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Research Note

Antimicrobial activity of a novel furan fatty acid, 7,10-epoxyoctadeca-7,9-dienoic acid against methicillin-resistant *Staphylococcus aureus*

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Abstract We analyzed the antimicrobial potential of a novel furan fatty acid, 7,10-epoxyoctadeca-7,9-dienoic acid (7,10-EODA) against methicillin-resistant and -sensitive *S. aureus* (MRSA and MSSA). The anti-staphylococcal activity of 7,10-EODA and its consequences on cell physiology was determined by disc diffusion, broth microdilution, and flow cytometry. Anti-virulence activity of 7,10-EODA was evaluated by bioassays. 7,10-EODA was anti-staphylococcal with minimum inhibitory concentration (MIC) range of 125-250 mg/L. 7,10-EODA exhibited a dose response and inhibited MRSA 01ST001 by 90.5% and ATCC 29213 (MSSA) by 85.3% at 125 mg/L. MIC of 7,10-EODA permeabilized >95 % of MRSA 01ST001 cells to small molecules. Sublethal dose of 7,10-EODA was non-toxic but markedly reduced the hemolytic, coagulase, and autolytic activities of MRSA and MSSA at 15.6 mg/L. The results provide a lead for the utilization of natural furan fatty acids as novel anti-MRSA agents.

Keywords: furan fatty acids, 7,10-epoxyoctadeca-7,9-dienoic acid, antimicrobial lipids, methicillin-resistant *Staphylococcus aureus*, virulence inhibition

Introduction

Staphylococcus aureus represents a serious public health threat due to the widespread nature of infections caused by this pathogen in humans and animals. Several *S. aureus* strains capable of secreting enterotoxins, nucleases and coagulases are implicated in food-borne illness (1).

Methicillin resistant *S. aureus* (MRSA) infections once confined to hospital are now prevalent in the community and are increasingly becoming hard to treat. Host adaptability of MRSA in livestock is potential threat for food security. Growing incidence of MRSA in the retail meat has been reported (2). The emergence of MRSA resistant to multiple antibiotics including vancomycin has led healthcare industries to explore the development of new antibiotics of natural origin. Food compatible dietary products used to attenuate MRSA have been reviewed (3). However, there is a large clinical demand for natural products with multiple health benefits for expanding the anti-microbial chemical arsenal against MRSA.

Furan fatty acids (F-acid), belonging to the furanoid class, are considered valuable minor components of several food products, such as fish, butter, milk, and seed oils, and have been reported to act as health-promoting agents (4). Recently, F-acids have been reported to possess anti-inflammatory, cardioprotective (4), radical scavenging (5), antithrombotic (6), neuroprotective (7), and hypolipidemic (8) properties. However, despite their health-related multifunctional benefits, relatively few studies have attempted to examine the potential utility of these bioactive lipids as antimicrobial agents. In our previous work, which attempted to identify novel functional fatty acids using the microbial biotransformation approach, we reported the production of a novel F-acid, 7,10-epoxyoctadeca-7,9-dienoic acid (7,10-EODA) with antioxidant property from 7,10-dihydroxy-8(Z)-octadecenoic acid (DOD) (9). In the present study, which was undertaken to address the need for novel non-antibiotic agents to control staphylococcal pathogens and mitigate drug resistance, we evaluated the antimicrobial potential of 7,10-EODA and its effects on the production of virulence factors (VF) by MRSA.

Materials and Methods

Materials 7,10-EODA was prepared according to a previous method (9). Mueller Hinton broth (MHB) and Tryptic Soy broth (TSB) was purchased from BD (Cockeysyvillae, MD, USA). All other chemicals

and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Microorganisms and growth conditions *Listeria monocytogenes* (ATCC 19111), *Escherichia coli* (ATCC 8739), and methicillin-sensitive *Staphylococcus aureus*; (ATCC 29213 and ATCC 6538) were purchased from American Type Culture Collection (Rockville, MD, USA). A clinical isolate of *S. aureus* SA1199 was provided by Dr. G.W. Kaatz (Wayne State University, Detroit, MI, USA). Methicillin-resistant *S. aureus* (MRSA) strains 01ST001, 032 and 136 were obtained from School of Medicine, Kyungpook National University, Daegu, South Korea. *Pseudomonas aeruginosa* (KACC 10186) and *Corynaebacterium glutamicum* (KACC 10784) were purchased from Korean Agriculture Culture Collection (Jeonju, Korea). *Salmonella enterica* subsp. Typhimurium (KCTC 2515) and *Streptococcus mutans* (KCTC 3065) were from Korean Collection for Type Culture (Daejeon, South Korea). *Bacillus brevis* (KCCM 11711) was purchased from Korean Culture Center of Microorganisms (Seoul, South Korea). Cultures were maintained at −80°C in TSB stocks containing glycerol (10%, MRSA 01ST001 v/v).

Screening for antimicrobial activity

***Agar disc diffusion assay*:** The antimicrobial activity of 7,10-EODA was examined using the disc diffusion method as per Clinical Laboratory Standards Institute (CLSI) guidelines. Briefly, MH agar plate (20 mL) was seeded with 0.1 mL of 1-2×10^5 colony forming units (CFU)/mL of the test bacterial strain. Sterile filter paper disks (6-mm diameter) were impregnated with 10 μL of 7,10-EODA (100 μg/disc) in dimethyl sulfoxide (DMSO). The inoculated plates were incubated at 37°C for 14-18 h. The size of zone of inhibition (ZOI) around the paper discs was determined.

***Determination of minimum inhibitory concentration (MIC)*:** The MIC of 7,10-EODA was determined by broth micro-dilution method. Overnight cultures of *S. aureus* were diluted 1:500 with MHB and further grown to a mid-log phase and the inoculum was adjusted to 1-2 ×10^5 CFU/mL. A 0.1 mL culture suspension was inoculated to 96 well plate (SPL Life Sciences, Pocheon, Korea) containing 0.1 mL of MHB adjusted with desired concentration of 7,10-EODA prepared from two fold serial dilution (range, 500-0.97 μg). The plates were incubated stationary at 37°C for 18 h and the turbidity (OD_{600} nm) was determined.

S. aureus *growth kinetics*: Growth kinetics of selected staphylococcal strains (MRSA 01ST001 and ATCC 29213) in presence of 7,10-EODA (rang, 125-15.6 μg) was determined by the method similar as mentioned in the above section except, that the OD_{600} of the culture was recorded 24 h with 2 h interval using a thermostat microplate reader (TECAN M200 Infinite Pro, Mannedorf, Switzerland).

***Flow cytometry*:** MRSA 01ST001 (10^5 CFU/mL) in phosphate buffer saline (PBS, pH 7.4) was incubated with 125 mg/L of 7,10-EODA for 2 h. The cells were harvested by centrifugation (4,200x*g*) at 4°C for 5 min, washed twice with PBS (pH 7.4) and re-suspended in 1 mL of PBS. To this, 5 μM propidium iodide (PI) was added and incubated for 10 min. Further, samples were analyzed through FL-1 channel (488 nm; blue argon laser) on flow cytometer (BD FACS Aria III, BD Biosciences, San Jose, CA, USA).

Analysis of virulence inhibition effect of 7,10-EODA

***Hemolytic assay*:** Hemolysin activity was assayed by measuring the hemolytic ability of the culture supernatants. Briefly, MRSA 01ST001, MRSA 032, and ATCC 29213 were cultured in the presence of 15.6-1.95 mg/L of 7,10-EODA in MHB for 12 h. A 0.1 mL cell supernatant was filter sterilized (0.2 μm membrane filter, Advantec, Toyo Roshi, Toyo, Japan) and diluted to 1 mL with PBS. To this a 0.1 mL of sheep red blood cells (RBC) was added and incubated for 15 min at 37°C. Upon brief centrifugation, the OD_{545} of the supernatant was determined for the hemolysis of the RBC.

***Coagulase assay*:** Filtered culture broth, as prepared in hemolytic assay was concentrated to 30-fold using Amicon Centrifugal Filter units (Millipore, Bedford, MA, USA) and the protein content was estimated using a Bradford method (Bio-Rad, Hercules, CA, USA). For coagulase activity, a 20 μg of the protein extract was serially diluted two-fold in PBS (pH 7.4) and incubated with 50 μL of rabbit plasma. The clotting was inspected visually and the titer value was recorded as the reciprocal of the highest dilution that caused clotting after 5 h of incubation at 37°C.

***Protease assay*:** Serine protease activity was determined by measuring the proteolytic activity of the supernatants. A 0.1 mL aliquot culture supernatant as prepared in hemolytic assay was incubated with 1 mL of azocasein at 37°C for 1 h. The reaction was stopped by 1 mL of trichloroacetic acid (5%, w/v). The OD of the supernatant was read at 328 nm. (1 Unit activity was equivalent to an absorbance of 0.001 over a 1 h incubation period).

***Autolysin activity*:** The autolysin activity of the *S. aureus* grown in the presence of 15.6 mg/L of 7,10-EODA for 6 h was subjected to autolysis induced by triton X-100 according to a previously described method (10).

Statistical analysis One-way analysis of variance (ANOVA) with Dunnett's post-test analysis was performed using 'GraphPad Prism' version 5.00 (GraphPad Software, San Diego, CA, USA).

Results and Discussion

Antimicrobial screening of 7,10-EODA Nine out of ten Gram-positive strains were inhibited with mean ZOI (range, 9.5-12.5 mm) and mean MIC (range, 31.2-2,000 mg/L) by 7,10-EODA (Table 1). The resistance of *L. monocytogens* (MIC>2,000 mg/L) was speculated to strain specific. The cell wall architectural difference between *B. subtilis*, *S. aureus*, and *L. monocytogenes* was reported (11). However, 7,10-EODA was consistently inhibitory towards both MRSA and MSSA with mean ZOI (range, 9.5-12.5 mm) and MIC (range, 125-250

Table 1. Antimicrobial screening of 7,10-epoxyoctadeca-7,9-dienoic acid (7,10-EODA) against *S. aureus*

Test microorganisms	ZOI[1)] (mm)	MIC[2)] (mg/L)
Gram-positive bacteria		
L. monocytogenes (ATCC19111)	ND[3)]	>2,000
B. brevis (KCCM 11711)	11.5	125
C. glutamicum (KACC 10784)	9.5	31.2
S. mutans (KCTC 3065)	12.5	62.5
MSSA strains		
ATCC 29213	11.5	125
ATCC 6538	10.5	125
SA 1199 (Clinical isolate)	12.5	125
MRSA strains		
01ST001 (Clinical)	12.5	125
032 (Clinical)	9.5	250
136 (Clinical)	12.0	125
Gram-negative bacteria		
P. aeruginosa (KACC 10186)	ND	ND
E. coli (ATCC 8739)	ND	1,250
S. Typhimurium (KCTC 2515)	ND	>2,000

[1)]Size of zone of inhibition in millimeters at 100 μg/disc of 7,10-EODA
[2)]Minimum inhibitory concentration of 7,10-EODA
[3)]ND, Inhibitory zone was not detected

Fig. 1. Antibacterial activity of 7,10-EODA against MRSA 01ST001. A; oxacillin (100 μg), B; 7,10-EODA (200 μg), C; oleic acid (200 μg), D; DOD (200 μg), E; vancomycin (100 μg), M; DMSO (20 μL). All the samples were completely dissolved in 20 mL of DMSO prior to application.

mg/L, corresponding to 0.42-0.84 mM). The MIC of 7,10-EODA against MRSA is comparable to the MIC value reported for oleic, linoleic, linolenic, docosahexaenoic (DHA), and eicosapentaenoic acid (EPA) (range, 0.2-0.4 mM) against *S. aureus* although their activities were limited to regular *S. aureus* (11,12). However, 7,10-EODA was specifically active against MRSA and it's activity was highly compatible with vancomycin which is a commercially available MRSA-treating antibiotic (Fig. 1). DOD (substrate for EODA production) and oleic acid (substrate for DOD production) were not active against MRSA. Gram-negative pathogens of the present study were resistant to 7,10-EODA (MIC, >2000 mg/L). This result is in good agreement with other reports in that the MICs of various long chain fatty acids were reported to be >1 mM against *E. coli* and *P. aeruginosa* (13). Resistance of the gram-negative bacteria against long chain fatty acid could be explained from the report in that the lipopolysaccharide content of the gram-negative bacteria prevented long chain fatty acid accumulation at the inner cell membrane at inhibitory concentration (14).

Anti-staphylococcal activity of 7,10-EODA MRSA 01ST001 and ATCC 29213 strains were selected as model MRSA and MSSA strains to study the anti-staphylococcal effects of 7,10-EODA. In growth curve study, a dose dependent growth inhibition of MRSA 01ST001 and ATCC 29213 was evident. At 125 mg/L, 7,10-EODA elicited >80% reduction in the cell dry weight (CDW) of MRSA 01ST001 and SA29213, with no recurrent growth till 24 h (Fig. 2A and 2B). An initial delay in the lag phase and a reduced growth of MRSA 01ST001 and ATCC 29213 were observed at 62.5 μg of 7,10-EODA and their growths during the exponential phase. Similar delay in lag phase of several bacteria including *S. aureus* by linoleate at 25 μg was reported (15). Flow cytometry data show a dose dependent membrane permeability of MRSA 01ST001 (Fig. 2C) with a >95% cell permeabilized at 125 mg/L of 7,10-EODA (Fig. 2D and 2E). Similar results were obtained with ATCC 29213 (data not shown). These results suggested that the disruption of integrity of cell membrane by 7,10-EODA was responsible for anti-staphylococcal effect of 7,10-EODA. This was supported by the report in that pore-forming anti-staphylococcal fatty acids could cause membrane permeability and inhibit macro-molecular biosynthesis and cell arrest in *S. aureus* (16).

Virulence inhibitory activity of 7,10-EODA in *S. aureus* It was found that, 7,10-EODA, at 15.6 mg/L, reduced the hemolytic activity of MRSA 01ST001, MRSA 032, and ATCC 29213 by 78.3, 69.1, and 88.6%, respectively (Fig. 3A). A dose dependent reduction in coagulase activity (p<0.01) was evident (Fig. 3B). *S. aureus* responded to glycerol monolaurate and cis-hexadecenoic acid by reducing the level of hemolysin expression (17,18). MRSA 01ST001 exhibited an increased protease activity (58.3%, p<0.01); in contrast, MRSA 032 exhibited a reduced protease activity (Fig. 3C) and this observation is in line with Arsic *et al*. (19) who reported that the sublethal linoleate at 25 μM differentially regulated proteases in various MRSA strains. An enhanced autolytic activity of *S. aureus* with fatty acids was reported (20). In contrast, 7,10-EODA at 15.6 μg inhibited autolysin activity of MRSA001, MRSA 032, and ATCC 29213 by 63.8, 58.6, and 57.4%, respectively (Fig. 3D). These results suggested that interaction of 7,10-EODA with exoproteins like hemolysin, protease, and autolysin could be different from that of linoleate. As explained above, the lipophilic property of 7,10-EODA (log D, 5.4) could partition the lipid

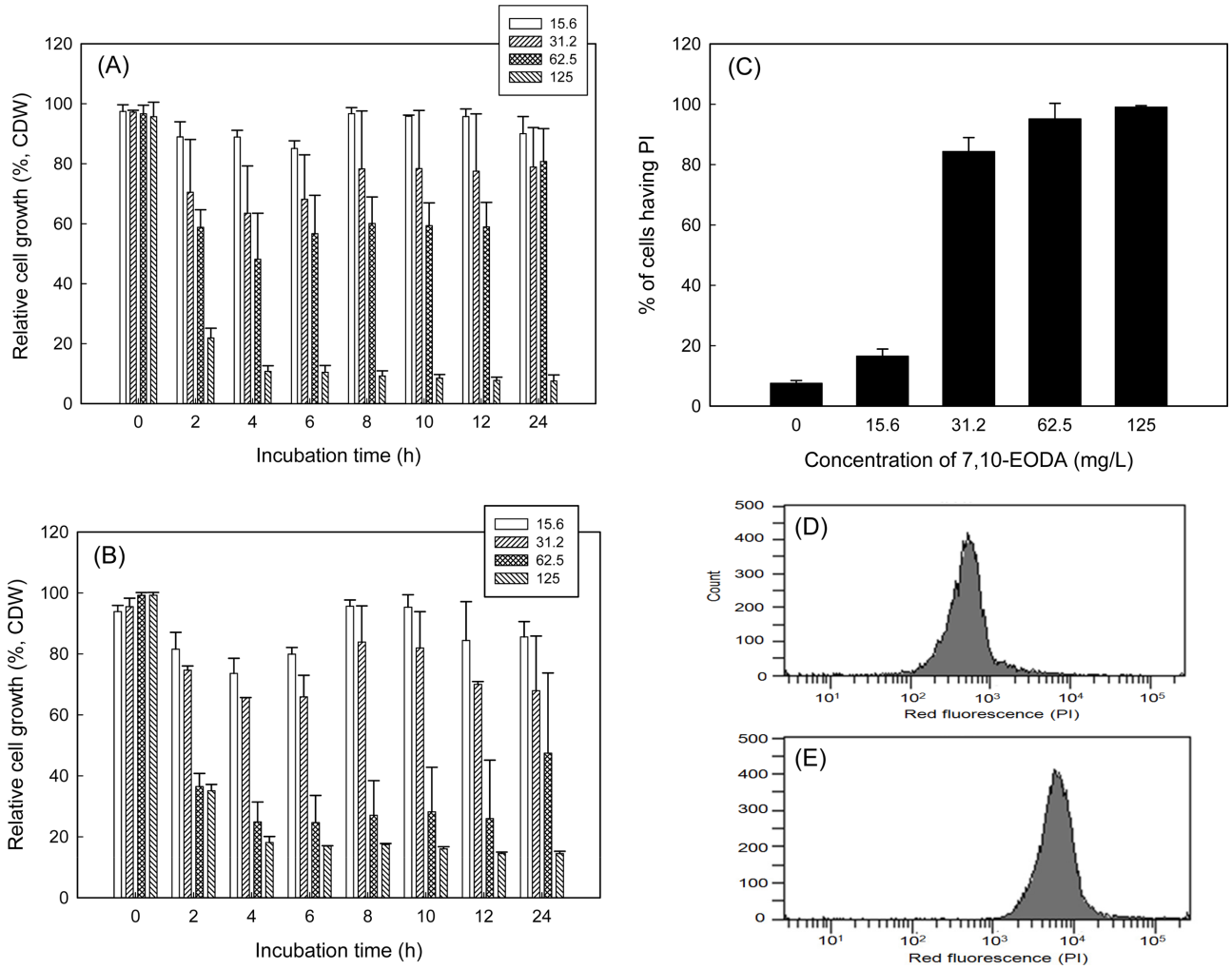

Fig. 2. Anti-staphylococcal effects of 7,10-epoxyoctadeca-7,9-dienoic acid (7,10-EODA) against *S. aureus*. Growth curves of MRSA 01ST001 (A) and ATCC 29213 (B) in the presence of 15.6-125 mg/L 7,10-EODA. Membrane permeability of MRSA 01ST001 in the presence of 15.6-125 mg/L of 7,10-EODA (C). Histograms of propidium iodide (PI) uptake in MRSAMRSA 01ST001001 in the absence (D) or presence (E) of 125 mg/L of 7,10-EODA.

layers of cell membrane and alter the integrity of staphylococcal phospholipids, resulting in alteration of exoproteins like hemolysin, protease, and autolysin. These suggested that 7,10-EODA could be related with staphylococcal quorum sensing accessory gene regulator (*agr*) which controls exoproteins like hemolysin, protease, and autolysin and regulate staphylococcal biofilm formation. Several components of *agr* locus interact with auto inducer proteins at membrane interface, a key event in the staphylococcal quorum sensing mechanism. It is conceivable, therefore, that 7,10-EODA could directly or indirectly interact with components of *agr* to influence the quorum sensing in *S. aureus*. Hence, a detailed study of 7,10-EODA on *agr* locus is further needed. The combined anti-staphylococcal and VF-disrupting potential of 7,10-EODA observed in the present study suggested that 7,10-EODA, as a natural multifunctional food compatible dietary product, can be exploited to prevent drug resistant MRSA infections.

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Disclosure The authors declare no conflict of interest.

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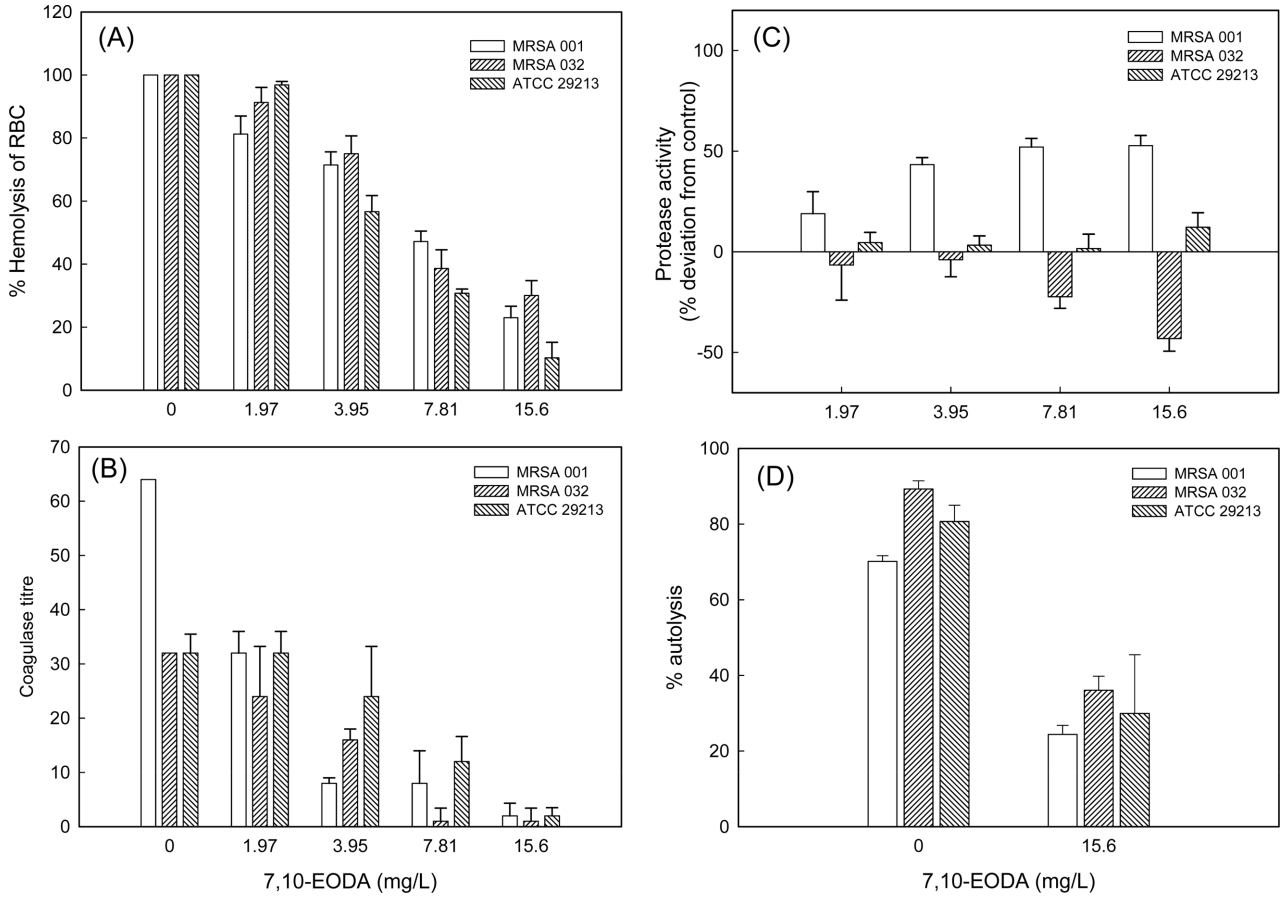

Fig. 3. Staphylococcal virulence inhibitory potential of 7,10-epoxyoctadeca-7,9-dienoic acid (7,10-EODA). Hemolytic (A), coagulase (B), proteolytic (C), and autolytic activity (D) of MRSA and MSSA grown in the presence of (0-15.6 mg/L) of 7,10-EODA.

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