetic compounds (5) on gram-negative *E. coli* and gram-positive *S. aureus* were observed 0.39 and 1.56 $\mu\text{g}/\mu\text{L}$, effectively inhibiting the growth of both bacteria. The MBC of compounds (5) in *E. coli* and *S. aureus* were observed to be respectively 078 and 3.12 $\mu\text{g}/\mu\text{L}$.

MIC, this compound at higher concentrations could kill gram positive bacterium *S. aureus*. In other words, to kill gram positive bacteria such as *S. aureus*, higher concentrations of compounds (5) is required. The reason for this was in the structural differences of cell wall in the two

gram positive and gram negative bacteria. It seems that penetration of compounds (5) into cell wall and its later attachment to target position is easier in a gram negative bacterium than a gram positive bacterium with a thicker peptidoglycane wall which could provide physical resistance to the penetration of compounds (5).

Conclusion

The antimicrobial test results revealed that the synthesized compound at an equivalent concentration exhibited a higher inhibitory impact on the Gram-negative *E. coli* bacteria than Gram-positive *S. aureus*. However, it demonstrated a more potent effect on the Gram-negative bacteria. The compound causes the rapid bactericidal effect of Gram-negative bacteria by destroying or disrupting the function of the outer membrane. This combination could be effective in treating infections caused by Gram-negative bacteria.

Abbreviation

MHB: Müller-Hinton nutrient medium

MIC: minimum inhibitory Concentration

MBC: minimum bactericidal. Concentration

Conflict of interest

None of the authors have any conflict of interest to declare.

Consent for publications

All authors approved the final manuscript for publication.

Availability of data and material

Data are available on request from the authors.

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