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Antibacterial Potential of Ethyl 3,5-Dibromoorsellinate, a Derivative of Diphenyl Ethers from *Graphis handelii*, against Methicillin-Resistant *Staphylococcus aureus*

Dao Dinh Nguyen, Thuc-Huy Duong, Thi-Phuong Nguyen, Huy Truong Nguyen,* and Chuong Hoang Nguyen*

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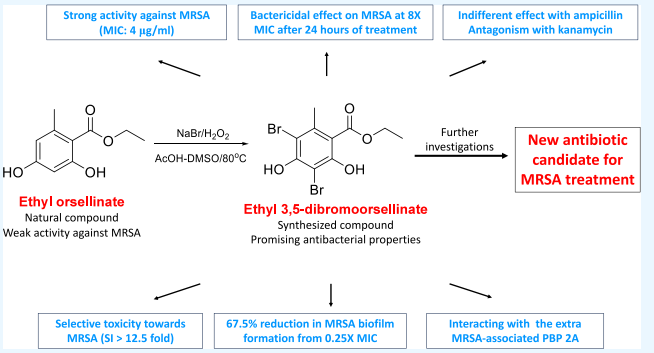
ABSTRACT: *Staphylococcus aureus* is a human pathogen responsible for a variety of diseases, from skin, soft tissue, and lung infections to severe cases such as meningitis, infective endocarditis, and bacteremia. The high level of antibiotic resistance in these pathogens, exemplified by methicillin-resistant *Staphylococcus aureus* (MRSA), necessitates the development of effective antibiotics. Thus, this work introduced the chemical synthesis of ethyl 3,5-dibromoorsellinate, a derivative of ethyl orsellinate from the lichen mycobiont of *Graphis handelii*, and its effectiveness against MRSA was assessed. Results showed that ethyl 3,5-dibromoorsellinate efficiently inhibited MRSA with a minimum inhibitory concentration (MIC) of 4 μg/mL, and the time-kill analysis showed the bactericidal effect of ethyl 3,5-dibromoorsellinate on MRSA at 8× MIC after 24 h. The compound also exhibited selective activity against MRSA compared with the human cell line, with a selectivity index of 12.5-fold. While ethyl 3,5-dibromoorsellinate exhibited an indifferent effect with ampicillin, this compound demonstrated antagonistic effects with kanamycin in the synergistic assessment. Additionally, ethyl 3,5-dibromoorsellinate demonstrated antibiofilm activity against MRSA starting from 0.25× MIC. The molecular docking investigation illustrated that ethyl 3,5-dibromoorsellinate binds with the penicillin-binding protein 2A of MRSA with a free energy of −42.5 to −45.7 kcal/mol. Given its promising antibacterial activities, ethyl 3,5-dibromoorsellinate warrants further investigation as a potential antibiotic option against MRSA.



INTRODUCTION

Staphylococcus aureus bore the name of methicillin-resistant *S. aureus* (MRSA) when *S. aureus* strains resistant to methicillin were clinically isolated in the United Kingdom in 1961 after only 2 years of introduction of methicillin in the treatment of *S. aureus* infection.[1] The term "MRSA" is still used to describe these bacterial pathogens even now when *S. aureus* has been found to become resistant to different classes of antibiotics[2] and not merely to methicillin, a beta-lactam antibiotic that is no longer used in clinical practice.[3] Actually, MRSA has also been considered a multidrug-resistant pathogen.[4] A recent systemic analysis of the burden of bacterial antimicrobial resistance (AMR) at a global scale showed that MRSA, which is one of the six leading bacterial pathogens, caused greater than 100,000 deaths in the total number of 929,000 deaths attributable to AMR by the six leading bacterial pathogens in the year of 2019.[5] MRSA possesses several AMR mechanisms, such as the production of modifying enzymes, modifications of target binding sites, efflux pumps, and biofilm formation,[6] with the latter involved not only in AMR but also in spreading infection in intrahost and interhost manners.[7,8] Due to its pathogenic properties and AMR, the World Health Organization in 2017 identified MRSA as one of the key antibacterial-resistant pathogens that urgently need new antibiotics for treatment.[9] And yet, new antibiotics introduced in clinical practice have not been sufficient to meet the critical requirement. The golden era of antibiotics from the 1950s and 1960s of the 20th century witnessed a huge number of antibiotics discovered, and one-half of them are currently used for the treatment of bacterial infection.[10] Unfortunately, there has been a sharp decline in antibiotic development since the golden era, and this unwanted decrease has been refractory up to now, as shown by the number

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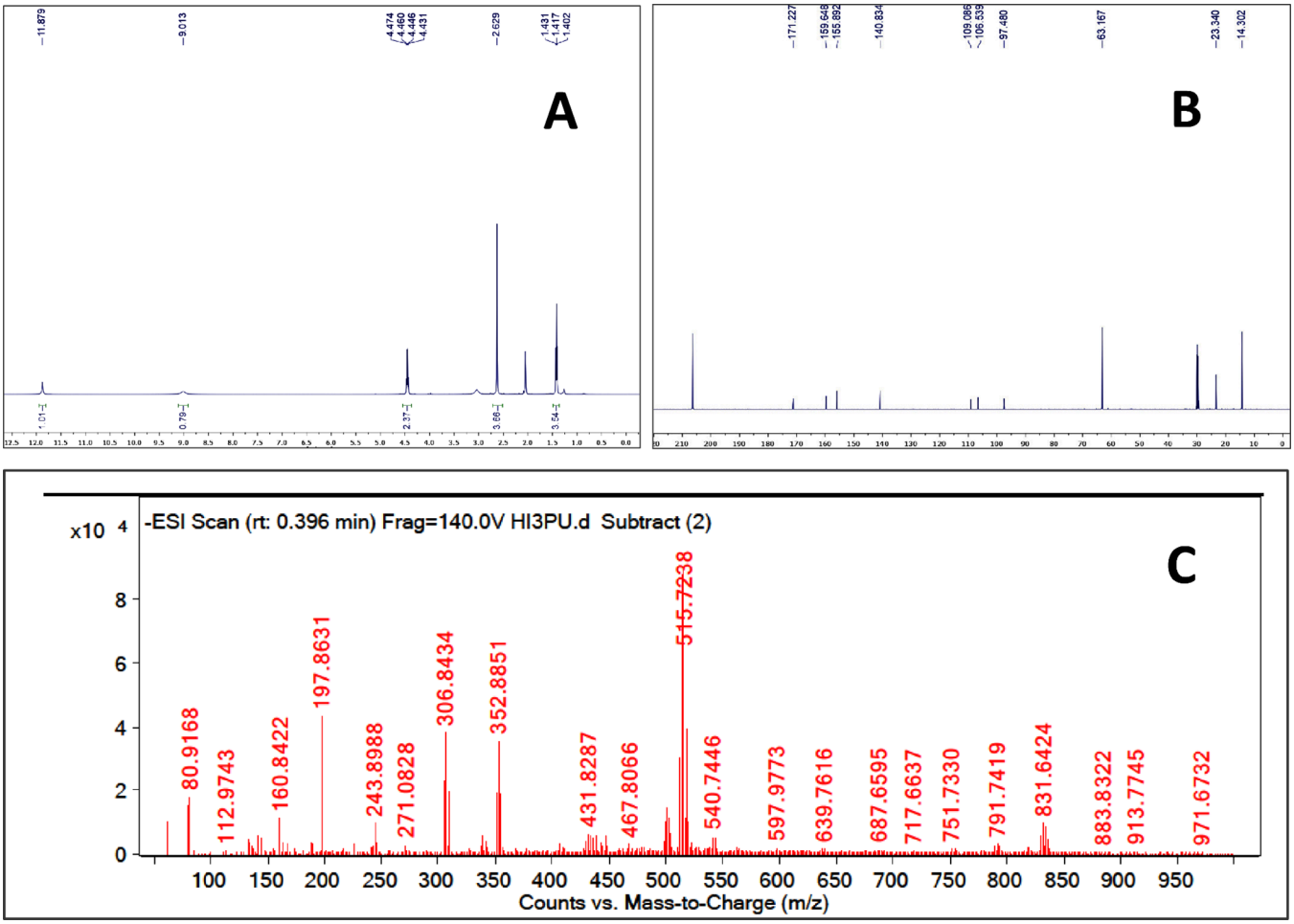

Figure 1. Spectral analysis of ethyl 3,5-dibromoorsellinate. (A) ^{1}H NMR (acetone-d_6, 500 MHz) spectrum. (B) ^{13}C NMR (acetone-d_6, 125 MHz) spectrum. (C) HRESIMS spectrum.

of antibiotics approved by the Food and Drug Administration (FDA) in the period from 2008 to 2017.[11] On the one hand, the shortage of antibiotics has worsened the treatment of infection caused by AMR pathogens,[12] and on the other hand, it has fueled resistance to antibiotics by their overconsumption.[13] If no action at a worldwide scale is taken, the annual death toll from AMR could reach 10 million by the year 2050.[14]

Hopefully, different initiatives for facilitating the early-stage discovery of new antibiotics have been implemented to respond to the antibiotic crisis.[15] The screening or generation of hits, which is the first step in the early-stage discovery, plays an important role in presenting a promising candidate for further steps.[16] In this step, antimicrobials are sought from several sources, such as plants, microbes, and lichens, to be developed as hits.[17] Although antimicrobial agents from natural origins are popular sources for the search for new antibiotics, those originating from the artificial modification of natural backbones demonstrate themselves to be reliable sources as well. Analogs of dihydrazones by chemical synthesis were shown to have remarkable antibacterial, antibiofilm, and cell membrane damaging properties, leading to the bactericidal effect on Gram-negative bacteria.[18] Similarly, the activities of quinazolinones, which are heterocyclic compounds with diverse biological activities, including antimicrobial activity, were considerably enhanced by different approaches, such as Schiff's base reaction[19] or coupling with amino acid by chemical conjugation.[20,21] Not only the modifications of natural backbones but also the combination between a drug delivery system, chitosan silver nanoparticles, with active compounds such as benzodioxane midst piperazine and thymol showed promising antibacterial and antibiofilm activities, in particular against MRSA.[22,23] In addition, several strategies in combinatorial chemistry have been proposed for existing antibiotics to overcome antibiotic resistance as well as to attack new targets in this pathogen.[24]

Still in the screening or generation of hits step, the following nonexhaustive experiments should be performed after an antimicrobial was selected, including the antimicrobial spectrum on relevant Gram-positive and Gram-negative bacteria, minimum inhibitory concentration against target bacteria, cytotoxicity against animal cell lines, bactericidal or bacteriostatic properties, mode of action, rate of resistance, stability, bioavailability, etc. These preliminary data were essential for defining a hit before it could undergo further stages of the drug discovery process.[25] In these evaluation experiments, the mode of action study is essential because it reveals the target−drug interaction for fulfilling the requirements of a preclinical candidate dossier and regulatory requirements. It also facilitates further rational optimization of chemical scaffolds for stronger effects but less toxicity.[26] A multitude of approaches in different areas of genetics, genomics, microbiology, chemical biology, biophysics, and bioinformatics were employed to elucidate the

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mode of action, with the latter one being extensively applied to clarify the structure−activity relationship (SAR) as mentioned in refs 21, 23, and 27.

Inspired by these above-mentioned ideas, the chemical synthesis of a diphenyl ether compound of the lichens *Graphis handelii* was carried out in this study, leading to a new derivative, ethyl 3,5-dibromoorsellinate. Then, the initial evaluation of the antibacterial activity of this compound against MRSA was performed to reveal its potency for future drug development.

RESULTS AND DISCUSSION

New Derivative Compound from Ethyl Orsellinate. In a previous work, the four compounds, including graphinone A, handelone, 4-*O*-methylhiascic acid, and ethyl orsellinate, isolated from the lichen *Graphis handelii* were tested for antimicrobial activity against *S. aureus*. The results showed that only ethyl orsellinate exhibited activity against MRSA with an inhibition zone of 13 mm.[28] However, this compound showed weak activity against *S. aureus* compared with those of other isolated compounds in the literature. Gyrophoric acid, a polyphenolic depside extracted from the lichen *Parmotrema indicum*, showed an inhibition zone to methicillin-resistant *S. aureus* (MRSA) of 20 mm at the concentration of 1 mg/mL, and the volume was 50 μL.[29] Vitexin, isovitexin, and orientin, the phytochemicals derived from *Santalum album*, showed *in vitro* antibacterial activity to *S. aureus* with inhibition zones of 19.8 mm (for vitexin), 18.53 mm (for isovitexin), and 18.16 mm (for orientin) at the 50 μg/disc concentration for each compound,[30] which was equivalent in quantity to the compounds graphinone A, handelone, 4-*O*-methylhiascic acid, and ethyl orsellinate in the previous study. Due to the weak activity, a structure modification strategy for improving its antibacterial activity was performed, leading to the derivative compound, and the spectral analysis of this compound is shown in Figure 1.

The derivative compound was a white, amorphous powder. Its structural elucidation was determined through HRESIMS and 1D-NMR. Spectroscopic data are shown as follows: HRESIMS m/z 352.8851 $[M + H]^+$ (calcd. for $C_{10}H_{11}Br_2O_4$ 352.9024). ^{1}H NMR (500 MHz, acetone-d_6) δ_H 11.88 (H, s, 2-OH), 9.01 (H, s, 4-OH), 4.45 (2H, q, J = 7.0 Hz, H-9), 2.63 (3H, s, H-8), 1.41 (3H, t, J = 7.0 Hz, H-10). ^{13}C NMR (125 MHz, acetone-d_6) δ_C 171.2 (C-7), 159.6 (C-2), 155.9 (C-4), 140.8 (C-6), 109.1 (C-1), 106.5 (C-5), 97.5 (C-3), 63.2 (C-9), 23.3 (C-8), 14.3 (C-10). The ^{1}H NMR spectrum revealed the presence of a hydrogen-bonded hydroxy group at δ_H 11.88 (1H, s, 2-OH), a phenolic hydroxy group at δ_H 9.01 (1H, s, 4-OH), and an ethyl group [δ_H 4.45 (2H, q, J = 7.0 Hz, H-9), 2.63 (3H, s, H-8), 1.41 (3H, t, J = 7.0 Hz, H-10)] that correspond precisely to the starting material, ethyl orsellinate. The ^{13}C NMR spectrum exhibited 10 carbon signals, including a carbonyl ester carbon at δ_C 171.2 (C-7) and six substituted aromatic carbons: δ_C 159.6 (C-2), 155.9 (C-4), 140.8 (C-6), 109.1 (C-1), 106.5 (C-5), and 97.5 (C-3). Among them, two carbons at δ_C values of 159.6 (C-2) and 155.9 (C-4) were oxygenated. The presence of two bromine atoms was determined by HRESI mass data, This provided isotopic values for two bromine atoms.

Brominated substitution was carried out using sodium bromide and hydroperoxide on ethyl orsellinate (**1**) to yield product **1a** (Figure 2). This reaction was chosen to enhance the alpha-glucosidase inhibition of brominated derivatives, as previously reported. Notably, the bromination of flavonoids, kamatakenin, and ayanin significantly increased their alpha-glucosidase inhibition.[31] Furthermore, brominated lichen

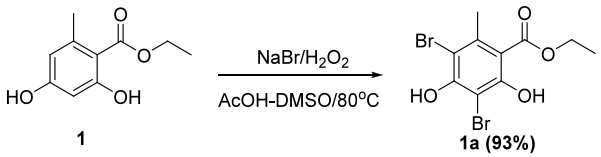

Figure 2. Synthesis scheme from ethyl orsellinate to ethyl 3,5-dibromoorsellinate.

metabolites exhibited significantly greater potency compared to their corresponding parent compounds.[32] The modification of ethyl orsellinate was undertaken with the objective of enhancing the limited antibacterial efficacy of the original compound against MRSA.

Antibacterial Activity of Ethyl 3,5-Dibromoorsellinate against *S. aureus*. The antibacterial activity of ethyl 3,5-dibromoorsellinate was tested against *S. aureus*, specifically on the MRSA strain and the *S. aureus* ATCC 25923 strain by agar diffusion technique, and it showed the inhibition zone of 30 mm for the MRSA strain and 31 mm for the *S. aureus* ATCC 25923 strain at the concentration of 1 mg/mL and the volume used of 50 μL (Figure 3). Roughly calculated, the antibacterial activity of

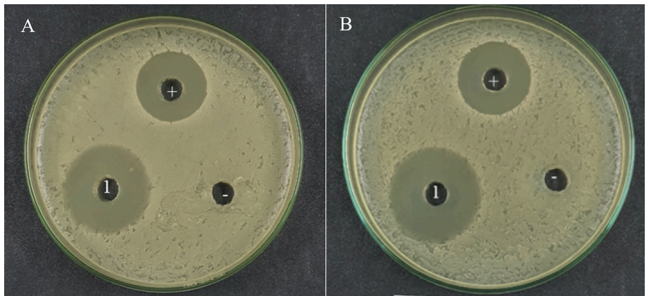

Figure 3. Antibacterial activity of ethyl 3,5-dibromoorsellinate against *S. aureus* strains. (A) *S. aureus* ATCC 25923. (B) MRSA.

the derivative from the compound ethyl orsellinate was 2.38-fold higher than that of the precursor, showing that the structural modification was effective. The successful enhancement of the antimicrobial activity by bromination was reported in the literature. Not only did chemical bromination of flavonolignans enhance the inhibition of quorum sensing and biofilm formation in *S. aureus* and *P. aeruginosa*, but also two of the brominated flavonolignans (6,8,21-tribromosilybins A and B) could sensitize the gentamicin-resistant *S. aureus*.[33] The presence of two bromine atoms on methyl orsellinate by chemical synthesis leading to methyl 3,5-dibromoorsellinate yielded a larger inhibition zone of 29 mm compared to that of the precursor, which was 13 mm.[34]

Besides the two *S. aureus* strains, the antibacterial activity of the new derivative was also tested on other human bacterial pathogens. Ethyl 3,5-dibromoorsellinate showed no inhibition zone to *Escherichia coli*, *Enterobacter cloaceae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Enterococcus faecium*, whereas apramycin (1 mg/mL) showed inhibition zones ranging from 17 to 27 mm on these bacterial pathogens (Table 1). Ethyl 3,5-dibromoorsellinate exhibited selective activity to *S. aureus* among seven bacterial pathogens in this study. The precursor of ethyl 3,5-dibromoorsellinate is a monoaromatic compound, and these compounds seemed to exert effects on Gram-positive bacteria rather than Gram-negative bacteria. In the work of Evans et al., a series of analogues

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Table 1. Antibacterial Spectrum of Ethyl 3,5-Dibromoorsellinate

		inhibition zones (mm)		
no.	bacteria	DMSO	apramycin	ethyl 3,5-dibromoorsellinate
1	*Escherichia coli*	0	22	0
2	*Enterobacter cloaceae*	0	22	0
3	*Acinetobacter baumannii*	0	20	0
4	*Pseudomonas aeruginosa*	0	27	0
5	*Klebsiella pneumoniae*	0	24	0
6	*Enterococcus faecium*	0	17	0
7	*S. aureus* ATCC 25923	0	22	31
8	MRSA	0	23	30

of totarol, which was a monoaromatic compound, were studied for their antibacterial activities on three Gram-positive bacteria comprising beta-lactamase-positive and high-level gentamycin-resistant *Enterococcus faecalis*, penicillin-resistant *Streptococcus pneumoniae*, and methicillin-resistant *Staphylococcus aureus* and one Gram-negative multidrug-resistant *Klebsiella pneumoniae*. There were about 7−10 compounds showing antibacterial activity against the three Gram-positive bacteria with MIC values from 2 to 8 μg/mL among 23 derivative compounds from totarol. For the Gram-negative bacterium, all of these compounds showed MIC values of over 32 μg/mL to *K. pneumoniae*.[35] The narrow spectrum of ethyl 3,5-dibromoorsellinate could be advantageous for the treatment of bacterial infection because it could avoid the drawbacks of spreading antibiotic resistance across multiple bacteria and deleterious effects on the host microbial flora, which are associated with broad-spectrum antibiotics,[36] although the broad-spectrum antibiotics offer treatment over a wide range of bacterial infections and conditions.[37]

Due to the selective antibacterial activity toward *S. aureus*, the preliminary antibacterial activity properties of ethyl 3,5-dibromoorsellinate such as minimum inhibitory concentration, time-kill analysis, synergistic effects with antibiotics, selective toxicity toward bacteria, inhibition of biofilm formation, and molecular docking analysis were performed on MRSA to elucidate the potential of this compound to be further developed as an antibacterial agent to MRSA.

The Minimum Inhibitory Concentration of Ethyl 3,5-Dibromoorsellinate to *S. aureus*. MIC is defined as the lowest concentration (in μg/mL) of an antimicrobial agent inhibiting completely the growth of a given strain of bacteria.[38] The lower the MIC is, the more potent is the antimicrobial agent. Ethyl 3,5-dibromoorsellinate had an MIC value of 4 μg/mL for both the *S. aureus* ATCC 25923 strain and the MRSA strain. Two antibiotics, ampicillin and kanamycin, were used as the controls for the MIC experiment. Ampicillin showed MIC values of 16 μg/mL to the *S. aureus* ATCC 25923 strain and 128 μg/mL to the MRSA strain. Kanamycin had an MIC value of 1 μg/mL for the two *S. aureus* strains. The higher MIC value of ampicillin on the MRSA strain than the ATCC strain was logical because MRSA was shown to be associated with resistance to nearly all beta-lactam antibiotics.[39] On the contrary, kanamycin is an aminoglycoside antibiotic acting primarily on the 30S ribosomal subunit, resulting in the prevention of protein elongation in the bacterial translation, which was different from the disruption of bacterial cell wall formation as done by beta-lactam antibiotics.[40]

The MIC value of ethyl 3,5-dibromoorsellinate of 4 μg/mL for the *S. aureus* strains was encouraging for the search for new antibiotic candidates against *S. aureus* infection. Some current antibiotics for the treatment of *S. aureus* infection have MIC values comparable to those of *S. aureus*, which are comparable to the MIC value of ethyl 3,5-dibromoorsellinate. Gentamicin, norfloxacin, linezolid tetracycline, doxycillin, and minocycline had an MIC breakpoint of 4 μg/mL for *S. aureus* to be susceptible to these antibiotics. Other antibiotics had even higher MIC breakpoints for *S. aureus* to be susceptible, such as trimethoprim, chloramphenicol, tecoplanin (breakpoint of 8 μg/mL) or nitrofurantoin (breakpoint of 32 μg/mL).[41] The MIC result of ethyl 3,5-dibromoorsellinate on *S. aureus* was used for the following time-kill analysis to evaluate the effect of the concentration range of this compound on the growth and death of MRSA.

Antibacterial Effects of Ethyl 3,5-Dibromoorsellinate on MRSA over Time. The MRSA strain was used in the time-kill experiment over a period of 24 h. At 4× and 8× MICs, the compound showed a bacteriostatic effect on MRSA as the $\log_{10}$ CFU/mL over time remained roughly the same as the starting log CFU/mL concentration from 0 to 12 h of culture. From the time point of 12 h of culture, there was a divergence in the MRSA cell counts between 4× MIC and 8× MIC of ethyl 3,5-dibromoorsellinate with increasing cell count at 4× MIC but

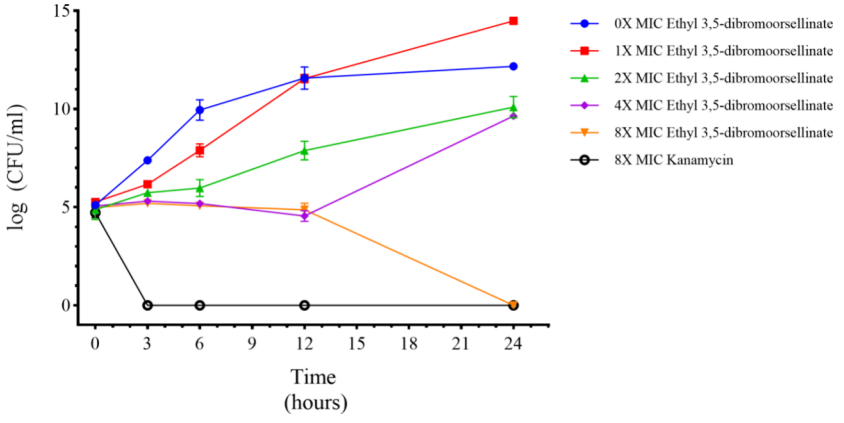

Figure 4. Time-kill analysis of ethyl 3,5-dibromoorsellinate to MRSA

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Table 2. Synergistic Study of Ethyl 3,5-Dibromoorsellinate with Antibiotics

no.	combination	MIC alone (μg/mL)	combined MIC (μg/mL)	FIC	interpretation
1	ethyl 3,5-dibromoorsellinate	4	2	1.5	indifference
	ampicillin	128	128		
2	ethyl 3,5-dibromoorsellinate	4	4	1.25	indifference
	ampicillin	128	32		
3	ethyl 3,5-dibromoorsellinate	4	2	8.5	antagonism
	kanamycin	1	8		
4	ethyl 3,5-dibromoorsellinate	4	4	2	antagonism
	kanamycin	1	1		

decreasing cell count at 8× MIC. The cell count continued declining up to 24 h of culture at 8× MIC, and a reduction in the MRSA viable cell count relative to the initial inoculum over 3 $\log_{10}$ CFU/mL was observed, demonstrating a bactericidal effect of ethyl 3,5-dibromoorsellinate at this concentration. For comparison, kanamycin at 8× MIC showed an early bactericidal effect when the bacterial cell count dropped more than 3 $\log_{10}$ CFU/mL after only 3 h of culture, showing the fast-killing rate of this antibiotic. The lower MIC values, i.e., 1× and 2× MICs, of ethyl 3,5-dibromoorsellinate had no effect on the growth of MRSA at all of the time points (Figure 4).

This compound showed a Janus face in activity against MRSA at 8× MIC in this study. It had a bacteriostatic effect from 0 to 12 h of culture and then a bactericidal effect starting from 12 to 24 h of culture. To the best of our knowledge, this was the first time-kill kinetics assay for a monoaromatic compound on MRSA hitherto being performed. In the case that ethyl 3,5-dibromoorsellinate may be developed as a new antibiotic candidate, the time-kill kinetics information of ethyl 3,5-dibromoorsellinate will be helpful to assess the pharmacodynamics of this compound in the treatment of *S. aureus* infection.

Synergistic Effect of Ethyl 3,5-Dibromoorsellinate with Antibiotics on MRSA. This characteristic was investigated when a bacterial infection needs to be treated with multiple antibiotics, such as in the case of combination therapy for multidrug-resistant bacterial infections.[42] In this study, the synergistic effect of ethyl 3,5-dibromoorsellinate with two antibiotics, ampicillin and kanamycin, was investigated on MRSA, which is also a multidrug-resistant bacterial pathogen. As seen in Table 2, indifferent effects with ΣFICI values of 1.25 and 1.5 were found between ethyl 3,5-dibromoorsellinate and ampicillin at the combination of 4 μg/mL of ethyl 3,5-dibromoorsellinate and 32 μg/mL of ampicillin and the combination of 4 μg/mL of ethyl 3,5-dibromoorsellinate and 128 μg/mL of ampicillin, respectively. Unexpectedly, the combination of ethyl 3,5-dibromoorsellinate and kanamycin showed antagonism effects with ΣFICI values of 2 for the combination of 4 μg/mL of ethyl 3,5-dibromoorsellinate and 1 μg/mL of kanamycin and 8.5 for the combination of 2 μg/mL of ethyl 3,5-dibromoorsellinate and 8 μg/mL of kanamycin.

The difference in the synergistic effect of ethyl 3,5-dibromoorsellinate with the two antibiotics ampicillin and kanamycin in this study was common in studies of synergistic effects between antimicrobial agents. Broadly, antibiotic classes with their diverse mode of action had different interactions, such as synergistic, additive, antagonistic, and suppressive effects between them, as seen in the review of Bollenbach.[43] In a similar manner, a combination of kanamycin and naturally occurring compounds was shown either to reduce the MIC values leading to synergistic effects or to increase the MIC values leading to antagonism.[44]

Due to no synergistic effect being found in the combination of ethyl 3,5-dibromoorsellinate either with ampicillin or with kanamycin, more antibiotics could be checked for the synergistic effect with ethyl 3,5-dibromoorsellinate with the hope of finding the combination with the extant antibiotics exhibiting synergy as seen in the classic example between beta-lactam antibiotics and aminoglycosides where beta-lactam antibiotics caused damage to the bacterial cell wall leading to the increased uptake of aminoglycosides, the antibiotics inhibiting bacterial protein synthesis.[45] Moreover, an antibiotic resistance breaker (ARB) could also be a purpose for the synergistic study between ethyl 3,5-dibromoorsellinate and existing antibiotics, which strengthens the potency of one to another on multidrug-resistant bacterial pathogens as mentioned in the literature. Berberine, a well-known plant-derived isoquinoline used in traditional medicine to treat diarrhea caused by bacteria,[46] had an additive effect with ampicillin and a synergistic effect with oxacillin against MRSA,[47] which decreased MRSA adhesion and intracellular invasion. This compound may also have ARB activity by increasing the host defense response by inhibiting the Toll-like receptor 4−nuclear factor κB−macrophage inflammatory protein 2 pathway in ileal cells[48] and blocking lipopolysaccharide−Toll-like receptor 4 signaling in murine macrophage-like cells.[49] The results on the ARB activity of berberine may suggest future studies of ethyl 3,5-dibromoorsellinate on inflammation and autophagy of animal cell lines, which modulate the host defense to break antibiotic resistance.

Selective Toxicity of Ethyl 3,5-Dibromoorsellinate toward MRSA. Selective toxicity toward bacteria of an antimicrobial agent is desirable, and the agent should be as highly effective as possible against bacterial pathogens but show minimal or no toxicity to the host. To express the selective toxicity in practice, the selectivity index (SI) of the antimicrobial agent was calculated by the ratio of the IC_{50} value on the animal cell line to the MIC value on bacteria; the larger the index is, the safer is the antimicrobial agent.[50] For calculating the IC_{50} of ethyl 3,5-dibromoorsellinate in this study, the human fibroblast cell line was used in the sulforhodamine B assay. The results showed that the number of human fibroblast cells was reduced by only 8.42% when treated with 50 μg/mL of ethyl 3,5-dibromoorsellinate compared to the untreated sample. Accordingly, it was supposed that the IC_{50} value of ethyl 3,5-dibromoorsellinate on this cell line was much more than 50 μg/mL. The positive control, camptothecin at 2.5 μg/mL, caused a reduction of 47.86% in human fibroblast cell number compared to the untreated sample.

The SI value of ethyl 3,5-dibromoorsellinate calculated from its IC_{50} and MIC values was higher than 12.5-fold, which was promising when compared to some antibiotics, in particular those used for the treatment of MRSA infection. Linezolid and doxycyclin showed cytotoxicity effects on the rat hepatocyte cell

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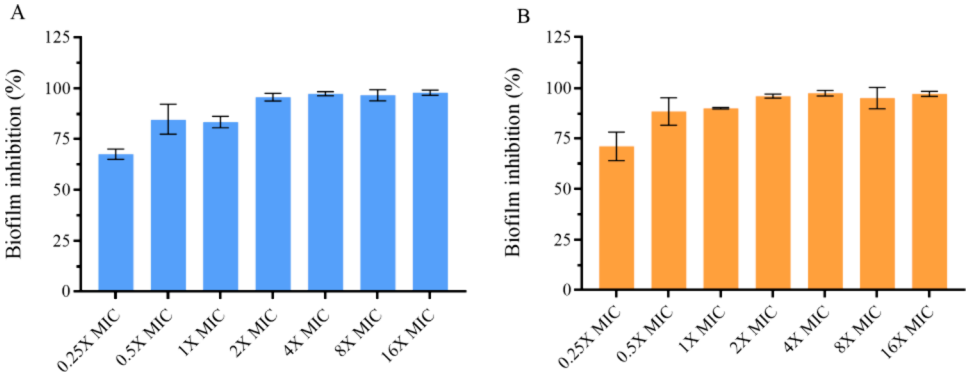

Figure 5. Antibiofilm activity against MRSA. (A) Ethyl 3,5-dibromoorsellinate. (B) Kanamycin.

line with IC_{50} values of 45.8 ± 2.45 μg/mL for each antibiotic revealed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.[51] As stated above, these antibiotics had the MIC breakpoints defined as sensitive for *S. aureus* of 4 μg/mL, inferring that the SI ratios between cytotoxicity against rat hepatocytes and antibacterial activity against *S. aureus* were 11.45-fold for these two antibiotics. The SI value, however, was just the preliminary data to interpret the safety for antibiotic usage in animals including human beings. Recently, the ignored risks of antibiotics currently used in human medicine have been summarized, and it was shown that the toxicological implications of long-term use of the majority of antibiotics had not yet been fully assessed.[52] It is found that several antibiotics showed effects with mechanisms identical to those of eukaryotic cells as in prokaryotes due to similar structures and functions between prokaryotic and eukaryotic targets. Aminoglycoside antibiotics suppress bacterial protein synthesis by binding to the 30S subunit of the ribosome. This action disturbs translation elongation, causing the production of aberrant proteins that kill bacteria.[53] Surprisingly, these antibiotics also interact with eukaryotic ribosomes on various sites of both subunits, disclosed by the analysis of crystal structures of the ribosome-aminoglycosides complex, which may suggest multiple possible effects on eukaryotic translation.[54] Therefore, future studies on the mechanism of action of ethyl 3,5-dibromoorsellinate should be performed not only in bacteria to clarify the cellular targets of this compound but also in eukaryotic cells to thoroughly evaluate the safety in using this compound for the treatment of human MRSA infection.

Inhibition of MRSA Biofilm Formation. Biofilm formation is a mechanism to protect bacteria from harmful physical, chemical, and biological factors.[55] For bacterial pathogens, biofilm formation increases the risk of infection and complicates treatment thereof. Biofilm formation is attributed to be responsible for nosocomial bacterial infection.[56] Moreover, antibiotic resistance is enhanced up to 1000-fold for bacteria living in biofilm compared to their planktonic states.[57] Thus, the inhibition of biofilm formation is a desirable characteristic in the search for compounds with antimicrobial activity. In this trend, ethyl 3,5-dibromoorsellinate was also investigated for its ability to prevent the formation of a biofilm of MRSA in this study using the crystal violet binding assay. The results in Figure 5 revealed a substantial reduction in the biofilm mass of MRSA at the different concentrations of ethyl 3,5-dibromoorsellinate. At 0.25× MIC, this compound began to show a suppressive effect on MRSA biofilm formation with a decrease of 67.41% in biofilm mass. Higher MIC values of ethyl 3,5-dibromoorsellinate increased the biofilm mass reduction, and the inhibition effect was saturated from 2× to 16× MIC, resulting in a more than 95% reduction in MRSA biofilm production. Similar results were obtained with the effect of kanamycin on the MRSA biofilm formation. This antibiotic showed a reduction of 70.98% in biofilm mass at 0.25× MIC and higher biofilm mass reduction at 0.5× and 1× MIC values before attaining a reduction of more than 95% from 2× to 16× MIC.

As far as is known, it has been the first time that a monoaromatic compound derived from natural compounds of lichens was reported to be associated with antibiofilm activity. Actually, ethyl 3,5-dibromoorsellinate showed an inhibitory effect on biofilm formation of MRSA immediately at its sub-MICs of 0.25× and 0.5×. This characteristic was useful in the context of biofilm formation in bacteria because antibiotics may not achieve their MICs at body compartments and tissues for several reasons.[58] Consequently, antibiotics at their sub-MICs were reportedly incapable of inhibiting bacteria proliferation,[59] and along with the inability to suppress bacteria, some antibiotics were also found to be associated with the induction of biofilm production at their sub-MIC values.[60] Conversely to the above, ethyl 3,5-dibromoorsellinate sharply decreased this crucial virulence factor in MRSA as low as 0.25× MIC. The ability of antibiotics to inhibit biofilm formation at sub-MIC values despite not inhibiting their growth offers a chance of suppressing or eliminating the important virulent factor of bacterial pathogens, thereby causing them to be vulnerable to the host defense system and more susceptible to antibiotics,[61] as well as diminishing their spreading to other parts in the human body that leads to recurrent of infection.[62]

Potential Target of Ethyl 3,5-Dibromoorsellinate in the MRSA Cell. The peptidoglycan is a crucial component of the bacterial cell wall, which has several functions, such as determining bacterial shape, conferring resistance to osmotic pressure, and serving as a scaffold for surface molecules.[63] These properties of the cell wall are important for Gram-positive bacteria because they lack the additional protective layer, which is the outer membrane, as seen in Gram-negative bacteria.[64] Penicillin-binding proteins (PBPs) are the enzymes involved in the assembly of peptidoglycan, and in particular, an extra PBP 2A with low affinity to b-lactam antibiotics was identified in MRSA in addition to four PBPs existing in all *S. aureus* strains.[65] The PBP 2A protein was therefore chosen to be the target for ethyl 3,5-dibromoorsellinate and its precursor ethyl orsellinate. After the screening from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), the crystal structures of PBP 2A were chosen, including PDB

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5M18,[66] 6Q9N,[67] 3ZG0,[68] for the study of interaction with ethyl orsellinate and ethyl 3,5-dibromoorsellinate. In this docking analysis, muramic acid, the native ligand of PBP 2A, was used as the control. The results of docking and energy estimation are shown in Table 3.

Table 3. Docking Results, MM/GBSA, and Interaction between Compounds Ethyl Orsellinate and Ethyl 3,5-Dibromoorsellinate and Target Protein in Antibacterial Activity

compounds	docking score	MM-GBSA (kcal/mol)	interaction
5M18			
muramic acid	−5.404	−28.4	2 h-bonds (Glu239, Arg151)
ethyl orsellinate	−4.4	−43.1	2 h-bonds (Ser149, His239)
ethyl 3,5-dibromoorsellinate	−4.6	−45.7	2 h-bonds (Glu239, Arg241)
			1 halogen bond (Arg151)
6Q9N			
muramic acid	−4.1	−25.2	3 h-bonds (Glu239, Lys148)
ethyl orsellinate	−4.6	−47.8	3 h-bonds (Arg241, Thr165)
ethyl 3,5-dibromoorsellinate	−4.2	−47.1	2 h-bonds (Glu239, Arg241)
			1 halogen bond (Arg151)
3ZG0			
muramic acid	−5.9	−35.3	4 h-bonds (Thr165, Arg241, Hie293)
ethyl orsellinate	−4.5	−42.8	2 h-bonds (Glu239, Ser149)
ethyl 3,5-dibromoorsellinate	−5.3	−42.5	2 h-bonds (Glu239, Arg151)

From the docking score and the molecular mechanics with generalized Born and surface area solvation (MM/GBSA) energy calculation results, it was evident that the antibacterial activity of the compounds ethyl orsellinate and ethyl 3,5-dibromoorsellinate against the three target proteins was quite similar, approximately −45 kcal/mol, indicating the stronger antibacterial efficacy than the muramic acid native ligand. Notably, ethyl 3,5-dibromoorsellinate demonstrated enhanced stability relative to ethyl orsellinate, as evidenced by the consistent interaction with the Glu239 residue across all three docking results, whereas ethyl orsellinate displayed varying interactions in these trials. This distinction can be attributed to the presence of a halogen bond between the bromo group and the Arg151 residue in proteins 5M18 and 6Q9N, facilitating a more secure binding of ethyl 3,5-dibromoorsellinate compared to ethyl orsellinate (Figure 6). Consequently, this difference potentially contributed to the observed 2.38-fold increase in antibacterial activity for ethyl 3,5-dibromoorsellinate over ethyl orsellinate in the experimental study.

Of the three experimental binding modes of muramic acid to PBP 2A, only two-thirds of the poses involved a repeated hydrogen bond with Glu 239, while the remaining poses constantly changed with different residues such as Arg151, Lys148, Thr165, Arg241, and His293. This suggested that muramic acid did not have a stable binding mode in the binding site and that its small size made it unstable at a specific position. This was also observed in the docking of ethyl orsellinate and ethyl 3,5-dibromoorsellinate, two molecules of similar size to muramic acid. In the binding behavior of these two compounds with PBP 2A (PDB: 5M18), the expansion of the two bromo groups led to the formation of a new halogen bond with Arg 151 and also caused a complete change in the hydrogen bond. Similarly, with PBP 2A (PDB: 6Q9N and 3ZG0), the expansion of the two bromo groups did not stabilize the binding site structure compared to the initial interactions of ethyl orsellinate. Instead, a rearrangement occurred to accommodate the size and interactions of the two bromo groups, but it still did not fully satisfy the maximum inhibitory capacity for PBP 2A. This explained why the docking score and MM-GBSA binding affinity did not meet expectations.

EXPERIMENTAL SECTION

Bacterial Strains, Cell Line, Chemical Reagents, and Culture Media. The bacterial pathogens including *Escherichia coli*, *Enterobacter cloaceae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterococcus faecium*, *Staphylococcus aureus* ATCC 25923, and methicillin-resistant *Staphylococcus aureus* (MRSA) were given by the Center for Research and Application in Bioscience. Solvents and chemical reagents were purchased from Sigma and Merck. Culture media for bacteria were purchased from HiMedia. Antibiotics were purchased from GoldBio. The fibroblast cell was supplied by the Department of Genetics, Faculty of Biology-Biotechnology, University of Science, VNUHCM.

Chemical Synthesis of Ethyl 3,5-Dibromoorsellinate from Ethyl Orsellinate. In a 2.0 mL volume of a mixture of acetic acid and DMSO (3:1, v/v), ethyl orsellinate (**1**) (10.0 mg, 0.051 mmol) and sodium bromide (15.76 mg, 0.153 mmol) were dissolved at room temperature. A volume of 0.5 mL of a 30% hydrogen peroxide solution (0.18 mmol) was added to the reaction mixture. The reaction was conducted for a duration of 30 min. The resulting solution was neutralized with saturated sodium hydrogen carbonate and subsequently extracted with ethyl acetate-water (1:1, v/v) to gain the organic layer. This layer was thoroughly washed with brine, dried, and subsequently applied to silica gel column chromatography. The elution was performed using a mixture of *n*-hexane, EtOAc, and acetone (10:1:2, v/v/v) to obtain **1a** (16.8 mg, 93%).

NMR spectra were recorded on a Bruker Avance III spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C NMR) with TMS as the internal standard. HRESIMS was recorded using a MicrOTOF-Q mass spectrometer on an LC-Agilent 1100 LC-MSD Trap spectrometer.

Evaluation of Antibacterial Activity. The antibacterial activity testing was performed using the agar diffusion method.[69] In brief, the pathogenic bacteria were cultured in a nutrient broth at 37 °C overnight with shaking, and the bacterial cultures were diluted with sterile 0.9% NaCl to the concentration of 1×10^8 CFU/mL before spreading on Mueller−Hinton agar plates. Ethyl 3,5-dibromoorsellinate was dissolved in DMSO at 1 mg/mL and put in wells on the surface of the MHA plates containing bacteria. These plates were incubated at 37 °C for 16−18 h, and inhibition zones surrounding each well were measured. DMSO and apramycin (1 mg/mL) were used as the negative and positive controls in this experiment.

Determination of the Minimum Inhibitory Concentration (MIC). The agar dilution method was employed[70] with some minor modifications. Ethyl 3,5-dibromoorsellinate in DMSO was dissolved in MHA to create the concentration range of 0, 1, 2, 4, 8, 16, 32, 64, and 128 μg/mL and put in wells of a 24-

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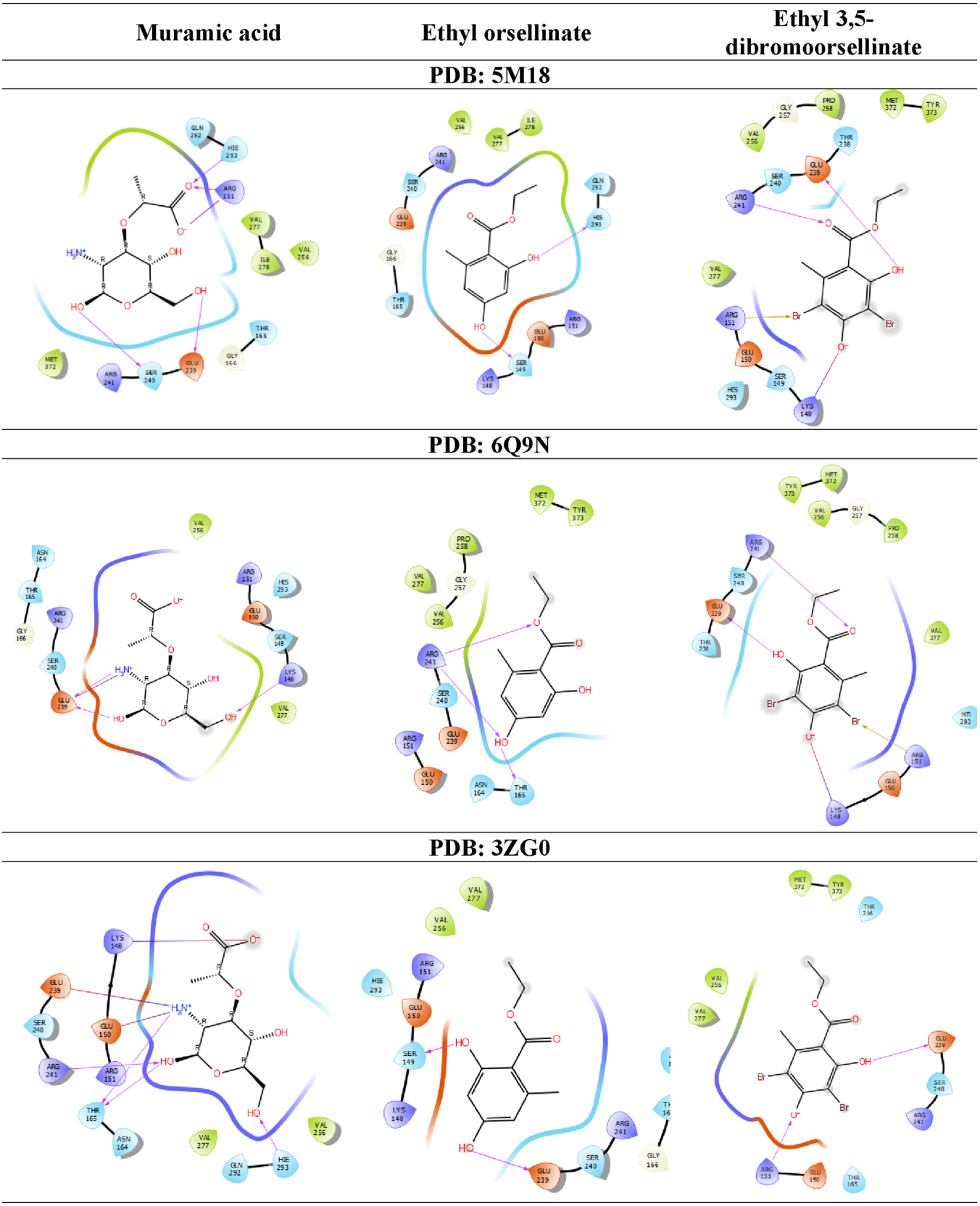

Figure 6. Binding modes of two ligands compared to muramic acid to the target proteins.

well plate. A total of 10^4 CFU of *S. aureus* ATCC 25923 and MRSA were placed on the surface of the wells containing the range concentration. The 24-well plate was incubated at 37 °C for 16−18 h. The MIC value was determined as the lowest concentration of ethyl 3,5-dibromoorsellinate that inhibits MRSA growth. Kanamycin and ampicillin were used as controls in this experiment.

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Time-Kill Kinetics Protocol. The time-kill assay[71] was used to evaluate the bactericidal kinetics of ethyl 3,5-dibromoorsellinate. Mueller−Hinton broths supplemented with this compound at concentrations of 0, 1, 2, 4, and 8× MIC were inoculated with 1×10^6 CFU/mL of the MRSA strain, and the bacterial cultures were incubated at 37 °C with shaking. A 100 μL aliquot of the bacterial cultures was spread on nutrient agar plates at each time interval of 0, 3, 6, 12, and 24 h. After incubation at 37 °C for 16−18 h, the bacterial colonies were counted, and the CFU/mL values were calculated for each culture with the corresponding MIC. Kanamycin was used as the control for this experiment.

Synergy Test. The synergistic effects between ethyl 3,5-dibromoorsellinate and the two antibiotics, ampicillin and kanamycin, were evaluated using the standard checkerboard assay.[72] Briefly, the MIC values of ethyl 3,5-dibromoorsellinate, ampicillin, and kanamycin alone or in their combinations were determined. Then, these values were used to calculate the fractional inhibitory concentration index (FICI) as follows: ΣFICI = MIC of antibiotic in combination/MIC of antibiotic alone + MIC of ethyl 3,5-dibromoorsellinate in combination/MIC of ethyl 3,5-dibromoorsellinate alone. The synergy was evaluated based on ΣFICI as synergism (ΣFICI ≤ 0.5), partial synergism (0.5 < ΣFICI < 1), indifference (1 ≤ ΣFICI < 2), and antagonism (ΣFICI ≥ 2).

Cytotoxicity Test. The colorimetric SulphoRhodamine-B (SRB) assay[73] on the fibroblast cell line was used to evaluate the cytotoxicity of ethyl 3,5-dibromoorsellinate. From maintenance in EMEM supplemented with essential elements at 37 °C, cells were seeded in 96-well plates with a density of 7.5×10^3 cells/well and incubated with complete media for 24 h before being treated with ethyl 3,5-dibromoorsellinate at different concentrations for 48 h. Then, cells were fixed with 150 μL of 10% trichloroacetic acid (TCA) and incubated at 4 °C for 1 h. The cells were washed five times with distilled water after the removal of the TCA solution. Aliquots of 70 μL 0.2% SRB solution were added, and the plate was incubated in a dark place at room temperature for 10 min. A second wash of three times with 1% acetic acid was performed, and the plate was allowed to air-dry overnight. Finally, 150 μL of 10 mM Tris base was added to dissolve the protein-bound SRB stain, and the absorbance was measured at 492 and 620 nm using a 96-well microtiter plate reader (Synergy HT, Biotek Instruments). The viability was calculated as (A_{540} of treated samples/A_{540} of the untreated sample) × 100, and the IC_{50} value was determined from the exponential curve of viability versus concentration.

Measurement of MRSA Biofilm Formation Inhibition. The crystal violet assay[74] was used to measure the biofilm formation inhibition percentage of ethyl 3,5-dibromoorsellinate on the MRSA strain. The Mueller−Hinton broth containing ethyl 3,5-dibromoorsellinate at 0, 0.25, 0.5, 1, 2, 4, 8, and 16× MICs in wells of a 96-well microtiter plate was inoculated with 10^7 CFU/mL of MRSA. The microtiter plate was incubated statically at 37 °C for 16−18 h for MRSA to form biofilm. The wells were washed with PBS to remove the planktonic cells followed by methanol fixation. The solvent was removed, and the plate was completely air-dried. The wells were stained with 0.1% crystal violet for 5 min followed by decolorization with 95% ethanol. The purple color in each well was measured by the microtiter plate reader at 595 nm, and the absorbance values were used to calculate the biofilm formation inhibition percentage according to the formula in ref 74. Kanamycin with the same MIC range was used as the control in the biofilm inhibition experiment.

Molecular Docking Study. To assess the activity of antibacterial compounds against MRSA, the ligand-protein binding ability between structures ethyl orsellinate, ethyl 3,5-dibromoorsellinate, and several important protein targets of penicillin-binding protein 2A from MRSA was evaluated. An *in silico* study was conducted utilizing the Maestro software Schrodinger.[75] Initially, target crystal proteins were selected from the RCSB Protein Data Bank and prepared by using the Protein Preparation Wizard methods.[76] This process entailed the incorporation of hydrogen atoms into target proteins, the construction of bonds like hydrogen bonds and disulfide bridges, the restoration of deficient loop regions and other atoms, and the elimination of water molecules. A grid box from Receptor Grid Generation was constructed as a binding pocket for interaction with the centroid ligands. Then, Glide Docking[77] was used to dock the experimental compounds to the target proteins. Finally, MM/GBSA was utilized to compute the free binding energy, thereby evaluating the energy values of the resultant complexes and comparing them with those of the native ligand of the protein.

CONCLUSIONS

In this study, we synthesized a novel compound, namely, ethyl 3,5-dibromoorsellinate, that exhibits promising antibacterial properties against MRSA, demonstrating significant efficacy at low concentrations and selective toxicity compared to human cells. Its ability to interact with PBP 2A and its antibiofilm activity further highlight its potential as a candidate for new antibiotic development. Continued research is warranted to fully explore and harness the therapeutic potential of this compound in combating MRSA infections.

AUTHOR INFORMATION

Corresponding Authors

Huy Truong Nguyen − *Faculty of Pharmacy, Ton Duc Thang University, Ho Chi Minh City 700000, Vietnam*; Email: nguyentruonghuy@tdtu.edu.vn

Chuong Hoang Nguyen − *Faculty of Biology-Biotechnology, University of Science, Vietnam National University Ho Chi Minh City, Ho Chi Minh City 749000, Vietnam*; orcid.org/0000-0001-8317-7298; Email: nhchuong@hcmus.edu.vn

Authors

Dao Dinh Nguyen − *Faculty of Biology-Biotechnology, University of Science, Vietnam National University Ho Chi Minh City, Ho Chi Minh City 749000, Vietnam*

Thuc-Huy Duong − *Department of Chemistry, Ho Chi Minh City University of Education, Ho Chi Minh City 748342, Vietnam*; orcid.org/0000-0001-9891-4326

Thi-Phuong Nguyen − *NTT Hi-Tech Institute, Nguyen Tat Thanh University, Ho Chi Minh City 700000, Vietnam*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.4c09518

Author Contributions

Thuc-Huy Duong, Huy Truong Nguyen, and Chuong Hoang Nguyen conceived and designed the experiments; Thi-Phuong Nguyen and Thuc-Huy Duong performed the chemical modification; Dao Dinh Nguyen and Chuong Hoang Nguyen performed characterization of the *in vitro* antibacterial activity;

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Huy Truong Nguyen performed molecular docking; Dao Dinh Nguyen, Thuc-Huy Duong, Huy Truong Nguyen, and Chuong Hoang Nguyen analyzed the data; and Huy Truong Nguyen and Chuong Hoang Nguyen prepared the manuscript.

Notes

The authors declare no competing financial interest.

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